ผลของน้ำทับทิมในการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรท และซุปไก่สกัดที่ทำปฏิกิริยากับไนไตรทโดยวิธีทดสอบเอมส์

นางสาวกุลธิดา เหลืองอ่อน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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EFFECT OF POMEGRANATE JUICE IN MODIFICATION OF MUTAGENICITY OF NITRITE TREATED AMINOPYRENE AND NITRITE TREATED CHICKEN EXTRACT USING AMES TEST

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Thesis Title	EFFECT OF POMEGRANATE JUICE IN MODIFICATION OF MUTAGENICITY OF NITRITE TREATED AMINOPYRENE AND NITRITE TREATED CHICKEN EXTRACT USING AMES TEST
By	Miss Kulthida Luang-on
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กุลธิดา เหลืองอ่อน : ผลของน้ำทับทิมในการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทและซุปไก่สกัดที่ทำปฏิกิริยากับไนไตรทโดยวิธีทดสอบเอมส์. EFFECT OF POMEGRANATE JUICE IN MODIFICATION OF MUTAGENICITY OF NITRITE TREATED AMINOPYRENE AND NITRITE TREATED CHICKEN EXTRACT USING AMES TEST) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.ลินนา ทองยงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.แก้ว กังสดาลอำไพ, 80 หน้า.

การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลของน้ำทับทิมคั้นสด, น้ำทับทิมพาสเจอร์ไรส์ ณ วันเริ่มต้นและหลังจากเก็บไว้เป็นเวลา 7 วัน และน้ำทับทิมสเตอริไลส์ ณ วันเริ่มต้นและหลังจากเก็บไว้เป็นเวลา 30 และ 90 วัน ในการก่อกลายพันธุ์และการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทและซุปไก่สกัดที่ทำปฏิกิริยากับไนไตรท โดยใช้เชื้อ *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100

จากการศึกษาฤทธิ์ก่อกลายพันธ์ของน้ำทับทิมทุกชนิดโดยวิธีทดสอบเอมส์ ไม่พบฤทธิ์ก่อกลายพันธุ์ของน้ำทับทิมสด ทั้งในสภาวะที่ทำปฏิกิริยาและไม่ทำปฏิกิริยากับไนไตรท น้ำทับทิมพาส-เจอร์ไรซ์ ณ วันเริ่มต้น และน้ำทับทิมสเตอริไลส์ทุกระยะการเก็บพบฤทธิ์ก่อกลายพันธุ์เพียงเล็กน้อยในเชื้อ TA98 ส่วนในสายพันธุ์ TA100 มีเพียงน้ำทับทิมสเตอริไลส์หลังจากเก็บไว้นาน 90 วัน ที่พบฤทธิ์ก่อกลายพันธุ์เล็กน้อยที่ความเข้นข้น 24.67 มิลลิกรัมต่อจานเลี้ยงเชื้อสูงสุดที่นำมาทดสอบ เมื่อศึกษาผลของน้ำทับทิมในการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของสารทดสอบ 2 ชนิดคืออะมิโนพัยรีนและซุปไก่สกัดที่ทำปฏิกิริยากับไนไตรท พบว่าน้ำทับทิมสดมีผลเพิ่มฤทธิ์การก่อกลายพันธุ์โดยสารไม่ทราบชนิดที่มีอยู่ในเมล็ดทับทิม สำหรับน้ำทับทิมที่ผ่านกระบวนการพาสเจอร์ไรซ์ไม่สามารถแสดงฤทธิ์ได้ชัดเจน (0-20%) ส่วนน้ำทับทิมที่ผ่านกระบวนการสเตอริไลส์เซชัน หลังจากเก็บไว้ที่อุณหภูมิห้อง 90 วัน เมื่อนำมาทดสอบ สามารถยับยั้งการก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทในระดับอ่อน (ร้อยละ 31-34) แต่มีผลเพิ่มการก่อกลายพันธุ์ของซุปไก่สกัดที่ทำปฏิกิริยากับไนไตรทได้ ซึ่งผลที่เกิดขึ้นอาจมาจากฟลาโวนอยด์หรือแทนนิน กระตุ้นการทำงานของเอนไซม์ nitroreductase และ *O*-acetyltransferase ในเซลล์แบคทีเรีย หรือเพิ่มการแทรกผ่านเยื่อหุ้มแบคทีเรียของสารก่อกลายพันธุ์

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KULTHIDA LUANG-ON: EFFECT OF POMEGRANATE JUICE IN MODIFICATION OF MUTAGENICITY OF NITRITE TREATED AMINOPYRENE AND NITRITE TREATED CHICKEN EXTRACT USING AMES TEST. ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., CO-ADVISOR: ASSOC. PROF. KAEW KANGSADALAMPAI, Ph.D., 80 pp.

The objectives of this study were to investigate the direct mutagenicity of fresh, pasteurized and sterilized pomegranate juices and their effects on mutagenesis induced by nitrite treated 1-aminopyrene and nitrite treated chicken extract in the absence of metabolic activation on *Salmonella typhimurium* strains TA98 and TA100. It was found that fresh juice was not mutagenic on both strains either with or without nitrite treatment. The pasteurized and sterilized juices exhibited slight mutagenicity on TA98, but on TA100, only day90 sterilized juice exhibited slightly mutagenic at 24.67 mg/plate. Fresh juice, pasteurized juices kept for 0 and 7 days and sterilized juices kept for 0, 30 and 90 days were evaluated for their antimutagenicity against nitrite treated 1-aminopyrene and nitrite treated chicken extract. Fresh pomegranate juice did not show antimutagenic activity but it presented co-mutagenic effect. It might be due to unknown natural compounds from arils and/or seeds. Pasteurized juices presented negligible effect (0-20%). On the other hand, sterilized juice kept for 90 days exhibited weak antimutagenic activity (31-34% inhibition) against nitrite treated 1- aminopyrene, but exhibited moderate co-mutagenic activity (46–57% enhancement) with nitrite treated 1- aminopyrene. This was possibly due to some flavonoids and tannins that could enhance activating enzymes of mutagens (nitroreductase and O-acetyltransferase) in *Salmonella* cell or induce the bacterial membrane permeability.

Department : Food and Pharmaceutical Chemistry... Student's Signature Kulthida Luang-on
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LIST OF ABBREVIATIONS

°C	degree Celsius
g	gram
HAs	heterocyclic amines
His^+	histidine prototrophy
h	hour
mg	milligram
mM	millimolar
ml	millilitre
mm	millimetre
M	molar
MI	Mutagenicity Index
min	minute
µl	microlitre
SD	standard deviation
et al.	*et alia* (and others)

CHAPTER I

INTRODUCTION

Because treatment options for advanced metastasized cancers remain inadequate, developing effective approaches for the prevention of cancer appears to be both a practical and promising strategy to reduce cancer burden. Chemoprevention is a rapid growing area of preventive oncology that focuses on cancer prevention by administration of one or more synthetic or naturally occurring agents to suppress, reverse or prolong the process of carcinogenesis (Mukhtar and Ahmad, 1999). It is becoming increasingly clear that chemopreventive compounds present in diet offer great potential in the fight against cancer by inhibiting the carcinogenesis through regulation of cell-defensive and cell death machineries. The chemopreventive effects of vegetables and fruits may be attributed to a combined effect of various phenolic phytochemicals which are generally antioxidants in nature, along with vitamins, dietary fibers, indoles, allium compounds and selenium (Khan *et al*., 2007).

Governments throughout the world advocate the inclusion of fruit juices in a healthy diet. A juice that is 100% derived from its parent fruit or fruits is almost universally regarded as a healthy and nutritious part of a human diet. The main emphasis in health-promoting dietary recommendations is increased consumption of fruits and vegetables (Esteve and Frigola, 2007).

Fruit juices contain a complex mixture of nutrients that are beneficial to the maintenance of good health, and they have intrinsic disease risk reduction properties. In addition to the major nutrients (e.g., vitamins, minerals) inherent in the fruit itself, juices also contain phytochemicals or phytonutrients derived from the fruit. The biological activity of phytochemicals has been studied in numerous *in vitro* and *in vivo* tests and in tests involving human.

Pomegranate juice is one of the most popular healthy drinks. Due to many health benefits, pomegranates (*Punica granatum*) have been used extensively in the folk medicine of many cultures (Longtin, 2003). Recently, pomegranate juice and even fermented pomegranate juice were demonstrated to be high in antioxidant activity (Schubert *et al*., 1999; Gil *et al.*, 2000). Pomegranate juice also displays

potent antiatherogenic action in atherosclerotic mice and humans (Aviram *et al*., 2000; Kaplan *et al.*, 2001). All these activities may be related to diverse phenolic compounds present in pomegranate juice, including punicalgin isomers, ellagic acid derivatives and anthocyanin (delphinidin, cyaniding and pelargonidin 3-glucosides and 3,5-diglucosides). These compounds are known for their properties in scavenging free radicals and inhibiting lipid oxidation *in vitro* (Gil *et al*., 2000; Noda *et al*., 2002).

In the human clinical trials, the pomegranate juice has been found effective in prolongation of prostate-specific antigen (PSA) rising time or stabilize the cancer (Pantuck *et al*., 2006). The juice also has potent antiatherogenic effects in healthy human that may be attributable to its antioxidative properties (Aviram *et al*., 2000) and daily consumption of pomegranate juice may improve stress-induced myocardial ischemia in patients who have coronary heart disease (Summer *et al*., 2005). However, the techniques used for their processing and subsequent storage may cause alterations in pomegranate juices so they do not provide the benefits expected by consumer and their toxicity test should be evaluated for safety aspect.

Therefore, the objectives of this study were to investigate the direct mutagenicity of fresh, pasteurized and sterilized pomegranate juices and their effects on mutagenesis induced by nitrite treated 1-aminopyrene and nitrite treated chicken extract in the absence of metabolic activation on *Salmonella typhimurium* strains TA98 and TA100. *S. typhimurium* strain TA98 was used for detection of frameshift mutation, whereas strain TA100 was used for detection of base-substitution.

CHAPTER II

LITERATURE REVIEW

2.1 Pomegranate

Pomegranate (*Punica granatum* L.) belongs to the *Punicaceae* family. It is one of the oldest known edible fruits and its association with ancient civilizations of the Middle East. The primary commercial pomegranate growing regions of the world are the near East, India and surrounding countries and southern Europe. Nearly all production in USA is centered in the southern San Joaquin Valley of California. The fruit is consumed fresh or processed into juice, syrup, jams or wine.

The leaves are opposite or sub-opposite, glossy, narrow oblong, entire, 3-7 cm long and 2 cm broad. The flowers are bright red, 3 cm in diameter, with five petals (often more on cultivated plants). The fruit is between an orange and a grapefruit in size, 7-12 cm in diameter with a rounded hexagonal shape, and has thick reddish skin and many seeds, demonstrating parietal placentation. The edible parts are the seeds and the red pulp surrounding them. There are some cultivars which have been introduced that have a range of pulp colors like purple.

The pomegranate fruit is nearly round, varying in diameter from about 6.25 to 12.5 cm (2.5 to 5 inches) with a prominent and persistent calyx and a hard, leathery skin (rind or husk). Skin color varies from yellow overlaid with light or dark pink to bright red, depending on variety. Botanically, the pomegranate is classified as a berry, but the edible portion develops not from the seedbox wall but from the outer seedcoat. The edible part is the pulp (aril) surrounding the seeds. The pulp, together with the locular septa (membranous walls and white, spongy tissues), represent the whole pericarp. The arils are filled with juicy red, pink or whitish (depending on variety) pulp. In each aril (juice sac), there is one angular, soft or hard seed. The taste differs depending on the variety of pomegranate and its state of ripeness. It can be very sweet or it can be very sour or tangy, but most fruits lie somewhere in between, which is the characteristic taste, laced with notes of its tannin (Kader, 2006).

2.1.1 Pomegranate Phytochemicals

The different types of phytochemicals have been identified from various parts of the pomegranate tree and from pomegranate fruits and seeds. The major class of

pomegranate phytochemicals is the polyphenols (phenolic rings bearing multiple hydroxyl groups) that predominate in the fruit. Pomegranate polyphenols include flavonoids (flavonols, flavanols and anthocyanins), condensed tannins (proanthocyanidins), and hydrolysable tannins (ellagitannins and gallotannins). Other phytochemicals identified from the pomegranate organic and phenolic acids, sterols and triterpenoids, fatty acids, triglycerides and alkaloids.

The major source of dietary pomegranate phytochemicals is a fruit. Pomegranates are popularly consumed as a fresh fruit, beverages (e.g., juices and wines), food products (e.g., jams and jellies) and extracts wherein they are used as botanical ingredients in herbal medicines and dietary supplements. Commercial pomegranate juice is obtained by a hydrostatic pressing process of whole fruits whereby two predominant types of polyphenolic compounds are extracted into pomegranate juice: flavonoids and hydrolysable tannins (Gil *et al*., 2000). The flavonoids include flavonols such as luteolin, quercetin and kaemferol found in the peel extract (Elswijk *et al*., 2004) and anthocyanins found in the arils (Santagati *et al*., 1984; Hernandez *et al*., 1999). Anthocyanins are the water-soluble pigments responsible for the bright red color of pomegranate juice. Pomegranate anthocyanins include pelargonidin 3, 5- diglucoside, cyanidin-3, 5-diglucoside and delphinidin 3, 5-diglucoside (Santagati *et al*., 1984; Hernandez *et al*., 1999).

The fresh juice contains 85% water, 10% total sugars, 1.5% pectin, ascorbic acid and polyphenolic flavonoids. In pomegranate juice, fructose and glucose are present in similar quantities, calcium is 50% of its ash content, and principle amino acids are glutamic and aspartic acids (Melgarejo *et al*., 2000). The soluble polyphenol content varies within the limits of 0.2 to 1.0% and includes mainly ellagic tannins, gallic and ellagic acid, anthocyanins and catechins (Seeram *et al*., 2006).

Hydrolysable tannins are found in the peels (rind, husk or pericarp), membranes and pith of the fruits. Hydrolysable tannins are the predominant polyphenols found in pomegranate juice and account for 92% of its antioxidant activity (Seeram *et al.*, 2005). The predominant pomegranate hydrolysable tannins is punicalgin, which is responsible for about half of the total antioxidant capacity of the juice. The soluble polyphenol content in pomegranate juice has been reported in vary within the limits of 0.2 to 1.0% (Narr *et al*., 1996). Hydrolysable tannins are gallic acid and ellagic acid esters of the core molecules that consist of polyols such as sugars. Hydrolysable tannins are susceptible to enzymatic and non-enzymatic

hydrolysis and are further classified according to the products of hydrolysis: gallotannins yield gallic acid and glucose while ellagitannins yield ellagic acid and glucose. This combination of monomers leads to large structural heterogeneity. For instance, pomegranate leaves, bark and fruit contain more than 18 individual hydrolysable tannin structures (Tanaka *et al.*, 1985; 1986). These structures are classified into gallotannins, ellagitannins (ellagic acid esters of D-glucose with one or more galloyl substitutions) and the more unique gallagoyl esters such as punicalagin and punicalin.

Polyphenols are relevant constituents regarding the organoleptic properties of pomegranate arils and juice as they are responsible for the distinctive red pigmentation and provide a mild astringency that is characteristic of pomegranate flavor. The juice obtained from these arils contains anthocyanidins (delphinidin, cyaniding and pelargonidin 3-glucosides and 3, 5- diglucosides), ellagic acid glycosides (ellagic acid glucoside, arabinoside and rhamnoside), free ellagic acid, ellagitannins (several punicalagin isomers, punicalin and some punicalagin polymeric forms) and gallotannins. The husk and fruit membranes contain mainly ellagitannins that are water soluble (punicalagins) and small amounts of procyanidins (prodelphinidins and gallocatechin) (Gil *et al.*, 2000). Anthocyanidins are also present in the skin, although the delphinidin derivatives are not generally observed and the cyaniding and pelargonidin derivatives are not generally observed and the cyaniding and pelargonidin derivatives coincide with those found in the juice (Gil *et al*., 1995). During industrial processing, the technological treatment allows the release of water-soluble husk punicalagins into the juice, which has been related to outstanding antioxidant activity observed in commercial pomegranate juice (Gil *et al.*, 2000).

Recently, there has been an increase in the use of pomegranate fruit extracts as botanical ingredients in herbal medicines and dietary supplements. In these extracts, both polyphenol and fatty acid constituents from various parts of the fruit such as its arils and juice, peels, pericarp and pith, and seeds may be present. The seeds of pomegranate have been well investigated. For example, pomegranate seed oil contains levels of >60% of punicic acid, a cis-9, trans-11, cis-13 conjugated linolenic acid (Kohno, 2004). Other fatty acids found in pomegranate seed oil include linoleic, oleic, palmitic, stearic, arachidonic and palmitoleic acids (Melgarejo, 1995). In addition, there have been reports of sex hormones such as estrone and coumestrol in pomegranate seeds (Moneam *et al.*, 1988; Melgarejo, 1995; Heftmann *et al*., 1996).

However, a recent paper did not detect the presence of the steroid hormones estrone, estradiol and testosterone in pomegranate seed, juice and commercial preparations.

2.1.2 Anticancer Potential of Pomegranate

The pomegranate's medicinal qualities have been known for thousands of years. References in the Bible and Roman mythology mention the tree's unique healing powers and some Middle Eastern, Asian and South American people still chew its bark, petals and peel to treat conditions as dysentery and disease of the mouth and gums (Longtin, 2003). Modern research has shown that the pomegranate contains polyphenols and anthocyanidins that are powerful free-radical scavengers and are more effective against disease than are those in red wine and green tea (Burton, 2003). It is widely used in traditional medicine to cure inflammation, diabetes, cardiac disease, AIDS, ischemia and cancer. On this basis, the possible anticarcinogenic effects of the pomegranate have been further explored. For example, the application of pomegranate extract to the skin of mice before they were exposed to a carcinogenic agent was shown to inhibit the appearance of erythemas and hyperplasia and the activity of epithelial ornithine decarboxylase (Burton, 2003). The pomegranate has also been shown to induce programmed cell death and to inhibit tumor invasion, proliferation and angiogenesis. It targets several proteins in the cell-signaling pathway

Various parts of the pomegranate fruit have been shown to exert antiproliferative effects on tumor cells. For example, the polyphenols in the fermented juice of pomegranates have been shown to exert anticancer effects on human breast cancer cells *in vitro* (Elswijk, 2004). Mehta and Lansky (2004) further showed that whole pomegranate seed oil was even more chemopreventive against breast cancer in a mouse mammary organ culture (MMOC) than are those polyphenols. Kim *et al*. (2002) studied on the chemopreventive and adjuvant therapeutic potential of pomegranate components in human breast cancer and found that all the components blocked endogenous active estrogen biosynthesis and aromatase activity by 60 to 80%. The inhibition of cell lines by fermented juice and pericarp polyphenols was highest in estrogen-dependent MCF-7 cells, somewhat lower in estrogen-independent MDA-MB-231 cells, and lowest in normal human breast epithelial MCF-10A cells. In both MCF-7 and MDA-MB-231 cells, fermented pomegranate juice polyphenols consistently exhibited approximately twice the antiproliferative effect that fresh pomegranate juice polyphenols showed. In addition, pomegranate seed oil effected at

90% inhibition of proliferation of MCF-7 cells at 100 μg/ml medium, a 75% inhibition of invasion of MCF-7 cells across a Matrigel membrane at 10 μg/ml and a 54% apoptosis in MDA-MB-435 estrogen receptor-negative metastatic cells at 50 μg/ml. Furthermore, in a murine mammary gland organ culture, fermented juice polyphenols effected at 47% inhibition of cancerous lesion formation induced by the carcinogen 7, 12- dimethylbenz [a] anthracene. These observations support the adjuvant therapeutic application of pomegranate in human breast cancer.

The seed oil, juice, fermented juice, and peel extract of pomegranate have also been shown to exert suppressive effects on human breast cancer cells *in vitro* (Settheetham and Ishida, 1995). Lansky *et al.* (2005) examined the various dissimilar biochemical fractions originating in anatomically discrete sections of the pomegranate fruit for their synergistic effect against the proliferation, metastatic potential and phospholipase A2 expression of human prostate cancer cells *in vitro*. They found that these fractions had supra-additive, complementary and synergistic effects. Similarly, Albrecht *et al*. (2004) examined the effects of pomegranate seed oil, fermented juice polyphenols and pericarp polyphenols on the growth of human prostate cancer cell xenografts *in vivo* and on the cell proliferation, cell cycle distribution, apoptosis, gene expression and invasion across Matrigel *in vitro*. All the three components acutely inhibited the *in vitro* proliferation of LNCaP, PC-3 and DU 145 human prostate cancer cell lines but had much less profound effect on normal prostate epithelial cells. These effects were mediated by changes in both the cell cycle distribution and the induction of apoptosis. In particular, all agents potently suppressed PC-3 invasion through Matrigel and PC-3 xenograft growth in athymic mice.

Pomegranate fruit extract treatment of highly aggressive human prostate cancer PC-3 cells resulted in a dose-dependent inhibition of cell growth/cell viability and induction of apoptosis. The antiproliferative effects of pomegranate fruit extract involves the induction of proapoptotic gene (Bax and Bak) products and down regulation of the antiapoptotic genes (Bcl-X_L and Bcl-2). Pomegranate fruit extract treatment also led to induction of WAF1/p21, KIP1/p27 and a decrease in cyclins D1, D2, E and cyclin-dependent kinase (cdk) 2, cdk4 and cdk6 expression. Oral administration of pomegranate fruit extract to athymic (*nu/nu*) nude male mice (obtained from NxGen Biosciences) implanted with androgen-sensitive CWR22Rnu1

cells resulted in a significant inhibition in tumor growth concomitant with a significant decrease in serum prostate-specific antigen levels (Malik *et al*., 2005).

Pomegranate juice has been demonstrated to inhibit the biosynthesis of cholesterol in macrophage cells (Fuhrman *et al*., 2005). This observation suggests that pomegranate juice may have cancer-chemopreventive as cancer-chemotherapeutic effects against prostate cancer. Because cholesterol accumulates in membrane domains called lipid rafts. Lipid rafts serve as membrane platforms for signal transduction mechanisms that mediate cell growth, survival, and a variety of other processes relevant to cancer. Several studies demonstrate that cholesterol accumulates in solid tumors and that cholesterol homeostasis breaks down in the prostate with aging and with the transition to the malignant state (Freeman and Solomon, 2004).

Pomegranate juice, ellagic acid, punicalagin, and total pomegranate tannins have been shown to induce apoptosis in HT-29 colon cells. However, in HCT116 colon cells, apoptosis was induced by ellagic acid, punicalagin and total pomegranate tannins but not by pomegranate juice (Seeram *et al*., 2005). Pomegranate seed oil is composed of more than 70% of the conjugated linolenic acid shown to suppress colon carcinogenesis. Not surprisingly, dietary pomegranate seed oil significantly inhibited the incidence of azoxymethane-induced colonic adenocarcinomas (Kohno *et al*., 2004).

Excessive human exposure to solar ultraviolet (UV) radiation, particularly to its UV-B component, causes many adverse effects, including erythema, hyperplasia, hyperpigmentation, immunosuppression, photoaging and skin cancer that the pomegranate can counter. In this regard, the anthocyanidins (such as delphinidin, cyaniding and pelargonidin) and hydrolysable tannins (such as punicalin, pedunculagin, punicalagin, and gallagic and ellagic acid esters of glucose) in the pomegranate possess strong antioxidant and anti-inflamatory properties that protect from ultraviolet radiation. In addition, the pomegranate fruit extract has been shown to inhibit UV-B-mediated phosphorylation of mitogen-activated protein kinase and nuclear factor (NF)-κB activation (Afaq *et al*., 2005). The dermal application of pomegranate fruit extract also suppressed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumor promotion in 7,12-dimethylbenz (a)anthracene-initiated CD-1 mice (Hora *et al*., 2003).

Pomegranate seed oil has also been shown to significantly decrease tumor incidence, tumor multiplicity and TPA-induced ornithine decarboxylase activity in a

two-stage skin carcinogenesis model in mice. These results highlight the potential of pomegranate as a safe and effective chemopreventive agent against skin cancer (Hora *et al*., 2003).

Flavonoid-rich polyphenol fractions from the pomegranate fruit also exert anti-proliferative, anti-invasive, antieicosanoid, and proapoptotic effects on breast and prostate cancer cells and have antiangiogenic activities *in vitro* and *in vivo*. For example, the flavonoid-rich fractions from pomegranate juice were shown to be potential differentiation-promoting agents of human HL-60 promyelocytic leukemia cells (Kawaii and Lansky, 2004). In addition, the effect of the boiled extract of pomegranate peel was examined in the human cell lines Raji and P3HR-1. The proliferation and viability of these tumor cells were dose-dependently reduced by the pomegranate extract. Collectively, these studies confirm the potent antitumor effects of the various components of the pomegranate fruit (Settheetham and Ishida, 1995).

2.2 Nitrite and Gastric Cancer

Nitrite is used as a food preservative to inhibit *Clostridium botulinum*, and is also secreted, sometimes in substantial quantities, in human saliva (Tannenbaum *et al*., 1974). Nitrate, especially which in starchy foods and in vegetables, is another source, being converted to nitrite when such foods are stored at room temperature (Weisburger and Raineri, 1975a). Many plants, such as leafy vegetables or certain roots, accumulate extremely high concentrations of nitrate under favourable condition of soil and water (Philips, 1968; Heisler *et al*., 1973; Huarte-Mendicoa *et al*., 1997). Since 1970s there has been concern about a possible link between nitrite and cancer. There is no conclusive evidence that nitrite is directly carcinogenic (Cantor, 1997) but in high doses it has been implicated as a co-carcinogen (Schweinsberg and Burkle, 1985).

Gastric cancer remains such a common neoplastic disease in many parts of the world (Kikugawa and Nagao, 1990). In 1990, Graham and co-workers found that high ingestion of nitrate or nitrite in processed meats and fish, heated fats and starch may be directly correlated with cancer risk. The correlation between nitrate ingestion and mortality from gastric cancer was reported (Fined *et al.*, 1976). In addition, an epidermiological study indicated that there was a high incidence of gastric cancer associated with nitrate intake via drinking water in China (Xu *et al*., 1992).

Gastric cancer is the most likely site of cancer induced by nitrosation reaction of alkylamide-type substrates (Marquardt *et al.*, 1977). The nitrosable products (amines and amide) also occur in our diet (Endo, Takahashi, Kinoshita, Utsunomiya & Baba, 1975). Many *N*-nitroso compounds have been shown to be carcinogenic in animal experiments (Mirvish, 1983). Such compounds may be formed in the human stomach from dietary nitrite or nitrate. Nitrite reacts with amines, amides and other proteins to form *N*-nitroso compounds. Nitrate itself is not a direct nitrosating agent, but is reduced to nitrite by many bacterial species harbouring in the mouth and achlorhydric stomach (Kono and Hirohata, 1996).

2.3 Nitrite as a Converter for Direct Mutagen

Several investigators (Marquard *et al*., 1977, Llanes and Tannenbaum, 1982 and Wakabayashi *et al*., 1985) suggested that direct-acting mutagens formed from nitrite and the precursors of mutagens in the acid condition of stomach were possible candidates for the causation of human gastric cancer. For instance, Fava beans (*Vicia fava* L.) commonly eaten in Columbia where gastric cancer incidence was high (Correa *et al*., 1983) showed direct acting mutagenicity toward *S.* strains after treated with nitrite in acidic solutions (Llanes and Tannenbaum, 1982; Hoeven *et al*.,1984; Correa *et al*., 1983).

Investigating on components of Japanese diet, Wakabayashi and his co-worker (1983, 1985) found that bean paste, fish sauce, pickled vegetables, sun-dried fishes and Chinese cabbage showed direct acting mutagenicity on *Salmonella typhimurium* TA100 after nitrite treatment. In addition, soy sauces widely used as a seasoning in Southeast Asia and Japan were strongly mutagenic to *Salmonella typhimurium* TA100 after being treated with 50 mM nitrite. Lin *et al*. (1979) found that soy sauce treated with nitrite in the range 1000 to 10000 ppm was mutagenic in direct relation to nitrite concentration. However, soy beans themselves and a soy sauce produced by acidic hydrolysis of soy beans showed no mutagenicity after nitrite treatment.

Various food produced in Asia were reported on their direct-acting mutagenicity after treatment with nitrite kimchis, sun-dried fish, 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 preformed nitroso compounds) were associated with an

increased risk of gastric cancer. Salted pickled cabbage eaten by Korean three times a day contained high level of *N*-nitroso compounds after treatment with nitrite under simulated human stomach condition. Thus, salted pickled cabbage may play an important role in the gastric cancer in Korea (Seel *et al.*, 1994). 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 metalbolic activation. All of them exhibited direct-acting mutagenicity in *Salmonella* assay (Hankimhun, 1997).

Many investigators examined the diets eaten mostly in European countries where the gastric cancer rate was also high (Marquardt *et al*., 1977). Ohshima *et al*. (1989) found that smoked foods, a frequent consumed food item, was associated with increased risk of gastric cancer; there were revealed to be direct acting genotoxicity after nitrosation *in vitro*. Munzner and Wever (1984) found that the products formed by the reation of beef extract with nitrite were assayed in the *Salmonella* /microsomae mutagenicity test on strains TA1538, TA98 and TA100. The products excibited mutagenic activity toward all test strains without metabolic activation. Marquardt *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 after treated with 50 mM sodium nitrite at pH 3.0 for 1 h at 37 °C and their mutagenicity were assayed on TA98 and TA100 without metabolic activation (Yano *et al*., 1988) (Table 1).

Table 1. Mutagenicity of cooked foods after nitrite treatment (Yano *et al.*, 1988)

Food	**Revertants / g 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 foods and charcoal-broiled foods are common foods which polycyclic aromatic hydrocarbons (PAHs) 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, and pork (medium fat) were mutagenic toward both strains TA98 and TA100 (Kangsadalampai *et al.*, 1996a). New substances were not *N*-nitroso compound but might be nitro-polycyclic aromatic hydrocarbons (nitro-PAHs). In addition, Kangsadalampai and Peerawong (1997) found that commercial chicken extracts in the presence of nitrite showed their mutagenicity towards *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation.

Kangsadalampai and Butryee (1995) found that nitrite treated products of natural Thai food colors from 5 plants, namely *Clitorea ternatea* Linn., *Hibiscus subdariffa* Linn., *Pandanus amaryllifolius* Roxb., Caramelized coconut sugar and *Carthamus tinctorius* Linn. and a synthetic color, Ponceau 4R excibited their mutagenicity on *Salmonella typhimurium* both strains TA98 and TA100. Mutagenicity of the extracts of Thai medicinal plants after nitrite treatment was found on *Andrographis paniculata* ness, *Carthamus tinctorius* Linn, *Cassia angustifolia* Vahl, *Cassia fistula* Linn, *Centella asiatica* Linn, *Oroxylum indicum* Vent and *Zingiber officinale* Roscoe (Kangsadalampai and Butryee., 1995). A food additive, namely sorbic acid could react with nitrite to yield mutagens (Namiki *et al*., 1981). Being treated with nitrite, pepper exhibited the strongest mutagenic activity in the Ames test. Nutmeg, chilli pepper and laurel also showed strong activities (Namiki *et al*., 1984). No mutagenicity was observed for spices alone. In addition, Shepard *et al*. (1993) reported that aspartame (artificial sweetener) nitrosated for 10-30 min with 40 mM nitrite (pH 3.5, 37°C) had mutagenic activity on *Salmonella typhimurium* strain TA100. Furthermore, the products formed by reacting with nitrite under gastric simulating condition (pH 3.0-3.5) of *Fomes japonica*, *Ganoderma applanatum*, Hed-Cha-Jean were mutagenic to both strains TA98 and TA100, while royal jelly was mutagenic to only strain TA98 (Katipagdeetham, 1996). Therefore, several nitrosable mutagen precursors in foods taken by people in high risk areas might be the ethiological factor of gastric cancer. Investigation must be continued to elucidate whether nitrosable compounds are involved in the development of human cancer, particularly of gastric cancer.

2.4 1-Aminopyrene – Nitrite Mutagenicity Model

1-Aminopyrene is a derivative of 1-nitropyrene in human gastrointestinal tract. Aerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. 1-nitropyrene is generally a product of incomplete combustion and is the predominant nitro-polycyclic aromatic hydrocarbon (nitro-PAH) emitted in diesel exhaust, exhaust of kerosene heaters and petroleum gas burners and some of food products (as a result of incomplete combustion or pyrolysis of fat in meat produced pyrene and NO_2 from burning of cooking gas) during barbecuing (Rosenkranz and Mermelstein, 1983; Honda *et al.*,1983; Tokiwa *et al*., 1985; Kinuchi *et al*., 1986; Edenharder *et al*., 1993). The most primary route of potential human exposure to 1-nitropyrene is inhalation.

The metabolism of 1-nitropyrene was shown to involve both nitroreduction and oxidation as shown in Figure 1 (Howard, Beland and Cerniglia, 1983; Howard, Reed and Koop, 1988; Kataoka *et al*., 1991; Kappers *et al*., 2000). Under aerobic conditions, cytochrome P-450 mediated C-oxidation results in the formation of expoxides and/or by conjugation with glutathione, glucoronic acid, sulfate and then excreted as conjugates (Djuric, 1987; Kataoka *et al*., 1991).

Nitro reduction of nitro-PAHs *in vivo* occurs mainly by bacteria in the intestinal tract (El-Bayoumy *et al*., 1983; Moller *et al*., 1988; Ball *et al*., 1991). Nitroreduction in mammalian cells is catalysed by xanthine oxidase, aldehyde oxidase or NADPH cytochrome P-450 reductase. The arylamine are formed by Nitroreduction of nitro-PAHs. Arylamines can be reactivated through *N*-oxidation by a cytochrome P-450- mediated reaction to form the *N*-hydroxylarylamine. Although reactive arylnitrinium 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 and Miller, 1981; Rosser *et al*., 1996).

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 aminopyrene treated with nitrite at pH 3.0 and 37°C showed mutagenicity on *Salmonella typhimurium* TA98 and TA100 without metabolic activation. The result agreed with the work of Kangsadalampai and Suharittamrong (1996) which stated that nitrite treated with 1-aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene toward *Salmonella typhimurium* both strains TA98 (frameshift mutation) and TA100 (base-pair substitution mutation) in the absence of metabolic

activation. The mutations appear to be due to the presence of nitroreductase (IARC, 1989) and *O*-acetyltransferase (Mermelstein *et al*., 1981) which are the two activating systems presented in bacterial cells for nitrite treated aminopyrene (supposed to be 1-nitropyrene). Such enzyme metabolise 1-nitropyrene to be arylhydroxylamine, which is active to interact with DNA. Evidence 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 aminopyrene and nitrite in acid condition has been established as a model for antimutagenicity studies of some chemical concerning the phenomenon occurred during stomach digestion.

2.5 Heterocyclic Amines

Common cooking procedures such as boiling, frying, barbecuing (flame-grilling), heat processing, and pyrolysis of protein-rich foods induce the formation of potent mutagenic and carcinogenic compounds (Felton *et al.*, 1997; Sugimura, 2000). The mutagens identified in cooked food are heterocyclic amines (Skogs, 1993). The heterocyclic amine is a family of mutagenic/carcinogenic compounds produced during the heating of creatine, amino acids and sugars.

Heterocyclic amines are classified into 2 groups by treatment with 2mM sodium nitrite (Robbana-Barnet *et al.*, 1996; Naccari *et al.*, 2009).

2.5.1 Amino-imidazoazaarenes AIAs (IQ-type, polar) (Figure 2).

The AIAs are thermic mutagens belonging to the “IQ type” compounds, 2 – amino – 3 - methylimidazo [4, 5- *f*] quinoline (IQ), 2 – amino - 3, 4 - dimethylimidazo [4, 5- *f*] quinoline (MeIQ), 2 – amino - 3, 8 - dimethylimidazo [4, 5- *f*] quinoxaline (MeIQx), 2 – amino - 3, 4, 8 - trimethylimidazo [4, 5-*f*] quinoxaline (4, 8 DiMeIQx), 2 – amino - 3, 7, 8 - trimethylimidazo [4, 5- *f*] quinoxaline (7, 8 DiMeIQx), 2 – amino -1 - methyl - 6 - phenylimidazo [4, 5- *b*] pyridine (PhIP) and amino-1, 6 – dimethylimidazo-[4, 5, *b*] pyridine (DMIP) (Schut *et al.*, 1999)(Figure 2). They are formed at the normal cooking temperature of 100 – 225 °C (i.e. cooking, broiling, frying, grilling, smoking) foodstuffs in the presence of creatinine, amino acids and sugars, involving maillard reaction (Ristic *et al.*, 2004). The amino group is not changed by treatment with 2 mM sodium nitrite but is converted to a nitro group with 50 mM sodium nitrite under acid condition (Sasagawa *et al.*, 1988). The nitro derivatives of IQ-type showed the same mutagenicity in the absence and presence of S9 mix as parent amino derivatives.

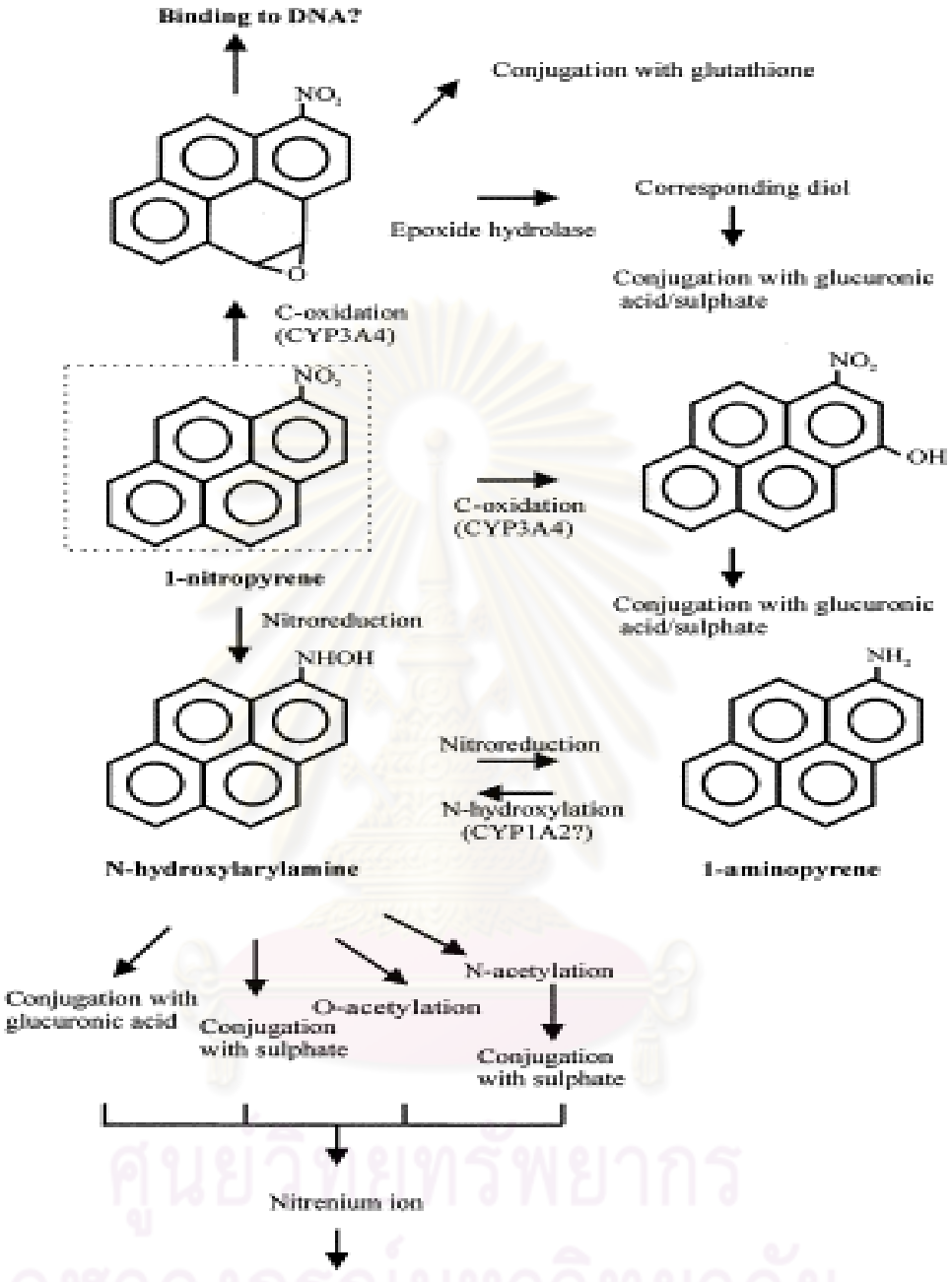

Figure 1. Potential pathways of 1-nitropyrene detoxification and metabolic activation (Kappers *et al.*, 2000)

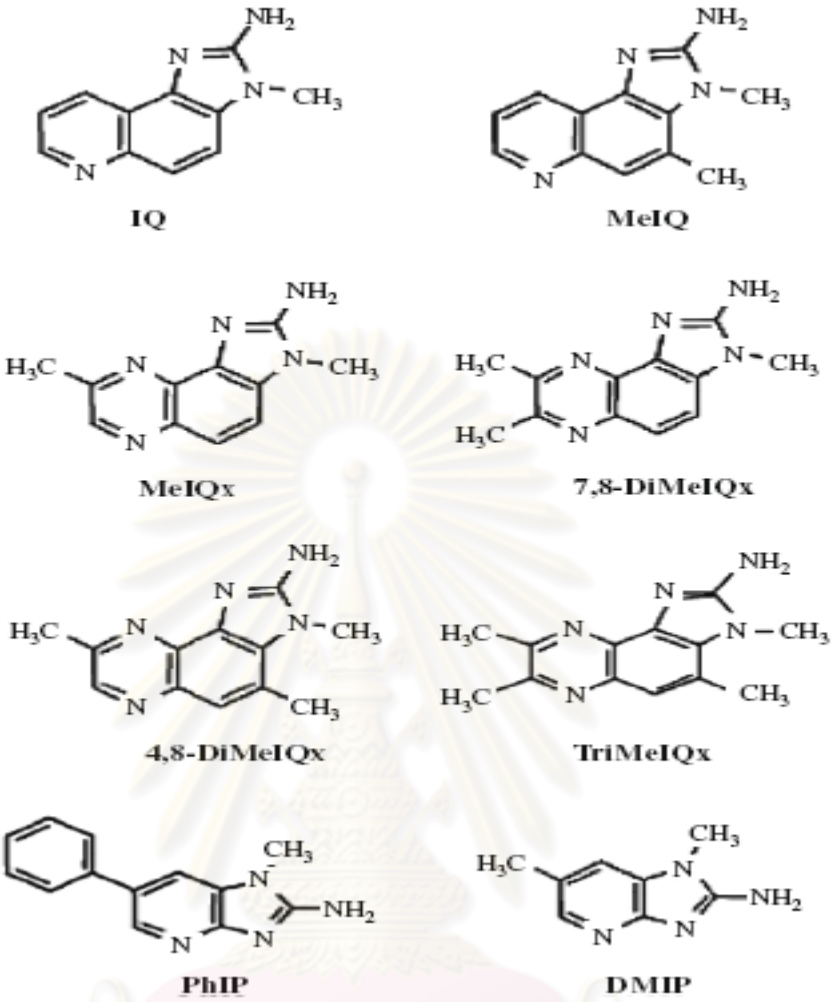

Figure 2. Aminoimidazoazaarenes AIAs (IQ-type heterocyclic amines).

2.5.2 Amino-carbolines (NON-IQ type, non polar) (Figure 3).

The Amino-carbolines are considered to be the pyrolytic amines because they are formed at high temperature pyrolysis of amino acids and protein, being produced at higher temperatures (above 300 °C) by the pyrolysis of proteins, amino-sugars and amino-acids (Tsuda *et al.*, 1985; Pais and Knize, 2000). The Amino-carbolines belong to the "NON-IQ type" compounds and include 3 – amino -1 – methyl- 5H- pyrido [4, 3- *b*] indole (Trp-P2), 3 – amino -1, 4 – dimethyl- 5H- pyrido [4, 3- *b*] indole (Trp-P1), 2 – amino - 9H- pyrido [2, 3- *b*] indole (AαC), 2 – amino -3- methyl- 9H- pyrido [2, 3- *b*] indole (MeAαC), 2 – amino - 6- methyldipyrido [1, 2- *a*: 3', 2'- *d*] imidazole (Glu-P1),), 2 – aminodipyrido [1, 2- *a*: 3', 2'- *d*] imidazole (Glu-P2) and co-mutagens

9H- pyrido [4, 3- *b*] indole (Norharman) and 1- methyl- 9H- pyrido [4, 3- *b*] indole (Harman) (Figure 3). The amino group is converted to a hydroxyl group with 2 mM sodium nitrite and this conversion is associated with loss of mutagenicity. They are considered to be the pyrolytic amines because they are formed at high temperature pyrolysis of amino acids and protein, being produced at higher temperatures (above 300 °C) by the pyrolysis of proteins, amino-sugars and amino-acids (Tsuda *et al.*, 1985; Pais and Knize, 2000).

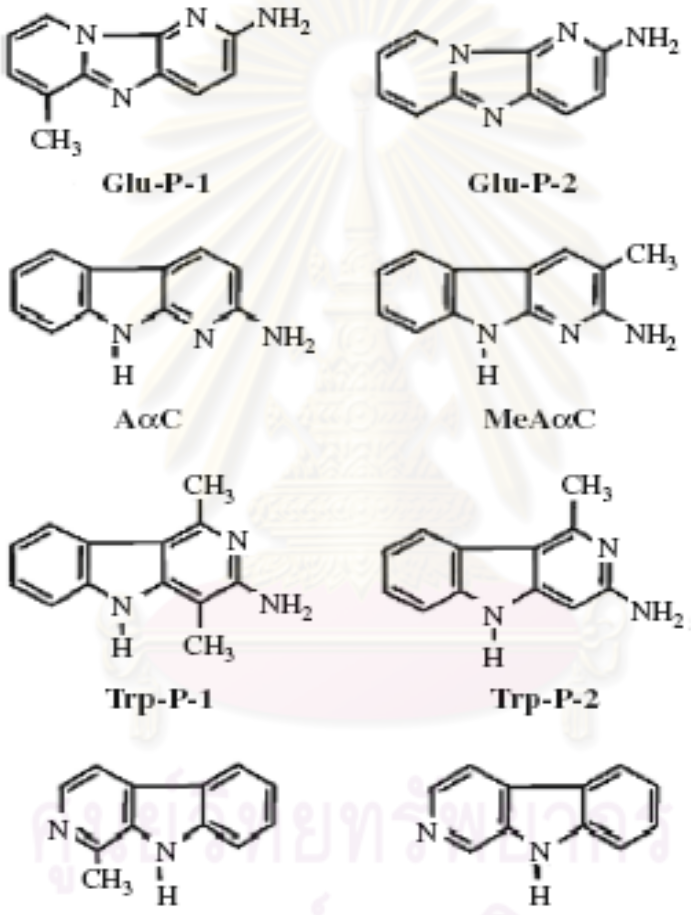

Figure 3. Carboline (Non-IQ type heterocyclic amines).

2.5.3 Conversion of Precursors in Chicken Extract to Direct Mutagens

Table 2 give a summary of the currently available data in the literature on the amounts of thermic mutagen in cooked meat and fish including chicken, etc. The mutagens namely, 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ), 2-amino-3,4

dimethylimidazo [4,5-*f*] quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-*f*] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo [4,5-*f*] quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) have all been found in cooked meat and fish. In broiled chicken, the amounts of MeIQx and DiMeIQx type compounds were quantified between 2.1-2.33 and 0.81 ng/g respectively while PhIP was 38.1 ng/g (Skog, 1993).

Direct acting mutagens that caused frameshift mutation were predominant in the nitrosated chicken extracts. The nitrosated products of chicken extracts were speculated to contain direct mutagens such as nitro compounds. Formation of nitro derivatives by nitrite treatment was observed with mutagenic and carcinogenic IQ. IQ was converted to 3- methyl- 2 nitroimidazo [4, 5-*f*] quinoline, showing mutagenicity toward *Salmonella* strains without metabolic activation after 50 mM nitrite treatment at pH 3.0 (Kangsadalampai and Peerawong, 1997). Sasagawa *et al*., (1988) also reported that IQ was converted to the direct acting mutagen 2- nitro IQ. Treatment of IQ with a much lower amount of nitrite (2mM) produced no effect (Tsuda *et al*., 1985). The reaction of nitrite under mildly acidic condition may have two aspects: one is the production of mutagens and the other is the destruction of mutagens (Tsuda *et al*., 1985). This result suggested that the non-enzymatic formation of direct-acting mutagens from indirect-acting mutagens such as IQ or MeIQ might be physiologically important, especially with regard to the etiology of human gastrointestinal tract tumors (Lin *et al*., 1992). IQ and MeIQ are very reactive to nitrite. The mutagenicity of nitrosated MeIQ and IQ is even higher than that of the parent compounds.

2.6 The *Salmonella* Mutagenic Assay (Ames test)

Bacterial mutagenicity assays, especially the Ames test (*Salmonella typhimurium his*$^-$ revesion assay) have been used worldwide, in research labolatories. Their application is motivated by several goals; the identification of genotoxic hazards; the quantitation and regulation of health risks result from environmental chemical exposure; and the elucidation of the biochemical mechanisms of mutagenesis. The potential of this method for use as a bioassay for the development of safe, useful chemicals raised many questions about the extent to which this kind of approach should be used in program aimed at cancer prevention.

Table 2. Thermic mutagens identified in cooked meat, fish and in food grade beef extract [a] (Skog, 1993)

Compound	Food	Amount (ng/g)[b]
IQ	Fried ground beef	0.02 – 20
(2–amino3methylimidazo	Broiled ground beef	0.5
[4,5-*f*] quinoline)	Broiled beef	0.19
	Fried ground beef	0.3 – 1.9
	Fried ground pork	nd – 0.04
	Broiled sardine	4.9 – 20
	Broiled salmon (flesh)	0.3 – 1.8
	Broiled salmon (skin)	1.1 – 1.7
	Broiled salmon	0.2 – 0.4
	Fried fish	0.16
	Beef extract	nd – 10
	Beef supernatant	nd – 6.2
	Meat extract	1.9 – 4.8
MeIQ	Fried ground beef	nd
(2 – amino - 3, 4 -	Fried ground pork	nd – 0.02
dimethylimidazo [4, 5-*f*]	Broiled sardine	16.6 – 20
quinoline)	Broiled salmon (flesh)	0.6 – 2.8
	Broiled salmon (skin)	1.5 – 3.1
	Broiled salmon	0.4 – 0.9
	Fried fish	0.03
MeIQx	Fried ground beef	nd – 12.3
(2 – amino - 3, 8 -	Broiled beef	0.5 – 2.11
dimethylimidazo [4, 5-*f*]	Fried beef	1.1 – 8.3
quinoxaline)	Fried hamburger	0.1 – 0.68
	Fried ground pork	nd – 1.4
	Broiled chicken	2.1 – 2.33
	Broiled mutton	1.01
	Smoked dried fish	0.8

Table 2. (continued) Thermic mutagens identified in cooked meat, fish and in food grade beef extract [a] (Skog, 1993)

Compound	Food	Amount (ng/g)[b]
MeIQx (continued)	Heated fish	5.2
	Fried fish	6.44
	Salmon (pan-broiled)	1.4 – 5
	Salmon (oven-baked)	< 1 – 4.6
	Canned roasted eel	1.1
	Beef extract	< 1 – 69
	Meat extract	6.2 – 28.3
4- MeIQx (2 – amino - 3, 4 - dimethylimidazo [4, 5-*f*] quinoxaline)	Fried ground pork	0.01 – 0.02
4, 8 – DiMeIQx (2 – amino - 3, 4, 8 - trimethylimidazo [4, 5-*f*] quinoxaline)	Fried ground beef	0.12 – 3.9
	Fried beef	1.3 – 2
	Fried hamburger	nd – 0.28
	Fried ground pork	nd – 0.6
	Broiled chicken	0.81
	Broiled mutton	0.67
	Smoked dried fish	0.08
	Heated fish	5.4
	Fried fish	0.10
	Beef extract	nd – 4.9
	Meat extract	2.9 – 3.6
7, 8 – DiMeIQx (2 – amino - 3, 7, 8 - trimethylimidazo [4, 5-*f*] quinoxaline)	Fried ground beef	0.7
	Canned roasted eel	5.3

Table 2. (continued) Thermic mutagens identified in cooked meat, fish and in food grade beef extract [a] (Skog, 1993)

Compound	Food	Amount (ng/g)[b]
PhIP	Fried ground beef	15
(2 – amino -1 - methyl - 6	Fried beef	0.56 – 48.5
- phenylimidazo [4, 5-*b*]	Broiled beef	15.7
pyridine)	Fried ground pork	nd – 4.5
	Broiled chicken	38.
	Broiled mutton	42.5
	Fried fish	69.2
	Salmon (pan-broiled)	1.7 – 23
	Salmon (oven-baked)	nd – 18
	Salmon (barbecued)	2 – 73
	Beef extract	3.62
4 – OH – PhIP	Broiled beef	21
(2 – amino -1 - methyl -6-		
(4-hydroxyphenyl)imidazo		
[4, 5-*b*] Pyridine)		
TMIP	Fried ground beef	0.5
(2–amino-n,n,n–		
trimethylimidazopyridine)		

[a] Food-grade beef extract are to be diluted before consumption.

[b] ng/g cooked food, is sometimes related to the weight before and sometimes after cooking.

nd = not determined

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 in the presence of a mutagen. The last 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. At present the most commonly used *Salmonella* strains are

TA1535, TA1537, TA1538, TA98 and TA100. The number and type of strains used depend upon the availability and type of sample, the focus of the study, and previous knowledge concerning the test material. In addition to having a mutation in one of the genes of the histidine operon, tester strains frequently have mutation that impart other specific characteristics to the tester strain. One mutation (*rfa*) leads to a detective lipopolysaccharide coat; another is a deletion of genes involved in the synthesis of vitamin biotin (*bio*) and in the excision repair of DNA damage (*uvr* B). The *rfa* mutation increase the permeability of the strains to large molecules, thereby increasing the mutagenicity and/or toxic effects of these chemicals. The *uvr* B mutation leads to a reduced level of error-free repair of some types of DNA damage and thereby enhances the strains sensitivity to certain chemical and physical mutagens. Strain TA100 is derived from TA1535 by the introduction of the plasmid pKM101, which increases the sensitivity of mutagen detection by enhancing error – prone DNA repair. The presence of this plasmid makes TA100 respond to some frameshift mutagens as well as base-pair substitution mutagens. Strain TA98 is derived from TA1538 by the introduction of plasmid pKM101. All tester strains should be maintained and stored according to published methods (Ames *et al*., 1975; Maron and Ames, 1983). They should be analyzed on a frequent and rational basis for each characteristic that could affect the test. For example, strain identification could include the following: histidine and biotin requirement, UV sensitivity (presence of the *uvr* B deletion), crystal violet sensitivity (presence of the *rfa* deletion), ampicillin and/or tetracycline resistance (present of the appropriate plasmid), spontaneous reversion frequency and reversion characteristics to various positive controls.

Three of the most important *his* $^{-}$ alleles found in the Ames tester strains (Hartman *et al*., 1986) are listed below, among with typical strains bearing the allele; the nature of the mutation in the target gene; and the most common pathway for its reversion :

- *hisD3052*; TA1538, TA98 : -1 frameshift; ΔGpC frameshift in $(GC)_4$ run
- *hisG46*; TA1535, TA100 : missence; base-substitution at G:C base-pair
- *hisG428*; TA102, TA104, TA2659 : ochre; base-substitution at A:T base-pair

Each Ames test strain evaluates mutagenic activity at a specific (reversion) target sequence. In the case of the frameshift allele *hisD3052* revertants bearing many different sequence changes (spanning a region of more than 50 bp) can be recovered: of course, each such event restores the correct reading frame. Multiple classes of

revertants of the base-substitution alleles can also recovered, including transitions, transversion and some extragenic suppressor mutations.

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

- 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.

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

- 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.

- 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 dietylnitrosamine 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 preincubation assay first described 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 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 methylmethane sulfonate has been determined using both plate incorporation and preincubation procedures and in all cases the preincubation 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 preincubation assay than in the standard plate incorporation test (Prival, 1979).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate corporation assay. Nevertheless, many laboratories use it routinely because of the increased sensitivity toward some compounds. Its use in screening assays has been recommended by De Serres and Shelby (1979).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

1-Aminopyrene (Sigma-Aldrich, St. Louis, USA) was used to interact with sodium nitrite (Sigma chemical, co., St. Louis, USA) in acid condition to produce a standard direct mutagen of the Ames test. Ammonium sulfamate ($NH_2SO_3NH_4$), d-biotin, sodium dihidrogen phosphate (NaH_2PO_4) and sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4 \cdot 4H_2O$) were purchased from Sigma Chemical, (St. Louis, USA). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), l-histidine monohydrochloride, potassium chloride (KCl), dipotassium hydrogen phosphate anhydrous (K_2HPO_4), Agar-Agar and crystal violet indicator were supplied by Merck (Darmstadt, Germany). Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), sodium chloride (NaCl), D (+)-glucose anhydrous, hydrochloric acid, di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) were purchased from BDH Chemical Ltd. (Poole, England). Ampicillin sodium salt was purchased from T.P.Drug Laboratories (1969) Co. Ltd. (Bangkok, Thailand). Oxoid nutrient broth No.2 was supplied by Oxoid Ltd. (Basingstoke, Hants., England). Acetonitrile was purchased from J.T. Baker (Phillibsburg, USA).

3.2 Experimental Design

Determination of the mutagenic modifying activity of pomegranate juice on nitrite treated 1-aminopyrene and nitrite treated chicken extract were carried out according to the experimental design shown in Figure 4.

First, the mutagenicity of chicken extract was assayed, nitrite treatment and no treatment, by using the standard Ames test in the absence of metabolic activation toward *Salmonella typhimurium* strains TA98 and TA100. Second, the mutagenicity of the fresh juice, pasteurized juices kept for 0 and 7 days and sterilized juices kept for 0, 30 and 90 days were assayed, nitrite treatment and no treatment, by using the standard Ames test in the absence of metabolic activation. Last, the modification effect of the fresh juice, pasteurized juices kept for 0 and 7 days and sterilized juices kept for 0, 30 and 90 days pomegranate juices on the mutagenicity of nitrite treated 1-

AP or nitrite treated chicken extract were investigated using Ames test in the absence of metabolic activation.

3.3 Preparation of Concentrated Chicken Extract

Chicken extract was purchased from a supermarket in Bangkok, Thailand (manufacturing date: 05/07) and then evaporated at 45°C until dried. For mutagenicity evaluation, the solution of 1 g of dried chicken extract in 1 ml of ultrapure water was sterilized by autoclaving at 121°C 20 min and stored in the freezer of refrigerator (-8°C) before use.

3.4 Preparation of Pomegranate Juices

Fresh pomegranate fruits were purchased from a supermarket in Bangkok, Thailand during January, 2008. For making pomegranate juice, the samples were cut open and discard the peel and white membranes to obtain the arils and the juice was made by using automatic juicer (National, MJ-68M). The juice was filtered through filter paper No.42 to remove the insoluble materials. The filtrate was separated into three parts, the first was fresh, the second was pasteurized at 68°C for 30 min (Fellow, 2000) and the third was sterilized at 121°C for 20 min. Before use, the fresh juice was steriled by filtrating through 0.20 μm-membrane (Sartorius, Minisart®). The pasteurized juices were stored in the refrigerator for 0 and 7 days and the sterilized juices were stored in room temperature for 0, 30 and 90 days before testing. All of the juices were protected from light.

3.5 Nitrosation of 1-Aminopyrene

1-Aminopyrene treated with nitrite in acid condition was used as a positive control because it has shown to give direct-mutagenicity (Kangsadalampai *et al*., 1996). In this study, appropriate volumes (20 and 40 μl for testing on *Salmonella typhimurium* strains TA98 and TA100 respectively) of 1-aminopyrene (0.0375 mg/ml) in a tube fitted with a plastic stopper was mixed with 730 and 710 μl of 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 250 μl of 2 M sodium nitrite to obtain the final volume of 1000 μl. The final concentration of nitrite was 500 mM. The reaction tube was shaken at 37ºC for 4 h and then placing this tube in an ice bath to stop the reaction. In order to decompose the residual nitrite,

250 µl of 2M ammonium sulfamate was added to the reaction mixture and then it was allowed to stand for 10 min in an ice bath before mutagenic assay (Ames test) as shown in Figure 5. The reaction mixture was then subjected to be tested as standard mutagen on *Salmonella typhimurium* strains TA98 and TA100 using Ames test with modified preincubation method.

3.6 Ames Test

Ames test, the screening test for checking mutagenicity of a chemical or a mixture of chemicals was used in this study to check the mutagenicity of chicken extract, pomegranate juices.

Salmonella typhimurium strains TA98 and TA100, which required histidine as growth factor, were used. They were provided by Assoc. Prof. Kaew Kangsadalampai, Ph.D. of the Institute of Nutrition, Mahidol University. The tester strains were manipulated as suggested by Maron and Ames (1983). Overnight cultures of bacteria inoculated from frozen stock culture in Oxoid nutrient broth No.2 at 37ºC 16 hr were used for mutagenic assay (Appendix). The cultures were kept in the refrigerator until use.

Minimal agar plate contained 30 ml of minimal glucose agar medium consisting of 1.5% agar and 2% glucose in Vogel-Bonner medium E. Top agar contained 0.6% agar and 0.5% sodium chloride (Appendix). It was autoclaved and kept warm in water bath (45ºC). Before use, 10 ml of a sterile solution of 0.5 mM L-histidine and 0.5 mM biotin was added to each 100 ml of the molten agar and mixed thoroughly by swirling.

3.6.1 Modified Pre-Incubation Method (Yahagi *et al*., 1975)

Modified method of Yahagi *et al*. (1975), including pre-incubation of the test samples without metabolic activation toward *Salmonella typhimurium* TA98 and TA100 was used throughout this study (Figure 6).

The 100 µl of reaction mixture (25 µl aliquots of nitrosated aminopyrene was taken to make volume with 75 µl distilled water for using in mutagenic modification test on TA98 and TA100) was mixed with 500 µl Na_3PO_4-KCl buffer (pH 7.4) and 100 µl of overnight culture of tester strain, then incubated at 37ºC in a shaking water bath for 20 min. After incubation, 2 ml of molten agar (45ºC) was added. The contents of the tube were well mixed and poured onto minimal agar plate. The plate

was evenly distributed by rotating the dish. After solidication, the plates were placed upside down in an incubator at 37ºC for 48 h. The aminopyrene concentrations 0.015 and 0.03 μg/plate and 0.03 and 0.06 μg/plate were used as standard mutagen for TA98 and TA100.

3.7 Mutagenicity of Chicken Extract

3.7.1 With Nitrite Treatment

The procedure was done as described in 3.5 and Figure 5. Aliquot volume of chicken extract (10, 50, 100 or 200 μl) was placed instead of 1- aminopyrene and after nitrosation, supernatant was collected by centrifugation at 3000 rpm for 20 min. The mutagenicity of nitrosated chicken extract was analyzed following the procedure described in 3.6.1 and Figure 6.

3.7.2 Without Nitrite Treatment

The procedure was done as described in 3.5 and Figure 5, Aliquot volume of chicken extract (10, 50, 100 or 200 μl) was placed instead of 1- aminopyrene and distilled water was placed instead of sodium nitrite and ammonium sulfamate. The mutagenicity of chicken extract was analyzed following the procedure described in 3.6.1 and Figure 6.

3.8 Mutagenicity of Pomegranate Juices

All pomegranate juices were evaluated for mutagenicity with and without nitrite treatment before mutagenic modification test.

3.8.1 With Nitrite Treatment

The procedure was done as described in 3.5 and Figure 5. Aliquots of pomegranate juices (10, 50, 100 or 200 μl) were placed instead of 1- aminopyrene. The mutagenicity of nitrosated pomegranate juices was analyzed following the procedure described in 3.6.1 and Figure 6.

3.8.2 Without Nitrite Treatment

The procedure was done as described in 3.5 and Figure 5, Aliquots of pomegranate juices (10, 50, 100 or 200 μl) were placed instead of 1- aminopyrene and distilled water was placed instead of sodium nitrite and ammonium sulfamate. The mutagenicity of chicken extract was analyzed following the procedure described in 3.6.1 and Figure 6.

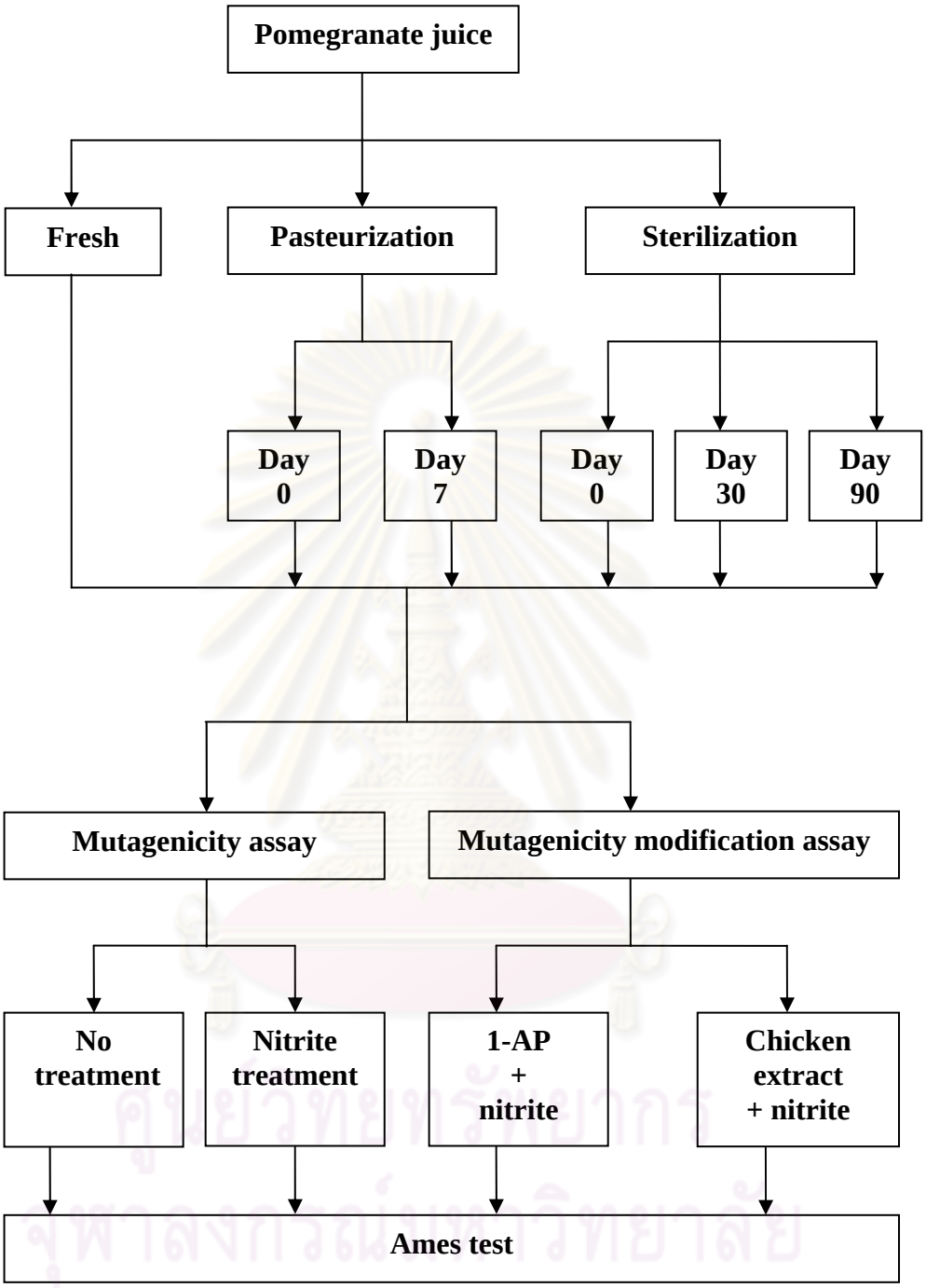

Figure 4. Experimental design for determining the mutagenicity modification of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and chicken extract

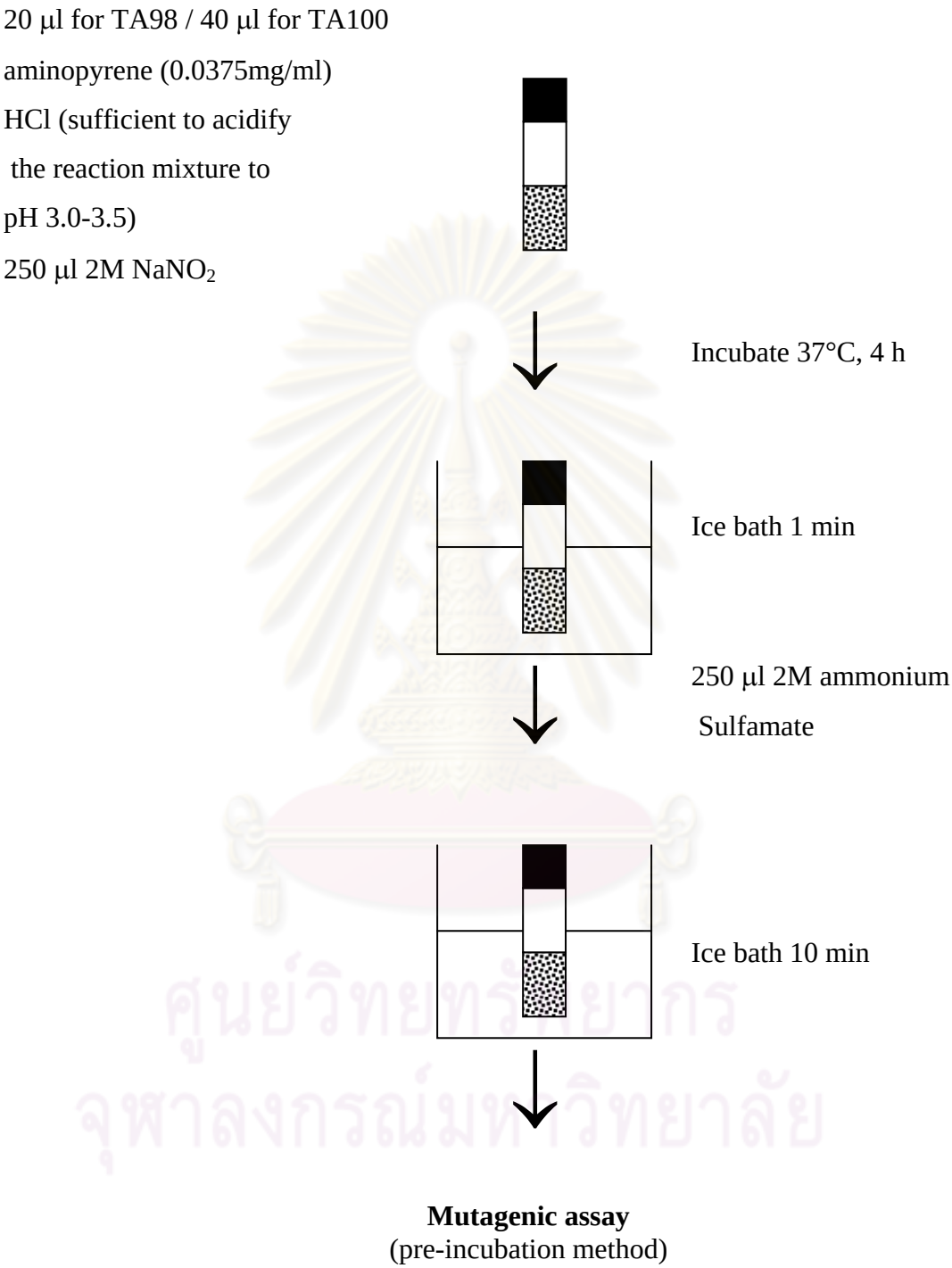

Figure 5. Nitrosation of 1-Aminopyrene**.**

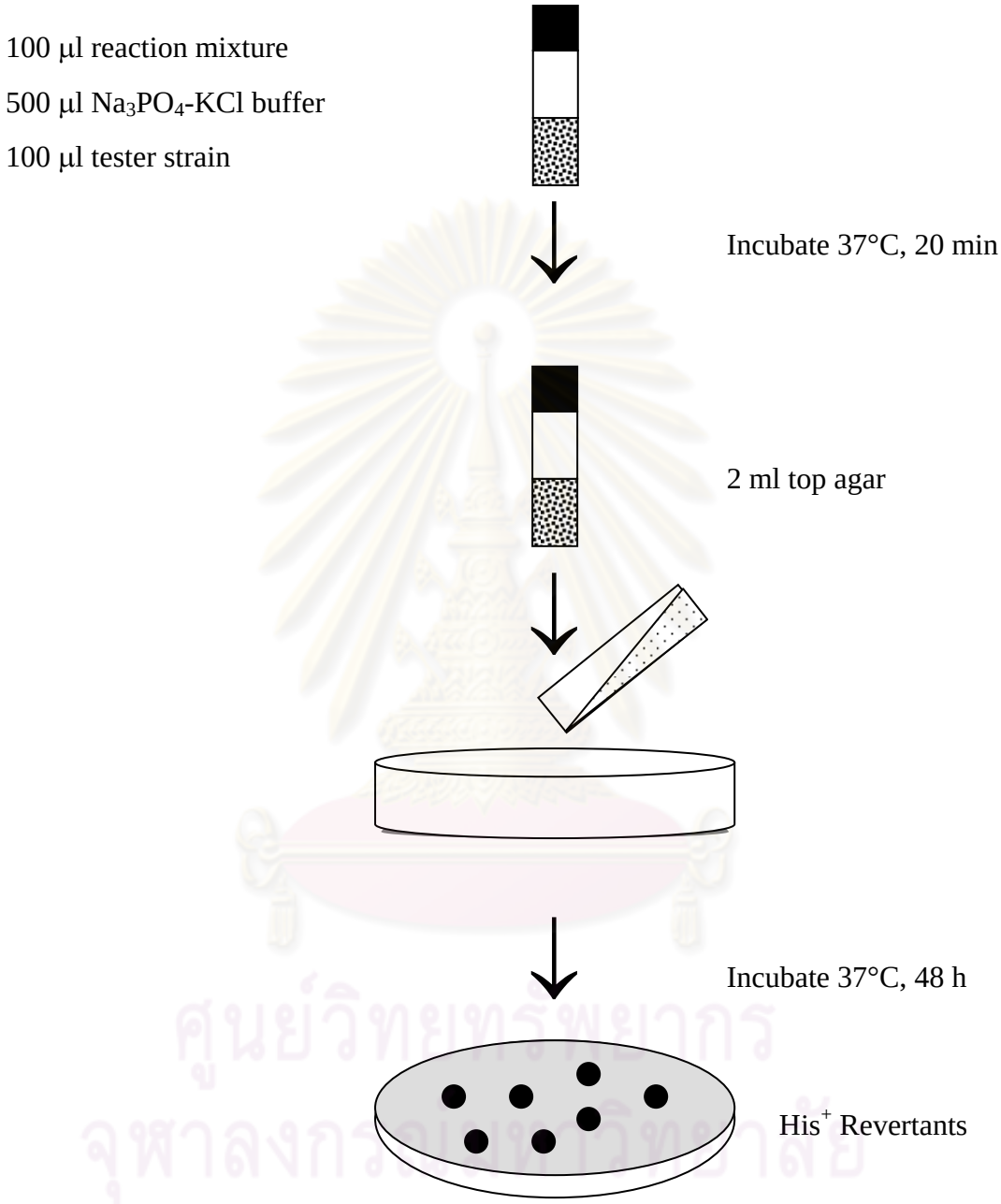

Figure 6. Steps of the mutagenicity evaluation using the Ames test (pre-incubation)

3.9 Mutagenic Modification of Pomegranate Juices

3.9.1 Modification on the Mutagenicity of Nitrite Treated 1- Aminopyrene (1-AP)

Nitrite treated 1-AP (from 3.5) was used as positive control. The procedure was performed as follows (Figure 9): 100 μl of the aliquot of nitrite treated 1-AP (final amounts of 1-Aminopyrene were 0.015 and 0.03 μg/plate for TA98 and TA100 respectively), an aliquot of fresh (day 0) or pasteurized (day 0 and 7) or sterilized kept for 0, 30 and 90 days pomegranate juices (25, 50 or 100 μl) were mixed together in a tube. The mixture was adjusted to 200 μl by distilled water, before adding 500 μl Na_3PO_4-KCl buffer (pH 7.4) and 100 μl of tester strain. After mixing, the tube of mixture was incubated at 37ºC for 20 min. After incubation, 2 ml molten agar (45ºC) was added. The contents of the tube were well mixed and poured onto minimal agar plate. The plate was evenly distributed by rotating the dish. After solidication, the plates were placed upside down in an incubator at 37ºC for 48 h and the number of His^+ revertant colonies was determined.

3.9.2 Modification on the Mutagenicity of Nitrite Treated Chicken Extract

The 100 μl nitrite treated chicken extract supernatant (16 mg of dried chicken extract/plate) (from 3.7.2) was used as positive control. The supernatant was collected by centrifugation of nitrosated chicken extract at 3,000 rpm for 20 min

The procedure was performed as follows (Figure 9): 100 μl of nitrite treated chicken extract supernatant, an aliquot of fresh (day 0) or pasteurized juice (day 0 and 7) or sterilized kept for 0, 30 and 90 days pomegranate juices (25, 50 or 100 μl) were mixed in a tube. The mixture was adjusted to 200 μl by distilled water, before adding 500 μl Na_3PO_4-KCl buffer (pH 7.4) and 100 μl of tester strain. After mixing together, the tube of mixture was incubated at 37ºC for 20 min. After incubation, 2 ml molten agar (45ºC) was added. The contents of the tube were well mixed and poured onto minimal agar plate. The plate was evenly distributed by rotating the dish. After solidication, the plates were placed upside down in an incubator at 37ºC for 48 h and the number of His^+ revertant colonies was determined.

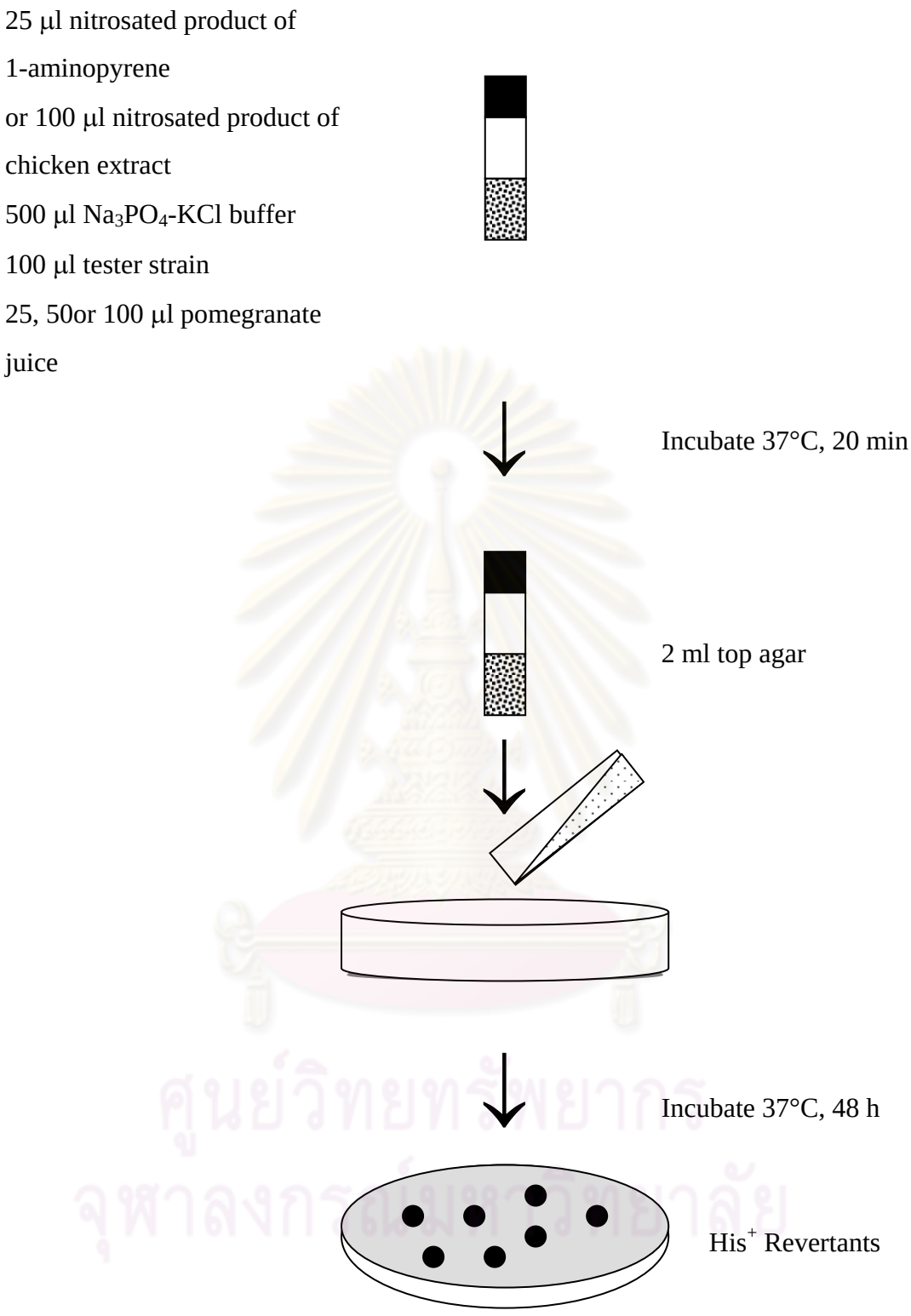

Figure 7. Steps for determining the mutagenic modification of the pomegranate juices on nitrosated products of 1-aminopyrene and nitrosated products of chicken extract using Ames test (pre-incubation method) in the absence of metabolic activation.

3.10 Data Manipultation

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

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

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 the 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) was calculated as following:

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

A = a number of histidine revertants induced by nitrite treated standard mutagen (1-AP) or chicken extract

B = a number of histidine revertants induced by mutagen in the present of pomegranate juice

C = a number of spontaneous revertants (negative control)

The inhibition (or enhancement) of mutagenicity may be divided into four classes (Calomme *et al*., 1996) as shown in Table 3.

Table 3. Criteria for evaluation as the inhibition or enhancement of mutagenicity

% modification	Inhibition or enhancement
± < 20	Negligible effect
± 20 - 40	Weak activity
± 40 - 60	Effective or moderate activity
± > 60	Strong or potent activity

- = enhancing effect, + = inhibitory effect

CHAPTER IV

RESULTS

4.1 Mutagenicity of Chicken Extract

The 120.73 g chicken extract was evaporated until dried (16.35 g). The solution of 1 g of dried chicken extract dissolved in 1 ml of ultrapure water was sterilized at 121°C for 20 min and stored in a refrigerator until use.

The study on the mutagenicity of chicken extracts before nitrite treatment in the absence of metabolic activation on *S. typhimurium* strains TA98 and TA100 (Table 4) showed that the chicken extract was not mutagenic on both strains. However, they showed mutagenicity after nitrite treatment on both TA98 and TA100. Therefore, 16 mg/plate of chicken extract with nitrite treatment was chosen to be the mutagen of these experimental strains for mutagenic modification test.

4.2 Mutagenicity of Pomegranate Juices

One hundred grams of pomegranate arils gave 64.85±1.81 ml per 100 g arils. The pomegranate juice was studied as fresh, pasteurized and sterilized juices.

The mutagenicity of fresh juice, pasteurized juices kept for 0 and 7 days and sterilized juices kept for 0, 30 and 90 days was tested. The mutagenic effects of all pomegranate juices with and without nitrite treatment on TA98 and TA100 were shown in Table 5 and 6, respectively. The mutagenicity index of all pomegranate juices treated with and without nitrite toward TA98 was shown in Figure 8 and 9 respectively. Figure 10 and 11 reveal the mutagenicity index toward TA100 of all pomegranate juices with and without nitrite treatment, respectively. The results showed that all types of pomegranate juice were not mutagenic without nitrite treatment; however, the slightly mutagenicity were also observed on pasteurized juice on the day of preparing, and sterilized juices kept for 0, 30 and 90 days after nitrite treatment toward TA98 (as shown in Table 5 and Figure 8). The weak mutagenicity was also observed on sterilized juice kept for 90 days after of nitrite treatment toward TA100 (as shown in Table 6 and Figure 10).

Table 4. Mutagenicity of chicken extract on *Salmonella typhimurium* TA98 and TA100 under acid condition (pH3-3.5) without metabolic activation

S. typhimurium strains	Amount (mg/plate)	No. revertants/plate[a]		Mutagenicity Index[b] (MI)	
		Without nitrite	With nitrite	Without nitrite	With nitrite
TA98	0[spontaneous]	15±6	15±6	1.00	1.00
	0.8	15±4	31±4	1.00	2.07
	4.0	17±4	59±11	1.13	3.93
	8.0	20±7	97±20	1.33	6.47
	16.0	24±6	132±33	1.60	8.80
TA100	0[spontaneous]	105±12	105±12	1.00	1.00
	0.8	92±14	127±20	0.88	1.21
	4.0	113±10	231±19	1.08	2.20
	8.0	134±16	334±23	1.28	3.18
	16.0	144±28	494±35	1.37	4.70

0.03 and 0.06 μg/plate of 1-aminopyrene induced 1351±91 and 935±18 revertants/plate for TA98 and TA100 respectively.

[a] Data are expressed as mean ± SD of triplicate plates from two independent experiments (N=6)

[b] Mutagenicity Index (MI) is calculated from the average value of a number of histidine revertants/plate of the chicken extract divided by that of spontaneous revertants.

4.3 Mutagenicity Modificating Activity of Pomegranate Juices

1-Aminopyrene or chicken extract are an indirect mutagens requiring metabolic activation. However, being treated nitrite in acid condition pH 3.0-3.5, they gave rise to positive mutagenicity in the Ames assay and were chosen to be the positive controls of the experiment.

Table 7 reveals the effects of pomegranate juices on the mutagenic activities of nitrite treated 1-aminopyrene and nitrite treated chicken extract toward TA98 in the absence of metabolic activation. All pomegranate juices were unable to inhibit mutagenic activity of nitrite treated 1-aminopyrene and presented co-mutagenic effect toward TA98 (Figure 12). The results showed that the mutagenicity of nitrite treated 1-aminopyrene was enhanced by the addition of pomegranate juices. Fresh pomegranate juice was weak to strong enhancer (-33 to -87 %) and pasteurized juices kept for 0, 30 and 90 days showed negligible to strong enhancing effect (-10 to -61%).

The sterilized juices kept for 0, 30 and 90 day showed surprisingly negligible inhibition (3-17%) at high dose (154.20 mg/plate).

Contrastingly, the effect of pomegranate juice showed no trend on nitrite treated chicken extract. The chicken extract treated with nitrite in this study was not appropriate for positive mutagen. The chicken extract precipitate occurred between nitrite treatment and number of His+ revetants induced by nitrite treated chicken extract was lower than optimal may be affect the results. However, Most of pomegranate juices showed negligible effect (0-20 %) as shown in Figure 13.

Table 8 reveals the effects of pomegranate juices (fresh juice, pasteurized juices kept for 0 and 7 days and sterilized juices kept for 0, 30 and 90 days) on the mutagenic activity of nitrite treated 1-aminopyrene and nitrite treated chicken extract toward TA100 in the absence of metabolic activation. Most of pomegranate juices inhibited the bacterial mutagenesis induced by nitrite treated 1 – aminopyrene at high dose (154.20 mg/plate). Pasteurized juices kept for 0 and 7 days exhibited 19 % inhibition. Sterilized juices kept for 0, 30 and 90 days exhibited weak inhibition (39, 31 and 34 %). However, the fresh juice weakly enhanced the bacterial mutagenesis (-1 to -28 %) as shown in Figure 14.

As shown in Figure 15, most of pomegranate juices enhanced the bacterial mutagenesis induced by nitrite treated chicken extract on TA100. Fresh juice showed weak enhancements (-8 to -26 %). Pasteurized juices on preparation day showed moderate enhancement (-46 to -57 %). Sterilized juices on preparation day also showed moderate enhancement (-36 to -54 %). Sterilized juices kept for 30 days exhibited negligible effect (-1 to 2 %) and sterilized juices kept for 90 days showed weak enhancement (-18 % to -25 %).

Table 9 reveals comparative summaries of pomegranate juices' effects against nitrite treated 1-aminopyrene and nitrite treated chicken extract without metabolic activation on TA98 and TA100. In this study, the effects of pomegranate juices were clearly observed on *S. typhimurium* strain TA100 more than TA98. The difference on the effect of pomegranate juices was clearly observed on nitrite – chicken extract model. Pomegranate juices exhibited moderate enhancing effect against nitrite treated chicken extract on TA100. However, they showed negligible effects on TA98. It indicated the predominant mutagens in nitrite–chicken extract model were base pair substitution mutagens. The difference on the effect of pomegranate juices between nitrite treated aminopyrene and nitrite treated chicken extract were found on TA100.

The mutagenicity of nitrite treated 1-aminopyrene was inhibited by pomegranate juices. However, the mutagenicity of nitrite treated chicken extract was enhanced. The difference between two mutagenic models was not observed on fresh juice.

Table 5. Mutagenicity of the pomegranate juices with and without nitrite treatment on *S. typhimurium* TA98 under acid condition (pH3-3.5) without metabolic activation

Juices	Storage duration (days)	Amount[a] (mg/plate)	No. of revertants/plate[b]		Mutagenicity Index[c]	
			Without nitrite	With nitrite	Without nitrite	With nitrite
Fresh	0	0[d]	13±4	13±4	1.00	1.00
		1.23	13±4	13±4	1.00	1.00
		6.17	15±4	19±8	1.15	1.46
		12.34	15±3	21±5	1.15	1.62
		24.67	14±3	24±3	1.08	1.85
Pasteurized	0	0[d]	13±4	13±4	1.00	1.00
		1.23	15±2	17±3	1.15	1.31
		6.17	14±3	18±4	1.08	1.38
		12.34	16±4	19±4	1.23	1.46
		24.67	17±4	27±1	1.31	**2.08**
	7	0[d]	30±7	30±7	1.00	1.00
		1.23	32±4	38±8	1.07	1.27
		6.17	31±4	45±13	1.03	1.50
		12.34	31±5	39±5	1.03	1.30
		24.67	29±7	52±9	0.97	1.73
Sterilized	0	0[d]	13±4	13±4	1.00	1.00
		1.23	12±3	21±3	0.92	1.62
		6.17	13±3	24±5	1.00	1.85
		12.34	16±4	23±4	1.23	1.77
		24.67	14±6	35±5	1.08	**2.69**
	30	0[d]	15±2	15±2	1.00	1.00
		1.23	11±5	19±2	0.73	1.27
		6.17	14±3	21±3	0.93	1.40
		12.34	11±3	27±11	0.73	1.80
		24.67	14±5	42±5	0.93	**2.80**
	90	0[d]	17±7	17±5	1.00	1.00
		1.23	16±8	12±3	0.94	0.71
		6.17	16±7	14±3	0.94	0.82
		12.34	17±8	39±31	1.00	**2.29**
		24.67	17±7	37±9	1.00	**2.18**

[a] Amount per plate of pomegranate arils.

[b] Data are expressed as mean ± SD of triplicate plates from two independent experiments (N=6)

[c] Mutagenicity Index (MI) is calculated from the average value of a number of histidine revertants/plate of the pomegranate juices divided by that of spontaneous revertants.

[d] Spontaneous revertants.

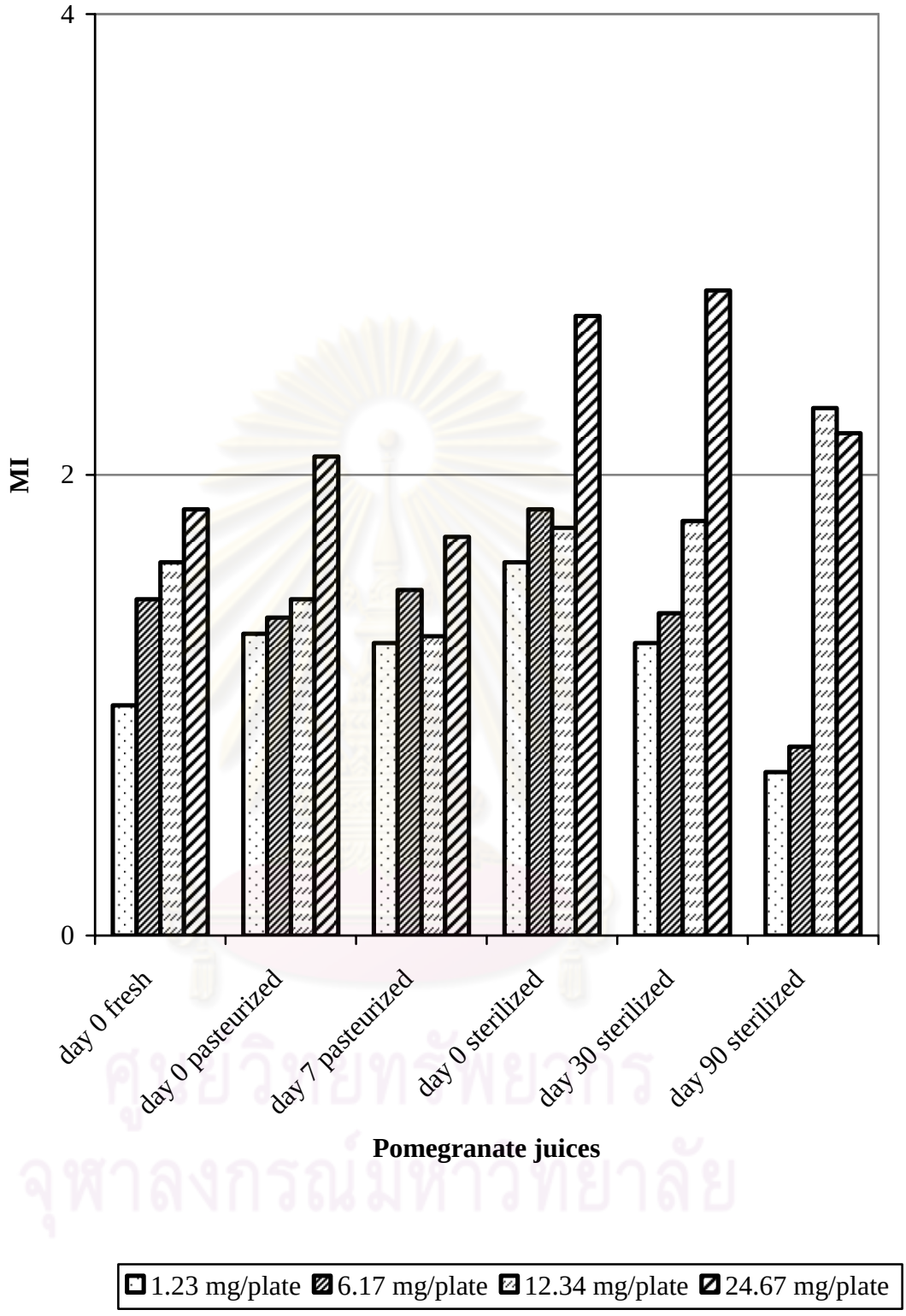

Figure 8. Mutagenicity Index (MI) of pomegranate juices with nitrite treatment on *S. typhimurium* TA98 under acid condition without metabolic activation using Ames test.

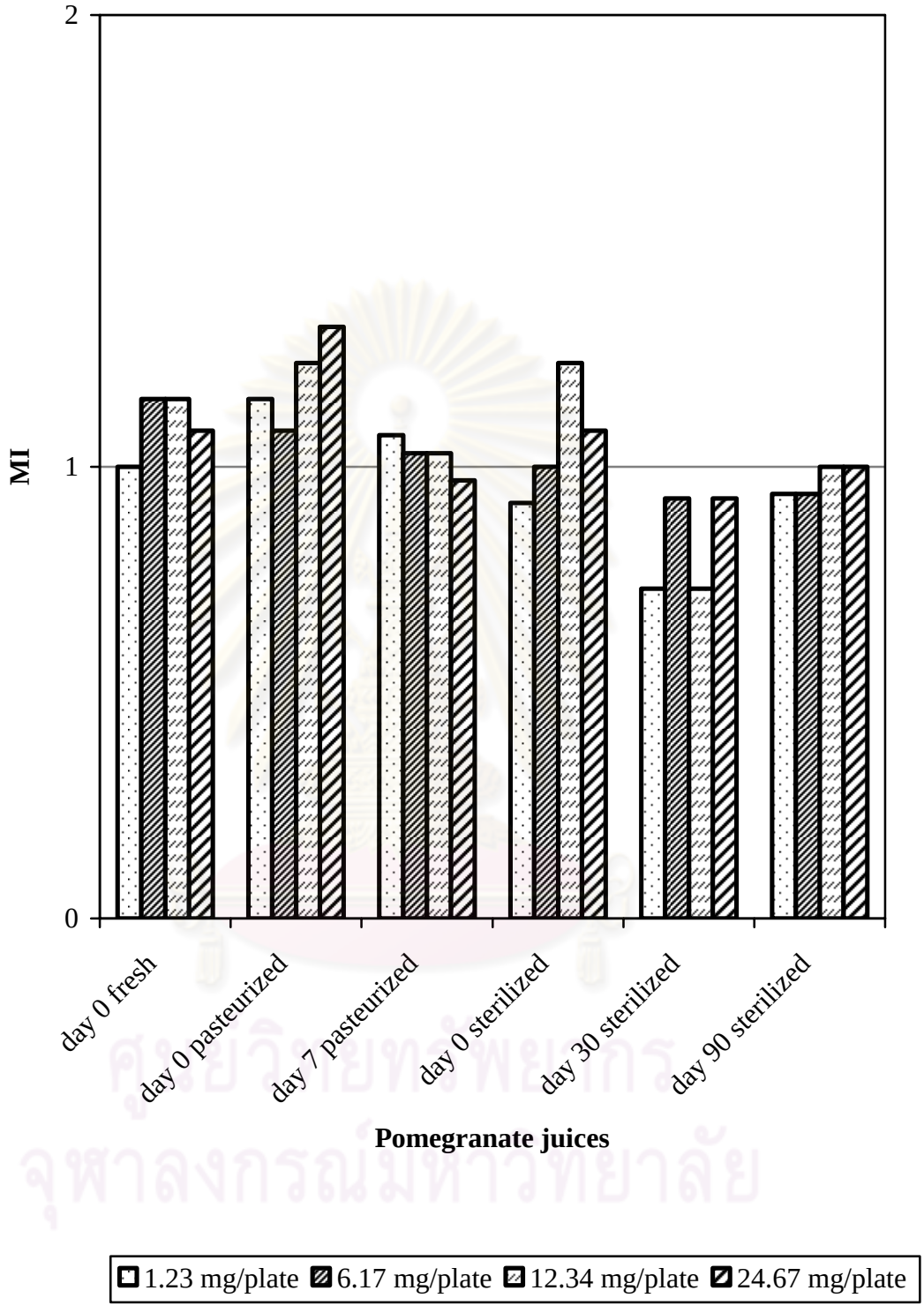

Figure 9. Mutagenicity Index (MI) of the pomegranate juices without nitrite treatment on *S. typhimurium* TA98 under acid condition without metabolic activation using Ames test.

Table 6. Mutagenicity of the pomegranate juices with and without nitrite treatment on *S. typhimurium* TA100 under acid condition (pH3-3.5) without metabolic activation

Juices	Storage duration (days)	Amount[a] (mg/plate)	No. of revertants/plate[b]		Mutagenicity Index[c]	
			Without nitrite	With nitrite	Without nitrite	With nitrite
Fresh	0	0[d]	124±7	124±7	1.00	1.00
		1.23	117±18	115±14	0.94	0.93
		6.17	106±6	122±12	0.85	0.98
		12.34	107±13	135±20	0.86	1.09
		24.67	98±15	158±9	0.79	1.27
Pasteurized	0	0[d]	124±7	124±7	1.00	1.00
		1.23	102±16	125±17	0.82	1.01
		6.17	108±17	124±6	0.87	1.00
		12.34	95±14	127±13	0.77	1.02
		24.67	105±12	155±19	0.85	1.25
	7	0[d]	121±13	121±13	1.00	1.00
		1.23	112±4	110±18	0.93	0.91
		6.17	121±12	133±30	1.00	1.10
		12.34	114±19	137±10	0.94	1.13
		24.67	125±23	161±20	1.03	1.33
Sterilized	0	0[d]	124±7	124±7	1.00	1.00
		1.23	102±13	134±33	0.82	1.08
		6.17	103±19	142±13	0.83	1.15
		12.34	105±10	160±9	0.85	1.29
		24.67	99±10	194±8	0.80	1.56
	30	0[d]	142±8	142±8	1.00	1.00
		1.23	131±18	160±21	0.92	1.13
		6.17	126±20	187±15	0.89	1.32
		12.34	130±26	195±18	0.92	1.37
		24.67	158±19	223±37	1.11	1.37
	90	0[d]	110±7	110±7	1.00	1.00
		1.23	119±22	131±18	1.08	1.19
		6.17	100±8	150±10	0.91	1.36
		12.34	116±21	165±17	1.05	1.50
		24.67	132±19	224±37	1.20	**2.04**

[a] Amount per plate of pomegranate.

[b] Data are expressed as mean ± SD of triplicate plates from two independent experiments (N=6)

[c] Mutagenicity Index (MI) is calculated from the average value of a number of histidine revertants/plate of the pomegranate juices divided by that of spontaneous revertants.

[d] Spontaneous revertants.

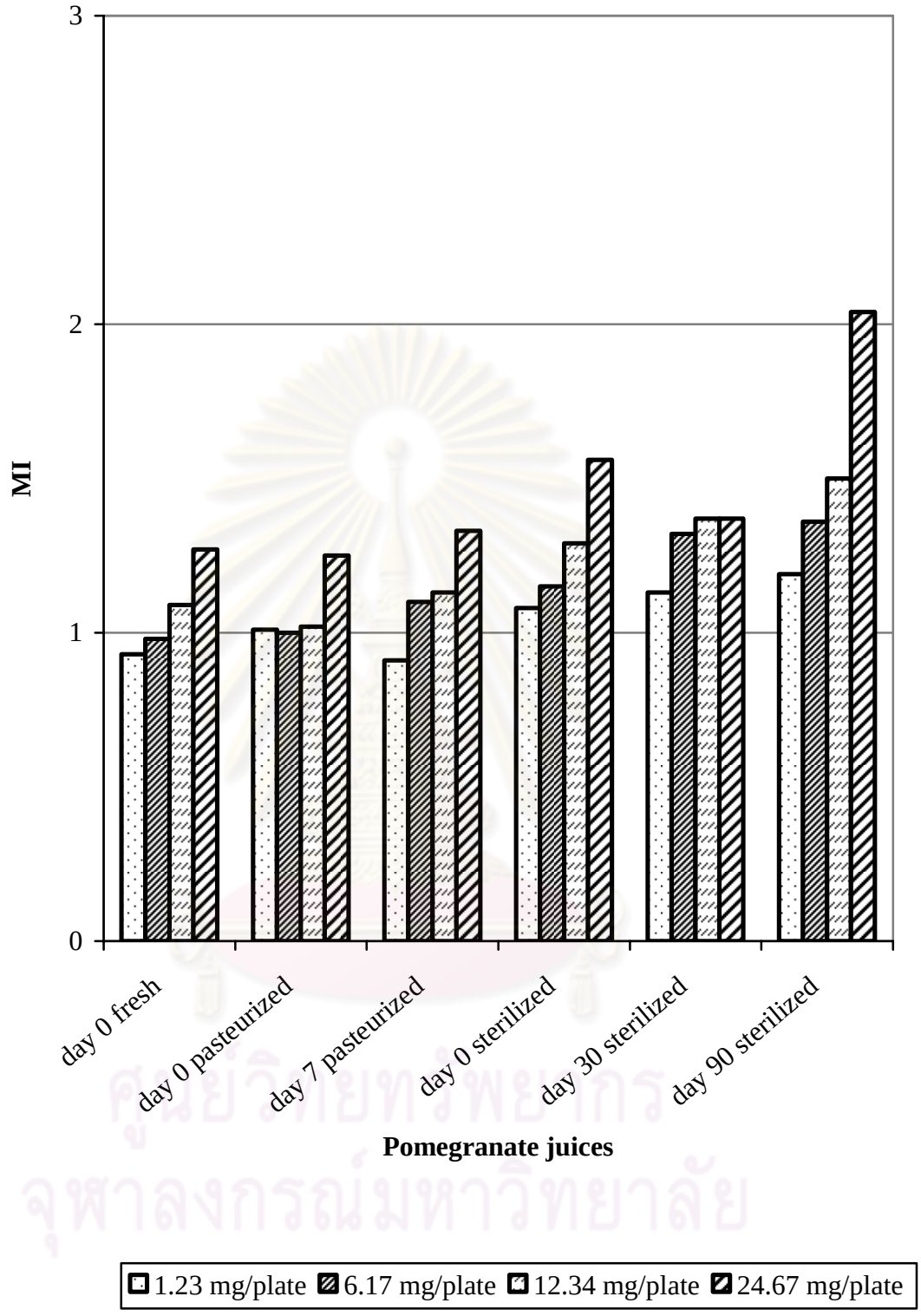

Figure 10. Mutagenicity Index (MI) of the pomegranate juices with nitrite treatment on *S. typhimurium* TA100 under acid condition without metabolic activation using Ames test.

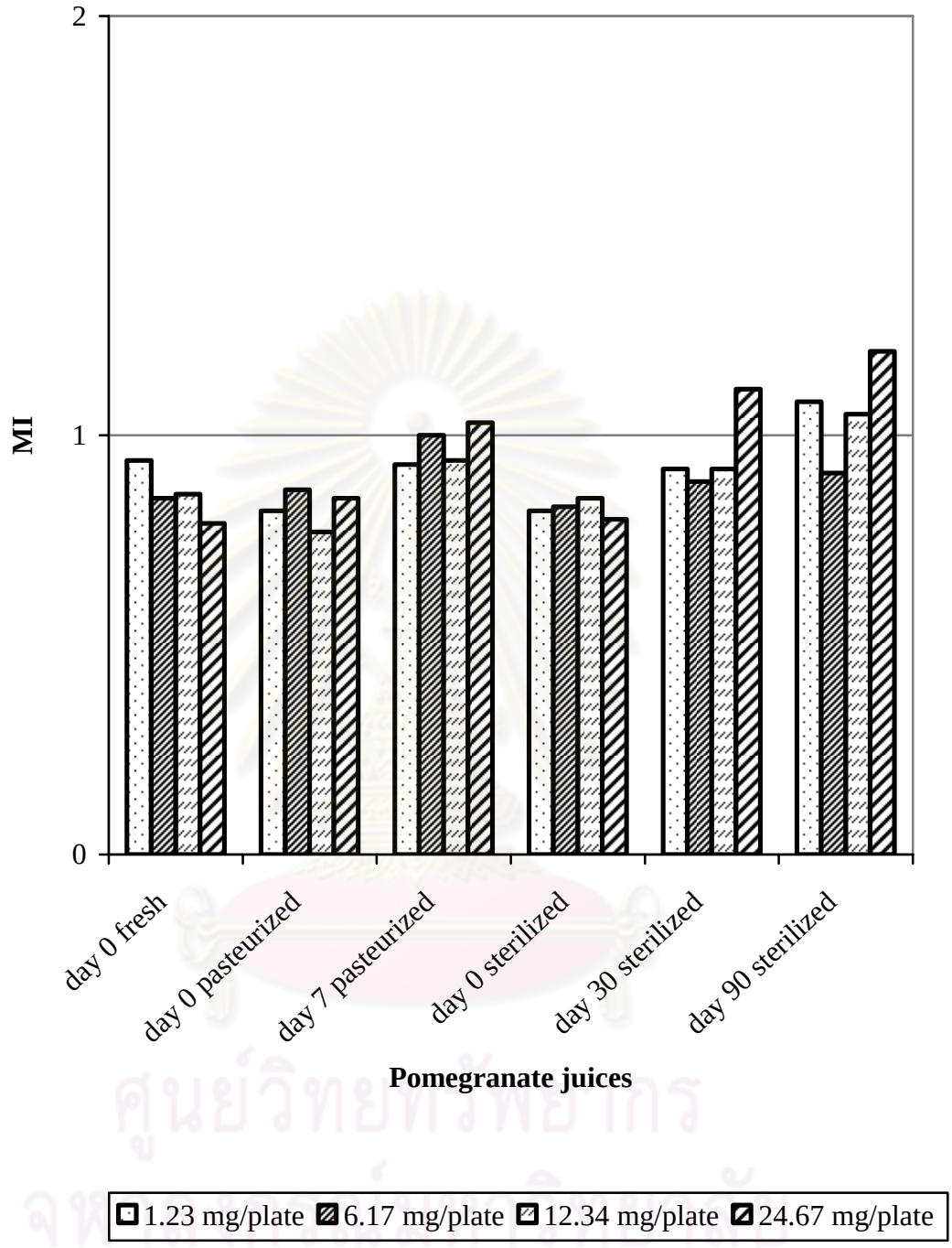

Figure 11. Mutagenicity Index (MI) of the pomegranate juices without nitrite treatment on *S. typhimurium* TA100 under acid condition without metabolic activation using Ames test.

Table 7. Effect of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract on *S. typhimurium* TA98 under acid condition (pH 3 - 3.5) and without metabolic activation.

Pomegranate juices	Storage duration (days)	Amount[a] (mg/plate)	Nitrite treated 1-AP			Nitrite treated chicken extract		
			Number of His+ revertants/plate[b]	%inhibition	%enhancement	Number of His+ revertants/plate[b]	%inhibition	%enhancement
Fresh		spontaneous	18±6			18±6		
		0[c]	423±66			108±29		
	0	38.55	776±175		-87	120±33		-13
		77.10	557±84		-33	105±40	+3	
		154.20	559±133		-34	103±23	+5	
Pasteurized		spontaneous	18±6			18±6		
		0[c]	423±66			108±29		
	0	38.55	670±85		-61	109±29		-1
		77.10	473±107		-24	88±22	+22	
		154.20	495±84		-18	108±46	0	0

Table 7 (continued). Effect of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract on *S. typhimurium* TA98 under acid condition (pH 3 - 3.5) and without metabolic activation.

Pomegranate juices	Storage duration (days)	Amount[a] (mg/plate)	Nitrite treated 1-AP			Nitrite treated chicken extract		
			Number of His+ revertants/plate[b]	%inhibition	%enhancement	Number of His+ revertants/plate[b]	%inhibition	%enhancement
Pasteurized (continued)		spontaneous	18±5			18±5		
		0[c]	364±27			125±24		
	7	38.55	525±56		-47	105±30	+19	
		77.10	424±27		-17	144±33		-18
		154.20	397±40		-10	124±22	+1	
Sterilized		spontaneous	18±6			18±6		
		0[c]	423±66			108±29		
	0	38.55	517±112		-23	103±32	+5	
		77.10	473±107		-12	131±16		-25
		154.20	359±46	+16		124±22		-17

Table 7 (continued). Modification effect of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract on *S. typhimurium* TA98 under acid condition (pH 3 - 3.5) and without metabolic activation.

Pomegranate juices	Storage duration (days)	Amount[a] (mg/plate)	Nitrite treated 1-AP			Nitrite treated chicken extract		
			Number of His+ revertants/plate[b]	%inhibition	%enhancement	Number of His+ revertants/plate[b]	%inhibition	%enhancement
Sterilized (continued)		spontaneous	23±6			23±6		
		0[c]	500±45			126±16		
	30	38.55	565±68		-29	119±22	+7	
		77.10	530±9		-15	135±15		-9
		154.20	462±4	+3		135±12		-9
		spontaneous	17±5			17±5		
		0[c]	586±50			144±16		
	90	38.55	688±52		-18	128±20	+13	
		77.10	584±44	0		128±33	+13	
		154.20	489±36	+17		137±20	+6	

[a] Amount per plate of pomegranate arils (mg/plate).

[b] Revertants/plate represents the mean of values of duplicate plates from two independent experiments± S.D.; spontaneous revertants have been already subtracted from the raw data.

[c] No extract was added to the standard mutagens, nitrite treated 1-aminopyrene or nitrite treated chicken extract.

- or + indicates that the extract increased or decreased the mutagenicity of the model, respectively.

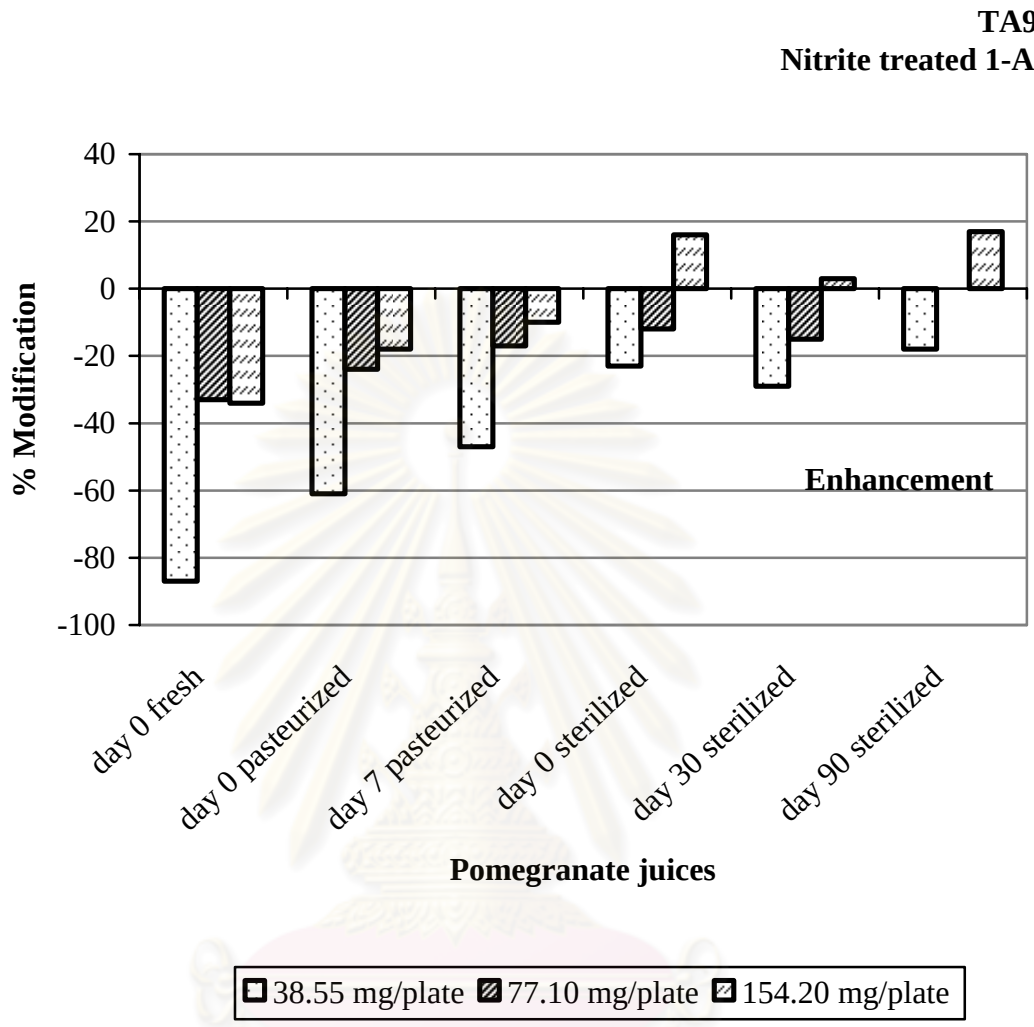

Figure 12. Effect of the pomegranate juices on the mutagenicity of nitrite treated 1-aminopyrene toward *S. typhimurium* TA98 under acid condition without metabolic activation using Ames test.

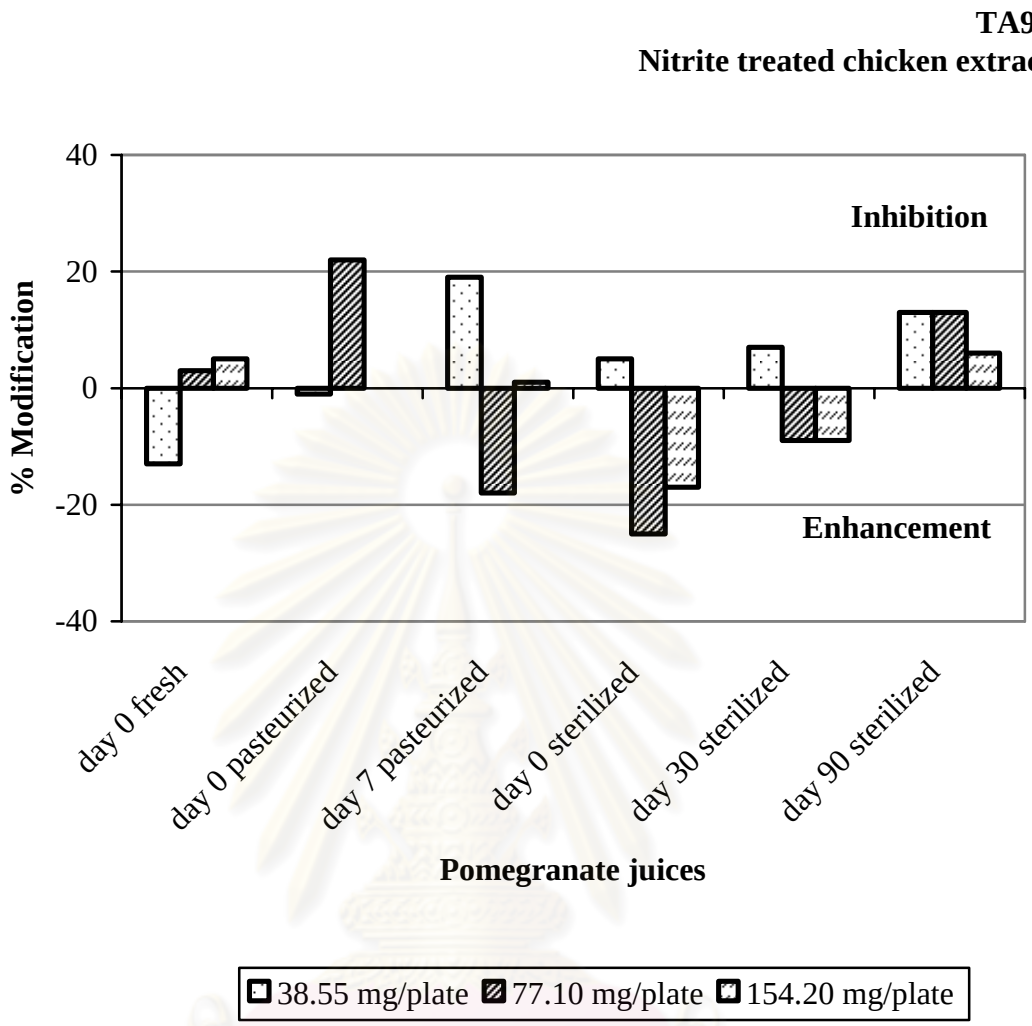

Figure 13. Effect of the pomegranate juices on the mutagenicity of nitrite treated chicken extract toward *S. typhimurium* TA98 under acid condition without metabolic activation using Ames test.

Table 8. Effect of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract on *Salmonella typhimurium* TA100 under acid condition (pH 3 - 3.5) and without metabolic activation.

Pomegranate juices	Storage duration (days)	Amount[a] (mg/plate)	Nitrite treated 1-AP			Nitrite treated chicken extract		
			Number of His+ revertants/plate[b]	%inhibition	%enhancement	Number of His+ revertants/plate[b]	%inhibition	%enhancement
Fresh	0	spontaneous	80±14			80±14		
		0[c]	366±24			420±98		
		38.55	445±50		-28	508±129		-26
		77.10	379±62		-5	447±130		-8
		154.20	369±49		-1	467±99		-14
Pasteurized	0	spontaneous	80±14			80±14		
		0[c]	366±24			420±98		
		38.55	411±40		-16	599±46		-53
		77.10	355±28	+4		613±63		-57
		154.20	311±48	+19		577±17		-46

Table 8 (continued). Effect of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract on *Salmonella typhimurium* TA100 under acid condition (pH 3 - 3.5) and without metabolic activation.

Pomegranate juices	Storage duration (days)	Amount[a] (mg/plate)	Nitrite treated 1-AP			Nitrite treated chicken extract		
			Number of His+ revertants/plate[b]	%inhibition	%enhancement	Number of His+ revertants/plate[b]	%inhibition	%enhancement
Pasteurized (continued)	7	spontaneous	111±9			111±9		
		0[c]	232±12			492±49		
		38.55	260±36		-23	559±25		-18
		77.10	262±31		-25	483±103	+2	
		154.20	209±33	+19		444±129	+13	
Sterilized	0	spontaneous	80±14			80±14		
		0[c]	366±24			420±98		
		38.55	362±27	+1		542±102		-36
		77.10	305±20	+21		602±59		-54
		154.20	255±37	+39		592±142		-51

Table 8 (continued). Effect of the pomegranate juice on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract on *Salmonella typhimurium* TA100 under acid condition (pH 3 - 3.5) and without metabolic activation.

Pomegranate juices	Storage duration (days)	Amount[a] (mg/plate)	Nitrite treated 1-AP			Nitrite treated chicken extract		
			Number of His+ revertants/plate[b]	%inhibition	%enhancement	Number of His+ revertants/plate[b]	%inhibition	%enhancement
Sterilized (continued)	30	spontaneous	142±8			142±8		
		0[c]	516±61			739±33		
		38.55	510±59	+1		733±18	+1	
		77.10	451±41	+17		736±67	+1	
		154.20	400±40	+31		751±98		-2
	90	spontaneous	110±7			110±7		
		0[c]	540±54			613±78		
		38.55	576±71		-8	717±98		-21
		77.10	501±85	+9		703±151		-18
		154.20	393±46	+34		735±165		-25

[a] Amount per plate of pomegranate arils (mg/plate).

[b] Revertants/plate represent the mean of values of duplicate plates from two independent experiments± S.D.; spontaneous revertants have been already subtracted from the raw data.

[c] No extract was added to the standard mutagens, nitrite treated 1-aminopyrene or nitrite treated chicken extract.

- or + indicates that the extract increased or decreased the mutagenicity of the model, respectively

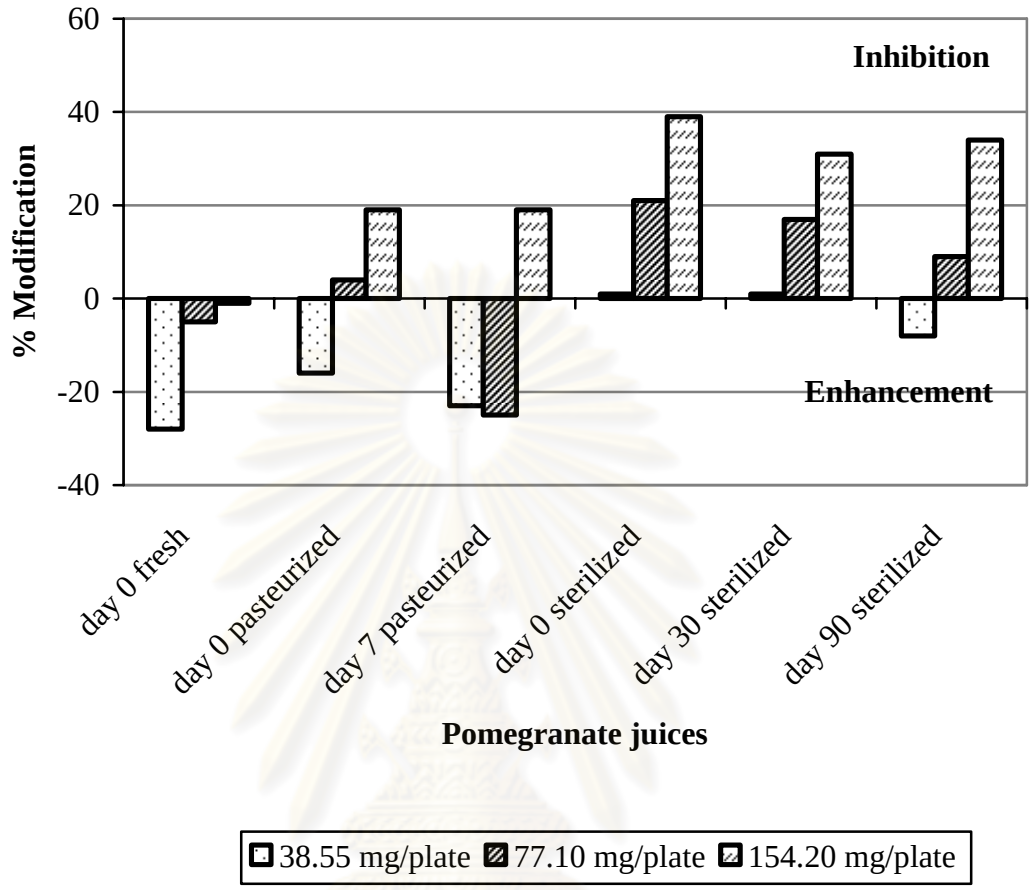

Figure 14. Effect of the pomegranate juices on the mutagenicity of nitrite treated 1-aminopyrene toward *S. typhimurium* TA100 under acid condition without metabolic activation using Ames test.

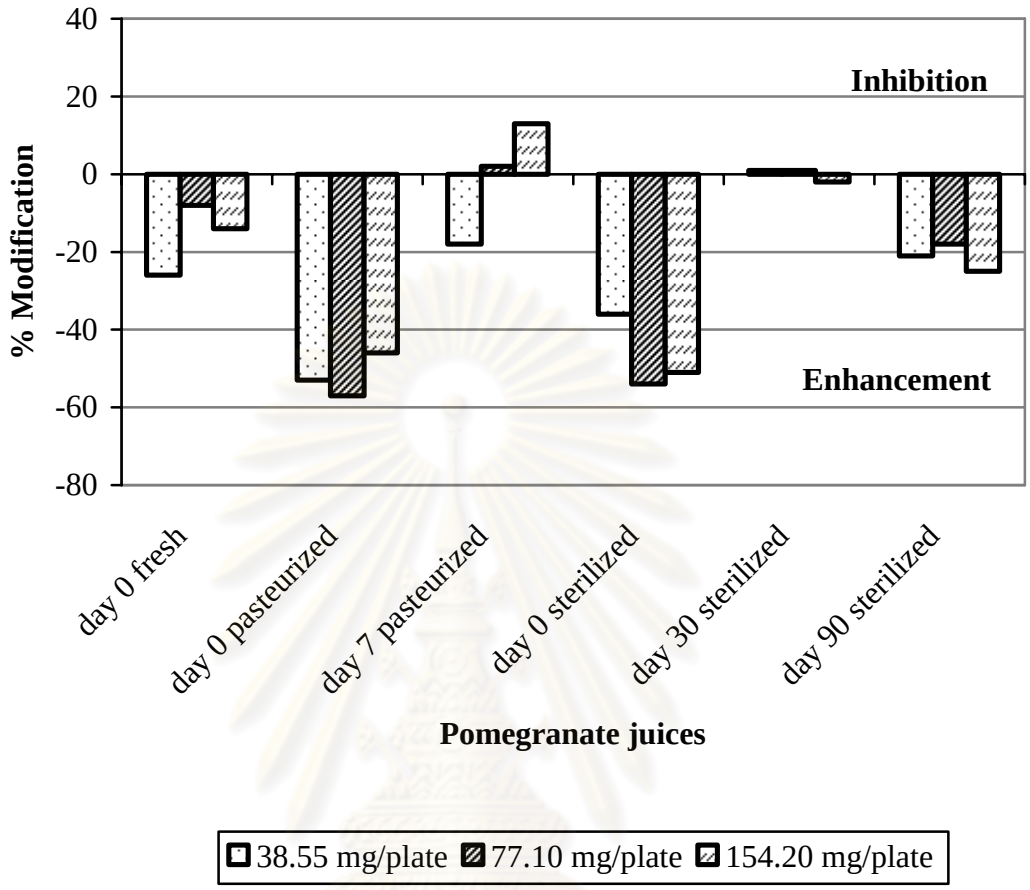

Figure 15. Effect of the pomegranate juices on the mutagenicity of nitrite treated chicken extract toward *S. typhimurium* TA100 under acid condition without metabolic activation using Ames test.

Table 9. Comparative summaries of effects of the pomegranate juices against nitrite treated 1-aminopyrene and nitrite treated chicken extract without metabolic activation**.**

Pomegranate Juices	Storage duration (days)	TA98		TA100	
		Nitrite-treated 1-AP	Nitrite-treated chicken extract	Nitrite-treated 1-AP	Nitrite-treated chicken extract
Fresh juice	0	Enh. -33 to -87 % (ALL)	Inh. +3, +5 % (MED, HI)	Enh. -1 to -28 % (ALL)	Enh. -8 to -26 % (ALL)
Pasteurized juice	0	Enh. -18 to 61 % (ALL)	Inh. 22 % (MED)	Inh. 4, 19 % (MED, HI)	Enh. -46 to -53 % (ALL)
	7	Enh. -10 to -47 % (ALL)	Inh. 19 , 1 % (LOW, HI)	Inh. 19 % (HI)	Inh. 13 % (HI)
Sterilized juice	0	Inh. +16 % (HI)	Enh. -25,-17 % (MED, HI)	Inh. 39 % (HI)	Enh. -36 to -54 % (ALL)
	30	Inh. +3 % (HI)	Enh. -9, -9 % (MED, HI)	Inh. 31 % (HI)	Enh. - 2 % (HI)
	90	Inh. -+17 % (HI)	Inh. 6 to 13 % (ALL)	Inh. 34 % (HI)	Enh. -18 to -25 % (ALL)

Enh. and Inh. referred to Enhancement and Inhibition, respectively.

ALL referred to all doses. LOW, MED and HI referred to low (38.55 mg/plate), medium (77.10 mg/plate) and high dose (154.20 mg/plate), respectively

CHAPTER V

DISCUSSIONS

5.1 Mutagenicity of Pomegranate Juices

Before mutagenic modification test, the mutagenicity of pomegranate juices was evaluated on TA98 and TA100. The results show that all pomegranate juices were not mutagenic before nitrite treatment. Being treated with nitrite, pasteurized juice kept for 0 day and sterilized juice kept for 0, 30 and 90 days exhibited slight mutagenicity on TA98; only sterilized juice kept for 90 days exhibited slight mutagenicity on TA100. It suggests that the juice might contain some compounds occurring during sterilization (121°C, 20 min) and/or during storage that can interact with nitrite and show their mutagenicity. Shinohara *et al*., (1980) and Powrie *et al*., (1986) reported the maillard products occurring during heating amino acid with sugars at 100ºC for 10 hours presented their mutagenicity on *Salmonella typhimurium* strains TA98 and TA100.

5.2 Mutagenicity Modifying Activity of Pomegranate juice

The study on the mutagenicity modifying activity of fresh, pasteurized and sterilized juices was carried out with *S. typhimurium* TA 98 and TA 100 in the Ames test against 2 mutagens (nitrite treated 1-aminopyrene and nitrite treated chicken extract) in simultaneous gastric condition. The first mutagen, 1-aminopyrene treated with nitrite at pH 3.0 and 37°C showed its mutagenicity toward *S. typhimurium* TA98 and TA100 without metabolic activation (Kato *et al*., 1991; Kangsadalampai *et al*., 1996).

Kato et al. (1991) identified that one of the product after nitrite treatment of 1-aminopyrene was 1-nitropyrene. It is the predominant nitropolycyclic aromatic hydrocarbon emitted in diesel exhaust, exhaust of kerosene heaters and petroleum gas burners. It is also released during barbecuing some foods as a result of incomplete combustion or pyrolysis of fatty meat produced pyrene and NO_2 from burning of cooking gas (Rosenkranz and Mermelstein, 1983; Honda *et al*., 1983; Tokiwa *et a*l., 1985; Kinuchi *et a*l., 1986; Edenharder *et al*., 1993). Thus, the finding that its mutagenicity was either enhanced or reduced was important for consumer health.

The second mutagen namely, nitrite treated chicken extract, represents nitro derivatives of IQ- type heterocyclic amines (HAs). The IQ- type heterocyclic amines (HAs) were formed at the normal cooking temperature of 100 – 225°C while heating (i.e. cooking, broiling, frying, grilling, smoking) foodstuffs in the presence of creatinine, amino acids and sugars, involving Maillard reaction (Ristic *et al*., 2004). The chicken extract treated with nitrite at pH 3.0 and 37°C for 4 h could demonstrate its mutagenicity towards strains TA98 and TA100 in the absence of metabolic activation. The amino group is converted to a nitro group with 50 mM sodium nitrite under acid conditions (Sasagawa *et al*., 1988). Thus, the product formed during the reaction of chicken extract and sodium nitrite in the gastric-liked condition, several heterocyclic amine compounds were found and exhibited direct-acting mutagenic towards both strains TA98 and TA100 (Kangsadalampai and Peerawong, 1998).

The major contribution to the total antioxidant capacity of pomegranate juice is attributed to punicalagin originating from the peels (Gil *et al.*, 2000; Tzulker *et al.*, 2007). However, it would not be expected large amount of bioactive compounds from the peels because only arils were used in this study. Therefore, pomegranate juice may not contain enough antioxidants to inhibit the mutagenicity of positive mutagens in this study. All pomegranate juices exhibited their effect with a biphasic-dose response curve. They were co-mutagenic on both mutagens with the lower dose but the enhancing effect decreased with the higher dose of the juices. Stavric *et al*. (1996) firstly stated this phenomenon. They found the significant stimulation of PhIP, Trp-P-1 and Trp-P-2 mutagenicity by the lower dose of tea. It was postulated that sufficient amounts of potentiating factors in diluted tea appeared to enhance the mutagenic activity of some heterocyclic amine while with the higher concentrations that might contain more inhibitory factors could eliminate the potentiating phenomenon. Ikken et al., (1998) also observed a similar trend in a study with aqueous extracts of fruit and vegetable against N-nitrosamines.

The pomegranate juice might require metabolic activation to present antimutagenic activity. Similarly, the plant extracts of *Peltastes peltatus*, *Maytenus ilicifolia*, *Vitex montevidensis* Cham., *Gochnatia cordata* and *Gochnatia polymorpha* rich in compounds of the flavonoids and tannins excibited direct co-mutagenic and cytotoxic effect on the mutagenicity of 4-oxide-1-nitroquinoline without metabolic activation. On the other hand, they exhibited inhibition effect on the mutagenicity of

2-aminofluorene (2AF) with metabolic activation (S9 mix) (Horn and Vargas, 2003; 2008).

Fresh pomegranate juice was co-mutagenic on both mutagens with the lower dose but the enhancing effect decreased with the higher dose of the juices, while heat processed pomegranate juices; pasteurized and sterilized juices, exhibited antimutagenic effect against nitrite treated 1-aminopyrene on both strains and this effect still remained after specific storing period. They showed no trend on the mutagenicity of nitrite treated chicken extract toward TA98. It was possibly due to the low number of His+ revertants induced by this mutagen, so the effect cannot be seen clearly. However, pasteurized and sterilized juices presented higher co-mutagenic effect with nitrite treated chicken extract on TA100 than the fresh one. This effect was not stable. It decreased when the juices were stored. The present result indicated that the heat processing may alter the effect of the pomegranate juices on the mutagens both inhibition and enhancement effect. It was not taught to be the effect of natural substances such as polyphenolic compounds in pomegranate juice due to the heat stability of them. Although, they found in pomegranate juice and were previously described as antimutagens and co-mutagens (Kada *et al*., 1993; Edenharder *et al*., 1993). Since the mutagenicity of sterilized pomegranate juices were increased after treated with nitrite and the nitrite counteracting by ammonium sulfamate on the nitrosation of the mutagen may not be complete, so the mutagenicity of pro-mutagens in sterilized juice interacted with residual nitrite would be take place. The synergistic effect on the mutagenicity of nitrite treated chicken extract and nitrite treated pro-mutagens in sterilized pomegranate juices should be developed toward TA100. However, sterilized juices at high dose show antimutagenic activity against nitrite treated 1-aminopyrene on the same strain. It was suggested that some antimutagens also occurred during heat processing in pomegranate juices. The maillard products were thought to be the active substances. The maillard products are the products from a reaction between carbonyl compounds and amino compounds, sugar degradation at high temperature (caramelization) and ascorbic acid degradation. Since pomegranate juices contained both carbonyl compounds and amino compounds, this reaction can occur between heating. Shinohara *et al*., (1980) and Powrie *et al*., (1986) reported the maillard products occurring during heating amino acid with sugars at 100ºC for 10 hours presented their mutagenicity on *Salmonella typhimurium* strains TA98 and

TA100. However, Chan *et al*. (1982) used the Ames/*Salmonella* assay to determine the antimutagenic effect of heated lysine-fructose solution (121ºC for 1 h) and caramelized sucrose (180ºC for 1.5 h). They had inhibitory effects on the mutagenicity of aflatoxin B_1 (AFB_1) and *N*-methyl-*N'*- nitro-*N*-nitroso-guanidine (MNNG) when the *Salmonella* organisms (TA98 and TA1535, respectively) were exposed to each mutagen in solution with a browning reaction system. Yen *et al*. (1992) study were also found that the maillard products obtained from xylose and lysine showed a strong antimutagenic effect against the mutagenicity of 2-amino-3-methylimidazole[4,5-*f*] quinoline (IQ), 2 – amino - 6- methyldipyrido [1, 2- *a*: 3', 2'-*d*] imidazole (Glu-P1), and 3 – amino -1, 4 – dimethyl- 5H- pyrido [4, 3- *b*] indole (Trp-P1) toward S. typhimurium TA98 and the antimutagenic effect of maillard products was also correlated with their antioxidative activity and reducing power. Therefore, this suggestion is required to be proved.

Nitro-polycyclic aromatic hydrocarbons and nitro-heterocyclic amines were the possible products of nitrosation of 1-aminopyrene or chicken extract required bacterial intracellular metabolic conversion to express their mutagenesis toward bacterial DNA, respectively (Kappers *et al*, 2000; Sriyapai, 2003). The mechanism of inhibition of genotoxicity by pomegranate juices may be due to their inhibitory effect on metabolic enzyme, i.e., nitroreductase and/or their ability to induce detoxification enzymes (Kuo *et al*., 1992). Pomegranate juices might reduce mutagenicity of these mutagens by inhibition of bacterial nitroreductase(s) and/or *O*-transferases since the nitro compounds are thought to require reduction of the nitro group to a hydroxyamine intermediate and esterification of the OH function by several transferases, especially the *O*-acetyltransferase, to metabolites that spontaneously decompose to an arylnitrenium ion reactive with DNA to initiate mutagenesis as shown in Figure 1 (Kappers *et al*., 2000). However, some compounds in pomegranate juices may promote activity of enzymatic systems and modified the permeability of bacterial membranes or by some extracellular physical and chemical interactions between flavonoids and mutagens (Edenharder and Tang, 1997) responsible for increasing mutagenicity. Further studies on these suggestions are required.

CHAPTER VI

CONCLUSION

The present result showed that all pomegranate juices were not mutagenic in the absence of nitrite on both *S. typhimurium* strains TA98 and TA100 in the Ames test. Nitrite treated sterilized juices were slightly mutagenic on both strains, while pasteurized juice was mutagenic on only TA98. It is hypothesized that the mutagenic of sterilized pomegranate juices may be due to maillard products from heating sugars and amino acids in pomegranate juice together.

All pomegranate juices also exhibited their effects with a biphasic-dose response curve on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract. They were co-mutagenic on both mutagens with the lower dose but the enhancing effect decreased with the higher dose of the juices. It was postulated that sufficient amounts of potentiating factors in pomegranate juices appeared to enhance the mutagenic activity of some mutagens while with the higher concentrations that might contain more inhibitory factors could eliminate the potentiating phenomenon.

Pasteurized and sterilized juices on the preparation day also exhibited higher enhancing effect against nitrite treated chicken extract on TA100 than the fresh one. On the other hand, they also exhibited higher inhibitory effect against nitrite treated 1-aminopyrene on the same strains. It was suggested that some co-mutagens and anti-mutagens occurred during heat processing in pomegranate juice together.

It should be considered that pomegranate juices are a mixture of substances with different characteristics and biological activities, whose action may modify by heat processing and storing period. Properties such as potentiation, synergism and antagonism of chemicals, interactions of a mutagenic and antimutagenic nature may be involved resulting in an activity with a specific response pattern. Avoidance of consumption pomegranate juices together with nitrite containing products and promutagens (PAHs and HAs) should done.

Limitation of this finding was bacterial cell (*in vitro*) and mammalian difference. For further studies, animal model evaluation and study of separated parts of pomegranate study were necessary for antimutagenicity or toxicity indicating.

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APPENDIX

APPENDIX

1. Preparation of stock solutions and media (Maron and Ames, 1983)

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

Ingredient	500 ml
Warm distilled water (45 °C)	336 ml
Magnesium sulfate ($MgSO_4.7H_2O$)	5 g
Citric acid monohydrate	50 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	250 g
Sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$)	87.5 g

Add salts in the order indicated to warm water in beaker placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume and filter the solutions into the glass bottles with screw caps and then autoclave at 121°C for 20 min.

1.2 Minimal glucose agar plate

Ingredient	300 ml
Agar-agar	4.5 g
Distilled H_2O	279 ml
VB salts	6 ml
40% glucose	15 ml

Agar was added to distilled water in a glass bottle and then autoclaved at 121°C for 20 min. When the solution has cooled slightly, sterile VB salts and sterile 40% glucose were added, mixed and poured 30 ml into each sterile petri plate. Minimal glucose agar plates were kept in incubator at 37°C before using.

1.3 Oxoid nutrient broth No.2

2.5 g of nutrient broth No.2 was dissolved in 100 ml distilled H_2O and 12 ml of nutrient broth was transferred into each flask (covered with sterile gauze). They were autoclave at 121°C for 20 min.

1.4 1 M L-histidine HCl stock

Ingredient	10 ml
L-histidine HCl	209.6 mg
Distilled water	10 ml

Dissolve L-histidine HCl (MW 209.63) in distilled water. Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water.

1.5 1 mM biotin stock

Ingredient	100 ml
Biotin	24.43 mg
Distilled water	100 ml

Dissolve biotin (MW 244.3) in distilled water. Warm it until dissolve completely. Autoclave at 121°C for 20 min.

1.6 0.5 mM L-histidine/biotin solution

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

Mix and autoclave at 121°C for 20 min.

1.7 Top agar

Ingredient	100 ml
Agar-agar	0.6 g
Sodium chloride	0.5 g
Distilled water	100 ml

Dissolve ingredients in distilled water. Store in a glass bottle. Autoclave at 121°C for 20 min and then add 0.5 mM L-histidine/biotin solution (10 ml for 100 ml of Top agar).

1.8 $NaPO_4$-KCl buffer

Ingredient	330 ml
0.5 M $NaPO_4$ pH 7.4	100 ml
1 M KCl	16.5 ml
Distilled water	213.5 ml

The ingredients were mixed and autoclave at 121°C for 20 min.

1.9 1 M potassium chloride

Ingredient	1000 ml
Potassium chloride	74.56 g
Distilled water	1000 ml

Mix and autoclave at 121°C for 20 min.

1.10 8 mg/ml ampicillin solution

Ingredient	10 ml
Ampicillin (sodium)	800 mg
Distilled water	10 ml

Ampicillin was dissolved into water and stored in glass bottle with screw cap at 0°C.

1.11 2 M sodium nitrite

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

Mix and autoclave at 121°C for 20 min.

1.12 2 M ammonium sulfamate

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

Mix and autoclave at 121°C for 20 min.

2. Procedure for Re-isolation and Growing Culture

Tester strains, TA 98 and TA 100 were grown in Oxoid nutrient broth No.2 and incubated overnight in a 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 spreaded with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1 M histidine HCl and 0.1 ml of 1 mM biotin. These plates were incubated at 37°C for 48 hours. After incubation, the 4 single colonies per strain of TA98 and TA100 were picked up and grown in Oxoid nutrient broth No.2 and shaken overnight at 37°C in shaking water bath. Each culture was confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1 ml of culture, 0.09 ml of spectrophotometric grade DMSO was added. Combine the culture and DMSO in a sterile tube and distribute 200 µl of the culture aseptically into sterile cryotube and store in freezer at -80°C

3. Confirming Genotype of Tester Strains

Broth cultures of TA98 and TA100 were used to confirm genotypes in the following ways.

3.1. Histidine requirement

The his^- character of the strains was 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 1mM 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 1mM biotin

Four minimal glucose agar plates were required for each tester strains. Each of the plates was applied on the surface with 0.1 ml of 1mM biotin, 0.3 ml of 0.1 M his-HCl, 0.3 ml of 0.1 M his-HCl plus 0.1 ml of 1mM biotin and no application (plate b, c, d, a respectively). A single streak of each strain was made across these plates. Four strains could be tested on the same plate. Incubated at 37°C for 48 hours. The growing of bacteria on histidine plus biotin plate was the result of histidine requirement.

3.2. R Factor

The R-factor strains (TA97, TA98, TA100 and TA102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria.

Procedure: For each tester strain (TA98 and TA100), 0.3 ml of fresh overnight culture was added to a tube containing 0.1 ml of 0.1 M histidine-HCl. And then 2 ml of molten top agar containing 0.5 mM histidine-HCl and 0.5 mM biotin were added, mixed and poured on a minimal agar plate. The plate was rotated in order to distribute the mixures 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 parts, one for R-factor and the other for rfa mutation. For R-factor, filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc was pressed lightly to embed in the overlay.

The plates were incubated at 37°C for 24 hours. The absence of the clear zones of inhibition around the disc indicates resistance to ampicillin.

3.3. rfa mutation

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

Procedure: 0.1% solution of crystal violet was pipetted to the sterile filter paper disc (1/4 inch) and transfer the disc to plates that seed with bacteria (the procedure was similar to R-factor). Incubated at 37°C for 48 hours. The clear zone appeared around the disc indicated the presence of the rfa mutation (crystal violet transferred into the cell and kill bacteria).

4. 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 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 and 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 minimal glucose agar plate. Plates were rotated and left it to become harden and incubated at 37°C for 48 hours. The his+ revertants colonies that grown on the minimal glucose agar plate were counted.

5. The Response to Standard Mutagen

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 that highly response to positive mutagens will be chosen.

Procedure: 0.02 and 0.04 ml of 0.0375 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 aminopyrene was 0.6 and 1.2 mg respectively, and 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 ($NH_4SO_3NH_4$) was added and standard for 10 min in ice bath. 0.1 ml of each mixture was pipetted to capped culture tube for testing the stock culture TA98 (equal to 0.06 mg of aminopyrene/ plate) and TA100 (equal to 0.12 mg 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 bacterial strain were done.

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