ฤทธิ์ต้านการก่อกลายพันธุ์ของข้าวหอมนิลและข้าวเหนียวดำทดสอบโดยวิธีโซมาติกมิวเตชันและรีคอมบิเนชัน

นางสาวณัฐนันท์ วิปัสสนาธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

ANTIMUTAGENIC ACTIVITY OF HOM NIL RICE AND BLACK GLUTINOUS RICE IN SOMATIC MUTATION AND RECOMBINATION TEST

Miss Nuttanun Vipassanatham

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Food Chemistry and Medical Nutrition
Department of Food and Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
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Thesis Title	ANTIMUTAGENIC ACTIVITY OF HOM NIL RICE AND BLACK GLUTINOUS RICE IN SOMATIC MUTATION AND RECOMBINATION TEST
By	Miss Nuttanun Vipassanatham
Field of Study	Food Chemistry and Medical Nutrition
Thesis Advisor	Assistant Professor Linna Tongyonk, D.Sc.
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ณัฐนันท์ วิปัสสนาธรรม: ฤทธิ์ต้านการก่อกลายพันธุ์ของข้าวหอมนิลและข้าวเหนียวดำทดสอบโดยวิธีโซมาติกมิวเตชันและรีคอมบิเนชัน. (ANTIMUTAGENIC ACTIVITY OF HOM NIL RICE AND BLACK GLUTINOUS RICE IN SOMATIC MUTATION AND RECOMBINATION TEST) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. ลินนา ทองยงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. แก้ว กังสดาลอำไพ, 85 หน้า.

การประเมินฤทธิ์ก่อกลายพันธุ์และฤทธิ์ต้านการก่อกลายพันธุ์ของข้าวหอมนิลและข้าวเหนียวดำในรูปแบบข้าวสาร ข้าวหุงและข้าวหมัก ต่อยูรีเทน และสารจากปฏิกิริยาระหว่างโซเดียมไนไตรทและเมทิลยูเรียทำโดยวิธี somatic mutation and recombination ในแมลงหวี่ *Drosophila melanogaster* โดยนำหนอนแมลงหวี่ trans-heterozygous (*mwh flr*$^{+}$*/mwh TM3*) อายุ 3 วัน ที่ได้จากการผสมพันธุ์ระหว่างแมลงหวี่ตัวเมียสายพันธุ์ *ORR flare hair* และแมลงหวี่ตัวผู้สายพันธุ์ *mwh/mwh* ไปเลี้ยงในอาหารทดลองที่มีการเติมตัวอย่างข้าวแทนที่แป้งข้าวโพด จากนั้นจึงนำปีกของแมลงหวี่ที่รอดชีวิตมาวิเคราะห์การเกิดจุดกลายพันธุ์ พบว่าทุกตัวอย่างข้าวไม่เหนี่ยวนำให้เกิดจุดกลายพันธุ์อย่างมีนัยสำคัญ การศึกษาฤทธิ์ต้านการก่อกลายพันธุ์ของข้าวแต่ละชนิดต่อยูรีเทน (20 mM) แสดงให้เห็นว่าทุกตัวอย่างสามารถลดฤทธิ์การก่อกลายพันธุ์ของยูรีเทนได้ และข้าวหอมนิลหมักมีฤทธิ์ต้านการก่อกลายพันธุ์สูงกว่าข้าวชนิดอื่น โดยอาจเป็นไปได้ว่าฤทธิ์ต้านการกลายพันธุ์ของตัวอย่างข้าวอาจเกิดจากฤทธิ์ต้านอนุมูลอิสระหรือสารประกอบโพลีฟีนอลในข้าวจับสารก่อกลายพันธุ์หรืออนุมูลอิสระที่เกิดขึ้นระหว่างการก่อกลายพันธุ์ นอกจากนี้การศึกษาฤทธิ์ต้านการก่อกลายพันธุ์ของข้าวแต่ละชนิดต่อสารซึ่งเกิดจากปฏิกิริยาระหว่างโซเดียมไนไตรท (36 mM) และเมทิลยูเรีย (10 mM) แบบ *in vivo* แสดงให้เห็นว่าข้าวหอมนิลและข้าวเหนียวดำแสดงผลต้านฤทธิ์การก่อกลายพันธุ์ของสารไนโตรโซเมทิลยูเรียที่เกิดจากปฏิกิริยาระหว่างโซเดียมไนไตรทและเมทิลยูเรีย โดยสารบางอย่างในข้าวอาจลดปริมาณการเกิดสารดังกล่าวได้ ดังนั้นจากผลการศึกษานี้จึงแสดงให้เห็นว่าการบริโภคข้าวหอมนิลและข้าวเหนียวดำนั้นมีความปลอดภัยซึ่งอาจมีประโยชน์ต่อผู้บริโภคที่ห่วงใยสุขภาพได้ ควรมีการศึกษาเพิ่มเติมถึงสารออกฤทธิ์และกลไกการต้านฤทธิ์ก่อกลายพันธุ์ของข้าว

ภาควิชา……อาหารและเภสัชเคมี..... ลายมือชื่อนิสิต……
สาขาวิชา..อาหารเคมีและโภชนศาสตร์ทางการแพทย์..ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก….....
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NUTTANUN VIPASSANATHAM: ANTIMUTAGENIC ACTIVITY OF HOM NIL RICE AND BLACK GLUTINOUS RICE IN SOMATIC MUTATION AND RECOMBINATION TEST. THESIS ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., THESIS CO-ADVISOR : ASSOC. PROF. KAEW KANGSADALAMPAI, Ph.D., 85 pp.

Raw, cooked and fermented Hom Nil rice and black glutinous rice were assessed for their mutagenicity and antimutagenicity against both urethane and *in vivo* formed nitrosomethylurea using somatic mutation and recombination test in *Drosophila melanogaster*. Three-day old, trans-heterozygous (*mwh flr*$^{+}$*/mwh TM3*) larvae obtained by mating virgin *ORR flare hair* females and *mwh/mwh* males were transferred to experimental media that contained each sample substituted for corn flour. The round wings of the surviving adult flies were analyzed for the occurrence of mutant spots. The results indicated that all samples did not significantly induce the frequencies of mutant spots at any testing amounts. The study of antimutagenicity of each sample against urethane (20 mM) showed that all samples reduced the mutagenicity of urethane, and the fermented Hom Nil rice possessed higher antimutagenicity than the other did. The antimutagenicity of each sample might possibly be due to their antioxidants or phenolic compounds which might scavenge the mutagen and/or free radicals occuring during mutagenesis. Furthermore, the study exhibited that Hom Nil and black glutinous rice showed antimutagenic effect against *in vivo* formed nitrosomethylurea, and the fermented black glutinous rice possessed the highest antimutagenicity. It is proposed that some components of rice might reduce the amount of *N*-nitrosomethylurea formed during the reaction between the methylurea and sodium nitrite. Therefore, this study suggests that Hom Nil rice and black glutinous rice are safe for health concerning consumers. Further study should be conducted to investigate the active compound(s) and mechanism of antimutagenic activity of pigmented rice.

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LIST OF ABBREVIATIONS

°C	degree Celsius
g	gram
hr	hour
I.U.	International Units
mg	milligram
mM	millimolar
ml	millilitre
mm	millimetre
M	molar
μg	microgram
μl	microlitre
μM	micromolar
SD	standard deviation
et al.	et alia (and others)

CHAPTER I

INTRODUCTION

1.1 Background and Significance of the Study

An imbalance caused by overabundant production of oxidants leaded to oxidative stress, which could increase the risk of chornic diseases such as cancer. Many studies indicated that phytochemicals in fruits, vegetables and cereal grains including rice were significantly associated with reducing the risk of development of chronic diseases such as cardiovascular disease, type 2 diabetes, and some cancers (Jacob et al., 1998; Mayer et al., 2000; Liu, 2007). These protective effects might be due to the phytochemicals which could contend the oxidative stress by balancing between oxidants and antioxidants in the body (Adom and Liu, 2002). The phytochemicals in grains, such as anthocyanins, exhibited the beneficial effects in reducing the risk of cardiovascular diseases diabetes, arthritis and cancers with its antioxidant and anti-inflammatory (Morse and Stoner, 1993; Prior and Wu, 2006). Numerous studies on the cancer preventative activities of anthocyanin such as the study using *in vitro* cell culture, *in vivo* animal model tumor systems, and human epidemiological studied (Hyun and Chung, 2004; Duthie et al., 2006; Xia et al., 2006). Chen et al. (2005) isolated and identified the two bioactive anthocyanins, peonidin-3-glucoside and cyaniding-3-glucoside, from black rice (*Oryza sativa* L.) could strongly inhibit the growth of cancer cells *in vitro*. In addition, the extracts from pigmented rice could inhibit phorbol ester-induced tumor promotion in marmoset lymphoblastoid cells B95-8 *in vitro* (Nam et al., 2005).

Rice is the staple food in many part of the world's population (Juliano, 1985; Friedman, 1996). Pigmented rice varieties could promote human health due to their antioxidative compounds that have the ability to inhibit the formation or to reduce the concentrations of reactive cell-damaging free radicals (Ichikawa et al., 2001; Oki et al., 2002; Toyokuni et al., 2002; Acquaviva et al., 2003). One of the important antioxidant in the pigmented rice is anthocyanin. There are many varieties of pigmented rice in Thailand, such as Hom Nil rice and black glutinous rice (อรอนงค์ นัยวิกุล, 2550).

Therefore, this investigation was proposed to evaluate whether anthocyanin pigmented rice in Thailand namely, Hom Nil rice and black glutinous rice as raw, cooked, and fermented rice could modify mutation induced by urethane or the reaction product of sodium nitrite and methylurea using the somatic mutation and recombination test.

1.2 Objectives of the Study

1.2.1 To elucidate the modulating effect of Hom Nil rice and black glutinous rice in raw rice, cooked rice and fermented rice on urethane or the reaction product of nitrite and methylurea using somatic mutation and recombination test

CHAPTER II

LITERATURE REVIEW

2.1 Rice

Rice is in the genus *Oryza* and comprised of many species. Only *Oryza sativa* and *Oryza glaberrima* are cultivated. *Oryza sativa* might originate in Southeast Asia while *Oryza glaberrima* might originated in West Africa (Juliano, 1985). In present, almost rice varieties grown and developed from *Oryza sativa*. *Oryza sativa* can be divided into three sub-species which is indica, japonica and javanica. Glutinous and nonglutinous varieties are in all sub-species which the amylose content is a major determination of the stickiness of rice. Rice with low amylose content is sticky while the rice with high amylose is firm (ประพาส วีระแพทย์, 2523).

Rice is the source of carbohydrates and it also contains protein and vitamin B including thiamin, riboflavin, niacin (Fresco, 2005). The nutrients composition of some types of rice and their major constitutes are shown in Table 1. One of the functional foods is whole grain pigmented rice since it contains high amounts of phenolic compounds, especially anthocyanin (Ryu et al., 1998; Abdel-Aal et al., 2006). Pigments in black rice are mixture of anthocyanin situated in the aleurone layer (Hu et al., 2003). These pigments could be separated into anthocyanin-rich fractions for using as functional colorants or functional food ingredients (Zeven, 1991; Abdel-Aal and Hucl, 1999).

Rye et al. (1998) reported that black rice consisted in a wide range of total anthocyanin content. Cyanidin-3-glucoside is the most common anthocyanin while peonidin-3-glucoside was the second dominant anthocyanin that found in most of the 10 varieties studied. Abdel-Aal et al. (2006) investigated the anthocyanin in the pigmented rice which total anthocyanin contents and the color of each extracts from pigmented rice is shown in Table 2.

Table 1 Nutrients composition of rice (composition per 100 g edible portion)

Rice	Moisture (g)	Fat (g)	CHO (g)	Fibre (g)	Protein (g)	Calcium (mg)	Phosphorus (mg)	Iron (mg)	A (I.U.)	B_1 (mg)	B_2 (mg)	Niacin (mg)
Rice												
raw[a]	11.8	0.8	80.4	0.3	6.4	24	135	1.9	0	0.10	0.05	2.1
parboiled[a]	12.4	1.0	79.3	0.6	6.7	7	135	1.2	0	0.20	0.08	2.6
steamed[a]	65.4	0.5	31.2	0.1	2.8	0	11	0.5	-	0.01	0	1.5
Glutinous rice												
raw[a]	13.9	1.6	75.4	0.5	8.4	16	130	1.2	0	0.16	0.06	2.4
steamed[a]	42.9	0.6	52.3	0.5	4.1	18	12	tr.	-	0.03	0.10	1.0
fermented[b]	60.3	0.1	37.7	0.3	1.8	12	29	0.6	0	0.01	0.03	0.8
Black glutinous rice												
raw[a]	11.8	3.0	76.1	4.9	8.2	26	65	2.3	3	0.55	0.29	0.6
Hom Nil rice												
raw[c]	-	-	70	6.17	7.22	11.93	-	1.15	-	0.41	0.02	-

[a]From Thai food composition table by Nutrition, Ministry of Public Health, Thailand; 2000

[b]From Thai food composition table by Nutrition, Ministry of Public Health, Thailand; 1987

[c] From evaluation of nutrition values in colored rice, 2007

- = not reported; tr. = trace

Table 2 Total anthocyanin contents and color of extracts from pigmented rice (Abdel-Aal et al., 2006)

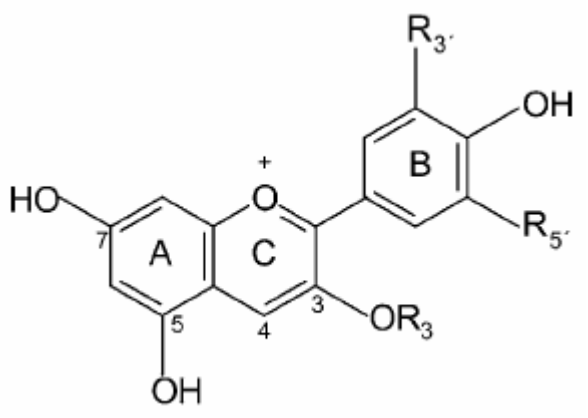
Rice	Anthocyanin[a] (μg/g)	Color of extract	
		Original	Diluted
Black	3276 ± 93.1	dark purple	dark purple
Red	93.5 ± 1.3	pink-orange	orange-red
Wild	27.2 ± 1.2	blackish green	yellowish brown

[a]Mean ± SD

There are some varieties of black rice cultivated in Thailand such as Hom Nil rice and black glutinous rice. Hom Nil rice, Khao Hom Nil (ข้าวหอมนิล), has been developed by Kasetsart University (Kukam-oo et al., 2008). It has been derived from the cross hybridization of Hom Mali rice and black rice from China. Black glutinous rice or black sticky rice, also called Khao-Kam (ข้าวก่ำ), has been generally cultivated in northern and north-eastern part of Thailand. Black glutinous rice is used as an ingredient in many snacks and desserts (อรอนงค์ นัยวิกุล, 2550).

2.2 Anthocyanin

Anthocyanin is a group of reddish to purple flavonoid. It is the primary pigment in the red and black cereal grains (Abdel-Aal and Hucl, 1999; Abdel-Aal and Hucl, 2003). The structures of anthocyanin are shown in Figure 1.



Anthocyanins	R_3	$R_{3'}$	$R_{5'}$
Peragonidin	H	H	H
Cyanidin	H	OH	H
Delphinidin	H	OH	OH
Peonidin	H	OCH_3	H
Petunidin	H	OCH_3	OH
Malvidin	H	OCH_3	OCH_3
Peragonidin-3-glucoside	Glycoside	H	H
Cyanidin-3-glucoside	Glycoside	OH	H
Delphinidin-3-glucoside	Glycoside	OH	OH
Peonidin-3-glucoside	Glycoside	OCH_3	H
Petunidin-3-glucoside	Glycoside	OCH_3	OH
Malvidin-3-glucoside	Glycoside	OCH_3	OCH_3

Figure 1 Basic structure of anthocyanins (modified form Stintzing and Carle, 2004)

Anthocyanin is highly soluble in water and alcoholic solution (Markakis, 1982). In aqueous solution, they are co-exist as four main equilibrium species (Figure 2). The relative amounts of each equilibrium form are varied, depending on the pH of the solution and the structure of a particular anthocyanin. The predominant form of anthocyanin is the flavylium cation which is red at low pH (de Pascual-Teresa and Sanchez-Ballesta, 2008). When pH increases most of the flavylium ions (red) change into quinonoidal forms (blue), pseudobases or carbinol, and chalcones (colourless) (Brouillard and Dangles, 1993). Anthocyanin is stable at low pH but it is easily oxidized and sensitive to many factors that may affect their stability and colour such as high pH, high temperature and UV radiation (Markakis, 1982).

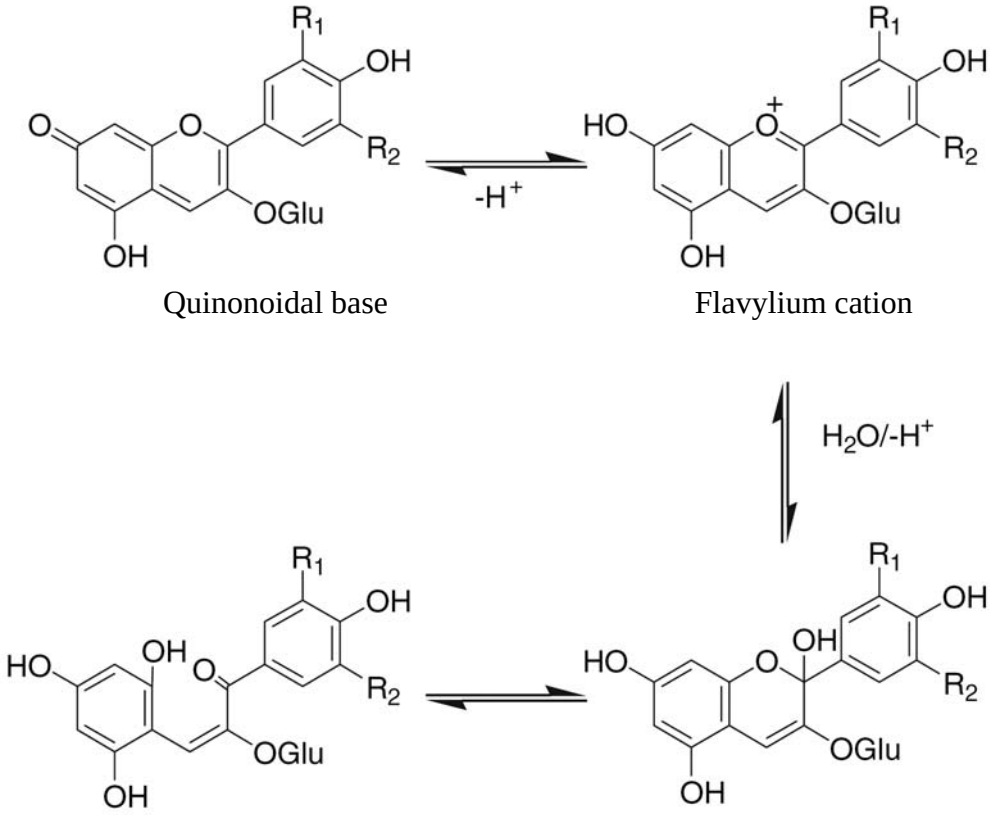


Figure 2 Chemical transformations of anthocyanins

(de Pascual-Teresa and Sanchez-Ballesta, 2008)

Anthocyanin possessed many biological properties. Consumption of blackcurrant had a positive effect on night vision (Nakaishi et al., 2000) and blackcurrant concentrates could inhibit the growth of different strains of *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecium* (Werlein et al., 2005). In addition, anthocyanin-enhanced extracts from chokeberry, bilberry, and elderberry showed endothelium-dependent relaxation capacity in porcine coronary arteries (Bell and Gochenaur, 2006) while blackberry anthocyanin could inhibit lipopolysaccharide (LPS) induced nitric oxide biosynthesis in macrophage (Pergola et al., 2006). Hibiscus anthocyanin could protect rat liver from hepatotoxicity induced by tertbutyl hydroperoxide (t-BHP) by lowering the serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and reducing oxidative liver damage (Wang et al., 2000). Anthocyanin had a protective effect against tumor necrosis factor-alpha (TNF-α) induced monocyte chemoattractant protein secretion in primary human endothelial cells (Garcia-Alonso et al., 2004). It also could effect insulin secretion from rodent pancreatic beta-cells *in vitro* (Jayaprakasam et al., 2005).

There were many studies of chemopreventive of anthocyanin. Some studies of anthocyanin in *in vitro* cell culture are shown in Table 3. Stoner et al. (2007), was a seminar group that discussed about the consumption of freeze-dried berries, which contained high anthocyanin that could inhibit the chemically-induced esophageal and colon cancer in rodent. The anthocyanin-rich tart cherry extracts are significantly reduced tumor development in the mice cecum and also inhibited the growth of human colon cancer cells *in vitro* (Kang et al., 2003). Hagiwara et al. (2001) demonstrated that anthocyanin in purple corn color reduced the promotion of 1,2-dimethylhydrazine (DMH) induced colon tumors in rats. The anthocyanin extracts from bilberry and chokeberry reduced colonic cellular proliferation in rats (Lala et al., 2006).

Table 3 Summary of growth inhibitory effects of anthocyanins (Cooke et al., 2005)

Agent	Cell line	Effects
Delphinidin-3-galactoside	HL60	Growth inhibition (~80%)
Delphinidin-3-glucoside	HCT116	Growth inhibition (~85%)
	HT29	Growth inhibition (87%)
	MCF-7	Growth inhibition (82%)
	HL60	Growth inhibition (~75%)
	HCT116	Growth inhibition (~80%)
Cyanidin-3-galactoside	LXFL529L	IC_{50} > 100 μM
	A431	IC_{50} > 100 μM
Cyanidin-3-glucoside	HT29	Growth inhibition (88%)
	MCF-7	Growth inhibition (85%)
	HL60	Growth inhibition (37%)
Malvidin-3-glucoside	LXFL529L	IC_{50} > 100 μM
	A431	IC_{50} > 100 μM
	HT29	Growth inhibition (90%)
	MCF-7	Growth inhibition (84%)

HL60 is human promyelocytic leukaemia cell. HCT116 and HT29 are human colon carcinoma cells. MCF-7 is human mammary cancer cell. LXFL529L is human lung carcinoma cells. A431 is human vulva carcinoma cell.

Anthocyanin form black rice also possessed some biological properties. Hu et al. (2003) indicated that black rice and specific anthocyanin components contributed to marked antioxidant activities in preventing DNA damage and suppressed the production of nitric oxide in the activated macrophage without introducing cytotoxicity. Cyanidin and malvidin isolated from black rice were cytotoxic through arrest of the G2/M phase of the cell cycle and induction of apoptosis on a human leukemia cell (Hyun and Chung, 2004). Moreover, cyanidin-3-glucoside and peonidin-3-glucoside from black rice had atheroprotective activity by inhibiting atherosclerotic plaque progression and enhancing the stability of the vulnerable plaque in old apoE-deficient mice (Xia et al., 2006).

2.3 Antioxidants in Rice and Human Health

2.3.1 Gamma-Oryzanol

Gamma-oryzanol (γ-oryzanol) is mainly comprised of esters of *trans*-ferulic acid (*trans*-hydroxycinnamic acid) with phytosterols (sterols and triterpenic alcohols) (Xu and Godber, 1999; Lloyd et al., 2000). The molecular structures of *trans*-ferulates of these four phytosterols are shown in Figure 3. Gamma-oryzanol had some health benefits, such as improvement of plasma lipid profile, reduction of total plasma cholesterol, increasing of HDL cholesterol levels and inhibition of the platelet aggregation (Cicero and Gaddi, 2001). Gamma-oryzanol also possessed antioxidant activities in *in vitro* systems (Kim et al., 1995), reducing serum total cholesterol in rats (Seetharamaiah and Chandrasekhara, 1989) and reducing lipid peroxidation in porcine retinal homogenate by ferric ion (Hiramitsu and Armstrong, 1991). Moreover, gamma-oryzanol has been used as multifunctional ingredient in food, pharmaceutical and cosmetic products.

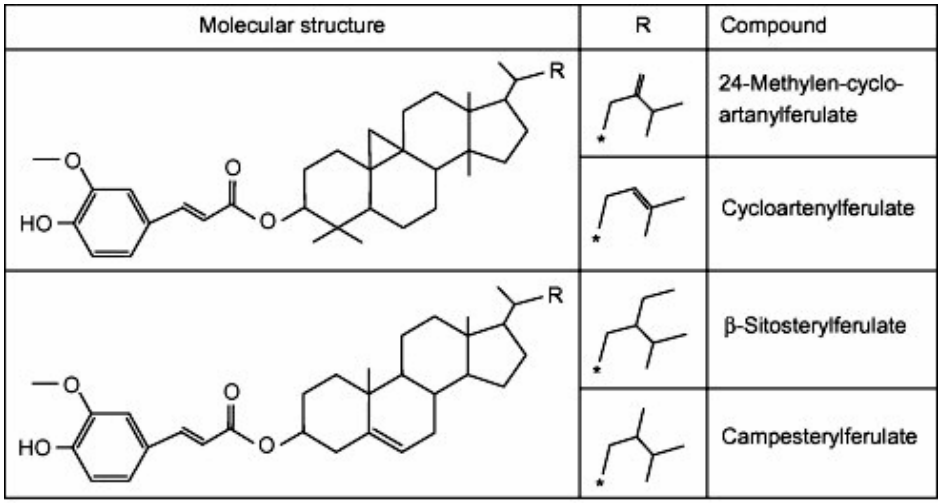
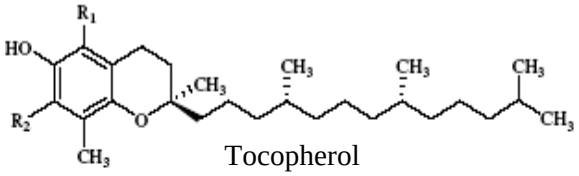


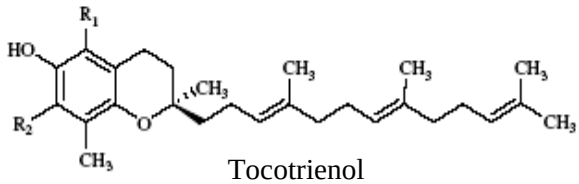
Figure 3 Chemical structures of the four main components of gamma-oryzanol (Lerma-García., 2009)

2.3.2 Vitamin E

Vitamin E compounds are found in many foods including rice especially rice bran (Imsanguana, 2008). Vitamin E is composed of eight lipid-soluble antioxidants with two types of structures, the tocopherols and tocotrienols in alpha (α), beta (β), gamma (γ), and also zigma (δ) forms (Zingg, 2007). The structures are shown in Figure 4. The tocopherols and tocotrienols could be used as radical scavengers, used for terminating the propagation of radical chain reactions by reacting with peroxyl radicals, and generating unreactive phenoxyl radicals as well as hydroperoxide products (Kitts, 1997). In addition, tocotrienols have shown biological properties such as antitumor properties (Guthrie et al., 1997), reduction of serum cholesterol (Qureshi et al., 2000), and anti-inflammation (Akihisa et al., 2000).



Tocopherol



Tocotrienol

Form	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 4 Chemical structures of tocopherols and tocotrienols (Zingg, 2007)

2.3.3 Carotenoids

Carotenoids are a family of compounds of fat-soluble plant pigments that are responsible for different colors of the foods (Parker, 1996). The structures of some major dietary carotenoids are shown in Figure 5. These pigments are important for plant in photosynthesis, reproduction, and protection (Liu, 2007). They also play an essential substance in the prevention of diseases and maintaining good health (Rao and Rao, 2007). Many epidemiologic studies supported the health-benefit properties of carotenoids in prevention of some diseases, including certain cancers (Charleux, 1996; Toniolo et al., 2001; Goodman et al., 2003) and eye disease (Ribaya-Mercado

and Blumberg, 2004). Johnson (2002) suggested that β-carotein and lycopene were inversely related to the risk of cardiovascular diseases and certain cancers.

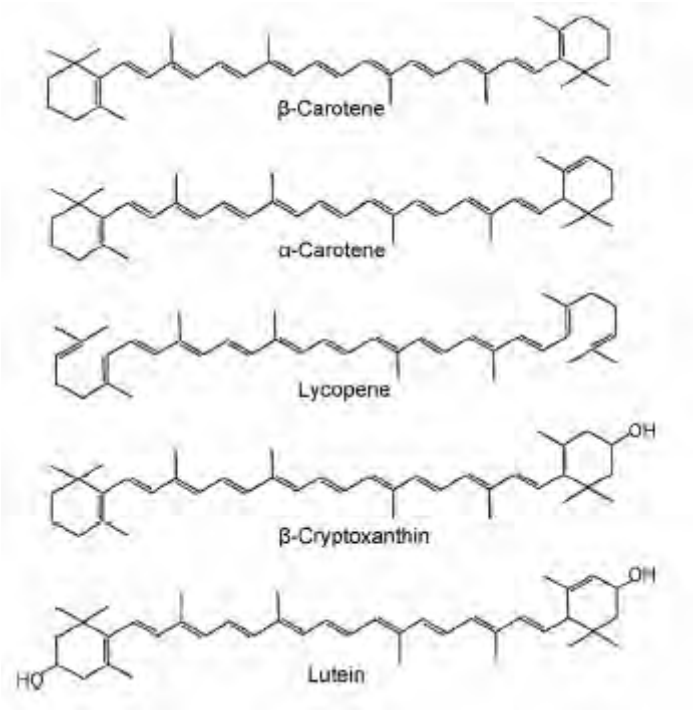


Figure 5 Structures of some major dietary carotenoids (Rao and Rao, 2007)

2.3.4 Phytic Acid

Phytic acid (myoinositol hexa-phosphoric acid) is the major phosphorus storage compound of many seeds, cereal grains and also rice (Garcia-Estepa et al., 1999). The contents of phytic acid in rice genotype are different (Liu, 2005). The structures are shown in Figure 6. Phytic acid has a potential to chelate multivalent metal ions, espicially zinc, calcium and iron. The result of binding is a very insoluble salts with poor bioavailability of minerals (Rhou and Erdman, 1995). Many studies showed that phytic acid had benefit for human health including anticancer and anti-oxidant functions (Ko and Gold, 1990; Thompon, 1993; Harland and Morris, 1995).

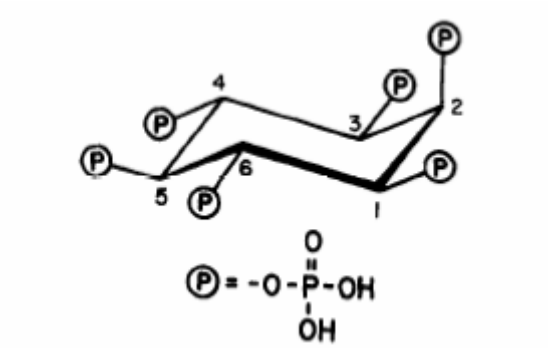


Figure 6 Structure of phytic acid (Grafs et al., 1987)

2.4 Cancer

Oxidative stress induced by reactive oxygen species (ROS) that are generated by through activities such as smoking, exercise and diet. They have been coperated with the causation and progression of many chronic diseases including cancer (Rao and Rao, 2007). Antioxidants that could reduce the damaging effects of ROS in chronic diseases are shown in Figure 7.

In 2000, the 10 million new cases of cancer diagnosed world-wide about 2.3 million were cancers of the pharynx, oesophagus, stomach or colorectum, and the colon and rectum carcinomas are the most common cancers in the alimentary tract (World Health Organisation, 2003). About 80% of colorectal cancer caused from the diet (Bingham, 2000). Stomach cancer is one of the most frequent causes of death. An important role in prevention of stomach cancer are food and nutrition (Catalano et al., 2009). World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) estimated that 30–40% of cancer incidence could be prevented through good nutrition and exercise. World Health Organisation World Cancer Report emphasizes the importance of nutrition as a goal of controlling the expected 50% rise in cancer incidence.

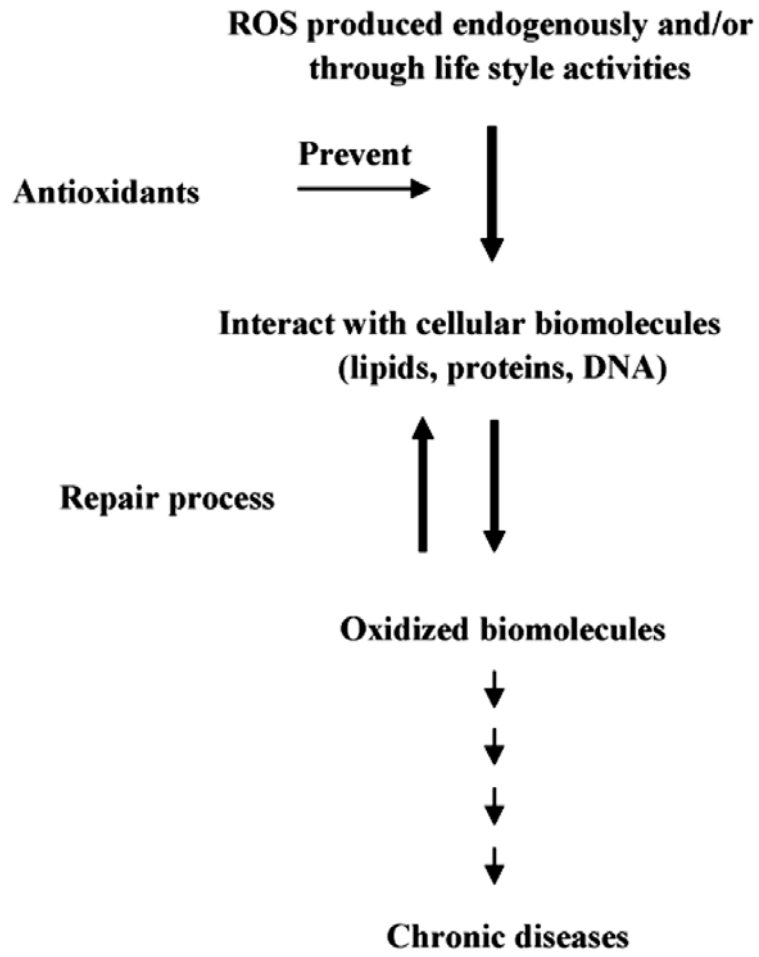


Figure 7 Oxidative stress, antioxidants and chronic diseases (Rao and Rao, 2007)

2.5 Somatic Mutation and Recombination Test (SMART)

The somatic mutation and recombination test (SMART) is one of the first assay systems used to test chemical and physical agents for recombinogenic effects and provides a number of methodological advantages to detect genotoxicity of chemical substances (Hiraizumi, 1979). SMART was designed to probe genetic damage in a sensitive, rapid and inexpensive way as an *in vivo* system. Eukaryotic organism was used with metabolic system similar to that found in mammalian cells (Vogel and Zijlstra, 1987; Graf et al., 1984, 1998).

Drosophila melanogaster is a 3 mm fruit fly that accumulates around spoiled fruit. It is also useful for biological research, particularly in genetics and development of biology. The main benefits of *Drosophila melanogaster* as a test organism for

detection of chemicals with genotoxic activity are as following (Sarıkaya and Çakır, 2005):

(1) a short life cycle (10 days at 25°C);

(2) simple detectable genetically controlled morphological characters;

(3) large numbers of mutants and genetically characterized strains;

(4) culture media are allow the breeding of large numbers of animals using simple facilities and inexpensive; and

(5) capable of activating enzymatically procarcinogens and promutagens *in vivo*.

This assay is based on inducing loss of the heterozygosity, which may occur through various mechanisms, such as point mutations, deletions, certain types of chromosome aberrations as well as mitotic recombination and gene conversion (Graf et al., 1984). Based on the treatment of larvae during the embryogenesis, the imaginal disc cells proliferate mitotically and some genetic results such as point mutation, deletion, somatic recombination and non-disjunction can be measured on the wing of adult flies (Würgler and Vogel, 1986). If genetic is changes in one imaginal disc cells during mitotic proliferation, it will create a clone of mutant cells expressing the phenotype controlled by the specific genetic markers. Using of improved high-bioactivation (HB) cross of *Drosophila melanogaster* is characterized by increased cytochrome P450-dependent bioactivation capacity facilitates the promutagens and procarcinogens of different chemical classes detection (Graf and Singer, 1989; Graf and van Schaik, 1992).

The SMART is also suited for measuring the mutagenicity and antimutagenicity of pure chemicals or mixtures (Negishi et al., 1989; Graf and van Schaik, 1992; Graf et al., 1998). Several researchs revealed that the modulation of genotoxicity in the wing somatic cells using various modulating agents such as enzyme inducers or inhibitors (Cederberg and Ramel, 1989; Romert et al., 1990).

2.5.1 Wing Spot Test in *Drosophila*

The wing spot test make use of the recessive markers multiple wing hair (*mwh*) and flare (*flr*^{3}) which alter the phenotypic expression of the hairs on the wing blade (Graf et al., 1984; 1989; Szabad et al., 1983). The two wing hair markers are both placed on the left arm of chromosome 3 (Graf and Würgler, 1986). The appearance of multiple wing hairs (*mwh*, 3-0.0) is a recessive, homozygously viable mutation and

produces multiple trichomes per cell instead of the normally unique trichome. The second marker, flare (*flr*^{3}, 3-39.0*)* is a recessive mutation that produces malformed wing hairs that look like a flare. All three mutant alleles of *flr* are recessive zygotic lethals. Nevertheless, homozygous cells in the wing imaginal discs are viable and lead to mutant wing cells. The *flr*^{3} allele is kept over a balancer chromosome carrying multiple inversions and a dominant marker that is a homozygous lethal (*flr*^{3}/*TM3*, *Bd*^{s}: Third Multiple 3, Beaded-Serrate). According to experimental series analyzed, the occurrence of the various types of spots were as follows: most frequent was single spots expressing the *mwh* phenotype, less frequent twin spots with both a recombination sub-clone and quite rare single spots with the *flr*^{3} phenotype (Lindsley and Zimm, 1992).

Many mechanisms lead to genetically marked clones (Figure 8). An important possibility is a mitotic recombination event between two non-sister chromatids. Twin spots are expected if recombination occurs between *flr*^{3} and the centromere (Becker, 1976). A recombination event between *mwh* and *flr*^{3} may resulted in a *mwh* single spot. If both types of recombination events (one between *flr*^{3} and the centromere, a second between *mwh* and *flr*^{3}) occur within the same cell, a *flr*^{3} single spot may result. Nondisjunctional or other loses of the chromosomes hold the aggressive type allele represents another mechanism that may lead to single spots. Mitotic recombination in the chromosome section between the centromere (spindle fiber attachment site) and the marker *flr*^{3} leads to two daughter cells, one homozygous for *mwh*, the other homozygous for *flr*^{3}. Clonal expansion to these two cells will be recognizable on the wing blade from the two multicellular adjacent clones, one exhibiting the *mwh* phenotype (multiple hairs), the other the *flr*^{3} phenotype (misshape hairs).

On the other hand, the origin of “single spots”, presenting either the *mwh* or the *flr*^{3} phenotype (mainly of the *mwh* phenotype, rarely also of the *flr*^{3} phenotype), cannot be clearly determined. Multiple wing hairs single spots may result from a recombination event that occur in the chromosome segment between the two marker genes. Moreover, a gene mutation or deletion of the *mwh*$^+$ gene will result in a *mwh* single spot. A *flr*^{3} single spot may either result from a gene mutation or a deletion of the *flr*^{3} gene, or from a rare double recombination with one recombination event to the left and the other event to the right of the *flr*^{3} locus (Würgler et al., 1991).

2.5.2 Approach of SMART

Three crosses of flies carrying the marker *mwh* and *flr*^{3} on the left arm of chromosome 3 are generally set up:

1. Standard cross (ST): *flr*^{3} */In (3LR) TM3, ri p*^{p} *sep bx*^{34e} *e*^{s} *Ser* virgin females mated to *mwh* males. This is the reciprocal cross of the standard cross used as previous (Graf et al., 1989; van Schaik and Graf, 1991).

2. High bioactivation (HB) cross: *ORR; flr*^{3} */TM3* females crossed with *ORR*; *mwh* males. This is the reciprocal cross of the one that described by Frölich and Würgler (1989). The strains contain chromosomes 1 and 2 of the DDT-resistant stock Oregon R(R) which exhibit a high constitutive level of cytochrome P450. A number of promutagens show increased genotoxicity when the HB cross is utilized, compared with ST cross (Frölich and Würgler, 1989; 1990a; 1990b). However, several problem for routine application are shown, such as disturbed wing hair patterns in certain areas of the wing, making spot classification difficult and a delay in development of the larvae.

3. Improved high bioactivation cross: *ORR, flr*^{3} */TM3* females crossed with *mwh* males. The main advantage of the improved HB cross is to combine the high bioactivation capacity with the ease of scoring the wings using the same criteria as for the standard cross. The hybrid larvae of the improved HB cross show P450-dependent activation capacity equal to or even slightly higher than those of the original HB cross In addition, the HB cross is more sensitive than the standard cross in measuring the genotoxicity of promutagens (Graf and van Schaik, 1992).

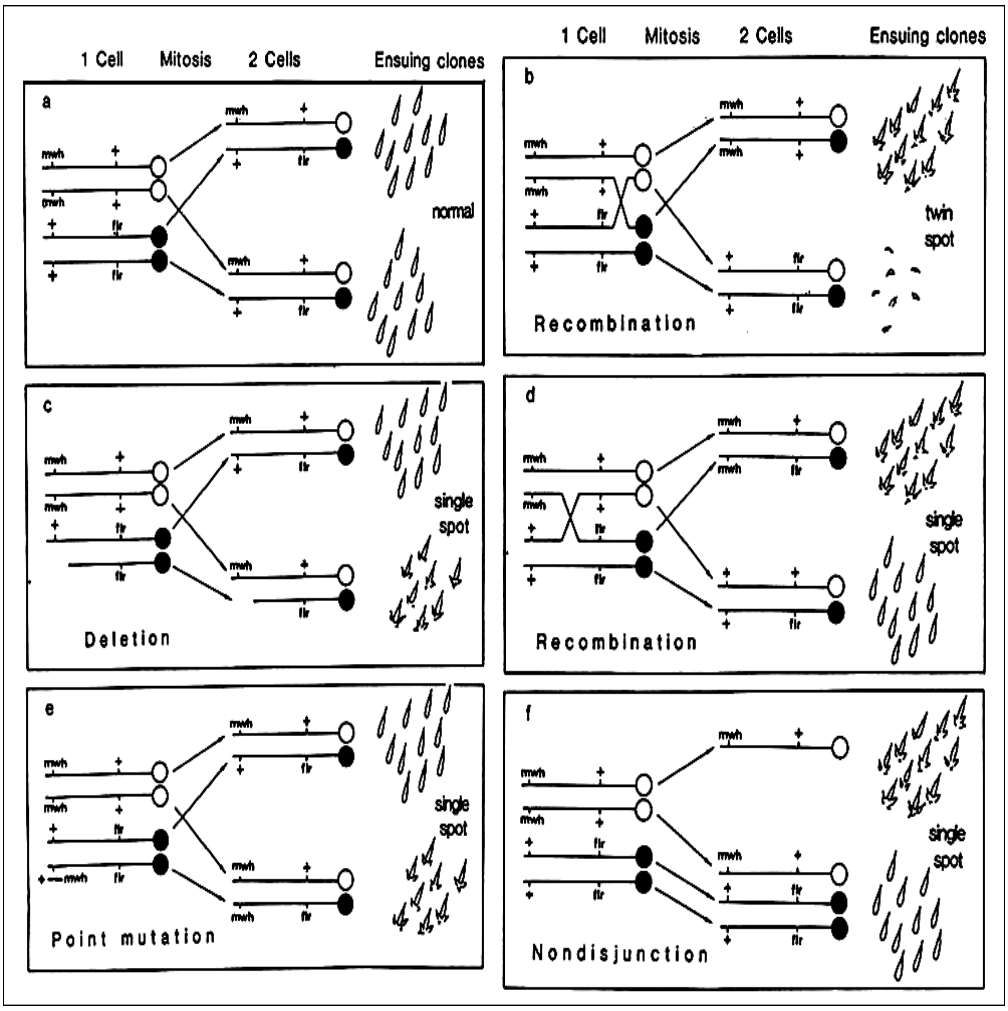


Figure 8 Genetics schemes illustrating various ways of spot formation in the somatic mutation and recombination test with the wing cell markers multiple wing hairs (*mwh*) and flare (*flr*^{3}). Twin spots are obtained by recombination proximal to the *flr*^{3} marker (b), while more distal recombination produces *mwh* single spots only (d). Deficiencies (c), point mutations (e) and nondisjunction events (f) give rise to *mwh* single spots or in analogous ways to *flr*^{3} single spots (not illustrated) (Graf et al., 1984).

2.5.3 Positive Mutagens of SMART

2.5.3.1 Urethane

Urethane ($NH_2COOCH_2CH_3$) (Figure 9), also called as ethyl carbamate, is the ethyl ester of carbamic acid (NH_2COOH) which may occur as a colorless, odorless crystal white, granular powder. It is slightly soluble in olive oil and soluble in water, ether, glycerol, chloroform and ethyl ether.

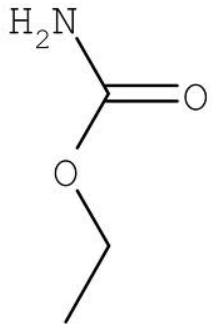


Figure 9 The structure of urethane

Urethane was concerned as a multiple carcinogen in various organs (Field and Lang, 1988) and animal species (Benson and Beland, 1997). Urethane could induce point mutation, gene conversion, intrachromosomal recombination, chromosomal aberrations and sister chromatid exchanges in yeast, plant systems and also mammalian cells (Schlatter and Lutz, 1990). In rodents, urethane can cause lung tumors, lymphomas, hepatomas, melanomas and vascular tumors (Mirvish, 1968; IARC, 1974; Mori et al., 2000). Urethane was classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer, but evidence of urethane carcinogenicity in humans was considered inadequate (IARC, 1974). Humans are exposed to urethane from some food, mainly fermented foods and alcoholic beverages such as stone-fruit brandies, sherry and table wines (Schlatter and Lutz, 1990; Stoewsand et al., 1991). In addition, urethane is used as an industrial chemical (Crout, 1976) and an animal anesthetic (Kotanidou et al., 1996; Norlen et al., 2000).

Urethane needs the bioactivation to vinyl carbamate epoxide that adducts to RNA and DNA and initiates tumorigenesis (Dahl et al., 1978; Leithauser et al., 1990). Urethane is metabolized by at least three pathways (Salmon et al., 1991; Park et al.,

1993) as shown in Figure 10. The major pathway of urethane accounting in rodents for over 90% is detoxification through the hydrolysis by liver microsomal esterase and amidases to ethanol, ammonia and carbon dioxide (Mirvish, 1968; IARC, 1974; Park et al., 1993). Approximately 0.1% of urethane is reversibly converted by cytochrome P-450 subtype 2E1 (CYP2E1) to 2-hydroxyethyl carbamate (Guengerich and Kim, 1991), which is not a carcinogen (Berenblum et al., 1959), and *N*-hydroxyethyl carbamate (Boyland and Nery, 1965; Nery, 1968), which is less carcinogenic than urethane (Mirvish, 1968). The two oxidation steps of urethane to the active vinyl carbamate epoxide is catalyzed primarily by (CYP2E1) (Guengerich et al., 1991). Less than 0.5% of urethane is metabolized by CYP2E1 to vinyl carbamate and the metabolite is more potent than its parent compound in its carcinogenicity. Vinyl carbamate, in turn, is converted by epoxidation to the putative ultimate carcinogen vinyl carbamate epoxide (Miller and Miller, 1983; Guengerich and Kim, 1991; Guengerich et al., 1991). Vinyl carbamate epoxide can covalently bind to DNA, RNA and proteins to form adducts and the initiation of tumorigenesis (Dahl et al., 1978; Miller and Miller, 1983; Leithauser et al., 1990). Vinyl carbamate can be inactivated by liver microsomal esterase and amidase, while vinyl carbamate epoxide can be inactivated though many pathways including glutathione-s-transferase (Park et al., 1993)

Urethane was genotoxic in the somatic mutation and recombination test in *Drosophila melanogaster* (Zimmerli et al., 1991), both standard strain and modified strain (ORR) in which genetic control of cytochrome P450 dependent enzyme systems were altered (constitutively increased P450 enzyme activities) (Frölich and Würgler, 1988; 1990a). Urethane is generally used as positive standard toxicants in evaluation genotoxicity of the unknown compounds in SMART (Abraham and Graf, 1996). It was shown to induce genotoxicity in *Drosophila melanogaster* (Zimmerli *et al*., 1991). The frequencies of spots per wing in high bioactivation cross were higher than that in standard cross (Frölich and Würgler, 1990a). The effects were dose-dependent and the modified strain (ORR) is more sensitive to urethane by about one fold than the standard strain. This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites.

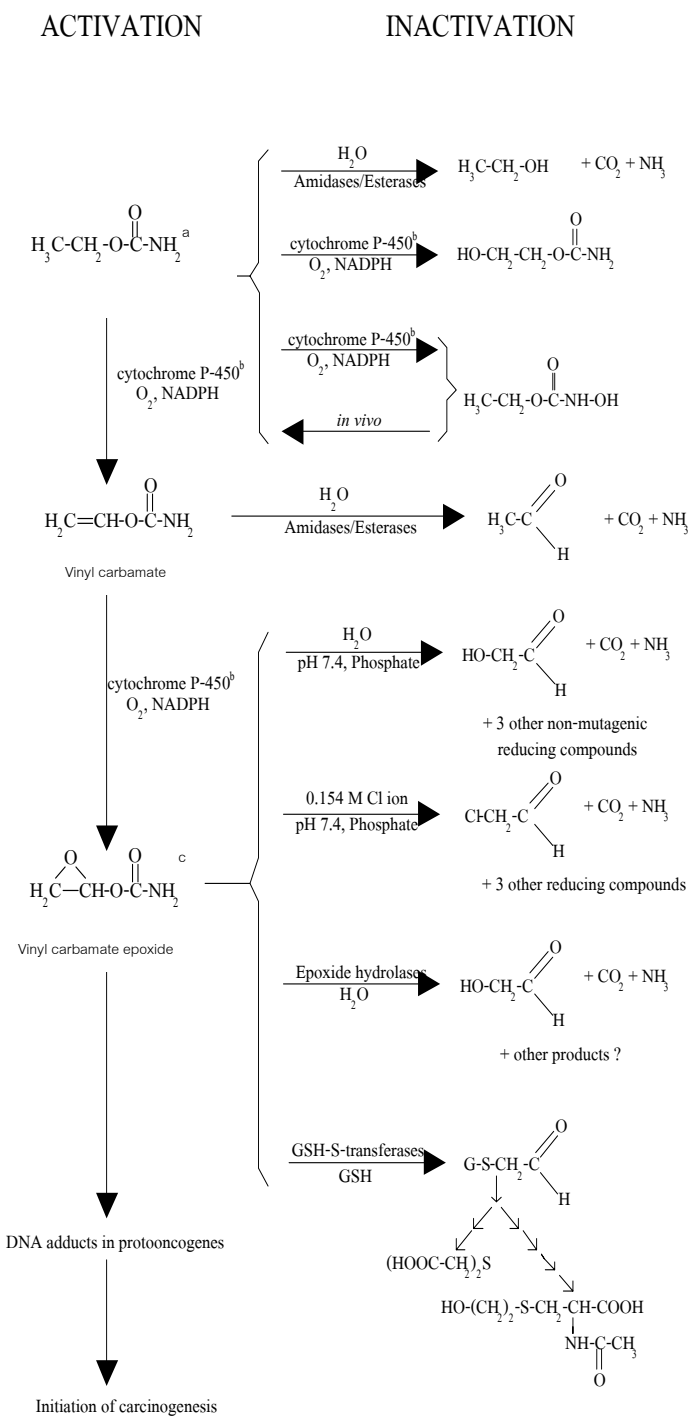


Figure 10 Known and probable activation and inactivation pathways of metabolism of urethane (ethyl carbamate), vinyl carbamate and vinyl carbamate epoxide; (a) Mouse liver microsomes + ethyl carbamate or vinyl carbamate + adenosine ⟶ 1,N^6-ethenoadenosine, (b) Human liver microsomal cytochrome P450 2E1, (c) Vinyl carbamate epoxide + adenosine ⟶ 1, N^6-ethenoadenosine. GSH = glutathione (Park et al., 1993)

2.5.3.2 *N*-Nitrosomethylurea

N-nitrosomethylurea (*N*-methylurea-*N*-nitrosourea or *N*-nitroso-*N*-methylurea; NMU) (Figure. 11) is a pale yellow crystals. It is soluble in water and polar organic solvents and insoluble in nonpolar organic solvents. This compound is highly reactive and sensitive to humidity and light. *N*-nitroso-*N*-methylurea is a human carcinogen, which exposure to endogenously formed *N*-nitroso compounds. They have been related to an increased risk of gastric, esophageal, nasopharyngeal, and bladder cancer based on sufficient evidence of carcinogenicity in experimental animals as shown in Table 4 (IARC, 1972; 1978; 1987). Deng (2000) suggested that *N*-nitrosoureas can be formed in the stomach of human when nitrite is consumed in the little amounts. Hence, high exposure levels of nitrite and nitrate, NMU could form endogenously in the stomach of residents in high-risk areas and might contribute to the causes of the high incidence of stomach cancer.

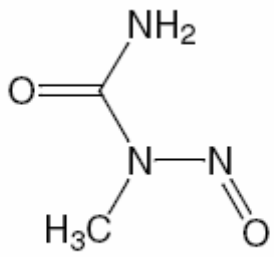


Figure 11 The structure of *N*-nitrosomethylurea

The substance of *N*-nitrosocompound (i.e. nitrate, nitrite, nitrosating agents) could expose to human through diet, tobacco smoke and drinking water. The *in vivo* formed of nitrosomethylurea can occur by acid-catalyzed and bacterial nitrosation in the stomach and via nitric oxide (NO) formation during inflammation. The amino compound (i.e. amide, carbamates, alkylureas) react with nitrosonium ion (NO^+) or its hydrated form in strong acidic condition (Bartsch et al., 1988).

The *in vivo* nitrosation capacity of the larvae was evaluated using SMART (Rincon et al., 1988). Larvae derived from two difference crosses, the standard cross (ST) and the high bioactivation cross (HB), treated with methyl urea was negative, while treated with sodium nitrite was weakly genotoxic. However, the reaction of

both compounds highly increased the frequencies of mutations and recombination predominantly in the HB crosses. Therefore, NMU will be used as positive mutagen in this study.

Table 4 The evidences of carcinogenicity of NMU in experimental animals (Modified from IARC, 1972; 1978; 1987)

Subject	Administration	Results
Rat	Diet	• squamous cell carcinomas of the forestomach • sarcomas and gliomas of the brain and neurosarcomas
Rat	Drinking water	• tumors of the brain • neurinoma of the spinal cord
Guinea pig	Drinking water	• carcinomas and sarcomas of the stomach • adenocarcinomas of the pancreas, malignant tumors of the ear duct • neurinoma of the lumbar nerve and leukemia

2.6 Antioxidant Activity and Total Phenolic Content Assay

A wide range of methods is currently used to evaluate antioxidant capacity (Halliwell et al., 1995), for instance, the measurement of prevention of oxidative damage to biomolecules such as lipids or DNA and methods assessing radical scavenging. Both *in vivo* and *in vitro* assays were used and all methods have their own advantages and limitations. Simple scavenging assays, such as the TRAP (total reactive antioxidant potential or total radical-trapping antioxidant parameter) and the TEAC (Trolox equivalent antioxidant capacity) assay, are popular because they empower high-throughput screening on potential antioxidant capacity. Such methods are used to assess antioxidant capacity of biological matrices, such as plasma, as well as single compounds, food components or food extracts.

2.6.1 2,2-Diphenyl-1-picrylhydrazyl Scavenging Capacity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is a commonly used assay in antioxidant studies and offers a rapid technique in which to screen the radical scavenging activity of pure synthetic compounds, crude plant extracts, food extracts, beverages and biological matrices such as plasma (Amarowicz et al., 2004). This assay based on the reaction between DPPH and Trolox. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), also known as 1,1-diphenyl-2-picrylhydrazyl or R,R-diphenyl-*â*-picrylhydrazyl, is a free radical used for assessing antioxidant activity results in a loss of absorbance at 520 nm. The degree of discoloration of the solution indicates the scavenging efficiency of the added sample (Fukumoto and Mazza, 2000).

2.6.2 Ferric Reducing Antioxidant Power (FRAP) Assay

This method is easy and cheap. It is assesses the total antioxidant level in a sample. It was carried out according to the method of Benzie and Strain (1996) with a little modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}- TPTZ). The reaction is nonspecific and any half-reaction which has a less-positive redox potential, under reaction condition, than the Fe^{3+}/ Fe^{2+}-TPTZ half-reaction will drive Fe^{3+}-TPTZ reduction. Test conditions favor reduction of the complex and, thereby, color is developed. Ferrozine (Stookey, 1970), a compound closely related to TPTZ, was widely used, with excess ascorbic acid, to measure iron. In the FRAP assay, excess Fe^{3+} was used and the rate-limiting factor of Fe^{2+}-TPTZ and hence, color formation is the reducing ability of the sample.

2.6.3 Total Phenolic Contents Assay

The total phenolic content of ethanol extract from each sample was determined according to method described by Swain and Hillis (1959), Naczk and Shahidi (1989), Amarowicz et al. (2004) and modified the procedures of measurement by using a microplate reader. The antioxidant activity of phenolic compounds is primarily due to their redox properties, which have ability to play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing

peroxides (Javanmardi et al., 2003). Total phenolic content was estimated by using the Folin-Ciocalteu colorimetric method described previously with a little modification. Finally, the appropriate dilutions of the extracts were oxidized with Folin-Ciocalteu reagent and then the reaction was neutralized with saturated sodium carbonate. The absorbance of the resulting blue color was measured with a spectrophotometer after incubation. Quantification was completed on the basis of the standard curve of gallic acid. Results were mentioned as gallic acid equivalent (GAE) (Cai et al., 2004).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

Urethane was purchased from Sigma Chemical (St. Louis, MO, and U.S.A.). Glycerol was bought from Farmitalia Carlo Erla (Milan, Italy). Sodium nitrite and gum arabic powder were purchased from BDH Chemical Ltd. (Poole, England). *N*-Methylurea was purchased from Fluka AG (Buch, Switzerland). Chloral hydrate was supplied by Srichand United Dispendary Co. Ltd. (Thailand). TPTZ (2, 4, 6-tripyridyl-s-triazine), ferric chloride hexahydrate, and ferrous sulfate heptahydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylether, potassium hydroxide, and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Propionic acid, DPPH (2, 2′ - diphenyl-1-picrylhydrazyl), gallic acid and Folin-Ciocalteu reagent were purchased from Fluka Chemika (Buchs, Switzerland). Trolox was purchased from Aldrich Chemical (Milwaukee, WI, Germany). Glacial acetic acid was purchased from J.T. Baker (Phillibsburg, USA). Sodium carbonate anhydrous was purchased from Riedel-De Haen AG (Seelze, West Germany). Hydrochloric acid was purchased from Lab Scan Ltd. (Dublin, Ireland).

3.2 Experimental Design

The overall experiment conducted to elucidate the antioxidant activity and the urethane and *N*-nitrosomethylurea modulating effect of two kinds of pigmented rice samples (Hom Nil rice and black glutinous rice) is shown in Figure 12. Firstly, antioxidant activity (using DPPH and FRAP assays) and total phenolic content were investigated. Then, toxicity of each sample was determined from data of survival rate of flies and its genotoxicity. After that antimutagenicity of each sample on urethane and *N*-nitrosomethylurea induced somatic mutation and recombination in *Drosophila melanogaster* was evaluated.

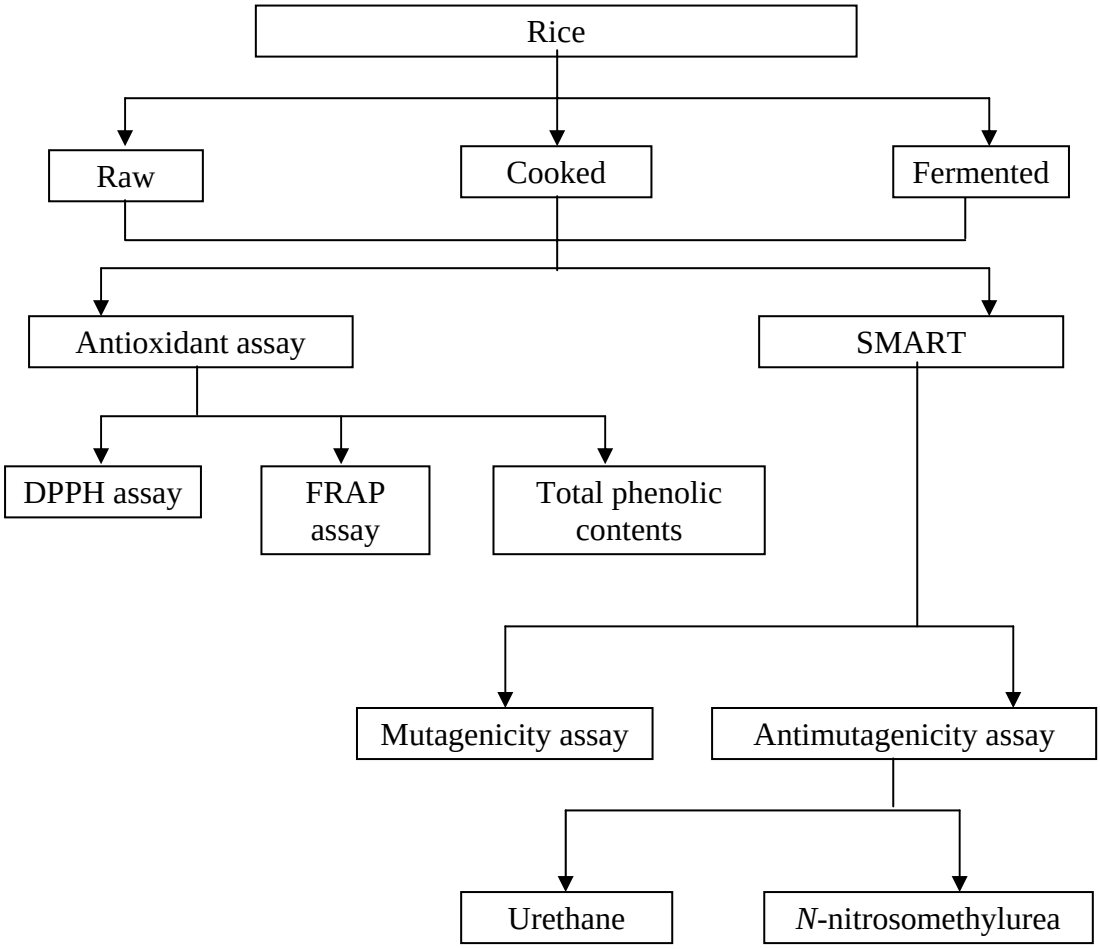


Figure 12 Overall experimental designed to determine the antioxidant activities and evaluate the antimutagenicity of Hom Nil rice and black glutinous rice in *Drosophila melanogaster*

3.3 Sample Preparation

Hom Nil rice and black glutinous rice were purchased from supermarket in Bangkok. Each sample (900 g) was divided into three portions; raw, cooked and fermented rice. Raw rice (300 g) was washed with tap water. Cooked rice was prepared by electric rice cooker using distilled water (2:1 w/v) and cooked to obtain cooked rice. Fermented rice was prepared by adding 0.3%w/w a traditional fungi-yeast (Loog-pang or yeast cake) in to cooked rice for 48 h at room temperature (อรอนงค์ นัยวิกุล, 2550). Each rice sample was lyophilized in a freeze dryer then it was protected from light and stored in desiccators. They were grounded in an electrical blender to fine powder before use.

3.4 Antioxidant Activity Assay and Determination of Total Phenolic Contents

3.4.1 Sample Extraction

Each rice sample powder (raw, cooked and fermented rice) was stirred with acid-alcohol (0.1 N acetic acid in 70% ethanol) 1.5 liter/1 kg of sample at room temperature for 24 h. The solution was filtered through Whatman filter paper No.1 and the extraction was repeated two times. The powdered extract was evaporated with vacuum rotary evaporator at 60°C. The concentrated solution was lyophilized in a freeze dryer. Each dried extract was protected from light and stored below 5°C until used. Each rice powder extract (200 mg) was stirred with 80% methanol (25 ml) at room temperature and assayed for its antioxidant activity.

3.4.2 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The antioxidant activity of the extract from rice sample was estimated by stable radicle 2,2′-Diphenyl-1-Picrylhydrazyl using the procedure described by Fukumoto and Mazza (2000) with slight modifications. An aliquot of 22 µl (in triplicate) of the rice extract or standard Trolox (28 mM Trolox in 80% Methanol) was added to 150 µl of DPPH in 80% methanol (200 µM) in a 96 well flat bottom microplate (Bibby Sterilin Ltd, UK). Standard Trolox was run in triplicate using several concentrations (1.28, 0.64, 0.32, 0.16, 0.08 mM). After the plate was covered in the dark at room temperature for 30 min, and then the absorbance of the solution was read by a microplate reader (Sunrise, Tecan Co., Austria) using a 520 mm filter. The radical scavenging activity was calculated as a percentage of DPPH scavenging activity using the equation:

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

Where A_E is the absorbance of the solution when an extract is added, and A_D is the absorbance of the DPPH solution without the extract.

3.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity was measured by its ability to reduce the Fe^{3+}/ferricyanide complex by forming ferrous products. Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 600 nm. Increased absorbance at 600 nm indicates a stronger reducing power. Aqueous solutions of known standard Fe^{2+} ($FeSO_4.7H_2O$) concentrations (62.5, 125, 250, 500, 1000 µM) were used for calibration. An aliquot (20 µl) of extract, standard or appropriate blank reagent was

added to each well in a 96 well flat bottom microplate and was run in triplicate. FRAP reagent (150 µl) freshly prepared by mixing the reagents (300 mM Acetate buffer pH 3.6: 10 mM TPTZ solution: 20 mM $FeCl_3.6H_2O$ with the ratio of 10:1:1) and warmed at 37^oC according to the procedure described by Griffin and Bhagooli (2004) was added to each well. Reading at 600 nm was performed after 8 min using a microplate reader (Sunrise, Tecan Co., Austria). The change in absorbance after 8 min from the initial blank reading was then compared to that of a standard that was run simultaneously. The FRAP values of the extracts were determined using this standard curve, expressed as mg of ferrous iron (Fe(II)) /g dry weight of rice extract. Data were presented as means ± SD of triplicate wells.

3.4.4 Determination of Total Phenolic Content

The total phenolic content of ethanol extract from rice sample was determined according to the method described by Swain and Hillis (1959), Naczk and Shahidi (1989) and Amarowicz et al. (2004) with some modification by microplate reader. Briefly, 10 µl of each extract was transferred into a 96 well microplate containing 160 µl of distilled water. After mixing the contents, 10 µl of Folin–Ciocalteu reagent and 20 µl of a saturated sodium carbonate solution were added and were mixed well. After 30 min, the absorbance of blue colored mixtures was recorded at 750 nm with microplate reader (Sunrise, Tecan Co., Austria). The readings of rice sample and reagent blanks were subtracted from the reading of reagent with extract. The amount of phenolic content was calculated as Gallic Acid Equivalent (GAE) from the calibration curve of gallic acid standard solutions (25, 50, 100, 200, 400 and 800 mg/l), and expressed as mg gallic acid equivalent/g dry weight of sample. All measurements were done in triplicate. Data were presented as means ± SD.

3.5 Somatic Mutation and Recombination Test (SMART)

3.5.1 *Drosophila* Stock

Two *Drosophila melanogaster* strains were used. Virgin females of *ORR/ORR; flr3/In(3LR)TM3, ri pp sep l(3)89Aa bx34e e Bds* were crossed with males of *mwh/mwh*. Prof. U. Graf (University of Zurich, Switzerland) kindly provided both strains. The stock cultures of the flies and also the treated larvae were maintained at 16±1ºC and 25±1ºC, respectively.

3.5.2 The Medium for Maintaining the Stock of Fly and Mating Fly (stock medium)

Stock medium was prepared accordingly to Robert (1986). Corn flour (125 g), sugar (100 g), baker's yeast (50 g) and agar (14 g) and water (1000 ml) were mixed. The mixture was heated in a water bath with magnetic stirrer to be homogeneous and propionic acid (3 ml) was added as a preservative. A portion (approximate 30 ml) of this standard medium was distributed into a 50 ml Erlenmeyer flask.

3.5.3 Experimental Medium

The composition of each medium used in this study is shown in Table 5. Each composite medium was mixed using a vortex mixture and heated in a water bath until it became sticky. These media was used for mutagenic and antimutagenic assay.

3.5.4 Mutagenicity Study

Each sample was evaluated for the mutagenicity assay as described by Graf et al. (1984). Virgin females of *ORR; flr*^{3} were mated with *mwh* males on the medium from 3.5.2. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water, and transferred (using a fine paintbrush) to sample medium, negative control medium and positive control medium. They were incubated at 25±1^{o}C until pupation. After metamorphosis, the surviving flies were collected on days 10-12 after egg laying and stored in 70% ethanol as suggested by Graf and van Schaik (1992).

Toxicity of each sample was determined from the survival rate of adult files from larvae fed on each sample medium. The sample that providing more than 50% survival of flies was determined for its mutagenic and antimutagenic investigations. Only the fly with the marker trans-heterozygous (*mwh+/+flr*^{3}), indicated with round wings, were mounted on a microscope slide. Wings were separated from the body with a fine paintbrush, and then lined up on a clean slide. A droplet of Faure's solution (30 g gum arabic, 20 ml glycerol, 50 g chloral hydrate and 50 ml deionized water) as suggested by Graf et al. (1984) was dropped on the slide before putting on a cover slip as a preservative. At least 40 round wings of selected flies (both the dorsal and ventral surface), were analyzed under a compound microscope at 400x magnification for the presence of clones of cells showing malformed wing hairs.

Figure 13 shows the position of the spots according to the sector of the wing. Single spots showing either the multiple wing hairs *(mwh)* or the flare (*flr*^{3}) phenotype, and twin spots showing abutting *mwh* and *flr*^{3} areas were recorded separately. The

resulting wing spots were classified accordingly into the following: (1) small single spots of 1 or 2 cells in size, (2) large single spots of 3 or more cells, and (3) twin spots. The spots were counted as two spots if they were separated by three or more wide-type cell rows. Multiple wing hairs (*mwh*) were classified when a wing cell contained three or more hairs instead of one hair per cell as in wide-type. Flare wing hairs exhibited a quite variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon-like extrusions of melanolic chitinous material.

Table 5 Composition and types of media was used for mutagenic and antimutagenic assay of Hom Nil rice and black glutinous rice.

Purpose of medium	Composition									
	Corn flour (g)	Sugar (g)	Agar (g)	Yeast (g)	Propionic (ml)	Water (ml)	Rice (g)	Urethane	Sodium nitrite	Methylurea
Negative control[a]	0.25	0.2	0.1	0.3	0.1	2	-	-	-	-
Positive control (urethane) [b]	0.25	0.2	0.1	0.3	0.1	-	-	20 mM 2 ml	-	-
Sample control[c]	0.25-A	0.2	0.1	0.3	0.1	2	A	-	-	-
Antimutagenicity against urethane[d]	0.25-A	0.2	0.1	0.3	0.1	-	A	20 mM 2 ml	-	-
Nitrite control[e]	0.25	0.2	0.1	0.3	0.1	-	-	-	18 mM 2 ml	-
In vivo interaction between sample and nitrite[f]	0.25-A	0.2	0.1	0.3	0.1	-	A	-	18 mM 2 ml	-
Methylurea control[e]	0.25	0.2	0.1	0.3	0.1	-	-	-	-	5 mM 2 ml
In vivo interaction between sample and methylurea[g]	0.25-A	0.2	0.1	0.3	0.1	-	A	-	-	5 mM 2 ml

Table 5 Composition and types of media was used for mutagenic and antimutagenic assay of Hom Nil rice and black glutinous rice (continued).

Purpose of medium	Composition									
	Corn flour (g)	Sugar (g)	Agar (g)	Yeast (g)	Propionic (ml)	Water (ml)	Rice (g)	Urethane	Sodium nitrite	Methylurea
Positive control (nitrosomethylurea) [h]	0.25	0.2	0.1	0.3	0.1	-	-	-	36 mM 1 ml	10 mM 1 ml
Antimutagenicity against nitrosomethylurea[i]	0.25-A	0.2	0.1	0.3	0.1	-	A	-	36 mM 1 ml	10 mM 1 ml

[a]used for negative control in mutagenicity and antimutagenicity studies; [b]used for positive control in antimutagenicity of sample on urethane; [c]used for evaluating the mutagenicity of sample; [d]used for evaluating the antimutagenicity of sample against urethane; [e]used for control in antimutagenicity study against the *in vivo* formed *N*-nitrosomethylurea; [f]used for evaluating the mutagenicity study sample treated with sodium nitrite; [g]used for evaluating the mutagenicity of sample interacted with methylurea; [h]used for positive control in antimutagenicity study of sample against the *in vivo* formed *N*-nitrosomethylurea; [i]used for evaluating the antimutagenicity study of sample the *in vivo* formed *N*-nitrosomethylurea; A=Suitable amounts of sample for antimutagenic study

The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$. A multiple decision procedure was used to decide whether a result was positive, weakly positive, inconclusive or negative according to Frei and Würgler (1988). Statistical consideration and step by step calculation are shown in Appendix A.

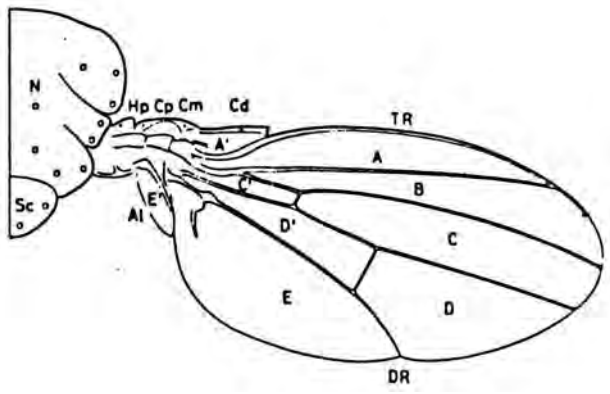


Figure 13 Normal half mesothorax showing the regions A-E of the wing surface scored for spots (Graf et al., 1984)

3.5.5 Antimutagenicity Assay

The amount of sample that provided more than 50% survival of adult files and did not express their genotoxicity were evaluated for antimutagenicity in co-administration study using urethane or the *in vivo* formed *N*-nitrosomethylurea as mutagen (Figure 14).

3.5.5.1 Against Urethane

Virgin *ORR;flr*^{3} females and *mwh* males were mated on the standard medium. After mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred (using a fine paintbrush) to experimental medium from 3.5.3 containing urethane (20 mM), negative control medium and positive control medium (urethane). They were incubated at 25 ± 1^{o}C until pupation. The surviving adult flies were collected after pupation and were preceded as of mutagenicity study.

3.5.5.2 Against *In Vivo* Formed *N*-Nitrosomethylurea

Virgin *ORR;flr*^{3} females and *mwh* males were mated on the standard medium. After mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred (using a fine paintbrush) to experimental medium from 3.5.3, negative control and positive control medium (nitrosomethylurea). The combination of sodium nitrite (36 mM, 1 ml) and methylurea (10 mM, 1 ml) was used as positive

control. Sodium nitrite solution (18 mM, 2ml) or methylurea solution (5 mM, 2 ml) were substituted for water in each experimental medium to served as the sample control (Rincon et al., 1998)

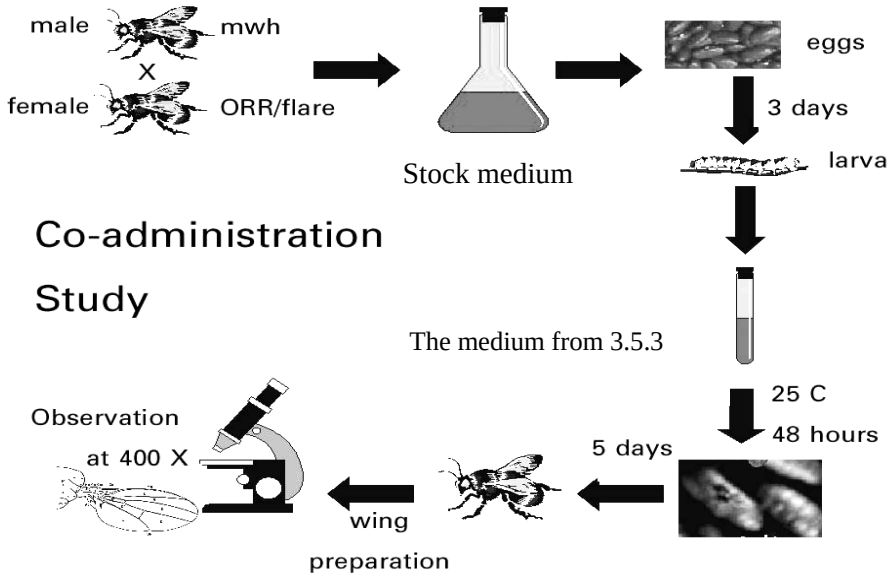


Figure 14 Antimutagenicity study of each rice on mutagenicity of urethane induced wing spots of *Drosophila melanogaster* in co-administration study
(By contesy of Assoc. Prof. Kaew Kangsadalampai)

3.5.6 Data Evaluation

The antimutagenicity was estimated as suggested by Abraham et al. (1994). The reduction of number of spots per wing induced by urethane or *in vivo* formed nitrosomethylurea will be calculated as following:

Percentage of inhibition = 100(a - b)/a

Where a is the frequency of spots induced by urethane or nitrosomethylurea alone and b the frequency of spots induced by urethane or nitrosomethylurea in the presence of rice sample. It is proposed that percentage of inhibition between 0-20 represented a negligible effect while expression of percent inhibition between 20-40, 40-60 and more than 60 are the evidences of weak, moderate and strong antimutagenicity, respectively.

CHAPTER IV

RESULTS

4.1 Antioxidant Activity and Total Phenolic Contents

The antioxidant activity of each pigmented rice extract expressed as mM of Trolox equivalent antioxidant capacity (TEAC) is shown in Table 6. The percentages of radical scavenging activity of the sample are between 1.75 and 33.76%. From all results of DPPH assay, fermented black glutinous rice showed the highest scavenging activity. In addition, the FRAP values (μM) are between 51.11 and 582.37; fermented black glutinous rice had the highest antioxidant activity.

Total phenolic contents are determined using the Folin-Ciocalteu reagent and expressed as milligram per liter of gallic acid equivalent (GAE). Total phenolic content of each sample were in the range from 4.38 to 32.86 mg/l. Cooked Hom Nil rice contained the least amount of phenolic compound while fermented Hom Nil rice had the highest value. Each result is given as the percent alteration from that of corresponding raw rice as shown in Table 6. The antioxidant activity of cooked rice is less than that of its corresponding raw rice in the range between 62.38 to 64.33% (DPPH assay), 64.78 to 81.02% (FRAP assay) and 62.77 to 68.80 (total phenolic content determination). Interestingly, the antioxidant activity of most fermented rices increase in the range of 321.90 to 564.14% (DPPH assay), 14.18 to 65.85 (FRAP assay) and 9.76 to 133.83% (total phenolic content determination).

Table 6 Antioxidant activity and total phenolic contents of each sample

Sample	DPPH assay			FRAP assay		Total phenolic content	
	TEAC[a]	%Scavenging	%Alteration[d] of %Scavenging	FRAP[b] value	%Alteration[d] of FRAP value	GAE[c]	% Alteration[d] of GAE
Hom Nil rice							
Raw	0.06	4.91	-	269.36	-	14.05	-
Cooked	0.02	1.75	-64.33	51.11	-81.02	4.38	-68.80
Fermented	0.10	20.71	321.90	307.56	14.18	32.86	133.83
Black glutinous rice							
Raw	0.06	5.08	-	351.15	-	23.68	-
Cooked	0.02	1.91	-62.38	123.67	-64.78	8.81	-62.77
Fermented	0.41	33.76	564.14	582.37	65.85	25.99	9.76

All values are the means of three measurements

[a]TEAC (Trolox equivalent antioxidant capacity) was expressed as mg/g dry weight of sample.

[b]FRAP assay = The FRAP values of the extracts were expressed as mg of ferrous iron (Fe (II))/g dry weight of sample.

[c]GAE (The gallic acid equivalent) was expressed as mg/g dry weight of sample.

[d]% Alteration = (value of cooked or fermented rice - value of raw rice) x 100/value of raw rice

4.2 Survival Rate of Adult Flies and Mutagenicity of Samples

The data in Tables 7 and 8 show the numbers of surviving flies and mutagenicity of rice sample obtained from the larvae brought up on sample medium containing varied amounts of rice sample, negative control medium, and urethane positive control medium (20 mM of urethane). The result indicated that each sample was neither mutagenic nor too toxic on the 3-day old larvae. They gave numbers of surviving flies higher than 50% and they did not significantly induce the frequencies of mutant spots at any testing amounts to be higher than that of the negative control medium. The experimental medium containing 0.25 g of each sample was chosen for further antimutagenicity test.

Table 7 The survival data of adult flies obtained from 100 of 3-days old larvae (*mwh*/*flr*^{3}) introduced to each sample medium, negative control medium, and urethane positive control medium containing 20 mM urethane

Medium	Amount of sample(g)	Percentage of surviving flies	
		Trial 1	Trial 2
Negative control		100	100
Positive control		87	81
Raw Hom Nil rice	0.05	95	92
	0.10	92	93
	0.15	97	90
	0.20	100	96
	0.25	94	96
Negative control		97	93
Positive control		79	74
Cooked Hom Nil rice	0.05	89	93
	0.10	90	83
	0.15	74	86
	0.20	77	76
	0.25	83	85
Negative control		82	91
Positive control		79	74
Fermented Hom Nil rice	0.05	94	83
	0.10	80	91
	0.15	93	97
	0.20	91	80
	0.25	87	86

Table 7 The survival data of adult flies obtained from 100 of 3-days old larvae (*mwh*/*flr*^{3}) introduced to each sample medium, negative control medium, and urethane positive control medium containing 20 mM urethane (continued)

Medium	Amount of sample (g)	Percentage of surviving files	
		Trial 1	Trial 2
Negative control		90	100
Positive control		80	72
Raw black glutinous rice	0.05	98	92
	0.10	96	97
	0.15	91	93
	0.20	100	94
	0.25	95	100
Negative control		95	98
Positive control		78	82
Cooked black glutinous rice	0.05	82	89
	0.10	80	77
	0.15	90	94
	0.20	92	91
	0.25	84	81
Negative control		98	100
Positive control		81	73
Fermented black glutinous rice	0.05	89	95
	0.10	92	96
	0.15	93	87
	0.20	86	91
	0.25	94	86

Table 8 Mutagenicity of each rice sample reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in trial 1

Sample			Spot per wing[a] (No. of spots from 40 wings)			
Rice	Medium	Amount (g/tube)	Small single m=2	Large single m=5	Twin m=5	Total m=2
Hom Nil						
Raw	Negative control	-	0.275 (11)	0.075 (3)	0.050 (2)	0.400 (16)
	Positive control (urethane)	-	7.900 (316)+	2.725 (109)+	0.400 (16)+	11.025 (441)+
	sample	0.05	0.225 (9)-	0	0	0.225 (9)-
		0.10	0.225 (9)-	0	0	0.225 (9)-
		0.15	0.225 (9)-	0.050 (2)-	0	0.275 (11)-
		0.20	0.300 (12)i	0	0.025 (1)i	0.325 (13)-
		0.25	0.300 (12)i	0.050 (2)-	0.025 (1)i	0.375 (15)-
Cooked	Negative control	-	0.175 (7)	0.075 (3)	0	0.250 (10)-
	Positive control (urethane)	-	9.300 (372)+	3.150 (126)+	0.550 (22)+	13.000 (520)+
	sample	0.05	0.375 (15)i	0	0	0.375 (15)i
		0.10	0.175 (7)i	0	0.025 (1)-	0.200 (8)-
		0.15	0.175 (7)i	0	0.025 (1)-	0.200 (8)-
		0.20	0.100 (4)i	0.025 (1)-	0	0.125 (5)-
		0.25	0.125 (5)i	0.025 (1)-	0	0.150 (6)-

Table 8 Mutagenicity of each rice sample reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in trial 1 (continued)

Sample			Spot per wing[a] (No. of spots from 40 wings)			
Rice	Medium	Amount (g/tube)	Small single m=2	Large single m=5	Twin m=5	Total m=2
Fermented	Negative control	-	0.275 (11)	0.075 (3)	0.050 (2)	0.400 (16)
	Positive control (urethane)	-	7.900 (316)+	2.725 (109)+	0.400 (16)+	11.025 (441)+
	sample	0.05	0	0.100 (4)i	0	0.100 (4)-
		0.10	0.100 (4)i	0.025 (1)i	0	0.150 (5)i
		0.15	0.050 (2)-	0.025 (1)i	0	0.075 (3)-
		0.20	0.025 (1)-	0.025 (1)i	0	0.050 (2)-
		0.25	0.025 (1)-	0	0.025 (1)i	0.050 (2)-
Black glutinous rice						
Raw	Negative control	-	0.275 (11)	0.075 (3)	0.050 (2)	0.400 (16)
	Positive control (urethane)	-	7.900 (316)+	2.725 (109)+	0.400 (16)+	11.025 (441)+
	sample	0.05	0.250 (10)i	0	0	0.250 (10)-
		0.10	0.100 (4)-	0	0	0.100 (4)-
		0.15	0.200 (8)-	0.025 (1)-	0	0.225 (9)-
		0.20	0.375 (15)i	0	0	0.375 (15)-
		0.25	0.350 (14)i	0.200 (8)i	0	0.550 (22)i

Table 8 Mutagenicity of each rice reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in trial 1 (continued)

Sample			Spot per wing[a] (No. of spots from 40 wings)			
Rice	Medium	Amount (g/tube)	Small single m=2	Large single m=5	Twin m=5	Total m=2
Cooked	Negative control	-	0.175 (7)	0.075 (3)	0	0.250 (10)
	Positive control (urethane)	-	9.300 (372)+	3.150 (126)+	0.550 (22)+	13.000 (520)+
	sample	0.05	0.325 (13)i	0.025 (1)-	0.025 (1)-	0.375 (15)i
		0.10	0.075 (3)i	0.025 (1)-	0	0.100 (4)-
		0.15	0.150 (6)i	0	0.100 (4)-	0.250 (10)i
		0.20	0.250 (10)i	0.025 (1)-	0.025 (1)-	0.300 (12)i
		0.25	0.150 (6)i	0	0	0.150 (6)-
Fermented	Negative control	-	0.150 (6)	0	0.025 (1)	0.175 (7)
	Positive control (urethane)	-	9.875 (395)+	4.625 (185)+	0.775 (31)+	15.275 (611)+
	sample	0.05	0.150 (6)i	0	0.025 (1)i	0.175 (7)i
		0.10	0.050 (2)-	0	0	0.050 (2)-
		0.15	0.150 (6)i	0	0.100 (4)-	0.250 (10)i
		0.20	0.075 (3)-	0	0	0.075 (3)-
		0.25	0.050 (2)-	0	0	0.050 (2)-

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparisons with distilled water: + = positive; - = negative; i = inconclusive; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$ and one-sided statistical test.

Table 9 Mutagenicity of each rice sample reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in trial 2

Sample			Spot per wing[a] (No. of spots from 40 wings)			
Rice	Medium	Amount (g/tube)	Small single m=2	Large single m=5	Twin m=5	Total m=2
Hom Nil						
Raw	Negative control	-	0.475 (19)	0.075 (3)	0	0.550 (22)
	Positive control (urethane)	-	9.875 (395)+	2.150 (86)+	0.550 (22)+	12.575 (503)+
	sample	0.05	0.225 (9)-	0	0	0.225 (9)-
		0.10	0.175 (7)-	0.025 (1)-	0	0.200 (8)-
		0.15	0.150 (6)-	0	0.075 (3)i	0.225 (9)-
		0.20	0.350 (14)-	0.025 (1)-	0	0.375 (15)-
		0.25	0.275 (11)-	0.250 (10)+	0.050 (2)i	0.575 (23)i
Cooked	Negative control	-	0.175 (7)	0.050 (2)	0	0.225 (9)
	Positive control (urethane)	-	10.300 (412)+	2.100 (84)+	0.450 (18)+	12.850 (514)+
	sample	0.05	0.275 (11)i	0.150 (5)i	0.050 (2)i	0.450 (18)+
		0.10	0.050 (2)-	0.025 (1)-	0	0.075 (3)-
		0.15	0.050 (2)-	0.025 (1)i	0	0.075 (3)-
		0.20	0.200 (8)i	0.025 (1)-	0	0.225 (9)i
		0.25	0.150 (6)i	0.075 (3)-	0.025 (1)i	0.25 (10)i

Table 9 Mutagenicity of each rice sample reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in trial 2 (continued)

Sample			Spot per wing[a] (No. of spots from 40 wings)			
Rice	Medium	Amount (g/tube)	Small single m=2	Large single m=5	Twin m=5	Total m=2
Fermented	Negative control	-	0.150 (6)	0	0.025 (1)	0.175 (7)
	Positive control (urethane)	-	8.450 (338)+	2.850 (114)+	0.675 (27)+	11.975 (479)
	sample	0.05	0.250 (10)i	0.075 (3)i	0	0.325 (13)i
		0.10	0.050 (2)-	0	0	0.050 (2)-
		0.15	0.050 (2)-	0.025 (1)i	0	0.075 (3)-
		0.20	0.075 (3)-	0.025 (1)i	0	0.100 (4)-
		0.25	0.025 (1)-	0.050 (2)i	0	0.075 (3)-
Black glutinous rice						
Raw	Negative control	-	0.475 (19)	0.075 (3)	0	0.550 (22)
	Positive control (urethane)	-	9.875 (395)+	2.150 (86)+	0.550 (22)+	12.575 (503)+
	sample	0.05	0.250 (10)-	0	0	0.250 (10)-
		0.10	0.200 (8)-	0.050 (2)-	0	0.250 (10)-
		0.15	0.250 (10)-	0	0	0.250 (10)-
		0.20	0.375 (15)-	0	0	0.375 (15)-
		0.25	0.350 (14)-	0.100 (4)i	0.025 (1)i	0.475 (19)-

Table 9 Mutagenicity of each rice reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in trial 2 (continued)

Sample			Spot per wing[a] (No. of spots from 40 wings)			
Rice	Medium	Amount (g/tube)	Small single m=2	Large single m=5	Twin m=5	Total m=2
Cooked	Negative control	-	0.175 (7)	0.075 (3)	0	0.250 (10)
	Positive control (urethane)	-	9.300 (372)+	3.150 (126)+	0.550 (22)+	13.000 (520)+
	sample	0.05	0.075 (3)-	0.025 (1)-	0	0.100 (4)-
		0.10	0	0.025 (1)-	0	0.025 (1)-
		0.15	0.150 (6)i	0	0.100 (4)i	0.250 (10)i
		0.20	0.350 (14)i	0.100 (4)i	0.025 (1)i	0.475 (19)+
		0.25	0.225 (9)i	0	0	0.225 (9)i
Fermented	Negative control	-	0.150 (6)	0	0.025 (1)	0.175 (7)
	Positive control (urethane)	-	8.450 (338)+	2.850 (114)+	0.675 (27)+	11.975 (479)+
	sample	0.05	0.200 (8)i	0	0	0.200 (8)i
		0.10	0	0.050 (2)i	0	0.050 (2)-
		0.15	0.150 (6)i	0	0.100 (4)i	0.250 (10)i
		0.20	0.150 (5)i	0	0.025 (1)i	0.150 (6)i
		0.25	0.075 (3)-	0	0	0.075 (3)-

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparisons with distilled water: + = positive; - = negative; i = inconclusive; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$ and one-sided statistical test.

4.3 Antimutagenicity of Samples against Standard Mutagens

4.3.1 Urethane The antimutagenicity of rice from two kinds of pigmented rice (raw, cooked and fermented rice) against urethane is shown in Table 9. All samples could decrease the frequencies of mutant spots in larvae treated with urethane. The percentage of inhibition on urethane mutagenicity (antimutagenicity) was calculated to show the relationship between the mutagenicity of urethane in the presence and absence of each treated rice in fly medium (Figure 16 and 17). Each sample possessed negligible to weak inhibitory effect on mutagenicity of urethane. Concerning to Hom Nil rice, it was indicated that the fermented one possessed higher antimutagenicity than the other did. However, it was not able to observe the same effect on the antimutagenicity of black glutinous rice.

4.3.2 The *in vivo* formed nitrosomethylurea Tables 10 and 11 show the effect of each rice sample (treated with sodium nitrite or methylurea) on number of wing spots of *Drosophila melanogaster*, respectively. The result indicated that no significantly increased the frequencies of total spots per wing to be higher than that of the negative control. The mixture of nitrite and methylurea administered to the 3-day larvae represented an example of *in vivo* nitrosation that the product was nitrosomethylurea as suggested by Rincon et al. (1998). The effect of each rice sample on the mutagenicity of the *in vivo* formed nitrosomethylurea is shown in Table 12 and Figure 18-19. The result indicated that Hom Nil rice had negligible inhibitory effect while black glutinous rice had weak to moderate inhibitory effect. In addition, fermented black glutinous rice possessed the highest antimutagenicity against the reaction product of sodium nitrite and methylurea.

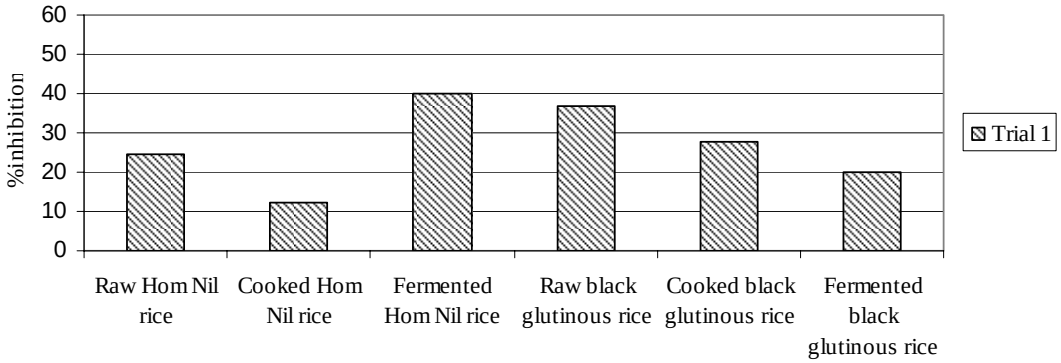


Figure 16 Antimutagenicity against urethane (20 mM) of Hom Nil rice and black glutinous rice in trial 1

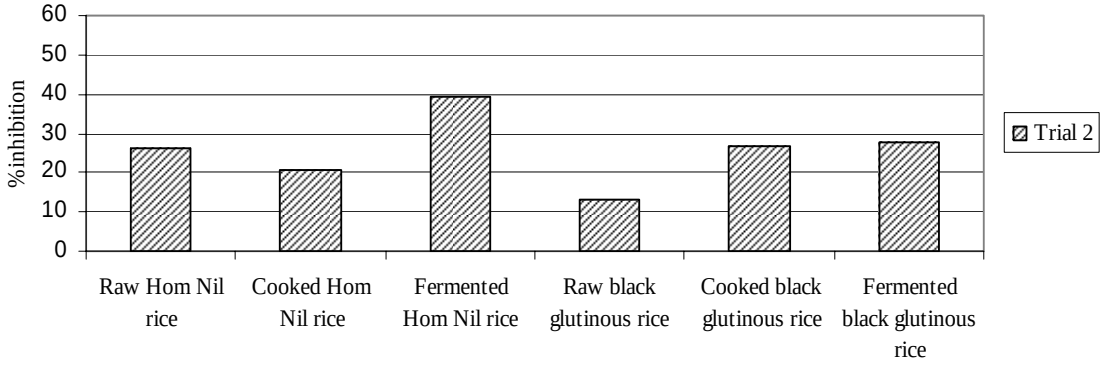


Figure 17 Antimutagenicity against urethane (20 mM) of Hom Nil rice and black glutinous rice in trial 2

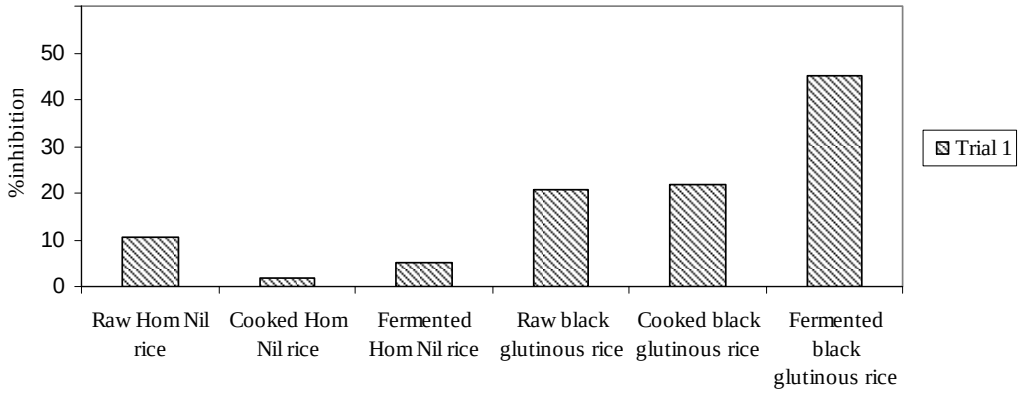


Figure 18 Antimutagenicity against the *in vivo* formed of nitrosomethylurea of Hom Nil rice and black glutinous rice in trial 1

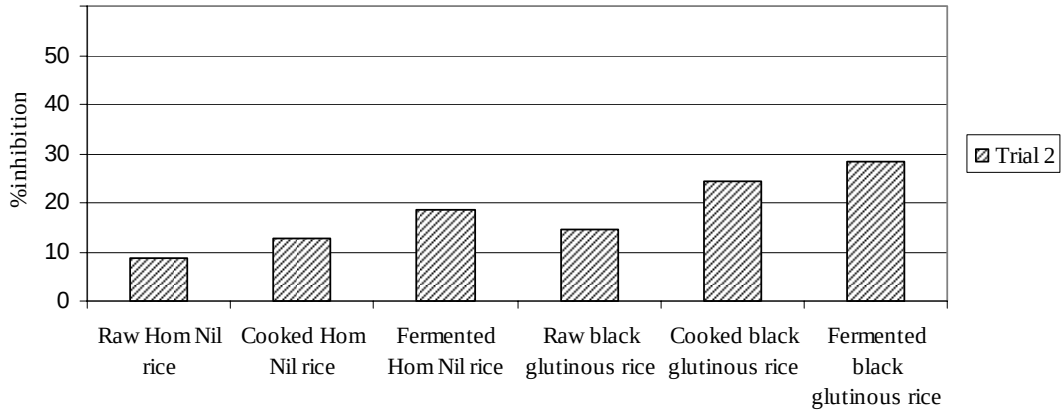


Figure 19 Antimutagenicity against the *in vivo* formed of nitrosomethylurea of Hom Nil rice and black glutinous rice in trial 2

Table 10 Antimutagenicity of each rice sample against urethane (20 mM) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in the co-administration study

Trial	Medium	Spot per wing[a] (No. of spots from 40 wings)				%inhibition (rate)[b]
		Small single m=2	Large single m=5	Twin m=5	Total m=2	
1	Negative control	0.300 (12)	0.025 (1)	0	0.325 (13)	-
	Positive control (urethane)	8.325 (333)+	5.200 (208)+	1.825 (73)+	15.35 (614)+	-
	Experimental (urethane+sample)					
	Hom Nil rice					
	Raw	6.750 (270)+	4.075 (163)+	0.075(30)+	11.575 (463)+	24.593 (w)
	Cooked	6.400 (256)+	5.725 (229)+	1.350 (54)+	13.475 (539)+	12.215 (n)
	Fermented	5.050 (202)+	2.850 (114)+	1.300 (52)+	9.200 (368)+	40.065 (m)
	Black glutinous rice					
	Raw	5.684 (216)+	3.395 (129)+	1.131 (43)+	10.210 (388)+	36.808 (m)
	Cooked	6.575 (263)+	3.750 (150)+	0.750 (30)+	11.075 (443)+	27.851(w)
	Fermented	6.450 (258)+	4.500 (180)+	1.350 (54)+	12.300 (492)+	19.870 (n)

Table 10 Antimutagenicity of each rice sample against urethane (20 mM) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in the co-administration study (continued)

Trial	Medium	Spot per wing[a] (No. of spots from 40 wings)				%inhibition (rate)[b]
		Small single m=2	Large single m=5	Twin m=5	Total m=2	
2	Negative control	0.350 (14)	0.025 (1)	0.025 (1)	0.400 (16)	-
	Positive control (20 mM urethane)	7.025 (281)+	4.800 (192)+	0.450 (18)+	12.275 (491)+	-
	Experimental (urethane+sample)					
	Hom Nil rice					
	Raw	4.050 (162)+	4.450 (178)+	0.650 (22)+	9.050 (362)+	26.273 (w)
	Cooked	4.450 (178)+	4.250 (170)+	1.025 (41)+	9.725 (389)+	20.773 (w)
	Fermented	3.763 (143)+	3.710 (141)+	0.395 (15)+	7.868 (299)+	39.107 (w)
	Black glutinous rice					
	Raw	4.900 (196)+	4.650 (186)+	1.100 (44)+	10.650 (426)+	13.238 (n)
	Cooked	4.250 (170)+	3.850 (154)+	0.900 (36)+	9.000 (360)+	26.680 (w)
	Fermented	3.500 (140)+	4.650 (186)+	0.700 (28)+	8.850 (354)+	27.902 (w)

[a]statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with distilled water:+ = positive; - = negative; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical tests. [b]n = negligible, w = weak antimutagenicity, m = moderate antimutagenicity, s = strong antimutagenicity

Table 11 Effect of mutagenicity of each rice sample simultaneously administered with sodium nitrite in *Drosophila melanogaster*

trial	Medium		Spot per wing[a] (No. of spots from 40 wings)			
			Small single m=2	Large single m=5	Twin m=5	Total m=2
1	Negative control		0.200 (8)	0.050 (2)	0	0.250 (10)
	Nitrite control (18 mM sodium nitrite)		0.425 (17)	0.075 (3)	0	0.500 (20)
	Experimental (Nitrite+sample)					
	Hom Nil rice					
		Raw	0.175 (7)i	0.025 (1)-	0.025 (1)i	0.225 (9)-
		Cooked	0.075 (3)-	0.075 (3)i	0	0.150 (6)-
		Fermented	0.225 (9)i	0.100 (4)i	0.050 (2)i	0.375 (15)i
	Black glutinous rice					
		Raw	0.125 (5)-	0.075 (3)i	0.025 (1)i	0.200 (8)-
		Cooked	0.250 (10)i	0.075 (3)i	0	0.325 (13)i
		Fermented	0.100 (4)-	0.050 (2)-	0.025 (1)i	0.175 (7)-

Table 11 Effect of mutagenicity of each rice sample simultaneously administered with sodium nitrite in *Drosophila melanogaster* (continued)

trial	Medium		Spot per wing[a] (No. of spots from 40 wings)			
			Small single	Large single	Twin	Total
			m=2	m=5	m=5	m=2
2	Negative control		0.375 (15)	0.050 (2)	0.025 (1)	0.450 (18)
	Nitrite control (18 mM sodium nitrite)		0.375 (15)	0.225 (9)	0.100 (4)	0.700 (28)
	Experimental (Nitrite+sample)					
	Hom Nil rice					
		Raw	0.225 (9)-	0.050 (2)i	0.050 (2)-	0.325 (13)-
		Cooked	0.450 (18)i	0.200 (8)+	0.050 (2)-	0.700 (28)+
		Fermented	0.325 (13)-	0.100 (4)i	0	0.425 (17)i
	Black glutinous rice					
		Raw	0.375 (15)i	0.025 (1)-	0.025 (1)i	0.425 (17)i
		Cooked	0.425 (17)i	0.050 (2)i	0.025 (1)i	0.500 (20)i
		Fermented	0.300 (12)-	0.075 (3)i	0	0.375 (15)i

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparisons with distilled water: + = positive; - = negative; i = inconclusive; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$ and one-sided statistical test.

Table 12 Effect of mutagenicity of each rice sample simultaneously administered with methylurea in *Drosophila melanogaster*

trial	Medium		Spot per wing[a] (No. of spots from 40 wings)			
			Small single	Large single	Twin	Total
			m=2	m=5	m=5	m=2
1	Negative control		0.200 (8)	0.050 (2)	0	0.250 (10)
	Methylurea control		0.150 (6)	0.025 (1)	0.025 (1)	0.200 (8)
	Experimental (Methylurea+sample)					
	Hom Nil rice					
		Raw	0.100 (4)-	0.050 (2)i	0.025 (1)i	0.175 (7)-
		Cooked	0.050 (2)-	0.075 (3)i	0.050 (2)i	0.175 (7)-
		Fermented	0.150 (6)-	0.050 (2)i	0	0.200 (8)-
	Black glutinous rice					
		Raw	0.150 (6)-	0.050 (2)i	0	0.200 (8)-
		Cooked	0.150 (6)-	0.075 (3)i	0	0.225 (9)i
		Fermented	0.150 (6)-	0.075 (3)i	0	0.225 (9)i

.

Table 12 Effect of mutagenicity of each rice sample simultaneously administered with methylurea in *Drosophila melanogaster* (continued)

trial	Medium	Spot per wing[a] (No. of spots from 40 wings)			
		Small single	Large single	Twin	Total
		m=2	m=5	m=5	m=2
2	Negative control	0.300 (12)	0.025 (1)	0	0.325 (13)
	Methylurea control	0.175 (7)	0.075 (3)	0	0.250 (10)
	Experimental (Methylurea+sample)				
	Hom Nil rice				
	Raw	0.075 (3)-	0.075 (3)i	0	0.150 (6)-
	Cooked	0.175 (7)-	0.050 (2)i	0.025 (1)i	0.250 (10)-
	Fermented	0.175 (7)-	0.025 (1)-	0.025 (1)i	0.225 (9)-
	Black glutinous rice				
	Raw	0.125 (5)-	0.075 (3)i	0.025 (1)i	0.200 (8)-
	Cooked	0.125 (5)-	0.075 (3)i	0	0.200 (8)-
	Fermented	0.100 (4)-	0.050 (2)i	0.025 (1)i	0.175 (7)-

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparisons with distilled water: + = positive; - = negative; i = inconclusive; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$ and one-sided statistical test

Table 13 Antimutagenicity of each rice sample against the reaction product of 36 mM sodium nitrite and 10 mM methylurea (*in vivo* formed nitrosomethylurea; NMU) in *Drosophila melanogaster* in the co-administration study

Trial	Medium	Spot per wing[a] (No. of spots from 40 wings)				%inhibition (rate)[b]
		Small single m=2	Large single m=5	Twin m=5	Total m=2	
1	Negative control	0.425 (17)	0.200 (8)	0	0.625 (25)	-
	Positive control (nitrosomethylurea)	3.925 (157)+	2.700 (108)+	0.625 (25)+	7.250 (290)+	-
	Experimental (NMU+sample)					
	Hom Nil rice					
	Raw	2.825 (113)+	2.425 (97)+	0.475 (19)+	6.475 (259)+	10.690 (n)
	Cooked	2.925 (117)+	3.550 (142)+	0.650 (26)+	7.125 (285)+	1.724 (n)
	Fermented	3.150 (126)+	3.000 (120)+	0.725 (29)+	6.875 (275)+	5.172 (n)
	Black glutinous rice					
	Raw	2.575 (103)+	2.225 (89)+	0.950 (38)+	5.750 (230)+	20.690 (w)
	Cooked	2.475 (99)+	2.675 (107)+	0.525 (21)+	5.675 (227)+	21.724 (w)
	Fermented	1.875 (75)+	1.725 (69)+	0.375 (15)+	3.975 (159)+	45.172 (m)

Table 13 Antimutagenicity of each rice sample against the reaction product of 36 mM sodium nitrite and 10 mM methylurea (*in vivo* formed nitrosomethylurea; NMU) in *Drosophila melanogaster* in the co-administration study (continued)

Trial	Medium	Spot per wing[a] (No. of spots from 40 wings)				%inhibition (rate)[b]
		Small single m=2	Large single m=5	Twin m=5	Total m=2	
2	Negative control	0.375 (15)	0.05 (2)	0.025 (1)	0.450 (18)	-
	Positive control (nitrosomethylurea)	4.900 (196)+	2.350 (94)+	0.900 (36)+	8.150 (326)+	-
	Experimental (NMU+sample)					
	Hom Nil rice					
	Raw	3.900 (156)+	3.075 (123)+	0.475 (19)+	7.450 (298)+	8.589 (n)
	Cooked	3.475 (139)+	3.025 (121)+	0.600 (24)+	7.100 (284)+	12.883 (n)
	Fermented	2.975 (119)+	2.850 (114)+	0.800 (32)+	6.625 (265)+	18.712 (n)
	Black glutinous rice					
	Raw	3.700 (148)+	2.750 (110)+	0.500 (20)+	6.950 (278)+	14.724 (n)
	Cooked	3.375 (135)+	2.125 (85)+	0.675 (27)+	6.175 (247)+	24.233 (w)
	Fermented	2.800 (112)+	2.450 (98)+	0.575 (23)+	5.825 (233)+	28.528 (w)

[a]statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with distilled water: + = positive; - = negative; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical tests. [b]n = negligible, w = weak antimutagenicity, m = moderate antimutagenicity, s = strong antimutagenicity

CHAPTER V

DISCUSSION

5.1 Effect of Cooking and Fermentation on Antioxidant Activity and Total Phenolic Contents

This study indicated that black glutinous rice and Hom Nil rice possessed the antioxidant activity and contained phenolic compounds. Previous studies reported the antioxidant activity and amount of polyphenolic compounds of black glutinous rice. Htwe (2009) reported that the antioxidant activity (DPPH radical scavenging activity) of black rice and black sticky rice are 83.91 and 81.15%, respectively while Tananuwong and Tewaruth (2010) found that antioxidant activities (FRAP assay) of black glutinous rice crude extracts are 9.31-11.46 μmol trolox/g flour. The differences of antioxidant activity and total phenolic content observed between the present study and other study might be due to variety. The antioxidant activity and polyphenolic compounds of black rice were also reported elsewhere. Therefore, the present investigation strongly support that consumption of pigmented rice should be good to most health concerning consumers.

Major antioxidant found in black rice is anthocyanin such as cyanindin-3-glucoside and peonidin-3-glucoside (Yawadio et al., 2007); they possess strong antioxidant activity (Tananuwong and Tewaruth, 2010; Tewaruth, 2007). Unfortunately, anthocyanin is relatively unstable and easily oxidized by many factors; for example, pH, temperature and UV radiation (Francis, 1989). Cooked rice in this investigation had less antioxidant activity and the content of phenolic compounds than those of the raw one; this confirmed that high temperature might degrade anthocyanin. The present result also confirmed Chanphrom (2007) who concluded that cooking rice in an electric rice cooker reduced antioxidant activity of purple rice (black glutinous rice) approximately 46.4% of the raw rice.

The present experiment has demonstrated that fermentation could increase the antioxidant activity and amount of polyphenolic compounds compared to that of its corresponding raw and cooked rice. In general, phenolic compounds are found in conjugated forms through hydroxyl groups with sugar and glycosides (Robbins, 1980). Yeast cake used as the starter organisms in the present study is the mixed

cultures containing yeast, mold and bacteria (Washirapa, 2002). It was found that Endomycopsis in Thai yeast cake could create extra cellular high glucosidase activity (มนตรี เชาวน์สังเกต, 2521). McCue and Shetty (2003); Lee and Chou (2006) suggested that fungi were ability of producing β-glucosidase that promoted cleavage of the β-glycosyl bond in the black soybean glucoside isoflavones and released aglycones. Juan et al. (2010) suggested that the increase of anthocyanins in the fermented black soybean might be due to the catalytic release of anthocyanins by the action of β-glucosidase produced during fermentation. It was supported by Kwak et al. (2007) who revealed that Chungkookjang, a ferment soybean paste in Korea had stronger antioxidant activity than that of unfermented steamed soybeans and they suggested that it was probably due to aglycone isoflavone and malonylglycoside isoflavone as well as total phenolic contents increased during fermentation. Lee and Chou (2006) also reported that fermentation increased the content of aglycone e.g. the bioactive isoflavone of the black soybeans while Đorđević et al. (2009) indicated that fermentation could enhance the levels of antioxidant activity and also improve the bioactive potential of the cereal. Since many studies revealed that the diet containing antioxidant might have antimutagenic property (Shon et al., 2004; Brahma et al., 2009; Zahin et al., 2010) and both types of rice possessed antioxidant activity and contained phenolic compounds, they might have a beneficial effect namely, antimutagenicity for consumers.

5.2 Antimutagenicity against Urethane of Pigmented Rice

The survival rate of adult flies fed on each sample was higher than 50% with normal size. The result indicated that none of the samples was mutagenic since they did not significantly induce the frequencies of mutant spots at any testing amounts higher than that of negative control medium. Such samples seem to be safe for consumers and possible to develop them in food industry.

The result presented that all samples could reduce the mutagenicity of urethane. Because of urethane needs metabolic activation using cytochrome P-450 enzyme system (Schlatter and Lutz, 1990) to be the carcinogenic active metabolite namely, vinyl epoxide (Dahl et al., 1978), which is detoxified with glutathione-*S*-transferase (GST) conjugation (Kemper et al., 1995). The antimutagenicity of the rice samples might be due to some active compounds acting as an enzyme modifier (an

inhibitor of cytochrome P-450 enzyme system and/or an inducer of GST) or as a urethane trapper. Some evidences showed that the levels of GST were induced by phenolics (Ferguson et al., 2004) and flavonoids (Galati and O'Brien, 2004; Moon et al., 2006). It was reported that the major pigments found in black rice were a group of natural compound named anthocyanin (Zhang et al., 2006), which is one class of flavonoids. Singletary et al. (2007) reported the evidence that anthocyanin crude extract from grapes and delphinidin were associated with a significant increasing activities of the phase II detoxification enzymes, namely GST and NAD(P)H: quinine reductase 1. Therefore, it might explain that the pigmented rice samples could act against the mutagenicity of urethane in this experiment.

Some antioxidant such as anthocyanin might act as free radical scavenger in diminishing the free radical generated during metabolism of urethane when each rice sample was incorporated into the fly medium in this study. *N*-hydroxyurethane, a urethane metabolite (Boyland and Nery, 1965; Nery, 1968), is hydrolyzed by esterase to generate hydroxylamine and exerts its carcinogenic effect in multiple organs via generating $O_2^{\cdot -}$ and $NO^{\bullet}$ to cause oxidation and depurination of DNA (Sakano et al., 2002). It was Stich et al. (1982) who suggested that some antioxidants had an inhibitory effect on genotoxic action of several known mutagens possibly by decreasing the level of cytochrome P-450.

5.3 Effect of Processing on Antimutagenicity of Rice

The result that the antimutagenicity against urethane of cooked Hom Nil rice was lower than that of raw rice might be due to the decreasing of active compound in cooked rice. The possible antimutagens might be anthocyanins. However, they are sensitive to many factors including high temperature (Francis, 1989). Henryk et al. (2001) also presented that bioactive compounds in grains such as tocopherols, tocotrienols, reduced glutathione and microelements (Cu, Zn and Mn) decreased by extrusion cooking (processing temperatures of 120-200 ºC).

The antimutagenicity of fermented Hom Nil rice was higher than that of its corresponding cooked rice. It might be due to higher content of total phenolics and aglycones in fermented rice. Edenharder and Tang (1997) suggested that the flavonoid glycosides were less antimutagenic in the Ames test than the corresponding aglycone. Some studies (Park et al., 2003; Hung et al., 2007) reported that the higher

antimutagenic activity found in fermented black soybean extracts was generally associated with the higher contents of total phenolics, anthocyanin and aglycones in fermented black soybean. However, it was also possible that some other antimutagenic metabolite might occur during the fermentation process of the rice samples. Researches on analysis of components of fermented products of rice are still required to explain their higher antimutagenic activity.

5.4 Antimutagenicity against *in vivo* formed Nitrosomethylurea

This study was designed to audit the effect of each sample on the mutagenicity of nitrosomethylurea, a direct acting mutagen (Deng, 2000), formed endogenously in gastric lumen of *Drosophila melanogaster* and could induce glandular stomach cancer in mice (Tomano et al., 1995). The negative result of nitrite treated samples indicated there might either no substance that could interact with nitrite to form mutagenic species in the lumen of the tester strain or the detoxifying system of the tester strain could handle such potential mutagen. In addition, the evaluation on the mutagenicity of each sample simultaneously administered with methylurea was to assure that no nitrite possibly contaminated to the sample during growing rice.

The antimutagenicity of rice samples against mutagenicity of nitrosomethylurea might possibly accompany by a reduction in the amount of *N*-nitrosomethylurea formed during the reaction between the methylurea and sodium nitrite (Cabllero-Salazar et al., 2002). Some phytochemicals in rice, such as flavonoids and phenolic compounds, might scavenge nitrite ions. Thus, nitrite ions were not-available for the methylurea nitrosation. Nagabhushan et al. (1989) found that hydroxychavicol, the phenolic compounds isolated from betel leaf (piper betel), had the inhibitory effect on the nitrosation of methylurea *in vitro* by scavenging of nitrite ions. Cabllero-Salazar et al. (2002) used Ames test to elucidate the anti-nitrosating properties of some vegetable extracts by adding each of them to the methylurea solution prior to the addition of the sodium nitrite. Their result showed that some compounds of vegetable extracts such as vitamin C, carotenoids and phenolics could reduce the amount of *N*-nitrosomethylurea and also its antimutagenicity.

The antimutagenicity of rice against nitrosomethylurea might also be competitively inhibited by other *in vivo* formed compounds. Some components of rice added to the fly medium might react with sodium nitrite and formed any

compounds in the same manner as of nitrosomethylurea; such compounds might compete with nitrosomethylurea in interacting with DNA but posed no or less mutagenicity. Ferguson et al. (2004) suggested that dietary antimutagens such as phenolics, including anthocyanins, could protect against mutation by inhibiting nitrosation and blocking or competition with mutagens.

The result that the antimutagenicity of Hom Nil rice was lower than that of black glutinous rice might be due to Hom Nil rice had lower or different active compounds. It was reported that black glutinous rice had higher anthocyanin contents than that of Hom Nil rice (สายสนม ประดิษฐ์ดวง, 2551). It was suspected that anthocyanins were the main antimutagens of this study. Pedreschi and Cisneros-Zevallos (2006) revealed that the anthocyanin-rich extracts from Andean purple corn showed a dose-dependent antimutagenicity against the food mutagen Trp-P-1 in Ames test. In addition, anthocyanins also exhibited multiple antitoxic and anti-carcinogenic effects namely, scavenging reactive oxygen species (ROS), reducing the formation of oxidative adducts in DNA, , increasing the oxygen-radical absorbing capacity of cells, stimulating the phase II detoxification enzymes, reducing lipid peroxidation, decreasing cellular proliferation by modulating signal transduction pathways and inhibiting mutagenesis by environmental toxins and carcinogens, (Wang and stoner, 2008).

The results of this investigation indicated that Hom Nil rice and black glutinous were safe and had some health benefits to consumers because they could counteract the mutagenicity of urethane and the *in vivo* formed nitrosomethylurea. Nevertheless, antimutagenic compounds in rice sample will be required to verify the possible compounds that respond to antimutagenicity. The results of this study suggested that these rice should be developed as dietary supplement to protect the consumer from some mutagens that have similar characteristic to urethane and the *in vivo* formed nitrosomethylurea.

CHAPTER VI

CONCLUSION

Nowaday, pigmented rice is popular among people; hence, the study on risk and benefit of pigmented rice is interesting. The present study showed that Hom Nil rice and black glutinous rice are good sources of antioxidants which might be responsible in antigenotoxic action of the mutagens both urethane and the *in vivo* formed nitrosomethylurea. It might be due to some active compounds such as polyphenolic compounds and flavoniods. Thus, Hom Nil rice and black glutinous rice provide healthy benefit to consumer. The results might encourage food industry to incorporate pigmented rice as food supplement. In addition, the higher antimutagenicity of fermented rice convinced that consumption of this kind of rice may benefits to consumer.

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APPENDICES

APPENDIX A
STATISTICAL CONSIDERATION

In assay designed to assess the mutagenicity of a chemical, most often a treatment series were compared with a control series. One might like to decide whether the compound used in the treatment should be considered as mutagenic or non-mutagenic. The formulation of 2 alternative hypotheses allowed one to distinguish among the possibilities of a positive, inconclusive, or negative result of an experiment.

In the null hypothesis one assumes that there was no difference in the mutation frequency between control and treated series. Rejection of the null hypothesis showed that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis postulated a priory that the treatment results in an increased mutation frequency compared to the spontaneous frequency. The alternative hypothesis was rejected if the mutation frequency was significantly lower than the postulated increased frequency. Rejection indicates that the treatment did not produce the increase requires to consider the treatment as mutagenic. If neither of the 2 hypotheses was rejected, the results were considered inconclusive, as one could not accept at the same time the 2 mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring the mutation frequency in the treated series be *m* times that in the control series and used together with the null hypothesis. It might happen in this case that both hypotheses had to be rejected. This should mean that the treatment was weakly mutagenic, but led to a mutation frequency which was significantly lower than *m* times the control frequency.

Testing against the null hypothesis (H_O) at the level α and against the alternative a hypothesis (H_A) at the level β led to the error probabilities for each of the possible diagnoses: positive, weakly but positive, negative, or inconclusive. The following four decisions were possible; 1) accept both hypotheses; these can not be true simultaneously, so no conclusions can be drawn--inconclusive result; 2) accept the first hypothesis and reject the second hypothesis--negative result; 3) reject the first hypothesis and accept the second hypothesis--positive result; 4) reject both hypotheses --weak effect (Frei and Würgler, 1988).

Calculation step by step

Estimation of spot frequencies and confidence limits of m_e

Particularly in the case that both hypotheses, H_O as well as H_A, had to be rejected, one might be interested in knowing the confidence interval of m_e, i.e., of the estimated multiple by which the mutation frequency in the experimental series was larger than the spontaneous frequency. The estimated value was

$$m_e = \frac{(n_t / n)\, N_c}{(n_c / n)\, N_t}$$

Where N_c and N_t represented the respective sample sizes in control and treatment series, n_c and n_t the respective numbers of mutations found, and n the total of mutations in both series together. Exact lower and upper confidence limits p_l and p_u for the proportion n_c/n on one hand, as well as q_l and q_u for the proportion n_t/n on the other hand, may be an easy method to calculate these values using an F-distribution table. To determined q_1 and p_u one-sidedly at the level α, and q_u and p_1 also one-sidedly at the level β. In this way and in agreement with the foregoing section, a confidence limit $m_1 > 1$ led to rejection of H_o, while a confidence limit $m_u < m$ led to rejection of H_A.

In the first step, F-distribution were used to determine the value $F_{\nu 1, \nu 2}$ at the level $\alpha = 0.05$, where the degrees of freedom (ν_1, ν_2) were given by the equations

$$\nu_1 = 2\,(n - nt + 1) \text{ and } \nu_2 = 2n_t$$

In the second step, the F-value so obtained was used to calculate the lower confidence limit (q_1) for the proportion of spots in the experimental series

$$q_1 = n_t / [n_t + (n - n_t + 1)\, F_{\nu 1, \nu 2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control, which was equal to

$$f_{t,1} = q_1 n / N_c$$

This was the following complementarily, namely that the lower confidence limit for the number of spots in the experimental series ($q_1 n$) plus the upper confidence limit for the number of spots in the experiment ($p_u n$) was equal to the total number of spots (n) found in experimental and control series together, i.e.,

$$P_u n = (1 - q_1)\, n$$

This gave an upper limit for the frequency of spots per wing for the control, which is

$$f_{c,u} = p_u n/N_c$$

The lower confidence limit m_1 of the multiple m_e was determined as the ratio between the lower confidence limit for the frequency in the treated series and the upper confidence limit for the frequency in the control, i.e.,

$$m_1 = \frac{f_{t,1}}{f_{c\,u}} = \frac{q_1\, n/N_t}{p_u n/N_c}$$

Only in the case that m_1, the lower confidence limit of m_e, was larger than 1.0 would reject H_o. Since this was not the case, H_o remains accepted.

In the same way, the lower confidence limit of the spot frequency may be determined in the control $f_{c,1}$ which will give $f_{t,u}$, the upper confidence limit of the spot frequency in the experimental series. This is also done one-sidedly, at the level $\beta = 0.05$. The inverse ratio of these values will provide the upper 5% confidence limit m_u for the multiple m_e.

Again, the F-distribution was used and determined the value $F_{v1,v2}$ at the level $\beta = 0.05$, where the degrees of freedom ($_{v1,v2}$) were given by the equations

$$\nu_1 = 2(n\text{-}n_c + 1) \text{ and } \nu_2 = 2\, n_c$$

The F-value so obtained was used to calculate the lower confidence limit (p_1) for the proportion of spots in the control

$$P_1 = n_c / [n_c + (n\text{-}n_c + 1)\, F_{v1,v2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control, which equal to

$$f_{c,\,1} = p_1 n/N_c$$

Again, there was complementarily, in that the lower confidence limit for the number of spots in the control ($p_1 n$) plus the upper confidence limit for the number of spots in the experiment ($q_u n$) was equal to the total number of spots (n), so that

$$q_u n = (1\text{-}p_1)n$$

This gave an upper limit for the frequency of spots per wing for this series, which is

$$f_{t,u} = q_u n/N_t$$

The upper confidence limit m_u of the multiple m_e can be determined as the ratio between the upper confidence limit for the frequency in the treated series and the lower confidence limit for the frequency in the control, i.e.,

$$m_u = \frac{f_{t,u}}{f_{c,1}} = \frac{q_u\, n/N_t}{p_1 n/N_c}$$

H_A was rejected if m_u, the upper confidence limit of m_e, was less than m (m=2 for the total of all spots and for the small single spots, and m=5 for the large single spots as well as for the twin spots). Substitution of m_e by m_1 or m_u in the above formulas provided the respective exact upper and lower confidence limits for the frequencies estimated

APPENDIX B

APPEARANCE OF RICE

Process of rice	Types of rice	
	Black glutinous rice	**Hom Nil rice**
Raw		
Cooked		
Fermented		

APPENDIX C

THE STANDARD CURVES OF ANTIOXIDANT ASSAYS

1. 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

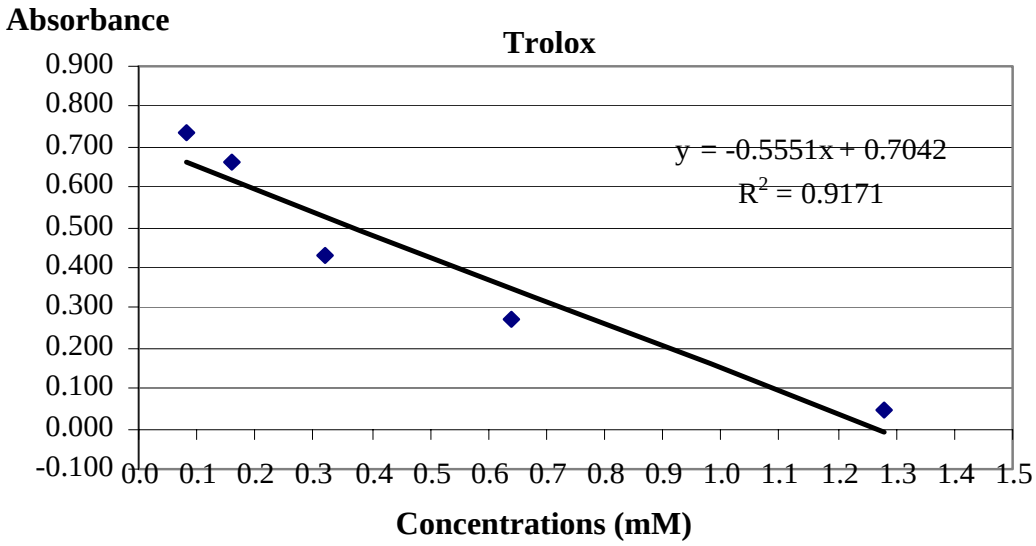

Figure C-1 Standard curves of DPPH assay

2 Ferric Reducing Antioxidant Power (FRAP) Assay

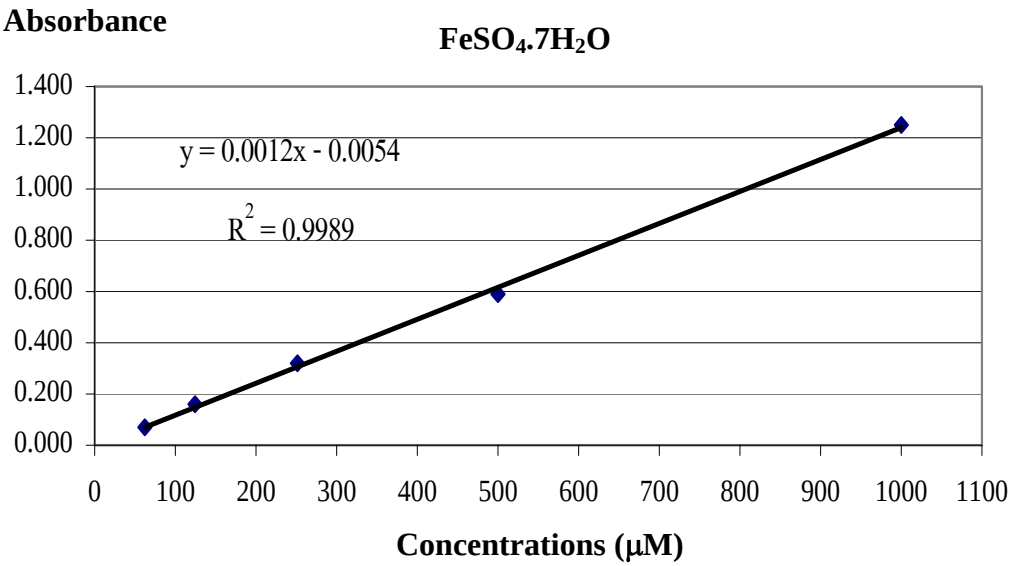

Figure C-2 Standard curves of FRAP assay

3. Determination of Total Phenolic Contents

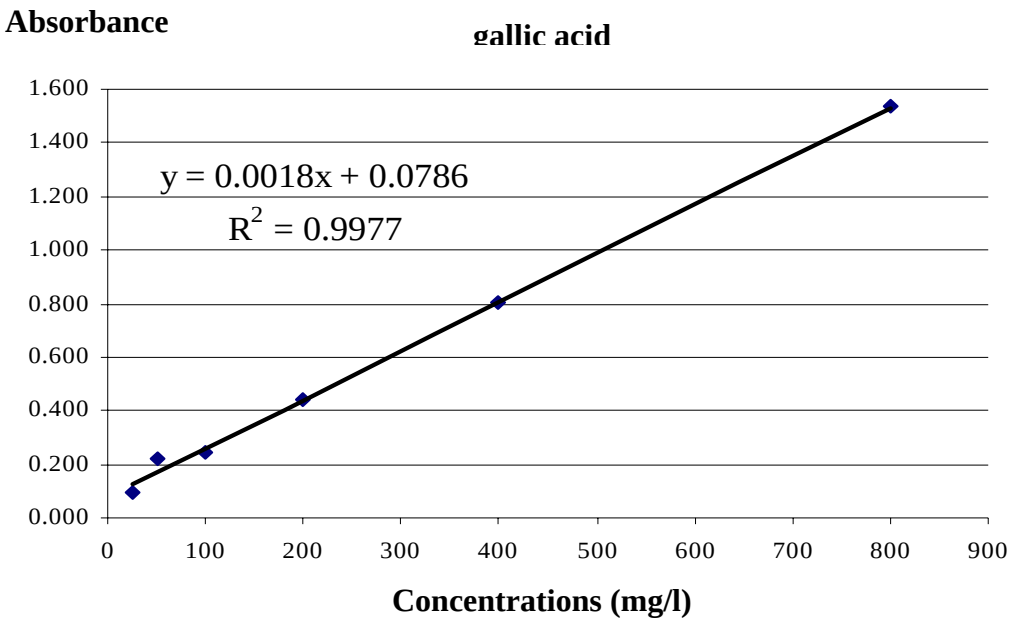

Figure C-3 Standard curves of determination of total phenolic contents

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