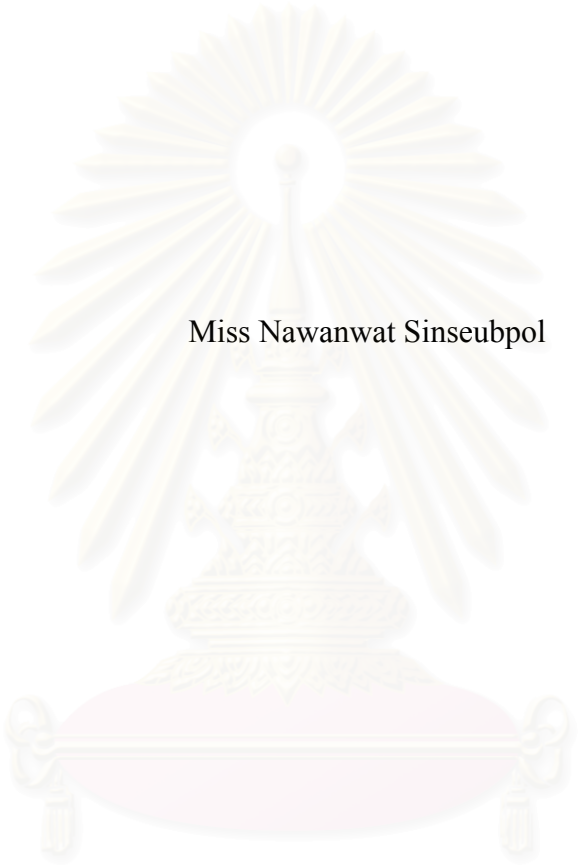
ฤทธิ์ต้านการก่อกลายพันธุ์ของสารให้สีแดงจากธรรมชาติบางชนิดเมื่อผสมในขนมแป้งนึ่ง ทดสอบโดยวิธีโซมาติกมิวเตชันและรีคอมบิเนชัน

นางสาวนวันวัจน์ สินสืบผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ภาควิชาอาหารเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

ANTIMUTAGENICITY OF SOME NATURAL RED COLORS INTRODUCED TO STEAMED STARCHY DESSERT BY SOMATIC MUTATION AND RECOMBINATION TEST

Miss Nawanwat Sinseubpol



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 Chemistry
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Thesis Title	ANTIMUTAGENICITY OF SOME NATURAL RED COLORS INTRODUCED TO STEAMED STARCHY DESSERT BY SOMATIC MUTATION AND RECOMBINATION TEST
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Field of Study	Food Chemistry and Medical Nutrition
Thesis Advisor	Assistant Professor Linna Tongyonk, D.Sc.
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นวันวัจน์ สินสืบผล : ฤทธิ์ด้านการก่อกลายพันธุ์ของสารให้สีแดงจากธรรมชาติบางชนิดเมื่อผสมในขนมแป้งนึ่งทดสอบโดยวิธีโซมาติกมิวเตชันและรีคอมบิเนชัน.
(ANTIMUTAGENICITY OF SOME NATURAL RED COLORS INTRODUCED TO STEAMED STARCHY DESSERT BY SOMATIC MUTATION AND RECOMBINATION TEST) อ. ที่ปรึกษา : ผศ.ดร.สินนา ทองยงค์, อ.ที่ปรึกษาร่วม : รศ.ดร. แก้ว กังสดาลอำไพ, 104 หน้า.

สีแดงจากธรรมชาติสี่ชนิด ซึ่งสกัดได้จากดอกกระเจี๊ยบ หัวบีท แก่นฝาง และเมล็ดคำแสด และขนมแป้งนึ่งที่ผสมสีแดงดังกล่าว ถูกนำมาทดสอบฤทธิ์ด้านการก่อกลายพันธุ์ต่อยูรีเทนในแมลงหวี่สายพันธุ์ *Drosophila melanogaster* ด้วยวิธีโซมาติกมิวเตชันและรีคอมบิเนชัน การศึกษาผลต่อการอยู่รอดของแมลงหวี่และฤทธิ์ก่อกลายพันธุ์ของตัวอย่าง ทำโดยนำหนอนแมลงหวี่ trans-heterozygous อายุ 3 วันที่ได้จากการผสมพันธุ์ระหว่างแมลงหวี่ตัวเมียสายพันธุ์ *ORR flare hair* กับแมลงหวี่ตัวผู้สายพันธุ์ *mwh/mwh* ไปเลี้ยงในอาหารทดลอง (อาหารที่มีการเติมสีหรือขนมผสมสี) นอกจากนี้ได้ทำการศึกษาฤทธิ์ด้านการก่อกลายพันธุ์ โดยศึกษาผลของหนอนที่ได้รับอาหารทดลองร่วมกับสารก่อกลายพันธุ์ (co-administration) ทำได้โดยนำหนอนแมลงหวี่อายุ 3 วัน ไปเลี้ยงในอาหารทดลองที่ผสมกับยูรีเทน ในขณะที่การศึกษาผลของหนอนแมลงหวี่ที่ได้รับอาหารทดลองตั้งแต่แรกเกิด (pre-feeding) ทำโดยนำแมลงหวี่ตัวเมียและตัวผู้ผสมพันธุ์กันในอาหารทดลองจนได้หนอนอายุ 3 วันจึงย้ายไปเลี้ยงในอาหารปกติที่ผสมกับยูรีเทน (การทดสอบแบบที่ 1) หรืออาหารทดลองที่ผสมกับยูรีเทน (การทดสอบแบบที่ 2) จนกระทั่งกลายเป็นตัวเต็มวัย จึงนำปีกของแมลงหวี่ (เฉพาะปีกบน) ที่รอดชีวิตมาวิเคราะห์ความผิดปกติของขน การศึกษาฤทธิ์ด้านอนุมูลอิสระของสารสกัดจากสีและขนมที่ผสมสีต่างๆ ด้วยเมทานอลทำโดย 2 วิธี คือ วิธี FRAP และวิธี DPPH ส่วนปริมาณของสารประกอบฟีนอลิกนั้นได้ทำการศึกษาโดยใช้ Folin-Ciocalteu เป็นตัวทำปฏิกิริยา ผลการศึกษาพบว่าทุกตัวอย่างไม่แสดงฤทธิ์ก่อกลายพันธุ์ การศึกษาใน co-administration พบว่าทุกตัวอย่างสีที่นำมาทดสอบสามารถลดฤทธิ์การก่อกลายพันธุ์ของยูรีเทนลงได้ในระดับที่แตกต่างกัน แต่เมื่อนำสีไปผสมในขนมแป้งนึ่ง พบว่ามีเพียงขนมที่ผสมสีจากกระเจี๊ยบและหัวบีทเท่านั้นที่สามารถต้านการก่อกลายพันธุ์ของยูรีเทนได้ ส่วนการศึกษาจาก pre-feeding พบว่าสีที่สกัดได้จากทุกตัวอย่างสามารถลดฤทธิ์การก่อกลายพันธุ์ของยูรีเทนลงได้ในการทดสอบแบบที่ 2 แต่เมื่อผสมสีดังกล่าวลงในขนมแป้งนึ่ง พบว่า มีเพียงขนมที่ผสมสีจากดอกกระเจี๊ยบและหัวบีทเท่านั้นที่ยังคงมีฤทธิ์ต้านการกลายพันธุ์ของยูรีเทนได้ ผลดังกล่าวอาจมาจากองค์ประกอบที่มีฤทธิ์ด้านการก่อกลายพันธุ์ในสีธรรมชาติเหล่านี้ นอกจากนี้ยังพบว่าสารสกัดตัวอย่างด้วยเมทานอลมีสารประกอบฟีนอลิกและมีฤทธิ์ด้านอนุมูลอิสระ สารประกอบในสีธรรมชาติเหล่านี้จึงอาจไปจับกับสารก่อกลายพันธุ์และ/หรืออนุมูลอิสระที่เกิดระหว่างการก่อกลายพันธุ์หรืออาจจะกระตุ้นระบบเอนไซม์ทำลายสารพิษ (phase II) และ/หรือยับยั้งการทำงานของเอนไซม์ในระบบไซโตโครมพี-450 (phase I)

ภาควิชา..อาหารเคมี..... ลายมือชื่อนิสิต.....นวันวัจน์ สินสืบผล.....
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา..2550..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

48765714 : MAJOR FOOD CHEMISTRY
KEY WORD: ANTIMUTAGENICITY / SMART / NATURAL COLORS / ROSELLE/ BEETROOT/ SAPPAN/ ANNATTO / URETHANE

NAWANWAT SINSEUBPOL: ANTIMUTAGENICITY OF SOME NATURAL RED COLORS INTRODUCED TO STEAMED STARCHY DESSERT BY SOMATIC MUTATION AND RECOMBINATION TEST. THESIS ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., THESIS CO-ADVISOR : ASSOC. PROF. KAEW KANGSADALAMPAI, Ph.D., 104 pp.

Four natural red colors from roselle calyx (*Hibiscus sabdariffa* L.), beetroot (*Beta vulgaris* L.), sappan heartwood (*Caesalpinia sappan* L.), and annatto seed (*Bixa orellana* L.) were introduced to their respective natural colored steamed starchy desserts. All of them, including each natural color extract, were determined for their antimutagenicity on urethane induced somatic mutation and recombination 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 an experimental medium (containing natural color extract or natural colored dessert) to determine the effect of each sample on the survival of adult files and its mutagenicity. Furthermore, the antimutagenicity of each sample was determined by transferring three-day old larvae to an experimental medium that had urethane as a co-administration study. In addition, pre-feeding studies were performed by mating the parent flies on the experimental medium containing each sample to obtain 3-day old larvae that were subsequently raised on the standard medium containing urethane as the type 1 study or the experimental medium containing urethane as the type 2 study. The wings of the surviving flies were analyzed for the occurrence of mutant spots. Antioxidant activity of methanolic extract from each sample was assessed by using two methods: 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay and Ferric Reducing Antioxidant Power (FRAP) Assay. The content of phenolic compound in the extracts was also determined using Folin-Ciocalteu reagent. The results showed that none of the samples was mutagenic at the concentration tested. In the co-administration study, all color extracts could reduce mutagenicity of urethane. However, when introduced these colors to the dessert, there were only roselle and beetroot dessert that showed the antimutagenicity. Moreover, while all colors could counteract the mutagenicity of urethane in pre-feeding type 2 study, there were only the desserts containing roselle and beetroot color that expressed antimutagenicity in this study. The protective effects of these natural colors may be due to the presence of antimutagenic components. In addition, the study showed that natural colors had phenolic compounds and antioxidant activity. It is proposed that these colors might scavenge urethane and/or free radicals that occur during mutagenesis, induce the enzymatic system to detoxify urethane (phase II) and/or inhibit the activity of cytochrome P-450 enzymes (phase I).

Department.. Food Chemistry.....Student's signature.....
Field of study Food Chemistry and Medical Nutrition Advisor's signature.....
Academic year..2007.....Co-advisor's signature.....

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LIST OF ABBREVIATIONS

SMART	Somatic Mutation and Recombination Test
GST	Glutathione-*S*-transferase
MI	Mutagenicity Index
°C	degree Celsius
$O_2^{\cdot -}$	superoxide radical
$NO^{\cdot}$	nitric oxide radical
g	gram
mg	milligram
mM	millimolar
ml	millilitre
mm	millimetre
µl	microlitre
µM	micromolar
ppm	part per million
min	minute
h	hour
SD	standard deviation
ND	non-detected
et al.	*et alia* (and others)
etc.	*et cetera* (and other similar things)

CHAPTER I
INTRODUCTION

1.1 Background and Significance of the Study

Color is the first notable characteristic of a food and often predetermines our expectation. Color was used as a way to identify a food and a way to judge the quality of a food. The colors of foods are the result of natural pigments or of added colorants. The latter are regulated as food additives, or call synthetic food colors. Since the number of permitted synthetic food colors have been banned because of undesirable toxic effects including mutagenicity and potential carcinogenicity, naturally pigmented foods and food products enhanced with pigments extracted from plants are, thus, overwhelmingly preferred by consumers (Francis, 1989). Natural pigments not only circumvent the putative health hazards posed by synthetic colorants but also confer substantive and nutraceutical value to the diet (Delgado-Vargas *et al*., 2000). Some natural pigments from plants such as carotenoids, anthocyanins, and betalains have been implicated in regimes to maintain human health, to protect against chronic disease incidence, or to restore wellness by repairing tissues after disease has been established (Lila, 2004).

In Thailand, food colors have been used for a long time, especially in Thai dessert. Most of these colors are extracted from various parts of plants such as calyces, leaves, heartwoods, roots, or seed coats and some are from animals (พเยาว์ เหมือนวงษ์ญาติ, 2530). The color that popularly used in Thai drinks and desserts is red because of its cheerful shade and it can be found in many plants. For instance, red colors from calyces of *Hibiscus sabdariffa* L. (Roselle, Krajeab-daeng, กระเจี๊ยบแดง), roots of *Beta vulgaris* L. (Beetroot, Hua-beet, หัวบีท), heartwood of *Caesalpinia sappan* L. (Sappan wood, Fang, ฝาง), and seed coats of *Bixa orellana* L. (annatto, Kam-sad, คำแสด).

Considerable amount of research had been conducted on the role of these red colors in cancer prevention. For example, the water extracts of roselle showed antimutagenic activity against urethane in *Drosophila melanogaster*. (Phanrattanamala, 2004). As well as the 80% ethanol extract of roselle which could

reduce about 60%-90% of the mutagenicity induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and other heterocyclic amines in the *Salmonella* mutation assay (Chewonarin *et al*., 1999). Furthermore, an *in vivo* antitumor evaluation of beetroot extract against the mice skin and lung bioassays revealed a significant tumor inhibitory effect (Kapadia *et al*., 1996). As well as the heartwood extraction of *Caesalpinia sappan* L. which had the antioxidant activity (Badami *et al*., 2003; Yingming *et al*., 2004) and could induce cell death in head and neck cancer cell lines (Kim *et al*., 2005). Moreover, annatto, a natural food color rich in carotenoid, had possible chemopreventive effect against dimethylhydrazine (DMH) of the cryptal cell proliferation in rat colon (Agner *et al*., 2005).

However, it is questionable that whether food processing can modulate the activities of these colors. Therefore, it would be of interest to investigate the antimutagenicity of these natural red colors that were incorporated into Thai dessert against the mutagenicity of urethane using the somatic mutation and recombination test (SMART).

1.2 Objectives of the Study

The specific objectives of the present study were as follow:

1.2.1 To determine the mutagenicity of natural red colors from *Hibiscus sabdariffa* L. (Roselle, Krajeab-daeng), *Beta vulgaris* L. (Beetroot, Hua-beet), *Caesalpinia sappan* L. (Sappan wood, Fang), and *Bixa orellana* L. (annatto, Kam-sad) both in the form of color extracts and when introduced these colors to steamed starchy dessert by somatic mutation and recombination test in the improved high bioactivation cross of *Drosophila melanogaster*

1.2.2 To elucidate the modulating effect of natural red colors from *Hibiscus sabdariffa* L. (Roselle, Krajeab-daeng), *Beta vulgaris* L. (Beetroot, Hua-beet), *Caesalpinia sappan* L. (Sappan wood, Fang), and *Bixa orellana* L. (annatto, Kam-sad) both in the form of color extracts and when introduced these colors to steamed starchy dessert on urethane induced somatic mutation and recombination test in *Drosophila melanogaster*

1.3 Benefits of the Study

1.3.1 This study provides the information regarding the mutagenicity modulation effect of natural red colors from calyces of *Hibiscus sabdariffa* L. (Roselle, Krajeab-daeng), roots of *Beta vulgaris* L. (Beetroot, Hua-beet), heartwood of *Caesalpinia sappan* L. (Sappan wood, Fang), and seed coats of *Bixa orellana* L. (annatto, Kam-sad) both in the form of color extracts and when introduced these colors to steamed starchy dessert.

1.3.2 The available results from this study can be applied to all Thai desserts that have steamed starchy characteristics such as Khanom Chan (ขนมชั้น), Khanom Nam Dok Mai (ขนมน้ำดอกไม้), Khanom Keng (ขนมเข่ง), Khanom Tien (ขนมเทียน).

CHAPTER II
LITERATURE REVIEW

2.1 Plant Pigments and Human Health

Pigments produce colors that can observe at each step of lives. Plants are the principal producers. They are in leaves, fruits, vegetables, and flowers. In addition, skin, eyes, and other animal structures also contribute to some edible colors. Natural and synthetic pigments are used in medicines, foods, clothes, furniture, cosmetics, and in other products. However, natural pigments have important functions other than the imparted beauty, a lot of pigments have a well-known pharmacological activity in sickness such as cancer and cardiovascular diseases, and this has stressed pigment importance for human beings (Hari *et al*., 1994; Koes *et al*., 1994).

A wide range of bioassays and tests have been forwarded to establish the biological efficacy of natural pigments in human health intervention, including *in vitro* bioassays, *in vivo*, epidemiological and more rarely, clinical trials (Table 1). Almost all early supports for the roles of pigments in biological systems were based on a diverse set of subjective or epidemiological evidence. However, increasingly with the interest in functional foods for health, the evidence is shifting towards replicated *in vivo* animal models and clinical trials, especially for the most widely researched pigments like anthocyanins and some carotenoids.

Table 1 Levels of research inquiry applied to natural plant pigments and human health interventions (Lila, 2004)

Pigment	Disease condition or health target	Selected research evidence supporting role of pigment in chronic disease prevention or therapy			
		In vitro	*In vivo*	Epidemiological	Clinical
Anthocyanins	**Vision disorders**	-	Mastsumoto *et al.,* 2001	-	Mastsumoto *et al.,* 2001
	Cardiovascular disease	Oak *et al.,* 2003 Serraino *et al*., 2003	Nielsen *et al.,* 2003	Havsteen, 2002 Duthie *et al*., 2000	Shanmuganayagam *et al.*, 2002
	Cancer	Kang *et al*., 2003 Naasani *et al*., 2003	Kang *et al.,* 2003 Naasani *et al*., 2003	Horvathova *et al.,* 2001 Duthie *et al.,* 2000	-
Betalains	**Cancer**	Wettasinghe *et al*., 2002	Kapadia *et al.,* 1996	-	-
Carotenoids	**Photooxidative damage**	Sies and Stahl, 2003	-	Khachik *et al*., 2002	Snodderly, 1995
	Cardiovascular disease	Fuhrman *et al.,* 1997	Fuhrman *et al.,* 1997	Delgado-Vargas *et al.,* 2000 Platz *et al*., 2003	Platz *et al*., 2003
	Cancer	Kotake-Nara *et al*., 2001 Liu *et al.,* 2003	Wang *et al.,* 1999 Jain *et al.,* 1999 Teplizky *et al.,* 2001	Goodman *et al*., 2003 Toniolo *et al.,* 2001 Nyberg *et al.,* 2003	Baron *et al*., 2003 Kucuk *et al.,* 2002

Despite the intense research efforts directed toward natural plant pigments and their manipulation, a number of key questions especially in health benefits remain to be answered. At present, neither scientists nor medical professionals can establish optimal levels for human intake. Requirements are likely to vary between individuals depending on their health status, gender, ethnicity, age, body mass index, and other factors. Given the potentiation effects of the interaction among phytochemicals, it is evident in many cases that the pigments intervene in human health maintenance most effectively when delivered as a component of the food matrix, rather than in supplement form. In addition, various forms of processing or cooking can impinge on the subsequent absorption and bioavailability of pigments and related phytochemicals. While in some cases, thermal processing, light, or oxygen may degrade these natural pigmented compounds. In others, the bioavailability may actually be enhanced.

2.2 Some Important Plant Pigments: Carotenoids, Anthocyanins, and Betalains

2.2.1 Carotenoids

In general, carotenoids are compounds comprised of eight isoprenoid units (ip) whose order is inverted at the molecule center (Figure 1). All carotenoids can be considered as lycopene ($C_{40}H_{56}$) derivatives by reactions involving: hydrogenation, dehydrogenation, cyclization, oxygen insertion, double bond migration, methyl migration, chain elongation, and chain shortening (Goodwin, 1980).

Carotenoids are the widest distributed group of pigments. They have been identified in photosynthetic and nonphotosynthetic organisms: in higher plants, algae, fungi, bacteria, and at least in one species of each form of animal life. Carotenoids are responsible for many of the brilliant red, orange, and yellow colors of fruits, vegetables, fungi, flowers, and also of birds, insects, crustaceans, and trout (Goodwin, 1980; Goodwin, 1992; Gordon *et al.,* 1982; Hari *et al.,* 1994; Wong, 1989). Only microorganisms and plants can synthesize carotenoids *de novo*; carotenoids in animals come from these two sources, although they can be modified during their metabolism to be accumulated in tissues (Goodwin, 1980).

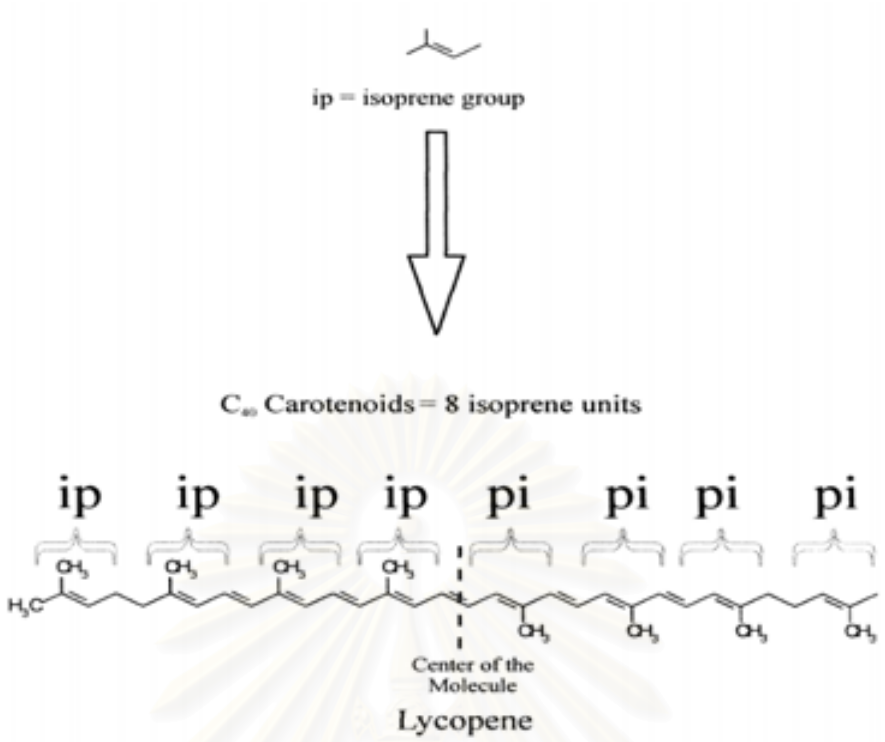


Figure 1. Carotenoid structure (Goodwin, 1980)

Many diseases, such as cancer and strokes, involve oxidative processes mediated by free radicals. Carotenoids, by their antioxidant effect, can show benefits in such diseases. There is evidence of the effectiveness of β-carotene in the treatment of certain kinds of cancer, such as smoking related cervical intraepithelial neoplasia and stomach cancer (Charleux, 1996). Siefer *et al.* (1981) showed that β-carotene affects the immune response in rats, and by this means tumor growth is inhibited.

More than 600 carotenoids are known, and 50 of them are consumed in meals to be transformed into the essential nutrient vitamin A. After their absorption, these carotenoids are metabolized by an oxidative rupture to retinal, retinoic acid, and small quantities of breakdown products (Parker, 1996). Vitamin A is required in the vision process, epithelial maintenance, mucous secretion, and reproduction (Olson, 1993; Radlwimmwer, 1997; Taylor-Mayne, 1996).

Carotenoids have been used as food colors for centuries: saffron, pepper, and red palm oil have carotenoids as their main color components. Color of carotenoids, together with beneficial properties such as vitamin A precursors and antioxidants, have led to their wide application in the food industry; preparations to apply them in

oily or aqueous media have been produced, including emulsions, colloidal suspensions, and complexes with proteins. These preparations have found applications to pigment margarine, butter, fruit juices and beverages, canned soups, dairy and related products, desserts and mixes, preserves and syrups, sugar and flour confectionery, salad dressings, meat, pasta and egg products (Kläui, 1979).

Stability of Carotenoids Retention or loss of carotenoids during processing and storage of food has been reported in numerous papers. The data are somewhat conflicting and often difficult to interpret. However, some conclusions can be drawn as in home preparation; losses of carotenoids generally increase in the following order: microwaving < steaming < boiling. Deep-frying, prolong cooking, combination of several preparations and cooking methods, baking and pickling all result in substantial losses of carotenoids (Rodriguez-Amaya, 1997).

2.2.2 Anthocyanins

Anthocyanins are one of the most important groups of pigment that can visible to the human eye (Harborne, 1988). Chemically, anthocyanins from the Greek *anthos*, a flower, and *kyanos*, dark blue are flavonoids (flavan like), and consequently based on a C15 skeleton with a chromane ring bearing a second aromatic ring B in position 2 (C6-C3-C6) and with one or more sugar molecules bonded at different hydroxylated positions of the basic structure (Figure 2). Anthocyanins are substituted glycosides of salts of phenyl-2-benzopyrilium (anthocyanidins) (Counsell *et al*., 1979).

Anthocyanins are responsible for many of the attractive colors, from scarlet to blue, of flowers, fruits, leaves, and storage organs (Harborne, 1988; Harborne and Grayer, 1988). They are almost universal in higher plants, but in general anthocyanins seem absent in the liverworts, algae, and other lower plants, although some of them have been identified in mosses and ferns.

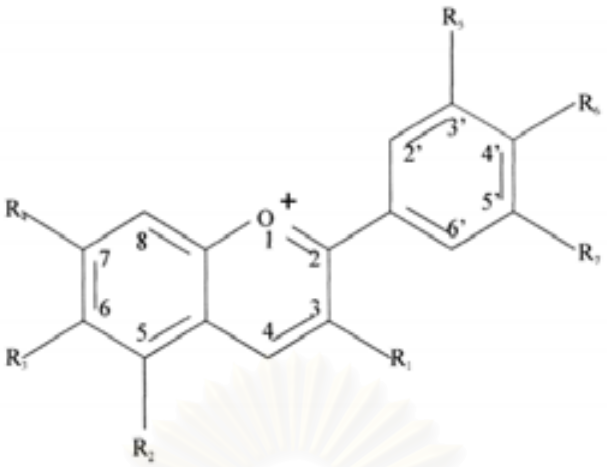


Figure 2. Basic structure of anthocyanidin pigments in which R_x could be H, OH, or OCH_3 depending on the considered pigment. The most common accepted nomenclature for numbering carbons is indicated inside the structure (Counsell *et al*., 1979)

Reports on biological activities of anthocyanins are scarce. Considering the wide distribution of anthocyanins, it is reasonable to assume that humans are well conditioned to large consumption of these compounds. In a survey with Italian subjects, anthocyanin daily intake was in the range of 25 to 215 mg/person, depending on gender and age, and this intake is largely enough to induce pharmacological effects. The consumption of wine flavonoids has been correlated with low incidences of coronary heart diseases (French paradox), and similarly, black chokeberry (*Aronia melanocarpa* Michx.) extracts have shown very strong nutraceutical properties. Moreover, anthocyanins possess bactericidal, antiviral, and fungistatic activities. They exhibit a strong antioxidant activity that prevents the oxidation of ascorbic acid, provides protection against free radicals, shows inhibitory activity against oxidative enzymes, and has been considered as important agents in reducing the risk of cancer and heart disease (Bridle and Timberlake, 1997). Also, it was demonstrated that anthocyanins have scavenging properties against ·OH and O_2- (Tsuda *et al*., 1996).

Stability of Anthocyanins During food processing, anthocyanin pigments are easily destroyed. High temperature, increased sugar level, pH, ascorbic acid and

other additives, and coating materials may affect the rate of destruction (Francis, 1989). Temperature is an important factor and pigment degradation is exponential.

2.2.3 Betalains

The term "betalains" introduced by Mabry and Dreiding (1968) was supported by structural and biogenetic considerations. In a strict sense, betalains do not belong to alkaloids because they are acidic in nature due to the presence of several carboxyl groups. Chemically, betalains definition embraces all compounds with structures based on the general formula shown in Figure 3; therefore, they are immonium derivatives of betalamic acid (Piatelli, 1981; Strack *et al*., 1993).

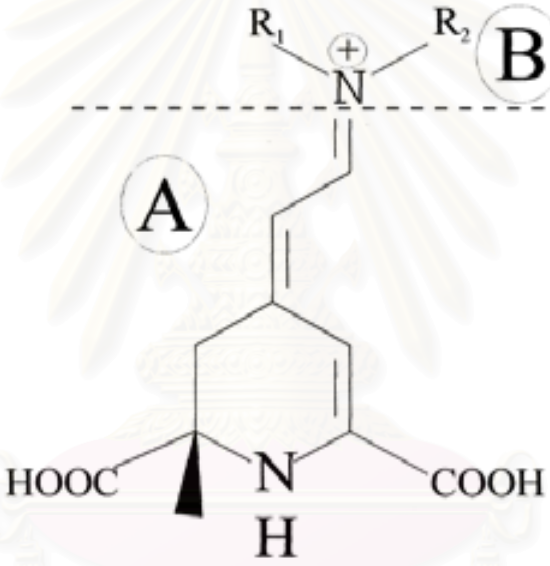


Figure 3. Betalain general formula. (A) Betalamic acid moiety is present in all betalain molecules. (B) The structure will represent a betacyanin or a betaxanthin, depending on the identity of the R_1 and R_2 residues (Böhm *et al*., 1988)

Betalain can be divided into two structural groups, the yellow betaxanthins and red-purple betacyanins, depending on R_1-N-R_2 moieties. More than 50 betalains are well known, and all of them have the same basic structure, in which R_1 and R_2 may be hydrogen or an aromatic substituent.

Although betalains are structurally related to alkaloids, they have no toxic effects in the human body. As they can be deduced from the fact that they are present in considerably high amounts in certain foodstuffs, such as red beet, prickly pear fruits, and *Amaranthus* seeds (Böhm *et al*., 1988); therefore, betalains represent a safe natural alternative to some synthetic color additives that are currently in use. Interestingly, there is no upper limit to the recommended daily intake.

Betanin had no mutagenicity in five *Salmonella typhimurium* strains of the Ames test (von Elbe and Schwartz, 1981). It did not initiate or promote hepatocarcinogenesis in rat liver at the levels up to 50 mg of pure betanin per kg body weight and diets containing betacyanin 2000 ppm also showed no positively toxic effect (Schwartz, 1983; Schwartz *et al*., 1983).

Notwithstanding, after ingestion of these products (particularly red beet), betanin occasionally appears in the urine. This effect is known as beeturia or betaninuria. The etiology and mechanism of this disorder are still controversial (Piatelli, 1976). Pszczola (1998) reviewed the importance of some natural pigments as nutraceutical ingredients. Betalains like anthocyanins, β-carotene, and various vegetable and fruit extracts are suggested to be used for their potential health benefits. For example, yellow betaxanthins, in addition to their potential role as natural food colorant, may be used as a means of introducing essential dietary amino acids into foodstuffs, giving rise to an “essential dietary colorant” (Leathers *et al*., 1992).

Stability of Betalains Betalains are afflicted with inferior stability compared to synthetic dyes. Temperature, pH, oxygen, and light are known to exhibit detrimental effects on betalain integrity, while certain antioxidants such as vitamin C and chelating agents may act as stabilizers (Herbach *et al*., 2006). In summary, color loss can be minimized during processing and storage of betalain sources by choosing the respective temperature and pH regimes as well as minimizing oxygen and light access (Delgado-Vargas *et al*., 2000).

2.3 Selected Natural Food Color Sources

2.3.1 *Hibiscus sabdariffa* L. (Roselle, Krajeab-daeng)

Roselle or *Hibiscus sabdariffa* L. is belonged to family Malvaceae (Figure 4). It is used in traditional medicines for decreasing of body temperature, health promotion, treatment of fatigue, biliary disease, disorder of urination, cough, as an antihypertensive, expectorant, blood tonic, element tonic (Sujamnong, 1981), antidiabetic, vasodilators (Chankhaao, 1983), alleviation of thirst and antihypercholesterolemic (Sujamnong, 1981; Chankhaao, 1983).

Figure 4. *Hibiscus sabdariffa* L. (roselle, krajeab-daeng)

2.3.1.1 Chemical Constituents (Hirunpanich *et al*., 2005)

Roselle is widely cultivated in tropical areas and its red persistent calyx is the major component possessing a sour taste that is used as beverage and food colorants. It contains many chemical constituents including alkaloids, *L*-ascorbic acid, anisaldehyde, anthocyanin, β-carotene, β-sitosterol, citric acid, cyanidin-3-rutinoside, delphinidin, galactose, gossypetin, hibiscetin, mucopolysaccharide, pectin, protocatechuic acid, quercetin, stearic acid and wax.

2.3.1.2 Pharmacological Activities and Clinical Trials of Roselle

A beverage containing roselle was bactericidal against *Escherichia coli, Bacillus subitlis, Salmonella typhosa,* and *Klebsiella pneumoniae* (Alian *et al*., 1983). An inhibitory effect of roselle flower on the growth and aflatoxin production of toxicgenic strain of *Aspergillus flavus* had been reported (El-Shayeb and Mabrouk, 1984).

Chang *et al*. (2005) and Hou *et al*. (2005) founded that delphinidin 3-sambubioside (Dp3-Sam), a *Hibiscus* anthocyanin isolated from the dried calyces of roselle, could induce a dose-dependent apoptosis in human leukemia cells (HL-60). These findings enhance our interesting for anticancer function of *Hibiscus* anthocyanins in herbal medicine.

Toxicity study of the aqueous fraction of an aqueous-alcoholic extract of roselle calyces given to Wistar albino rats orally was reported by Akindahunsi and Olaleye (2003). The rats, which were fed with commercial diet and water *ad libitum*, were divided into six groups of four rats each. Treatments 1 through 6 received 0, 1, 3, 5, 10 and 15 doses of 250 mg/kg each, respectively; the control group received physiological saline (0.9% NaCl). Results of the studies showed that the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased in all the treatments compared with the control group. However, the serum levels of alkaline phosphatase, and lactate dehydrogenase were not significantly affected. Only the group with 15 doses had their serum level of albumin significantly increased. However, the results of histopathological studies showed that both the livers and hearts gave no pathological features for all the treatments. Besides, prolong usage of this extract at 15-dose level could cause liver injury while the effect was mild at small dose levels (1-10). Though the average consumption of 150-180 mg/kg per day appears safe, the extract should be taken with caution bearing in mind that higher doses could affect the liver.

The 80% ethanol extract of roselle calyces was examined for antimutagenic activity (Chewonarin *et al.,* 1999). It reduced about 60-90% of the mutagenicity induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and other heterocyclic amines; 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), at

a concentration of 12.5 mg/plate in the *Salmonella* mutation assay. The extract showed no mutagenicity and no antibacterial activity below this dose. Mutagenicity of methylazoxymethanol (MAM) acetate, which, like PhIP, is a colon carcinogen,was also efficiently inhibited by the roselle extract. These results indicate that roselle has antimutagenic activity against MAM acetate and other heterocyclic amines.

2.3.2 *Beta vulgaris* L. (Beetroot, Hua-beet)

Beta vulgaris L., commonly known as beetroot is a species in the family Chenopodiaceae (Figure 5). Beetroot is an excellent source of red and yellow pigments (Bokern *et al.,* 1991), which provide a natural alternative to synthetic red dyes and have attracted the interest of the natural colorant industry. Beet pigments, collectively known as "betalains" and beetroot powder have been used as natural colorant in food products such as processed meat, ice cream, baked goods, candies, and yogurt (Vereltzis and Buck, 1984; Vereltzis *et al*., 1984; Delgado-Vargas *et al*., 2000). Betalains have been successfully used in commercial food coloring for a number of years, and continue to be an important source of red color in the food industry. There are two distinct types of betalains, namely betacyanins, the red betalains, and betaxanthins, the yellow betalains (Kobayashi *et al*., 2000); both are highly water soluble. These differ by conjugation of a substituted aromatic nucleus to the 1,7-diazaheptamethinium chromophore, which is present in betacyanin. The betalains have a limited distribution in the plant world and it would appear that betalains and anthocyanins are mutually exclusive. Plants producing betalains do not contain anthocyanins.

Most varieties of beetroot contain the red betacyanin (betanin and betanidin) as the predominant coloring compound and this represent 75% to 90% of the total color present. Vulgaxanthin I and II are the principal yellow betaxanthins.

Figure 5. *Beta vulgaris* L. (Beetroot, Hua-beet)

2.3.2.1 Chemical Constituents

Besides betalains, *Beta vulagris* L. roots also contain significant amounts of vitamin C, whilst the leaves are an excellent source of vitamin A. The roots are also high in folate, soluble and insoluble dietary fiber, phenolic acids and antioxidants. It is among the sweetest of vegetables, containing more sugar even than carrots or sweet corn. The content of sugar in beetroot is no more than 10% (USDA, 2006).

2.3.2.2 Pharmacological Activities and Clinical Trials of Beetroot

Besides imparting attractive color to food products, crude preparations of beet pigments are known to confer free radical scavenging/antioxidant activities (Escribano *et al*., 1998). It is possible that the antioxidant activities may arise from an array of chemically diverse compounds that include tocopherols, phenolic acids, and their esters, pigments, aromatic peptides, hydrocarbons, and other naturally occurring antioxidants. Antioxidants from various sources have also been implicated in cancer chemoprevention due mainly to their direct involvement in eliminating carcinogens, such as free radicals in humans (Wattenberg, 1996).

The *in vitro* inhibitory effect of beetroot extract on Epstein-Barr virus early antigen (EBV-EA) induction using Raji cells revealed a high order of activity

compared to capsanthin, cranberry, red onion skin and short and long red bell peppers. An *in vivo* anti-tumor promoting activity evaluation against the mouse skin and lung bioassays also revealed a significant tumor inhibitory effect. The combined findings suggest that beetroot ingestion can be one of the useful means to prevent cancer (Kapadia *et al*., 1996).

Aqueous and ethanolic extracts of beetroot have been reported to have free radical scavenging activity, reducing the radical cations and phase II enzyme-inducing activities in murine hepatoma cell *in vitro* study (Prajapati *et al*., 2003). Agarwal *et al*. (2006) founded that ethanolic extract of beetroots given orally at doses of 1000, 2000, and 4000 mg/kg exhibited significant dose-dependent hepatoprotective activity against carbontetrachloride (CCl_4)-induced hepatotoxicity in rats.

2.3.3 *Caesalpinia sappan* L. (Sappan, Fang)

Sappan wood (*Caesalpinia sappan* L.), native to India and Malaysia, belongs to family Caesalpiniaceae. It had been imported into Europe since the Middle ages under the name of “Brazil”, derived from the Portuguese word *braza* (of Arabic origin) suggesting “red” (Ferreira, *et al*., 2004) (Figure 6).

Figure 6. *Caesalpinia sappan* L. (Sappan, Fang)

2.3.3.1 Chemical Constituents

The coloring matter of sappan wood is brazilin (Figure 7). The leaves, bark, and fruit walls contain tannin. Volatile oil is present in the leaves most are d-α-phellandrene, terpene, and methyl alcohol. (Moon, *et al*., 1988)

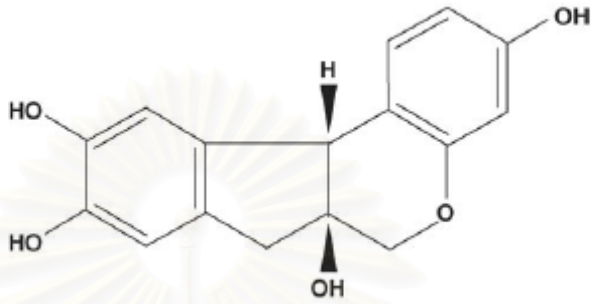


Figure 7. Structure of Brazilin

2.3.3.2 Pharmacological Activities and Clinical Trials of Sappan

In Thailand, a decoction of sappan heartwood is traditionally used as coloring agent in bevarage, food, garment and cosmetics. It is mixed with the extracts of *Carthamus tinctorius* L., *Crocus sativus* L., *Cinnamomum loureirii* Nees., *Jasminum sambac* (L.)Ait., *Mesua ferrea* L., *Nelumbo nucifera* Gaertn., and so on for indigenous coloring solution called Namya-utai, which has antithrist and cardiotonic properties (Wetwitayaklung *et al*., 2005).

In China, the wood of sappan is used to heal wounds, stop haemorrhage and haemoptysis, regulate menstruation after childbirth, and to soothe bruises. In Vietnam, a decoction of about 25 g of wood is ingested daily to stop dysentery, intestinal and uterine haemorrhage, heal wounds and furuncles, regulate menstruation, soothe contusion, and to treat impetigo and leucorrhoea (Wiart, 2002).

Brazilin, the major component of sappan wood, shows hypoglycemic action in experimental diabetic animals, and this hypoglycemic effect is the result of increased glucose metabolism, including glucose uptake into soleus muscles and adipocytes (Moon *et al*., 1988; Moon *et al*., 1993; Lee *et al*., 1994). You *et al*. (2005) found that brazilin inhibited hepatic gluconeogenesis by elevating the F-2,6-BP level in hepatocytes, possibly by elevating cellular F-6-P/H-6-P levels and PFK-2 activity and increased pyruvate kinase activity which also play a role in the anti-gluconeogenic action of brazilin. Brazilin is also shown to exert many biological

effects, which include being an antibacterial principle against antibiotic-resistant bacteria, notably methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), multi-drug resistant *Burkholderia cepacia* as well as a number of other bacteria. (Xu and Lee, 2004), preventing the induction of immunological tolerance caused by high dose sheep red blood cells (SRBC) which suppresses the elevation of suppressor cell activity and inhibits the decrease in IL-2 production in C57BL/6 female mice (Mok *et al.,* 1998). Its effects also include augmenting cellular immunity in type I diabetic mice (Yang *et al*., 2000) induced by multiple low dose streptozotocin (MLD-STZ), and modulating immune function mainly by augmenting T cell activity in halothane administered mice (Choi *et al*., 1997).

The main constituent of sappan wood is brazilin, but brazilein is also present, which is the oxidized product of brazilin and can be isolated in large quantities when the organic extract is exposed to air and light (Oliveira *et al.,* 2002). As for the biological effects of brazilein, Oh *et al.* (1998) have reported that brazilein have anti-complementary activity on the complement system. Besides brazilein also could inhibit the proliferation of T lymphocyte stimulated by Concanavalin A (Con A) and the proliferation of B lymphocyte stimulated by lipopolysaccharides (LPS), and could suppress mice humoral immune response by plaque forming cell (PFC) test (Ye *et al*., 2006). This suggested that brazilein from *Caesalpinia sappan* L. has been shown to have interesting immunosuppressive properties.

2.3.4 *Bixa orellana* L. (Annatto, Kam-sad)

Bixa orellana L. (annatto or Kam-sad), family Bixaceae, is a shrub or small evergreen tree, native to northern South America and wildly cultivated for its seeds or as an ornamental plant in the west Indies, tropical Asia and Africa (Wiart, 2002) (Figure 8).

Figure 8. *Bixa orellana* L. (annatto, Kam-sad)

2.3.4.1 Chemical Constituents

Analysis of annatto seeds indicates that they contain 40% to 45% cellulose, 3.5% to 5.5% sucrose, 0.3% to 0.9% essential oil, 3% fixed oil, 4.5% to 5.5% pigments, and 13% to 16% protein, as well as alpha- and beta-carotenoids and other constituents. Annatto oil is extracted from the seeds and is the main source of pigments named bixin and norbixin (Figure 9), which are classified as carotenoids. *Cis*-bixin is the major color component of annatto seed, accounting for over 80% of the annatto pigment (Preston and Richard, 1980). Water-soluble annatto extracts are prepared by alkaline hydrolysis whereby bixin is converted to norbixin. In addition to bixin and norbixin, annatto contains bixaghanene, bixein, bixol, crocetin, ellagic acid, isobixin, phenylalanine, salicylic acid, threonine, tomentosic acid, and tryptophan.

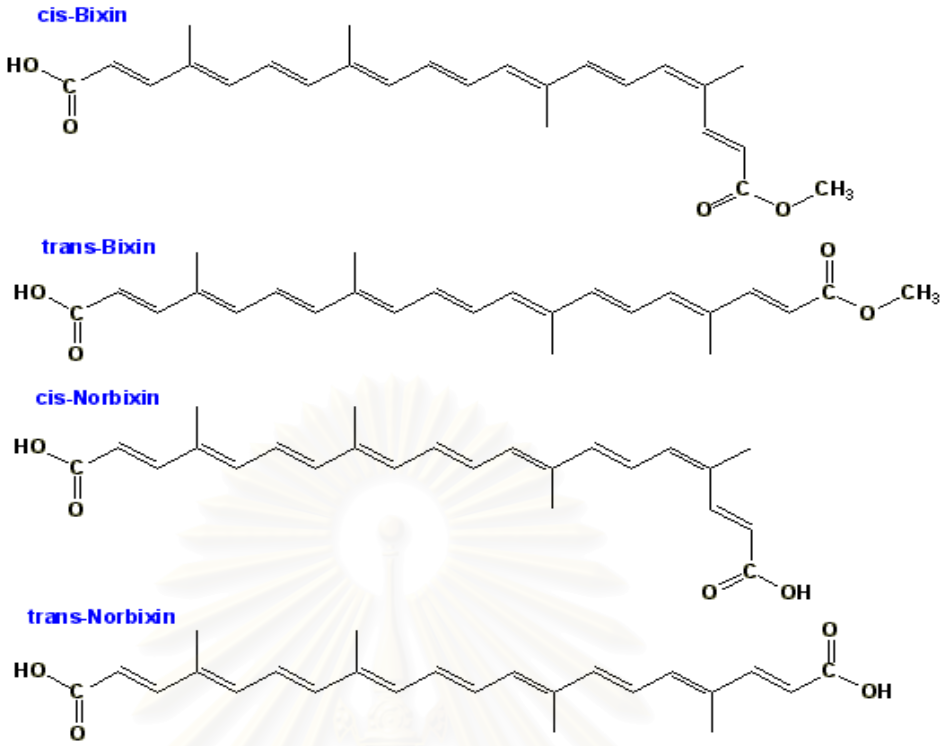


Figure 9. Structures of bixin (*cis*-and *trans*-) and norbixin (*cis*- and *trans*-)

2.3.4.2 Pharmacological Activities and Clinical Trials of Annatto

Annatto seeds have long been used in traditional medicine of the South American Indians to promote the healing of wounds, against skin eruptions, and in the healing of burns and have been given internally to subdue diarrhea and asthma (Morton, 1989). It is not clear if these effects are attributable to bixin or to some other compounds in the annatto seeds.

Bixin, the major component of annatto seeds, has strong physical quenching activity of singlet molecular oxygen and thus may excert a protective action against some types of cancer (DiMascio *et al.,* 1990). As a protectant against biological membrane oxidation, bixin is a potent inhibitor of lipid peroxidation at the same level of lutein and canthaxanthin and is only surpasses by α-tocopherol (Zhang *et al.,* 1991).

Annatto extracts seem to be of low acute oral toxicity in rodents (JECFA, 1982; Hallagan *et al.,* 1995) and annatto pigments are considered not to be genotoxic, based mainly on *in vitro* screening data (Sasaki *et al.,* 1980; Haveland-Smith, 1981; Ishidate *et al.,* 1984; Fujita *et al.,* 1988). Kovary *et al.* (2001) recently demonstrated that fibroblasts treated with norbixin *in vitro* were rendered either resistant or susceptible to DNA damage induced by hydrogen peroxide, as measured

by the comet assay. The *in vivo* genotoxic potential of the annatto pigments was also investigated and founded that norbixin ingestion did not induce any detectable DNA breakage in liver and kidney (Fernandes *et al.,* 2002). Taking into account the available toxicological data on annatto extracts, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established an acceptable daily intake (ADI) for man as low as 0–0.065 mg/kg body weight (expressed as bixin) (JECFA, 1982).

2.4 Effect of Food Processing on Antimutagenic Activity

Processing may reintroduce a novel compound having activity on antimutagenicity or having pro-antimutagen. For example, the least processed form of tea, that called Exotica China White tea, showed more antimutagenic activity than premium green tea. Their different processes may affect the active ingredients that have an antimutagenic activity. Green tea was pan-fried or steamed, rolled or shaped and dried but white tea was steamed only and then dried. (Santana-Rios *et al*., 2001). Butz *et al*. (1997) studied the antimutagenic activities of high pressure processing fruit and vegetable juice against 2-amino-3-methyl-imidazo [4,5-*f*] quinoline (IQ) in Ames test. The results showed that thermal stability of the antimutagenic factors of each sample is varied considerably.

Turkmen *et al*. (2006) studied the effects of cooking methods on chlorophylls, pheophytins and color of selected green vegetables. As a result, chlorophylls a and b content of these vegetables were reduced in various extents to their derivatives, pheophytin a and pheophytin b, which also had an antimutagenic effect. From this nutritional point of view, cooking causes a loss of main active chemicals which are known to have health benefits such as antimutagenicity and anticarcinogenicity. They concluded that nutritional properties of vegetables or fruits might have been maintained since the degradation products might also have the similar health effects.

2.5 Steamed Starchy Dessert as a Model of Thai Dessert

Traditionally, Thai desserts are composed of three main ingredients, namely flour, sugar and coconut. The classification of Thai desserts depends on various criteria such as cooking method, region and festival (Harntrikanon, 2006). Steaming is one of the favorite cooking methods in making Thai desserts, namely Khanom Chan, Khanom Nam Dok Mai, Khanom Taan, Khanom Pui Fai, Khanom Tien.

Steaming is a preferred cooking method for health conscious individuals because no cooking oil is needed, thus resulting in a lower fat content. Steaming also results in a more nutritious food than boiling because fewer nutrients are destroyed or leached away into the water (which is usually discarded) (กระยาทิพย์ เรือนใจ, 2549). Besides, Harntrikanon (2006) studied the effects of cooking method on the antimutagenicity activity of Thai desserts containing natural food colors derived from *Monascus* spp. against urethane. The result showed that moist heat dessert (by steaming at 100^{o}C, 10 min) had stronger protective effect than that of dry heat dessert (by baking at 180^{o}C, 15-20 min).

2.6 Somatic Mutation and Recombination Test (SMART)

Drosophila melanogaster is a fruit fly, a little insect about 3 mm long, of the kind that accumulates around spoiled fruit. It is also one of the most valuable of organism in biological research, particularly in genetics and developmental biology. *Drosophila* has been used as a model organism for research for almost a century. Originally, it was mostly used in genetics, for instance to discover that genes were related to proteins and to study the rules of genetic inheritance. More recently, it is used mostly in developmental biology, looking to see how a complex organism arises from a relatively simple fertilized egg. Embryonic development is where most of the attention is concentrated, but there is also a great deal of interest in how various adult structures develop in the pupa, mostly focused on the development of the compound eye, but also on the wings, legs and other organs. Today, several thousand scientists are working on many different aspects of the fruit fly.

Somatic mutation and recombination test (SMART) of *Drosophila*, the rapid, inexpensive and sensitive testers, have received increasing interest because of its well-known array of genotoxicity test systems. These assays are able to detect a wide spectrum of genetic end points, such as point mutations, deletions, certain types of

chromosome aberrations as well as mitotic recombination and gene conversion (Graf *et al.,* 1984; Würgler and Vogel, 1986; Vogel and Zijlstra, 1987). This eukaryote, namely *Drosophila melanogaster*, presents several advantages. The main points are: it is a eukaryotic organism with a short generation time (approx. 10 days at 25°C); it has easily detectable genetically controlled morphological characters; large numbers of mutants and genetically characterized strains are available; culture media are inexpensive and allow the breeding of large numbers of animals using simple facilities; it is capable of activating enzymatically promutagens and procarcinogens *in vivo*. It is well established that *Drosophila* possesses a versatile system for the metabolism of xenobiotics. The SMART provides a suitable substitute or at least a complementary *in vivo* method to mammalian *in vivo* investigation. *Drosophila* has detoxification-activating systems in many respects closely resembling the corresponding systems in mammals, which makes it possible to extrapolate data to mammals. The use of SMART assays is based on the treatment of larvae, and besides the number of mutated spots appearing in the adult flies, indicating the frequency of genetic events, the size of the spots indicates the time of action during embryogenesis.

Two different SMART systems have been vastly investigated, namely the wing spot test and the eye spot test. Both are based on the fact that during early embryonic development, groups of cells (imaginal discs) are set apart. They proliferate mitotically during the larval development until they differentiate during metamorphosis into structures of the body of the adult fly (eyes, wings, *etc*.). The somatic assays take advantage of the possibility to expose such large populations of mitotically growing cells in the imaginal discs of larvae. If genetic alteration occurs in one of these imaginal disc cells, this alteration will be present in all the descendent cells and will form a clone of mutant cells. If the alteration causes a visible change in the phenotype, the mutant cell clone can be detected as a spot of mutant cells on the body surface of the adult flies. The SMART assays were developed to detect the loss of heterozygosity of suitable gene markers which determine detectable phenotypes expressed on the eyes or the wings of the flies. The somatic assays can be performed in only one fly generation in contrast to the classical test for sex-linked recessive lethals in germ cells which need at least one month for completion (Vogel and Zijlstra, 1987). Owing to these advantages, the SMART assays have become a very

suitable approach for genotoxicity testing of chemical and physical agents (Würgler and Vogel, 1986; Graf *et al*., 1989).

The SMART assays are not only useful to analyze single pure compounds for genotoxic activity, but also to investigate genotoxicity of complex mixtures of various origins. The wing spot test has been used for genotoxicity studies with several kinds of beverages used for human consumption, such as different types of coffees, various herbal teas as well as wines and brandy (Graf and van Schaik, 1992). The wing spot test has also been employed to investigate the genotoxic potential of airborne contaminations. Extracts from differently located building ventilating filters which were exposed or not exposed during a warehouse fire in Switzerland were evaluated (Graf and Singer, 1989). More recently, extracts of airborne particulate matter collected at two different sites on two dates in Mexico City were evaluated using the standard (ST) and the high bioactivation (HB) crosses. The extracts showed genotoxic activity predominantly in the HB crosses which indicated the presence of indirectly acting genotoxins. On dates, the contamination with air particles and the resulting genotoxic activities were higher at the downtown location than at the suburban site. Furthermore, there was a good correlation with the results obtained with the same extracts in the *Salmonella* microsome assay. This study demonstrates the sensitivity of the *Drosophila* wing spot test to the organic fraction of airborne particles. The wing spot test is also a suitable model for the study of *in vivo* nitrosation reaction and their modulation.

Furthermore, the SMART assays have been used for investigations of antigenotoxicity of specific compounds or mixtures, such as coffee, chlorophyll, chlorophyllin and vitamin C. The first published paper by Negishi *et al*. (1989) indicated inhibitory effect of chlorophyllin on the genotoxicity of Trp-P-2 (3-amino-1-methyl-*5H*-pyrido[4,3-*b*]indole) in the wing spot test. In the same year, Katz (1989) showed that sodium thiosulfate is an efficient inhibitor of cisplatin-induced mutagenesis, also using the wing SMART. A series of papers were then published on the protective effects of chlorophyllin against gamma rays (Zimmering *et al*., 1990), chromium (VI) oxide (Olvera *et al*., 1993), and a number of carcinogens (Negishi *et al*., 1994). Hayatsu *et al*. (1992) showed the suppressing effect of carcinogens in the wing spot test by (-)-epigallocatechin gallate, a flavonoid compound. Furthermore, the protective effect of ascorbic acid (Vitamin C) against the genotoxicity of gamma rays and chromium (VI) oxide (Olvera *et al*., 1995), and the effects of bio-

antimutagens against chlorambucil and methotrexate (Sato *et al*., 1995) in wing somatic cells have also been exhibited. With respect to the antigenotoxicity of complex mixtures, Abraham and Graf (1996) indicated that instant coffee demonstrates protective effects against series of known mutagens and carcinogens. In addition to these antigenotoxicity studies, several researchers have published some papers on the modulation of genotoxicity in the wing somatic cells by various modulating agents such as enzyme inhibitors or inducers (Cederberg and Ramel, 1989; Romert *et al*., 1990).

2.6.1 Principle of the Somatic Assays *Drosophila melanogaster*, a dipteran insect, develops through successive developmental stages of different duration. *Drosophila* undergoes complete metamorphosis: (duration 1 day at the optimal culture temperature of 25°C), 1st larval instar (L1, 1 day), 2nd larval instar (L2, 1 day), 3 rd larval instar (L3, 2 days), metamorphosis in pupal stage (prepupa 4 hr, pupa 4.5 days) and adult stage (imago, up to 40 days) (Würgler *et al*., 1991).

During embryogenesis primarily larval tissues (cuticle, gut, fat body, nervous system, *etc*.) are found, and during the larval period these tissues enlarge and finally form the body of a large L3 larva ready for pupation. The adult structures (wings, legs, eyes, etc.) are formed in the pupal stage from the so-called imaginal discs. These develop during embryogenesis. Such discs grow during the larval period by cell proliferation. The cells of the imaginal wing disc derived from a sample of about 50 nuclei of the primitive egg syncytium, which happen to migrate to given region of egg cortex. After these nuclei have been surrounded by cytoplasm and membranes, the corresponding cells become developmentally segregated from the neighboring ectodermal cells. They do not divide during embryonic development but can already be detected histologically, grouped in a discrete wing imaginal disc, in the newly hatched larva. Proliferative growth starts in the first instar and continues throughout the larval period. Cell proliferation is logarithmic in all the presumptive adult cuticular cells, although the number of cells per clone deviates from 2^n. On average, cells divide every 8.5 hours and growth is complete after 9-10 cell divisions. After pupation, mitoses are still dectecable, but somatic crossing over initiated at this time result in single marked cells. Twenty-four hours later, mitosis ceases altogether and visible cell differentiation begins. During differentiation about 50,000 cells give rise

to single identifiable cuticular processes organized in the typical adult pattern (Würgler *et al*., 1991).

During disc growth in the larval stage if a wing imaginal disc cell is genetically altered into a mutant form, a group of mutant cells will result from clonal expansion during disc growth. After pupation, in the course of metamorphosis of the imaginal disc into an adult wing the mutant phenotype will become expressed. The mutant clone will be recognizable as a group of phenotypically altered wing blade structure (called a "spot"), e.g. showing multiple hairs instead of the single hair formed by each wild-type wing cell (Würgler *et al*., 1991).

2.6.2 Wing Spot Test the wing spot test makes 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. The two wing hair markers are both located on the left arm of chromosome 3. The appearance of multiple wing hairs (*mwh*, 3-0.3) is a recessive, homozygously viable mutation and produces multiple trichomes per cell instead of the normally unique trichome. The second marker flare^3 (*flr*^{3}, 3-38.8*)* is a recessive mutation that produces malformed wing hairs that have the shape of a flare. All three mutant alleles of *flr* are recessive zygotic lethals. However, 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). In all the experimental series analyzed, the occurrence of the various types of spots was as follows: most frequent were 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).

There exist several mechanisms that lead to genetically marked clones (Figure 10). 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 result in a *mwh* single spot. If both types of recombination events (one between *flr*^{3} and the centromere and a second between *mwh* and *flr*^{3}) take place within the same cell, a *flr*^{3} single spot may result.

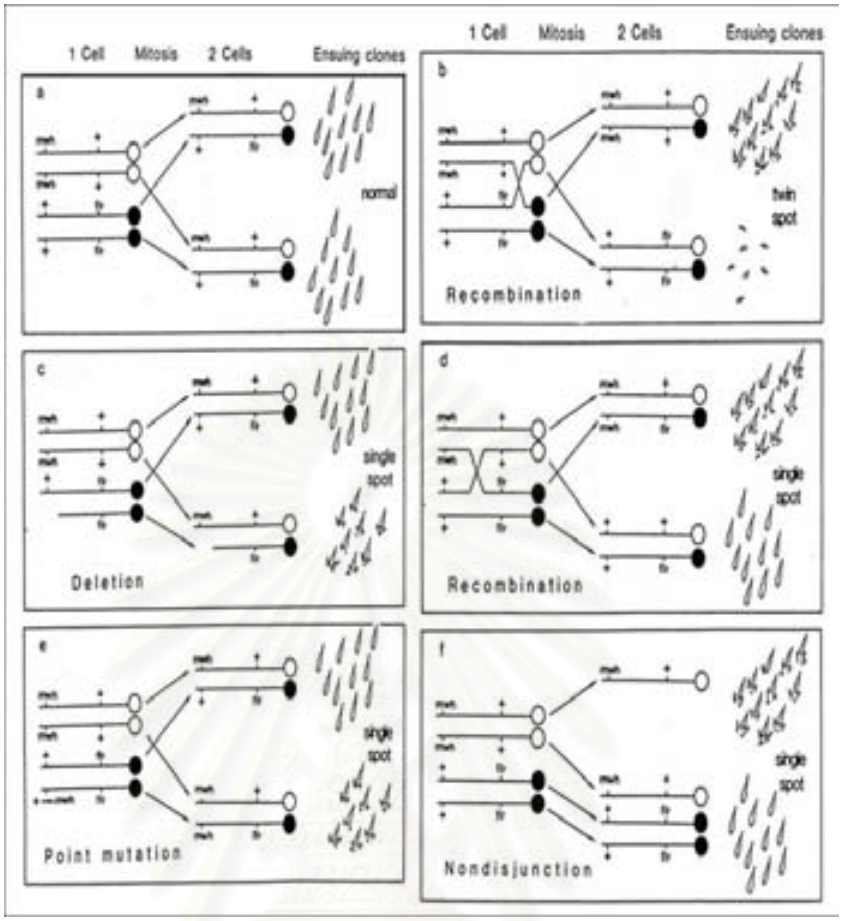


Figure 10. Genetics schemes illustrating various ways of spot formation in the somatic mutation and recombination test with the wing cell markers multiple wing hairs (*mwh*) and flare (*flr*^{3}) (a). Twin spots are obtained by recombination proximal to the *flr*^{3} marker (b), while more distal recombination 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)

A “twin spot” is an indicative of a recombinational event. Nondisjunctional or other loses of the chromosomes carrying the wild 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", showing 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 recombinational event occurring in the chromosome segment between the two marker genes. But also 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).

2.6.3 Approach of SMART

Three crosses of flies carrying the marker *mwh* and *flr*^{3} on the left arm of chromosome 3 were 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 previously (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 described by Frölich and Würgler (1989). A number of promutagens show increased genotoxicity when the HB cross is used, compared with ST cross (Frölich and Würgler, 1989; Frölich and Würgler 1991). These are: (1) The presence of an irregular whirling in the pattern of wing hairs making spot classification difficult, especially for inexperienced scores, (2) an undesirably high variation in results from repeated experiments, (3) the low egg production of the females used and the delay in development of the larvae of HB cross.

3. Improved HB 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 cytochrome P-450 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 evaluating the genotoxicity of promutagens (Graf and van Schaik, 1992).

2.6.4 Standard Mutagens for Mutagenicity of SMART

Urethane ($NH_2COOCH_2CH_3$), also known as ethyl carbamate, is the ethyl ester of carbamic acid (NH_2COOH). Urethane may occur as a colorless, odorless crystal white, granular powder. It is slightly soluble in olive oil and soluble in water, ethane, ether, glycerol, chloroform, and ethyl ether. In the 1940s, urethane was used as a hyponotic in man at doses of 1 g/person/day and as an anesthetic for laboratory animals. In 1943, it was discovered that urethane had a carcinogenic effect in animals. It is regarded as an initiator, but also as a co-carcinogen and specifically as a promoter of radiation-induced cancer. Since 1948 it has been known that urethane is mutagenic in *Drosophila melanogaster*. Urethane is generally used as positive standard toxicants in evaluation genotoxicity of the unknown compounds in SMART (Abraham and Graf, 1996). This chemical requires metabolic activation via the cytochrome P-450 enzyme system (Schlatter and Lutz, 1990) to express its mutagenic activity (Frölich and Würgler, 1990a). Today, humans are exposed to urethane in their food, mainly fermented foods and alcoholic beverages such as stone-fruit brandies, sherry and table wines (Schlatter and Lutz, 1990; Stoewsand *et al*., 1991).

2.6.5 Metabolic Activation and Detoxification of Urethane

Urethane was found to induce point mutation, gene conversion, intrachromosomal recombination, chromosomal aberrations and sister chromatid exchanges in yeast, plant systems and mammalian cells (Schlatter and Lutz, 1990). Urethane exerts its carcinogenic effect following bioactivation to vinyl carbamate epoxide which forms RNA and DNA adducts and initiates tumorigenesis (Dahl *et al*., 1978; Leithauser *et al*., 1990). The activation of urethane is important in exerting its carcinogenic effect. The two steps oxidation of urethane to the active vinyl carbamate epoxide is catalyzed primarily by cytochrome P-450 subtype 2E1 (Guengerich *et al*., 1991).

Urethane is metabolized by two different pathways (Figure 11). The major pathway, which accounts in rodents for over 90%, is the hydrolysis of urethane by microsomal esterase and amidases to ethanol, ammonia and carbon dioxide (Mirvish, 1968; Park *et al*., 1993).

This major pathway is probably one for detoxification. The minor pathway involves the oxidation of urethane via cytochrome P-450 2E1 (CYP2E1) to 2-

hydroxyethyl carbamate, to *N*- hydroxyethyl carbamate and to vinyl carbamate, which is in turn converted by epoxidation to the putative ultimate carcinogen vinyl carbamate epoxide (Guengerich and Kim, 1991; Guengerich *et al*., 1991; Miller and Miller, 1983). Vinyl carbamate epoxide is a major strong ultimate reactive electrophilic, mutagenic and carcinogenic metabolite of urethane and vinyl carbamate in mouse (Park *et al*., 1993). Generation of the electrophillic vinyl carbamate epoxide leads to the formation of RNA and DNA adducts and the initiation of tumorigenesis (Leithauser *et al*., 1990).

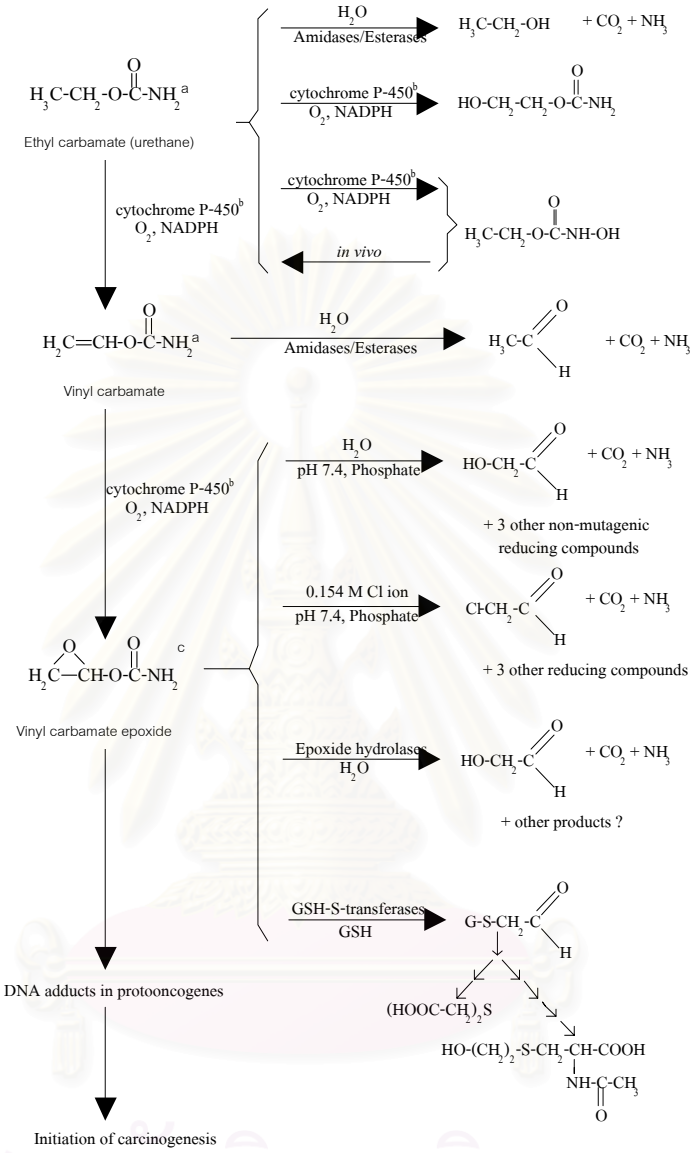


Figure 11. Known and probable activation and inactivation pathways of metabolism of urethane (ethyl carbamate), vinyl carbamate and vinyl carbamate epoxide. (a) Mouse liver microsomes + ethyl carbamate or vinyl carbamate +adenosine ⟶ 1,N^6-ethenoadenosine. (b) Human liver microsomal cytochrome P-450 2E1. (c) Vinyl carbamate epoxide + adenosine ⟶ 1, N^6-ethenoadenosine. GSH = glutathione (Park *et al.*, 1993)

2.6.6 Mutagenicity of Urethane

Many reports were published concerning the mutagenicity of urethane in a wide range of organisms (Field and Lang, 1988). In tests with eukaryotic cells, positive and negative findings were about equal in frequency. It seemed that positive results were obtained only under conditions of appropriate metabolic activation. Urethane was genotoxic in the somatic mutation and recombination test in *Drosophila melanogaster* (number and shape of wing hairs after treatment of larvae), in a standard strain and in a strain in which genetic control of cytochrome P-450 dependent enzyme systems were altered (constitutively increased P-450 enzyme activities) (Frölich and Würgler, 1988; Frölich and Würgler, 1990b). The effects were dose-dependent and the modified strain was more sensitive to urethane by about one order of magnitude than the standard strain. This further suggested that the P-450 enzyme system involved in the activation of urethane. More than 10 publications are available with quantitative data on DNA adduct formation by urethane. Several authors proposed a metabolic pathway which leads to the formation of vinyl carbamate and, after epoxidation, to DNA and RNA adducts (Miller and Miller, 1983; Leithauser *et al*., 1990). In addition, there is a vast literature on urethane carcinogenicity (Mirvish, 1968; IARC, 1974) and urethane is a pluripotent carcinogen with respect to tumor induction in different species, organs, and the stages of development of the animals (Zimmerli and Schlatter, 1991).

2.6.7 Modification the Mutagenicity of Urethane

A number of compounds found to be good inducers of cytochrome P-450 subtype 2E1 (CYP2E1) such as ethanol can increase the metabolism of urethane or decrease it depending upon the condition of exposure (Yamamoto *et al*., 1990; Kurata *et al*., 1991). Acute administration of high doses of ethanol may postpone the metabolism of urethane, possibly by blocking metabolizing enzymes, including the group of cytochrome P-450 (Waddell *et al*., 1987; Yamamoto *et al*., 1988). Chronic administration of ethanol, in contrast to the acute situation, may lead to induction of metabolizing enzymes systems such as P-450 (Lieber *et al*., 1987), and thus modulate the carcinogenicity of urethane. Mirvish (1968) reported that degradation of urethane was inhibited up to 90% by blocking esterase activity, which indicated that ethanol may be formed in near equimolar amounts to the administered urethane dose. It was also shown whether the ethanol thus formed and modulated the further

metabolism of urethane. Kurata *et al*. (1991) demonstrated that acetone was a very potent, acute initiator of the *in vivo* metabolism of urethane when metabolically derived from 2-propanol. Conversely, pretreatment using acetone for 24 and 48 hours before urethane administration accelerated the clearance of urethane, indicating that enzyme metabolizing urethane was induced by acetone. Urethane is also metabolized by esterase to yield ethanol, carbon dioxide and ammonia. Carlson (1994) indicated that the cytochrome P-450 inducers, Phenobarbital and β-naphtholflavone, and esterase inhibitor, paraoxon, were without effect to the conversion of (carbonyl ^{14}C) urethane to $^{14}CO_2$ while the CYP2E1 inhibitor, diethyldithiocarbamate, decreased metabolism. Pyridine, was also shown previously to enhance this metabolism in microsomal preparations, greatly inhibited it *in vivo*.

Kemper *et al*. (1995) investigated the role of glutathione in protection against vinyl carbamate epoxide-mediated adduct formation, and the involvement of glutathione-*S*-transferase in detoxification of vinyl carbamate epoxide. They reported that glutathione inhibited formation of ethenoadenosine, a measure of vinyl carbamate epoxide toxicity, in a concentration-dependent manner at concentration ranging from 1 to 8 mM. This effect was significantly enhanced by addition of rat liver glutathione-*S*-transferase. In addition, pretreatment of mice with 1% dietary butylated hydroxyanisole (BHA) caused parallel increases in cytosolic glutathione-*S*-transferase activity and cytosolic enhancement of detoxification of vinyl carbamate epoxide by glutathione. The major conclusions of the study were (1) vinyl carbamate epoxide could be detoxified by spontaneous conjugation with glutathione, (2) conjugation of vinyl carbamate epoxide with glutathione could be catalyzed by glutathione-*S*-transferase, (3) pretreatment with BHA protected against binding of active urethane metabolites *in vivo* and *in vitro,* and (4) the protective effect of BHA against urethane metabolite was mediated by increases in glutathione-*S*-transferase activity and glutathione concentration. De flora *et al*. (1986a) reported that *N*-acethylcysteine (NAC), a precursor of intracellular glutathione, induced a significantly and counteracted the mutagenicity of direct acting compound (such as epichlorohydrin, hydrogen peroxide), as a result of its reducing and scavenging properties. At high concentrations, NAC completely inhibited the mutagenicity of procarcinogens such as cyclophosphamide, cigarette smoke condensate and aflatoxin B1 by binding their electrophilic metabolites. In contrast, decreasing NAC concentrations stimulated their metabolic activation, especially when liver

preparations from enzyme-induced rat were used. In addition, when administered with the diet, NAC markedly inhibited the induction of lung tumors in mice by urethane (De flora *et al*., 1986a; De flora *et al*., 1986b). Abraham *et al*. (1998) investigated the change in glutathione-*S*-transferase activity in relation to the observed *in vivo* antigenotoxicity of fresh vegetables, spices, tea and coffee. This experiment showed that treatment with urethane alone resulted in inhibition of glutathione-*S*-transferase activity. In addition, only coffee could moderately increase glutathione-*S*-transferase activity, while extracts of vegetables, spices and tea did not exert any significant effect on glutathione-*S*-transferase level. However, combination of urethane with extracts of vegetables, spices, and coffee attenuated the inhibitory effects observed with urethane alone in the mouse bone marrow micronucleus test. They concluded that there was no indication of a firm association between the observed protection against genotoxicity and glutathione-*S*-transferase activity (Abraham *et al*., 1998).

A dose-dependent increase in the genotoxicity activity of urethane was observed in SMART (Frölich and Würgler, 1990a). The frequency of induction of mutations in the modification strain with increased P-450 enzyme activities was increased by about one order of magnitude compared with the standard strain. The frequencies of spots per wing in high bioactivation cross were higher than those of standard cross (Frölich and Würgler, 1990b). This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites.

2.6.8 Application of SMART

The wing test system can detect a great variety of genotoxins belonging to different chemical classes, including compounds directly or indirectly interacting with DNA. Detection of direct acting mutagens: Strong direct acting mutagens such as methylating and ethylating monofunctional alkylating agents, e.g., methyl nitrosourea, ethyl nitrourea, and EMS, or polyfuntional alkylating agents are readily detected in the wing spot test. In these cases, short acute treatments of just a few hours duration are sufficient to detect the genotoxic activity.

To confirm possible genotoxic role of retinol, the result show that the SMART test with *Drosophila melanogaster* gave a possible effect in the marker-herterozygous flies, producing statistically significant increase in the frequencies of

total spots, indicating that retinol at 96 μM is active in this test system (Klamt *et al*., 2003).

SMART was used to test antibiotic, doxorubicin (DOX), which has been clinically used for more than 30 years, especially against solid tumors (Lehmann *et al*., 2003), and use to check safety of the vinca alkaloids; vincristine (VCR), vinblastine (VBL), vinorelbine (VNR), and chemotherapeutic drugs. All drugs assessed induced genetic toxicity, causing increments in the incidence of mutational events as well as in Somatic recombination (Tiburi *et al*., 2002).

SMART test was also used to detect mutagenic activity of fungicides, namely captan and captafol under *in vivo* condition and the ability of both compounds. The results presented in the work demonstrated the weak overall mutagenic activity (Rahden-Staron, 2002).

Detection of promutagens: biochemical studies as well as genetic tests with a large variety of promutagens and procarcinogens showed that *Drosophila* possesses a potent xenobiotics metabolism. Corresponding enzymatic activities were demonstrated in homogenates of adult flies as well as larvae. Genetic studies indicated an active metabolism in germ cells of adults and larvae.

Detection of unstable mutagens: Unstable compound can be detected in feeding experiments already with a few hours of exposure. This clearly illustrates the ease of detecting the mutagenicity of an unstable compound in the wing assay by means of an acute feeding regime.

The *Drosophila* wing spot test has been used as well for the study of the modulation of genotoxicity by chemopreventive agents. Negishi *et al*. (1989) for the first time showed that chlorophyll and Cu-chlorophyllin inhibit Trp-P-2 mutagenicity in the wing spot test, on feeding the larvae with the green pigments together with Trp-P-2. Later, with the use of the *Drosophila* wing spot test, protective effects of chlorophyllin against γ-ray irradiation (Zimmering *et al*., 1990), against chromium mutagenesis (Olvera *et al*., 1993) and against various carcinogenic mutagens were shown (Negishi *et al*., 1994). Various antimutagens such as sodium thiosulphate (Katz, 1989), ascorbic acid (Olvera *et al*., 1995), instant coffee (Abraham, 1991; Abraham and Graf, 1996) have modulating effects on genotoxic agents in the wing somatic cells of *Drosophila.*

The genotoxicities of a series of *N*-nitrosamines, *N*-nitrosodiethyamine, *N*-nitrosodiethylammine, *N*-nitrosodi-n-butylamine, *N*-nitrosomorpholine,

nitrosopiperidine and *N*-nitrosopyrolidine were in the wing spot test. All showed clearly genotoxic in somatic cells of *Drosophila* in this study (Negishi *et al*., 1991).

Chromium (VI) oxide and chromium (II) chloride were tested in *Drosophila melanogaster* according to standard procedures. The hexavalent compound was highly genotoxic whereas the trivalent was clearly non-genotoxic. Furthermore, over 90% of the spots induced by Chromium (VI) oxide are due to mitotic recombination (Graf *et al*., 1992a).

The genotoxicity of three polycylic aromatic hydrocarbons (PAHs) and of three of their nitro derivatives was evaluated in the wing somatic mutation and recombination test in *Drosophila melanogaster*. These results demonstrate that the genotoxic activity of these PAHs and their nitro derivatives can be detected with the somatic cells of the wing imaginal discs of larvae with high bioactivation capacity (Delgado-Rodriguez *et al*., 1999). The SMART was also used to study the mutagenic potential of three benzo[*c*]phinanthridines with antileukemic properties, fagaronine, nitidine and *O*-methylfagaronine, as compared with that of two structurally related aromatic hydrocarbons: 7, 12-dimethylbenzo[*a*]anthracene and chrysene (Perez-Chiesa and Rodriguez, 1993).

The potent food mutagen and carcinogen 2-amino-3-methylimidazo[4,5*f*] quinoline (IQ), the structurally related heterocyclic aromatic amines 2-aminoimidazo [4,5*f*]quinoline (dimethyl-IQ) and 2-amino-1methylimidazo[4,5*f*]quinoline (iso-IQ) with 3-nitroaromatics 3-amino-2methylimidazo[4,5*f*]quinoline (nitro-IQ), 2-nitroflurorene and 1,8diniropyrene were assayed for genotoxicity in SMART. Iso-IQ, IQ, dimethyl-IQ and niro-IQ exhibited genotoxic activities (Graf *et al*., 1992b).

The *in vivo* nitrosation capacity was assayed and demonstrated that the wing spot test is well suited for the determination of genotoxicity produced by *in vivo* nitrosation processes and for the study of their modulation by individual compounds or complex mixtures (Guzmen *et al*., 1998).

2.7 Antioxidant Assays

A wide range of methods are currently used to assess antioxidant capacity (Halliwell *et al*., 1995), for example for 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 are 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, have gained popularity because they enable 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.

2.7.1 2, 2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

DPPH assay is a common employed assay in antioxidant studies and offers a rapid technique in which to screen the radical scavenging activity of pure synthetic compounds, crude plant extracts and foods. The reaction of DPPH was used to compare the radical scavenging activity of a compound with Trolox, a watersoluble vitamin E analogue (van den Berg *et al*., 1999; Pietta *et al*., 1988). 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. Reduction of DPPH by an antioxidant or by a radical species results in a loss of absorbance at 520 nm. Thus, the degree of discoloration of the solution indicates the scavenging efficiency of the added substance (Fukumoto and Mazza, 2000).

2.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out by the method of Benzie and Strain (1996) with minor 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 development, provided that a reductant (antioxidant) is present. Ferrozine (Stookey, 1970), a compound closely related to TPTZ, has been widely used, with excess ascorbic acid, to measure iron. In the FRAP assay, excess Fe^{3+} is used, and

the rate-limiting factor of Fe^{2+}-TPTZ, and hence color, formation is the reducing ability of the sample.

2.7.3 Determination of Total Phenolic Contents

The total phenolic contents was determined according to the method described by Swain and Hillis (1959), Naczk and Shahidi (1989), Amarowicz *et al*. (2004) with minor modification. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Javanmardi, 2003). Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method. Briefly, 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 done on the basis of the standard curve of gallic acid. Results were expressed 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, USA). Glycerol was bought from Farmitalia Carlo Erla (Milan, Italy). Gum arabic powder was purchased from BDH Chemical Ltd. (Poole, England). 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-picrylhydrazl), 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). Other chemicals were of laboratory grade.

3.2 *Drosophila melanogaster* Strains

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 stocks were kept on the standard medium of Robert (1986) and maintained at a constant temperature 16±1ºC.

3.3 Sample Preparation

3.3.1 Natural Color Extracts

Herbs (Table 2) were purchased from a traditional drugstore in Bangkok, Thailand, except beetroot which was obtained from a local supermarket. Each sample (20 g), except annatto seed, was grinded coarsely and extracted with 70% ethanol (100 ml) for 1 h at 60^{o}C (วีระชัย ธารมณีวงศ์ และคณะ, 2532; เพียง อุดมเกียรติกูล, 2533; บุษรารัตน์

สายเชื้อ, 2545). The extraction procedure was repeated three times. Annatto seeds (20 g) were soaked in 70% ethanol with 3% potassium hydroxide (400 ml) and swirled every 15 min for 1 h and left in the dark at room temperature for 24 h. Each extract was filtered through cotton mesh and filter paper. The solvents (ethanol and water) were removed under reduced pressure in a vacuum rotary evaporator at the temperature lower than 60^{o}C. The residue was lyophilized, stored and protected from light in a desiccator.

Table 2. Natural colors in this study

English Name	Thai Name	Scientific Name	Color	Part Used
Roselle	Krajeab-daeng	*Hibiscus sabdariffa* L.	Purple-red	Calyx
Beetroot	Hua-beet	*Beta vulgaris* L.	Red	Root
Annatto	Kam-sad	*Bixa orellana* L.	Orange-yellow	Seed coat
Sappan	Fang	*Caesalpinia sappan* L.	Pink	Heartwood

3.3.2 Preparation of Steamed Starchy Dessert

Steamed starchy dessert was select to be a model for evaluation the stability of the natural colors during heat processing. The main ingredients of the dessert composed of arrowroot flour (15 g), tapioca flour (5 g), rice flour (1.25 g), sugar (20 g), coconut milk (10.5 g) and water (40 ml). The amount of each natural color incorporated into the dessert was depended on its solubility in water and/or toxicity to the flies. Amount of coconut milk and sugar used in these dessert models were added enough to maintain good appearance of each dessert. The original steamed starchy dessert (without any color) was studied as a negative control.

The amount of each natural color powder used in making the steamed starchy dessert were 2.56 g, 1.28 g and 0.64 g for roselle (Krajeab-daeng) powder and beetroot (Hua-beet) powder; 0.08 g, 0,04 g and 0.02 g for sappan (Fang) powder and 0.04 g, 0.02 g and 0.01 g for annatto (Kam-sad) powder. The preparation (step by step) of the steamed starchy dessert is shown in Appendix A. Each sample (dessert containing each color, namely roselle dessert, beetroot dessert, annatto dessert, sappan dessert and original dessert) was lyophilized, homogenized in a blender and stored in a desiccator until used. It was assumed that the amount of coconut milk was negligible after lyophilization.

The desserts were named after the colors and their concentrations namely high, medium, and low. For example, the roselle dessert contained roselle color powder 2.56 g was named “high roselle dessert” while “medium roselle dessert” and “low roselle dessert” were contained 1.28 g and 0.64 g of roselle color, respectively.

3.4 Somatic Mutation and Recombination Test (SMART)

3.4.1 Standard *Drosophila* Medium It was prepared accordingly to Robert (1986). Corn flour (125 g), sugar (100 g), baker’s yeast (50 g), agar (14 g) were mixed and water (1000 ml) was added. The mixture was heated in a water bath to be homogeneous and propionic acid (5 ml) was added as a preservative. A portion (approximate 30 ml) of this standard medium was distributed into a 50 ml Erlenmeyer flask for maintaining the stock of fly, mating and collecting larvae for mutagenesis study. The standard medium was served as a negative control and a positive control when it contained 1380 ppm urethane.

3.4.2 Experimental Media There were two types of experimental media. The amounts of each natural color powder, namely 30.0 mg (11628 ppm), 15.0 mg (5814 ppm), or 7.50 mg (2907 ppm) for roselle powder and beetroot powder; 1.0 mg (388 ppm), 0.5 mg (194 ppm), or 0.25 mg (97 ppm) for sappan powder; 5.0 mg (1938 ppm), 2.5 mg (969 ppm), or 1.25 mg (484.5 ppm) for annatto powder, were mixed in the standard medium using vortex mixer and this medium was named “natural color medium”. The other experimental medium, namely “dessert medium” was prepared by substituting 0.48 g each lyophilized dessert (described in 3.3.2) for corn flour in the standard medium. Each dessert medium contained the same amount of color powder as in natural color medium, but the concentration of color in this medium was lesser because other ingredients in the dessert such as sugar was included. List of experimental media is shown in table 3. All media were studied for their mutagenicity and antimutagenicity.

Table 3. Concentration of color in the experimental medium

Medium		Concentration of color (ppm)
Roselle color	Low	2907
	Medium	5814
	High	11628
Beetroot color	Low	2907
	Medium	5814
	High	11628
Sappan color	Low	97
	Medium	194
	High	388
Annatto color	Low	484.5
	Medium	969
	High	1938
Roselle dessert	Low	2669
	Medium	5338
	High	10676
Beetroot dessert	Low	2669
	Medium	5338
	High	10676
Sappan dessert	Low	89
	Medium	178
	High	356
Annatto dessert	Low	445
	Medium	890
	High	1780
Original dessert		0

3.5 Experimental design

The overall experiment to elucidate the urethane modulating effect of samples is shown in Figure 12. Toxicity of each sample was firstly determined from data of survival rate of flies and their genotoxicity. Secondly, antimutagenicity of each sample on urethane induced somatic mutation and recombination in *Drosophila melanogaster* was evaluated using co-administration and pre-feeding studies. Afterwards, antioxidant activity (using DPPH and FRAP assays) and total phenolic content were investigated.

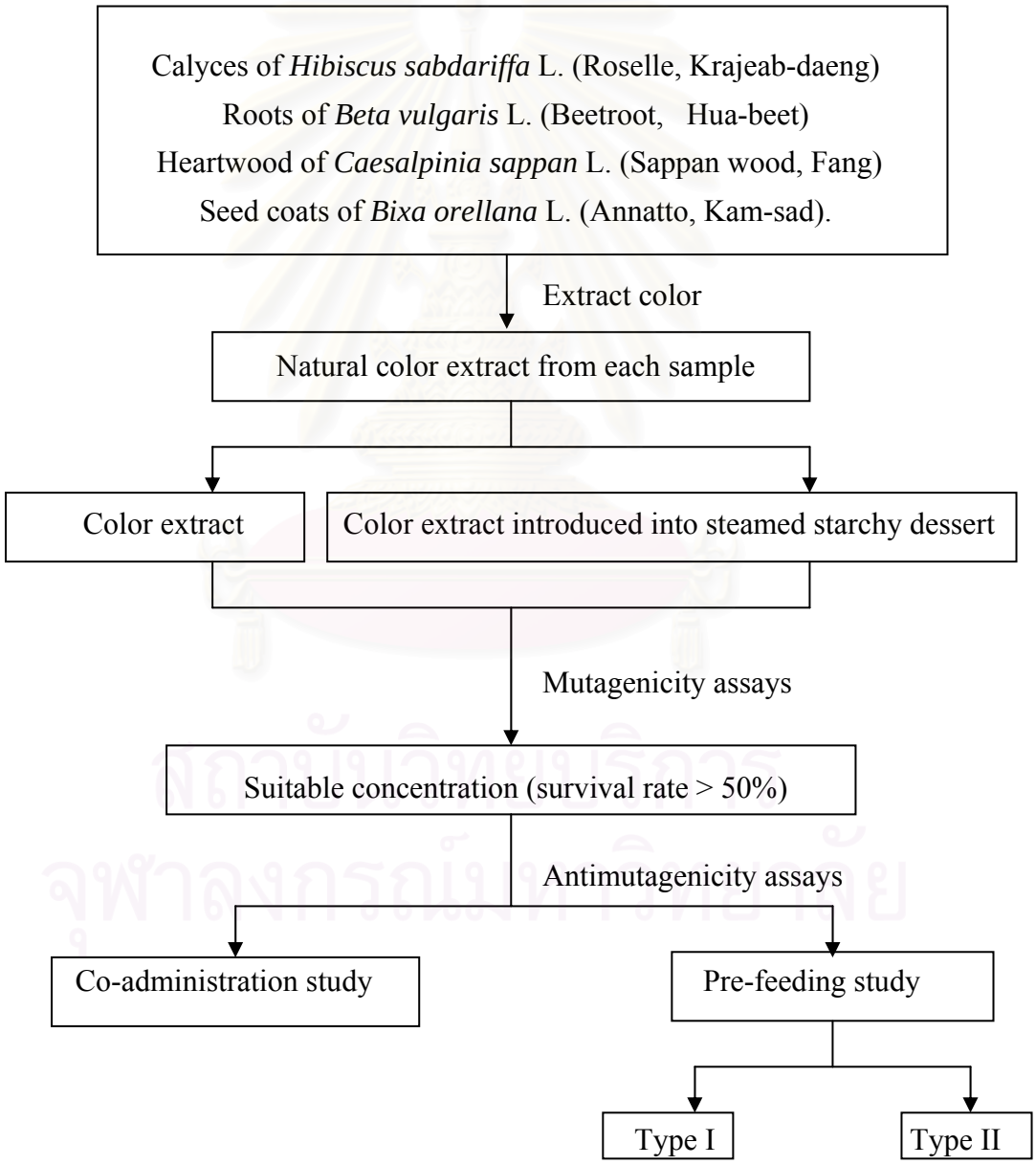


Figure 12. Overall experiment to elucidate the effect of natural color extracts

3.5.1 Mutagenicity Study Each sample was studied for its mutagenicity as described by Graf *et al*. (1984). Virgin females of *ORR; flr*^{3} were mated with *mwh* males on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water, and transferred (with the help of a fine artist's brush) to medium containing each color and their respective colored desserts, negative control and medium containing urethane 1380 ppm (positive control). They were incubated at 25$\pm$1^{o}C until pupation. After metamorphosis, the surviving flies were collected and stored in 70% ethanol. Survival rate of adult flies from larva fed on each natural color medium or dessert medium were collected to determine the toxicity of each sample. Only the medium that provide more than 50% survival of flies was determined for its mutagenicity.

The insect bearing 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, 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 and a cover slip was put on. The round wings of surviving flies (both the dorsal and ventral surface), at least 40 wings, were analyzed under a compound microscope at 400x magnification for the presence of clones of cells showing malformed wing hairs. The position of the spots was noted according to the sector of the wing (Figure 13). Different types of spots namely, single spots showing either the multiple wing hairs *(mwh)* or the flare (*flr*^{3}) phenotype, and twin spots showing adjacent *mwh* and *flr*^{3} areas were recorded separately. The size of each spot was determined by counting the number of wing cells (hairs) exhibiting the *mwh* or the *flr*^{3} phenotype. 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. Different types of wing hair mutations are shown in Figure 14.

The wing spots data were evaluated using the statistical procedure described by Frei and Würgler (1988). Frequencies of induced wing spots of both the treated groups and the negative control (deionized water treated group) were compared. 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 (Figure 14). 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 calculation step by step are shown in Appendix B.

3.5.2 Antimutagenicity Study All samples that provided more than 50% survival of adult flies and did not express their genotoxicity were evaluated in co-administration (Figure 15) and pre-feeding studies (Figure 16). The experimental medium was prepared by adding each lyophilized color powder or substituting lyophilized dessert for starch in the standard medium.

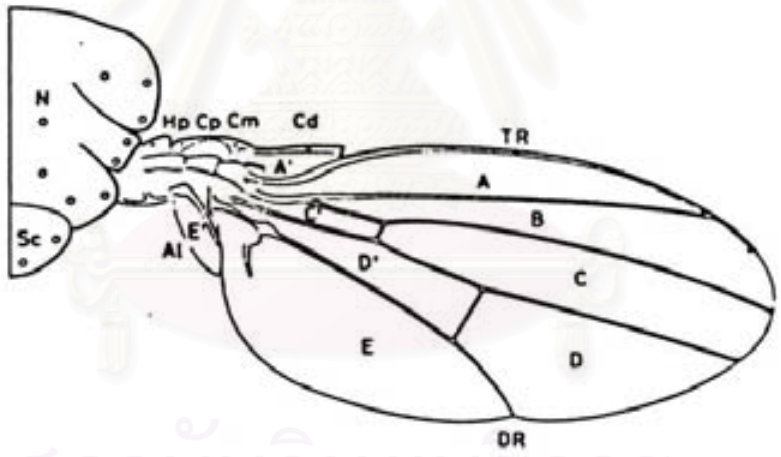


Figure 13. Normal half mesothorax showing the regions A-E of the wing surface scored for spots according to Graf *et al*. (1984).

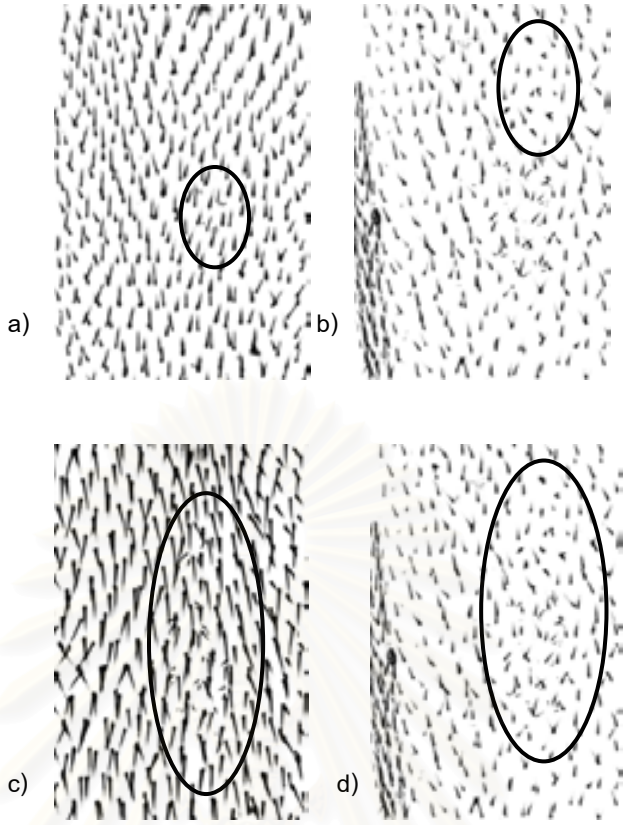


Figure 14. Marker mutations of wing surface to show clone of cuticle secreted by cells homozygous for multiple wing hairs, a) small single spots of *mwh* on wing, b) large single spots of flare on wing, c) large single spots of *mwh* on wing, d) twin spots.

3.5.2.1 Co-administration Study Virgin *ORR;flr*^{3} females and *mwh* males were mated on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred (with the help of a fine artist's brush) to each experimental medium containing urethane 1380 ppm, negative control and positive control medium. They were incubated at 25$\pm$1^{o}C until pupation. The surviving adult flies were collected after pupation and were progressed as of mutagenicity study.

3.5.2.2 Pre-feeding study Virgin *ORR;flr*^{3} females and *mwh* males were mated on each experimental medium. Six day after mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred (with the help of a fine artist's brush) to standard medium containing urethane 1380 ppm as type 1 study and experimental medium containing urethane 1380 ppm as type 2 study. Negative and

positive controls were conducted similarly to those in co-administration study. They were incubated at 25±1°C until pupation and were progressed as of mutagenicity study.

The Antimutagenicity by each sample was estimated as suggested by Negishi *et al*. (1994). Mutagenicity index of urethane obtained from co-administration and pre-feeding were compared with that from a standard urethane group. Mutagenicity index (MI) is calculated as described below.

$$MI = \frac{\text{Spots per wing induced with urethane administrated with sample}}{\text{Spots per wing induced with urethane}}$$

Generally, MI of positive control is 1. The MI of positive control reduces if the sample contains any antimutagen or increases in the presence of potentiator. It is proposed that MI between 0.8-0.6 represented weak antimutagenicity while expression of MI between 0.6-0.4 and less than 0.4 are the evidences of moderate and strong antimutagenicity, respectively. On the other hand, the potentiation is pronounced when MI is higher than 1. It is proposed that MI between 1.2-1.4 represented weak mutagenicity potentiator while expression of MI between 1.4-1.6 and higher than 1.6 are the evidences of moderate and strong potentiation, respectively.

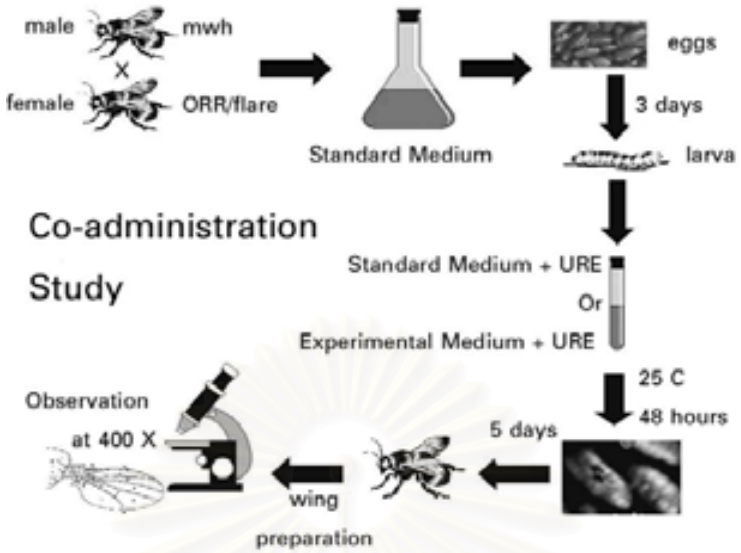


Figure 15. Co-administration study of each natural color extracts and the added color steamed starchy desserts on mutagenicity of urethane induced wing spots of *Drosophila melanogaster*.

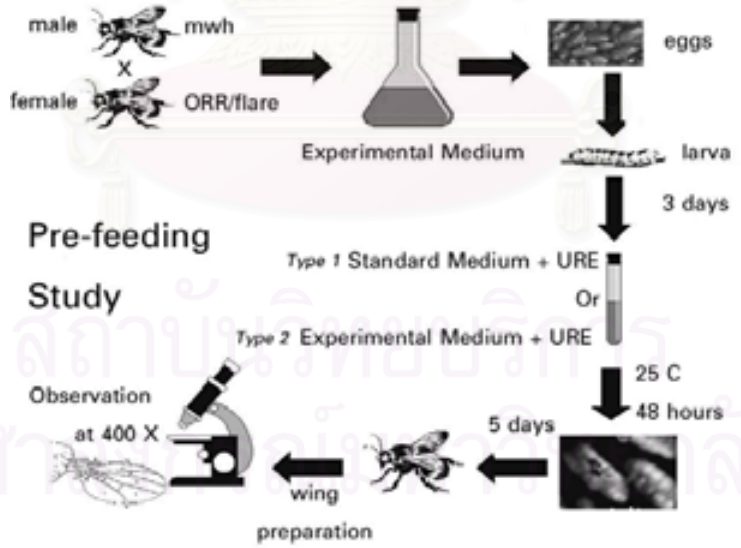


Figure 16. Pre-feeding study of each natural color extracts and the added color steamed starchy desserts on mutagenicity of urethane induced wing spots of *Drosophila melanogaster*.

3.6 Antioxidant Assay

3.6.1 Sample Extraction Five grams of each high colored dessert powder were stirred with 80% methanol (25 ml) at room temperature for 2 h. The extraction procedure was repeated three times (Fukumoto and Mazza (2000); then, it was filtered through Whatman no.5 filter paper. The volume of combined filtrate was adjusted to 75 ml with 80% methanol and assayed for its antioxidant activity. Each color extract was run simultaneously using as same concentration of color as in their chosen colored dessert.

3.6.2 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay The antioxidant activity of the methanolic extract from each sample on DPPH was estimated using the procedure described by Fukumoto and Mazza (2000) with slight modifications. An aliquot of 22 µl (in triplicate) of each extract or standard Trolox was transferred into a 96-well flat-bottom microplate (Bibby Sterilin Ltd, UK). The solution of 150 µM DPPH in 80% methanol (200 µl) was added to each microplate well. The plate was then covered and left to stand in the dark at room temperature. After 30 min, the absorbance of the solution was read in microplate reader (Sunrise, Tecan Co., Austria) using a 520 nm filter. Standard curve was constructed by using several concentrations of Trolox (0.08 - 1.28 mM in 80% methanol). The antioxidant activity of the extracts was determined using the standard curve expressed as mg of Trolox Equivalent Antioxidant Capacity (TEAC)/g dry weight of sample. The more value of TEAC, the more the antioxidant activity of the sample is. The radical scavenging activity was also calculated as a percentage of DPPH scavenging activity using the equation: % scavenging activity = $100 \times (1\text{-}A_E/A_D)$, where A_E is the absorbance of the solution when an extract is added, and A_D is the absorbance of the DPPH solution with nothing added. Data were presented as means ± SD of at least triplicate experiments.

3.6.3 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. Each (20 µl) extract or standard or blank reagent was added to each well in a 96-well microtiter plate in triplicate. FRAP reagent (150 µl), freshly prepared (see Appendix C) and warmed at 37^{o}C according to the procedure described

by Griffin and Bhagooli (2004), was added to each well. The mixture was left at room temperature for 8 min. The absorbance was read at 600 nm using a microplate reader. The change in absorbance after 8 min from the initial blank reading was compared to that of a standard that was run simultaneously. Aqueous solutions of known standard Fe^{2+} ($FeSO_4.7H_2O$) concentrations (62.5, 125, 250, 500, 1000 µM) were used for calibration. The FRAP values of the extracts were determined using this standard curve, expressed as mg of ferrous iron (Fe (II))/g dry weight of sample. Data were presented as means ± SD of triplicate wells.

3.6.4 Determination of Total Phenolic Contents The total phenolic content of methanol extract from each sample was determined according to method described by Swain and Hillis (1959), Naczk and Shahidi (1989) and Amarowicz *et al*. (2004) with slight modification by using a microplate reader. Briefly, 10 µl of each extract was transferred into a 96-well flat-bottom microplate containing 160 µl of distilled water. After mixing the contents, 10 µl of Folin-Ciocalteu reagent and 20 µl of saturated sodium carbonate solution were added. The microplate was vortexed and absorbance of blue colored mixtures recorded after 30 min at 750 nm with microplate reader (Sunrise, Tecan Co., Austria). The amount of total polyphenols was calculated as a Gallic Acid Equivalent (GAE) from the calibration curve of gallic acid standard solutions (concentration range between 25 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.

CHAPTER IV
RESULTS

Four natural red colors were extracted from calyces of *Hibiscus sabdariffa* L. (roselle, Krajeab-daeng), roots of *Beta vulgaris* L. (beetroot, Hua-beet), heartwood of *Caesalpinia sappan* L. (sappan, Fang), and seed coats of *Bixa orellana* L. (annatto, Kam-sad). These colors and their respective colored desserts were determined their toxicity and modifying effect on somatic mutation and recombination of *Drosophila* wing spots induced by urethane. All experiments were performed twice in order to observe the reproducibility of the effect of each sample.

The appearances of these colors introduced to steamed starchy dessert are shown in Figures 17-21.

Figure 17. Appearances of *Hibiscus sabdariffa* L. (roselle) color introduced to steamed starchy dessert from left to right: Low roselle dessert, Medium roselle dessert, High roselle dessert.

Figure 18. Appearances of *Beta vulgaris* L. (beetroot) color introduced to steamed starchy dessert from left to right: Low beetroot dessert, Medium beetroot dessert, High beetroot dessert.

Figure 19. Appearances of *Caesalpinia sappan* L. (sappan) color introduced to steamed starchy dessert from left to right: Low sappan dessert, Medium sappan dessert, High sappan dessert.

Figure 20. Appearances of *Bixa orellana* L. (annatto) color introduced to steamed starchy dessert from left to right: Low annatto dessert, Medium annatto dessert, High annatto dessert.

Figure 21. Appearance of control steamed starchy dessert (no color added)

4.1 Survival Rate of Adult Flies and Mutagenicity of Samples

Data in Tables 4 and 5 show the number of surviving flies obtained from the larvae brought up on each experimental medium, negative control medium, and positive control medium. The percentages of surviving adult flies fed on experimental medium containing roselle color were in the range of 88 to 99, beetroot and sappan color were in the range of 90 to 99, annatto color were in the range of 89 to 97; whereas those fed on negative and positive control media were in the range of 81 to 92 and 68 to 73, respectively. In addition, the percentages of surviving flies fed on experimental media containing steamed starchy dessert substituted for corn flour

in the standard media were between 88 to 100 for control dessert (original dessert), 77 to 100 for roselle desserts, 87 to 96 for beetroot desserts, 82 to 100 for sappan desserts, 73 to 100 for annatto desserts, and those fed on negative and positive control media were 88 and 51 to 82, respectively. Overall results indicated that none was too toxic to testing on the 3-day old larvae because the survival rate of flies fed on each experimental medium was higher than 50%.

Mutagenicity of each sample was evaluated using the data of total spots per wing. The data shown in Tables 6 and 7 indicated that none was mutagenic since they did not significantly induce the frequencies of mutant spots at any testing concentrations to be higher than that of the negative control medium.

Table 4. Survival data of adult flies obtained from 100 of 3-days old larvae (*mwh*+/+*flr*^{3}) introduced to the standard medium containing distilled water, urethane (1380 ppm) or each natural color powder (added to the standard medium).

Sample		Concentration of color (ppm)	Percent of surviving flies (%)	
			Trial 1	Trial 2
Water		0	81	92
Urethane		0	73	68
Roselle color	High	11628	99	90
	Medium	5814	98	92
	Low	2907	99	88
Beetroot color	High	11628	95	96
	Medium	5814	98	95
	Low	2907	99	90
Sappan color	High	388	99	95
	Medium	194	95	90
	Low	97	99	99
Annatto color	High	1938	91	92
	Medium	969	97	97
	Low	484.5	92	89

Table 5. Survival data of adult flies obtained from 100 of 3-days old larvae (*mwh*+/+*flr*^{3}) introduced to the standard medium containing distilled water, urethane (1380 ppm) or each sample (steamed starchy desserts containing each color) substituted for corn flour in standard medium.

Sample		Concentration of color (ppm)	Percent of surviving flies (%)	
			Trial 1	Trial 2
Water		0	88	88
Urethane		0	82	51
Original dessert		0	100	88
Roselle dessert	High	10676	94	95
	Medium	5338	100	77
	Low	2669	92	96
Beetroot dessert	High	10676	90	94
	Medium	5338	91	90
	Low	2669	96	87
Sappan dessert	High	356	83	92
	Medium	178	90	85
	Low	89	100	82
Annatto dessert	High	1780	100	81
	Medium	890	87	73
	Low	445	91	77

Table 6. Mutagenicity of each natural color powder (added to the standard medium) reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross.

Trial	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)			
				Small single m=2	Large single m=5	Twin m=5	Total m=2
1	Water		0	0.450 (18)	0	0	0.450 (18)
	Urethane		0	9.600 (384)+	3.150 (126)+	0.575 (23)+	13.325 (533)+
	Roselle color	High	11628	0.250 (10)-	0.075 (3)i	0	0.325 (13)-
		Medium	5814	0.283 (11)-	0	0	0.283 (11)-
		Low	2907	0.307 (12)-	0	0.025 (1)i	0.325 (13)-
	Beetroot color	High	11628	0.375 (15)-	0.025 (1)i	0	0.400 (16)-
		Medium	5814	0.250 (10)-	0.075 (3)i	0.025 (1)i	0.350 (14)-
		Low	2907	0.200 (8)-	0	0	0.200 (8)-
	Sappan color	High	388	0.475 (19)-	0.025 (1)i	0	0.500 (20)i
		Medium	194	0.250 (10)-	0.025 (1)i	0.025 (1)i	0.307 (12)-
		Low	97	0.100 (4)-	0.025 (1)i	0.025 (1)i	0.150 (6)-
	Annatto color	High	1938	0.307 (12)-	0.050 (2)i	0.050 (2)i	0.400 (16)-
		Medium	969	0.250 (10)-	0	0.025 (1)i	0.283 (11)-
		Low	484.5	0.175 (7)-	0.050 (2)i	0	0.225 (9)-

Table 6. Mutagenicity of each natural color powder (added to the standard medium) reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross (continued).

Trial	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)			
				Small single m=2	Large single m=5	Twin m=5	Total m=2
2	Water		0	0.475 (19)	0	0	0.475 (19)
	Urethane		0	9.175 (367)+	3.275 (131)+	0.450 (18)+	12.900 (516)+
	Roselle color	High	11628	0.275 (11)-	0.025 (1)i	0	0.300 (12)-
		Medium	5814	0.200 (8)-	0.100 (4)i	0	0.300 (12)-
		Low	2907	0.225 (9)-	0.025 (1)i	0.025 (1)i	0.275 (11)-
	Beetroot color	High	11628	0.425 (17)-	0.025 (1)i	0	0.450 (18)-
		Medium	5814	0.300 (12)-	0.050 (2)i	0.025 (1)i	0.375 (15)-
		Low	2907	0.225 (9)-	0	0	0.225 (9)-
	Sappan color	High	388	0.300 (12)-	0.050 (2)i	0.075 (3)i	0.425 (17)-
		Medium	194	0.225 (9)-	0.050 (2)i	0.075 (3)i	0.350 (14)-
		Low	97	0.125 (5)-	0	0	0.125 (5)-
	Annatto color	High	1938	0.450 (18)-	0	0.025 (1)i	0.475 (19)-
		Medium	969	0.350 (14)-	0.025 (1)i	0	0.375 (15)-
		Low	484.5	0.250 (10)-	0	0	0.250 (10)-

[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 7. Mutagenicity of steamed starchy desserts (containing each color) substituted for corn flour in standard medium reported as wing spot induction on *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross.

Trial	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)			
				Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
1	Water		0	0.450 (18)	0.025 (1)	0	0.475 (19)
	Urethane		0	8.000 (134)+	3.350 (134)+	1.300 (52)+	12.650 (506)+
	Original dessert		0	0.350 (14)-	0.050 (2)i	0	0.400 (16)-
	Roselle dessert	High	10676	0.325 (13)-	0	0	0.325 (13)-
		Medium	5338	0.275 (11)-	0	0.025 (1)i	0.300 (12)-
		Low	2669	0.200 (8)-	0	0	0.200 (8)-
	Beetroot dessert	High	10676	0.200 (8)-	0.025 (1)i	0	0.225 (9)-
		Medium	5338	0.150 (6)-	0.025 (1)i	0	0.175 (7)-
		Low	2669	0.073 (3)-	0	0	0.075 (3)-
	Sappan dessert	High	356	0.400 (16)-	0.025 (1)i	0	0.425 (17)-
		Medium	178	0.375 (15)-	0	0.025 (1)i	0.400 (16)-
		Low	89	0.300 (12)-	0.025 (1)i	0	0.325 (13)-
	Annatto dessert	High	1780	0.425 (17)-	0.050 (2)i	0	0.475 (19)-
		Medium	890	0.250 (10)-	0.025 (1)i	0.025 (1)i	0.300 (12)-
		Low	445	0.150 (6)-	0	0	0.150 (6)-

Table 7. Mutagenicity of steamed starchy desserts (containing each color) substituted for corn flour in standard medium reported as wing spot induction on *Drosophila melanogaster* from 100 *trans*-heterozygous(*mwh+/+flr*^{3})larvae of improved high bioactivation cross(continued).

Trial	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)			
				Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
2	Water		0	0.325 (13)	0	0	0.325 (13)
	Urethane		0	8.450 (338)+	4.725 (189)+	1.475 (59)+	14.650 (586)+
	Original dessert		0	0.250 (10)-	0	0	0.250 (10)-
	Roselle dessert	High	10676	0.475 (19)i	0.025 (1)i	0	0.500 (20)i
		Medium	5338	0.300 (12)-	0.050 (2)i	0.025 (1)i	0.375 (15)i
		Low	2669	0.300 (12)-	0.025 (1)i	0.025 (1)i	0.350 (14)i
	Beetroot dessert	High	10676	0.300 (12)-	0.075 (3)i	0	0.375 (15)i
		Medium	5338	0.225 (9)-	0.025 (1)i	0.075 (3)i	0.325 (13)i
		Low	2669	0.175 (7)-	0.025 (1)i	0	0.200 (8)-
	Sappan dessert	High	356	0.575 (23)i	0.050 (2)i	0	0.625 (25)+
		Medium	178	0.350 (14)i	0.125 (5)i	0	0.475 (19)i
		Low	89	0.300 (12)-	0	0	0.300 (12)-
	Annatto dessert	High	1780	0.550 (22)i	0.075 (3)i	0	0.625 (25)+
		Medium	890	0.450 (18)i	0.050 (2)i	0	0.500 (20)i
		Low	445	0.375 (15)i	0	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.

4.2 Antimutagenicity of Samples

The number of urethane induced total spot per wing reduced after administered each sample to 3-day-old larvae in co-administration study. Each mutagenicity index (MI) of urethane was calculated to show the relationship between the mutagenicity of urethane in the presence of each sample and that of only urethane (Tables 8 and 9). Results in Table 8 indicated that all color extracts could decrease the frequencies of mutant spots in larvae treated with urethane. The MIs of urethane obtained from flies derived from larvae fed on urethane with each color of both trials decreased to lower than 1 (of the positive control); roselle, beetroot, and sappan colors showed strong to moderate antimutagenic activity while the antimutagenicity of annatto color was weak.

The data in Table 9 showed that the original steamed starchy dessert may have no antimutagenic activity while the antimutagenicity of the desserts containing each color in both trials were varied in strong to negligible. The desserts contained roselle and beetroot colors still showed strong to moderate antimutagenic activity, whereas the desserts added sappan and annatto colors showed weak to negligible antimutagenicity. The latter result indicated that steaming process may influence the antimutagenicity of these colors.

The data shown in Table 10 indicated the results obtained from pre-feeding of color extracts to newborn larvae for 3 days before they were transferred to the standard medium which containing urethane (pre-feeding type1) or the medium containing each color (experimental medium) and urethane (pre-feeding type2). All colors in this study could counteract the mutagenicity of urethane in type 2 study. They showed moderate to weak antimutagenicity (MIs were between 0.535 to 0.767 in both trials). However, in type 1 study, there were only roselle and annatto colors that could slightly inhibit the mutagenicity of urethane while the antimutagenicity activities of beetroot and sappan color were negligible. No mutagenicity from all colors was observed in these studies.

Table 11 revealed the antimutagenic effect of steamed starchy desserts containing each color in the pre-feeding study. It was surprisingly that the desserts containing roselle and beetroot colors had stronger antimutagenicity activity in both trials of type 1 and type 2 study than the colors themselves (MIs were between 0.302 to 0.630), while the original dessert had weak antimutagenicity. On the other hand, the antimutagenicity activities of the desserts containing sappan and annatto colors were negligible. No mutagenicity from all of the desserts was observed in these studies.

Table 8. Antimutagenicity of each natural color on urethane (1380 ppm) 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	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
				Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water		0	0.475 (19)	0.025 (1)	0	0.500 (20)	-
	Urethane		0	17.150 (686)+	8.100 (324)+	2.300 (92)+	27.550 (1102)+	1
	Roselle color	High	11628	5.950 (238)+	3.525 (141)+	1.875 (75)+	11.350 (454)+	0.412 (m)
		Medium	5814	7.125 (285)+	4.000 (160)+	2.175 (87)+	13.300 (532)+	0.483 (m)
		Low	2907	9.175 (367)+	3.875 (155)+	1.425 (57)+	14.475 (579)+	0.525 (m)
	Beetroot color	High	11628	7.875 (315)+	4.300 (172)+	1.575 (63)+	13.750 (550)+	0.499 (m)
		Medium	5814	7.925 (317)+	4.075 (163)+	2.275 (91)+	14.275 (571)+	0.518 (m)
		Low	2907	7.850 (314)+	4.850 (194)+	1.650 (66)+	14.350 (574)+	0.521 (m)
	Sappan color	High	388	6.225 (249)+	2.775 (111)+	1.025 (41)+	10.025 (401)+	0.364 (s)
		Medium	194	6.000 (240)+	3.450 (138)+	0.825 (33)+	10.275 (411)+	0.373 (s)
		Low	97	7.000 (280)+	3.225 (129)+	1.275 (51)+	11.500 (460)+	0.417 (m)
	Annatto color	High	1938	9.000 (360)+	6.625 (265)+	1.300 (52)+	16.925 (677)+	0.614 (w)
		Medium	969	13.350 (534)+	5.100 (204)+	0.950 (38)+	19.400 (776)+	0.704 (w)
		Low	484.5	14.950 (598)+	5.550 (222)+	2.125 (85)+	22.625 (905)+	0.821 (n)

Table 8. Antimutagenicity of each natural color on urethane (1380 ppm) 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	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
				Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
2	Water		0	0.300 (12)	0.025 (1)	0	0.325 (13)	-
	Urethane		0	17.575 (703)+	7.300 (292)+	2.050 (82)+	26.925 (1077)+	1
	Roselle color	High	11628	5.875 (235)+	3.100 (124)+	0.950 (38)+	9.925 (397)+	0.369 (s)
		Medium	5814	7.150 (286)+	4.850 (194)+	1.500 (60)+	13.500 (540)+	0.501 (m)
		Low	2907	8.750 (350)+	4.475 (179)+	1.375 (55)+	14.350 (574)+	0.533 (m)
	Beetroot color	High	11628	4.825 (193)+	2.675 (107)+	0.725 (29)+	8.225 (329)+	0.305 (s)
		Medium	5814	7.700 (308)+	3.700 (148)+	1.475 (59)+	12.875 (515)+	0.478 (m)
		Low	2907	8.100 (324)+	3.700 (148)+	1.300 (52)+	13.100 (524)+	0.487 (m)
	Sappan color	High	388	5.875 (235)+	3.225 (129)+	1.225 (49)+	10.325 (413)+	0.383 (s)
		Medium	194	7.625 (305)+	3.350 (134)+	1.300 (52)+	12.275 (491)+	0.456 (m)
		Low	97	8.375 (335)+	4.175 (167)+	1.500 (60)+	14.050 (562)+	0.522 (m)
	Annatto color	High	1938	10.325 (413)+	5.325 (213)+	1.575 (63)+	17.225 (689)+	0.640 (w)
		Medium	969	13.750 (550)+	4.600 (184)+	1.500 (60)+	19.850 (794)+	0.737 (w)
		Low	484.5	14.300 (572)+	5.025 (201)+	2.000 (80)+	21.325 (853)+	0.792 (w)

[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.

[b]Mutagenicity Index (MI) obtained from number of total spots per wing induced by urethane in the presence of each sample divided by that of urethane positive control group. Antimutagenic potential: n = negligible; w = weak, m = moderate, s = strong antimutagenic

Table 9. Antimutagenicity of steamed starchy desserts (containing each color) on urethane (1380 ppm) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae in the co-administration study.

Trial	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
				Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water		0	0.325 (13)	0.075 (3)	0	0.400 (16)	-
	Urethane		0	20.300 (812)+	7.875 (315)+	2.925 (117)+	31.100 (1244)+	1
	Original dessert		0	16.350 (654)+	6.175 (247)+	2.950 (118)+	25.475 (1019)+	0.819 (n)
	Roselle dessert	High	10676	6.100 (244)+	4.450 (178)+	1.300 (52)+	11.850 (474)+	0.381 (s)
		Medium	5338	7.300 (292)+	6.600 (264)+	1.400 (56)+	15.300 (612)+	0.492 (m)
		Low	2669	10.675 (427)+	6.325 (253)+	2.175 (87)+	19.175 (767)+	0.617 (w)
	Beetroot dessert	High	10676	7.650 (306)+	3.100 (124)+	1.250 (50)+	12.000 (480)+	0.386 (s)
		Medium	5338	8.600 (344)+	5.400 (216)+	1.650 (66)+	15.650 (626)+	0.503 (m)
		Low	2669	10.500 (420)+	4.650 (186)+	2.075 (83)+	17.225 (689)+	0.554 (m)
	Sappan dessert	High	356	16.100 (644)+	5.350 (241)+	2.025 (81)+	23.400 (936)+	0.752 (w)
		Medium	178	14.150 (566)+	7.225 (289)+	2.650 (106)+	24.025 (961)+	0.773 (w)
		Low	89	15.475 (619)+	6.800 (272)+	2.875 (115)+	25.150 (1006)+	0.809 (n)
	Annatto dessert	High	1780	17.325 (693)+	5.425 (217)+	0.925 (37)+	23.675 (947)+	0.761 (w)
		Medium	890	19.075 (763)+	8.825 (233)+	2.675 (107)+	27.575 (1103)+	0.887 (n)
		Low	445	17.925 (717)+	6.500 (260)+	3.650 (146)+	28.075 (1123)+	0.903 (n)

Table 9. Antimutagenicity of steamed starchy desserts (containing each color) on urethane (1380 ppm) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae in the co-administration study (continued).

Trial	Sample		Concentration of color (ppm)	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
				Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water		0	0.350 (14)	0	0	0.350 (14)	-
	Urethane		0	16.925 (677)+	9.450 (378)+	2.925 (117)+	29.300 (1172)+	1
	Original dessert		0	16.325 (653)+	7.125 (285)+	3.850 (154)+	27.300 (1092)+	0.932 (n)
	Roselle dessert	High	10676	8.000 (320)+	5.100 (204)+	2.150 (86)+	15.250 (610)+	0.520 (m)
		Medium	5338	9.000 (360)+	6.450 (258)+	1.175 (47)+	16.625 (665)+	0.567 (m)
		Low	2669	9.700 (388)+	5.650 (226)+	1.800 (72)+	17.150 (686)+	0.585 (m)
	Beetroot dessert	High	10676	7.450 (298)+	3.850 (154)+	2.450 (98)+	13.750 (550)+	0.469 (m)
		Medium	5338	7.100 (284)+	3.825 (253)+	3.350 (134)+	14.275 (571)+	0.487 (m)
		Low	2669	9.300 (372)+	4.500 (180)+	2.100 (84)+	15.900 (636)+	0.543 (m)
	Sappan dessert	High	356	11.900 (476)+	7.450 (298)+	5.325 (213)+	24.675 (987)+	0.842 (n)
		Medium	178	10.375 (415)+	6.175 (247)+	3.025 (121)+	26.575 (1063)+	0.907 (n)
		Low	89	14.675 (587)+	7.550 (302)+	5.375 (215)+	27.600 (1104)+	0.945 (n)
	Annatto dessert	High	1780	19.200 (768)+	5.375 (215)+	2.175 (87)+	26.750 (1070)+	0.913 (n)
		Medium	890	16.875 (675)+	5.575 (223)+	2.750 (110)+	25.200 (1008)+	0.860 (n)
		Low	445	16.700 (668)+	7.450 (298)+	3.350 (134)+	17.500 (1100)+	0.939 (n)

[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.
[b]Mutagenicity Index (MI) obtained from number of total spots per wing induced by urethane in the presence of each sample divided by that of urethane positive control group.
Antimutagenic potential: n = negligible; w = weak, m = moderate, s = strong antimutagen

Table 10. Antimutagenicity of each natural color on urethane (1380 ppm) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in the pre-feeding study.

Sample	Trial	Pre-feeding type	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
			Small single (m= 2)	Large single (m= 5)	Twin (m= 5)	Total (m= 2)	
High roselle color[c] (11628 ppm)	1	1	5.300 (212)+	3.150 (126)+	1.425 (57)+	9.875 (395)+	0.658(w)
		2	5.800 (232)+	3.275 (131)+	1.275 (51)+	10.350(414)+	0.690(w)
	2	1	6.350 (254)+	3.150 (126)+	1.200 (48)+	10.700(428)+	0.713(w)
		2	4.700 (188)+	2.200 (88)+	1.125 (45)+	8.025 (321)+	0.535(m)
High beetroot color[c] (11628 ppm)	1	1	8.175 (327)+	3.500 (140)+	1.600 (64)+	13.275(531)+	0.885(n)
		2	6.900 (276)+	3.375 (135)+	1.050 (42)+	11.325(453)+	0.755(w)
	2	1	5.775 (231)+	5.325 (213)+	1.225 (49)+	12.325(493)+	0.822(n)
		2	5.800 (232)+	2.750 (110)+	1.325 (53)+	9.875 (395)+	0.658(w)
High sappan color [d] (388 ppm)	1	1	8.925 (357)+	3.325 (133)+	0.850 (34)+	13.100(524)+	1.038(n)
		2	4.550 (182)+	2.200 (88)+	0.900 (36)+	7.650 (306)+	0.605(w)
	2	1	7.700 (308)+	2.500 (100)+	0.900 (36)+	11.100(444)+	0.877(n)
		2	4.450 (178)+	2.400 (96)+	0.450 (18)+	7.300 (292)+	0.577(m)

Table 10. Antimutagenicity of each natural color on urethane (1380 ppm) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross in the pre-feeding study (continued).

Sample	Trial	Pre-feeding type	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
			Small single (m= 2)	Large single (m= 5)	Twin (m= 5)	Total (m= 2)	
High annatto	1	1	5.750 (230)+	3.875 (155)+	1.250 (50)+	10.875(435)+	0.725(w)
color [c]		2	7.825 (313)+	2.325 (93)+	1.350 (54)+	11.500(460)+	0.767(w)
(1938 ppm)	2	1	5.075 (203)+	2.875 (115)+	1.300 (52)+	9.250 (370)+	0.617(w)
		2	5.850 (234)+	2.850 (114)+	1.150 (46)+	9.850 (394)+	0.657(w)

The mutagenicity of each sample evaluated by using the data of total spots per wing indicated that none was mutagenic. MIs of each control medium were between 0.325-0.500 and did not significantly higher than that of the negative control medium.

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with negative control: + = positive; - = negative; i = inconclusive; Probability level: $\alpha = \beta = 0.05$. One-sided statistical tests.

[b]Mutagenicity Index (MI) obtained from number of total spots per wing induced by urethane in the presence of each sample divided by that of urethane positive control group.

c = MI of these colors are the ratio obtained by dividing the total spots per wing of the experimental group by that of the respective positive control run in the same day. Number of total spots per wing of negative control is 0.500 and number of total spots per wing of positive control group is 15.000.

d = MI of this color is the ratio obtained by dividing the total spots per wing of the experimental group by that of the respective positive control run in the same day. Number of total spots per wing of negative control is 0.350 and number of total spots per wing of positive control group is 12.650.

Antimutagenic potential: n = negligible; w = weak, m = moderate, s = strong antimutagenic

Table 11. Antimutagenicity of steamed starchy desserts (containing each color) on urethane (1380 ppm) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae in the pre-feeding study.

Sample	Trial	Pre-feeding type	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
			Small single (m= 2)	Large single (m= 5)	Twin (m= 5)	Total (m= 2)	
Original dessert[c]	1	1	8.700 (348)+	1.275 (51)+	0.325 (13)+	10.300(412)+	0.703(w)
		2	6.975 (279)+	2.150 (86)+	0.825 (33)+	9.950 (398)+	0.679(w)
	2	1	8.875 (355)+	1.500 (60)+	0.625 (25)+	11.000(440)+	0.751(w)
		2	7.500 (300)+	2.000 (80)+	1.000 (40)+	10.500(420)+	0.717(w)
High roselle dessert[d]	1	1	5.850 (234)+	3.825 (153)+	4.325(173)+	14.000(560)+	0.520(m)
(10676 ppm of		2	7.125 (285)+	4.250 (170)+	1.150 (46)+	12.525(501)+	0.465(m)
roselle color)	2	1	8.700 (348)+	5.100 (204)+	3.175(127)+	16.975(679)+	0.630(w)
		2	5.400 (216)+	3.075 (123)+	1.650 (66)+	10.125(405)+	0.376(s)
High beetroot dessert [d]	1	1	6.125 (245)+	3.625 (145)+	0.500 (20)+	10.250(410)+	0.381(s)
(10676 ppm of		2	6.975 (279)+	1.925 (77)+	0.825 (33)+	9.725 (389)+	0.361(s)
beetroot color)	2	1	4.425 (177)+	4.250 (170)+	1.250 (50)+	9.925 (397)+	0.369(s)
		2	4.825 (193)+	2.175 (87)+	1.125 (40)+	8.125 (325)+	0.302(s)

Table 11. Antimutagenicity of steamed starchy desserts (containing each color) on urethane (1380 ppm) induced wing spots of *Drosophila melanogaster* derived from 100 *trans*-heterozygous (*mwh+/+flr*^{3}) larvae in the pre-feeding study (continued).

Sample	Trial	Pre-feeding type	Spots per wing[a] (No. of spots from 40 wings)				MI[b]
			Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
High sappan dessert [c]	1	1	8.350 (334)+	4.775 (191)+	1.350 (54)+	14.475(579)+	0.988(n)
(356 ppm of		2	6.650 (266)+	4.900 (196)+	2.350 (94)+	13.900(556)+	0.949(n)
sappan color)	2	1	9.500 (380)+	3.200 (128)+	1.250 (50)+	13.950(558)+	0.952(n)
		2	9.650 (386)+	1.950 (78)+	2.300 (92)+	13.900(556)+	0.949(n)
High annatto dessert [c]	1	1	7.125 (285)+	5.100 (204)+	2.175 (87)+	14.400(576)+	0.983(n)
(1780 ppm of		2	6.750 (270)+	4.875 (195)+	2.575(103)+	14.200(568)+	0.969(n)
annatto color)	2	1	9.075 (363)+	2.650 (106)+	1.350 (54)+	13.075(523)+	0.892(n)
		2	8.500 (340)+	4.225 (169)+	1.925 (77)+	14.650(586)+	1.000(n)

The mutagenicity of each sample evaluated by using the data of total spots per wing indicated that none was mutagenic. MIs of each control medium were between 0.150-0.535 and did not significantly higher than that of the negative control medium.

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with negative control: + = positive; - = negative; i = inconclusive; Probability level: $\alpha = \beta = 0.05$. One-sided statistical tests.

[b]Mutagenicity Index (MI) obtained from number of total spots per wing induced by urethane in the presence of each sample divided by that of urethane positive control group.

c = MI of these colors are the ratio obtained by dividing the total spots per wing of the experimental group by that of the respective positive control run in the same day. Number of total spots per wing of negative control is 0.325 and number of total spots per wing of positive control group is 14.650.

d = MI of this color is the ratio obtained by dividing the total spots per wing of the experimental group by that of the respective positive control run in the same day. Number of total spots per wing of negative control is 0.425 and number of total spots per wing of positive control group is 26.925.

Antimutagenic potential: n = negligible; w = weak, m = moderate, s = strong antimutagenic

4.3 Antioxidant Activity and Total Phenolic Contents

Antioxidant activity and total phenolic content of methanol extract of each natural color and different steamed starchy desserts are shown in Table 12. The reduction of DPPH by antioxidants in the samples expressed as the percentage of free radical scavenging activity was between 6.83 to 89.41%. While antioxidant activity expressed as mg of Trolox equivalent antioxidant capacity (TEAC)/g dry weight (DW) of sample was between 37.63 to 1125.00 mg TEAC/g DW of natural color and non-detected to 2.31 mg TEAC/g DW of colored dessert. Most colors exhibited pretty good scavenging activity, except annatto and annatto dessert that seemed to have very little activity as same as the original one. The FRAP (Ferric Reducing Antioxidant Power) values were between 35.51 to 1982.17 mg Fe(II)/g DW of natural color and 0.20 to 4.14 mg Fe(II)/g DW of colored dessert. All samples showed good potential except annatto color and annatto dessert as well as the original one. The amounts of total phenolic content of each natural color was varied from non-detected to 2236.21 mg GAE/g DW and non-detected to 2.05 mg GAE/g DW of colored dessert. Most samples showed high value of total phenolic content except annatto color and annatto dessert, beetroot dessert, and original dessert that had GAE values less than the lowest concentration of the standard.

Figure 22 showed a good correlation between the antioxidant activity and the total phenolic content. The correlation coefficient (R^2) obtained from the linear regression among antioxidant activity expressed as TEAC and total phenolic content of samples was $R^2 = 0.933$ (Figure 22(a)), whereas for the correlation between antioxidant activity expressed as FRAP value and total phenolic contents was $R^2 = 0.9134$ (Figure 22(b)). This suggested that phenolic compounds, which are generally methanol soluble, might have an important role in antioxidant activities.

Table 12. Antioxidant activity and total phenolic contents of methanolic extracts of each sample.

Sample	Concentration of color in the extract (mg/ml)	DPPH assay		FRAP[b] assay	Total phenolics assay
		mg TEAC[a]/g DW	% Scavenging activity	mg Fe(II)/g DW	mg GAE[c]/g DW
Roselle color	1.86	85.57±4.32	89.41	149.19±4.89	301.96±14.98
Beetroot color	3.71	38.63±2.06	78.98	73.85±4.54	48.96±6.06
Sappan color	0.12	1125.00±75.11	75.37	1982.17±229.63	2236.21±473.40
Annatto color	0.62	37.63±6.15	6.83	35.51±5.08	UD[d]
Roselle dessert	1.86	1.86±0.11	68.89	4.14±0.07	2.05±0.34
Beetroot dessert	3.71	0.76±0.06	24.17	1.25±0.05	UD[d]
Sappan dessert	0.12	2.31±0.10	86.73	4.03±0.17	1.33±0.13
Annatto dessert	0.62	UD[d]	0	0.20±0.01	UD[d]
Original dessert	0	UD[d]	0	0.25±0.02	UD[d]

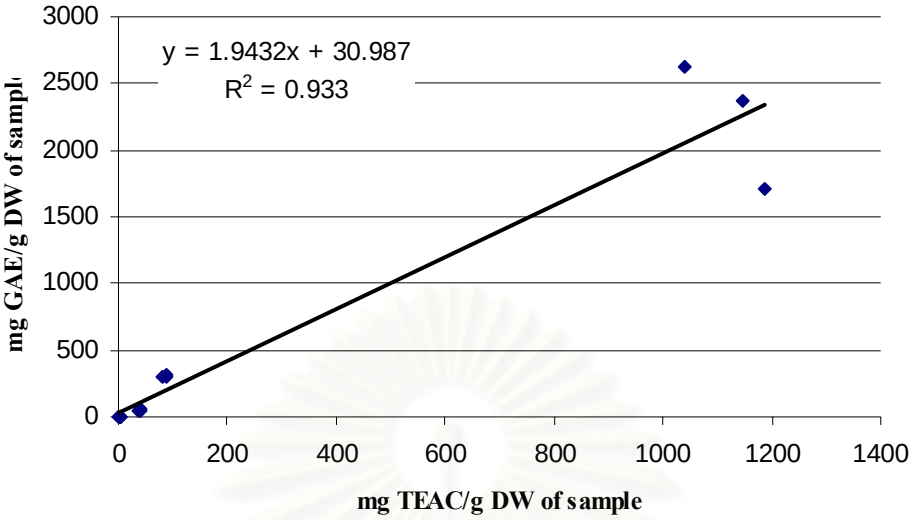
All values are the means of three measurements.

[a]TEAC (Trolox equivalent antioxidant capacity) in mg/g dry weight (DW) of sample.

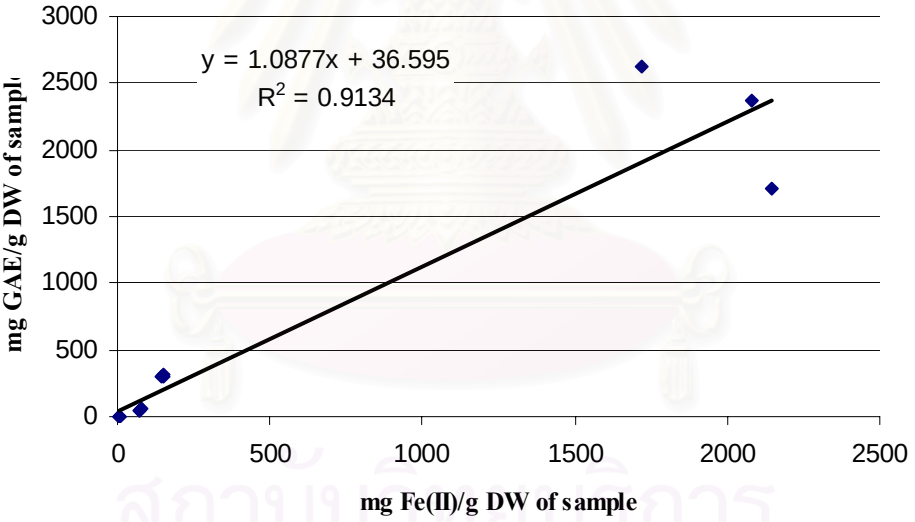
[b]FRAP assay = The FRAP values of the extracts were expressed as mg of ferrous iron (Fe (II))/g dry weight (DW) of sample.

[c]GAE (The gallic acid equivalent) in mg/g dry weight (DW) of sample

[d]Undetectable (UD) = Value was less than the lowest concentration of standard.



(a)



(b)

Figure 22. Correlation between antioxidant activity in DPPH assay and total phenolic contents (a), and antioxidant activity in FRAP assay and total phenolic contents (b) of methanolic extracts from all samples

CHAPTER V
DISCUSSION

5.1 The Survival of Adult Flies and Mutagenicity of Samples

The survival of adult flies fed on either color extracts or steamed starchy desserts containing these colors were more than fifty percents and the flies had normal size. It indicated that no sample was toxic at the doses used in this study. These results represented that selected natural colors are safe to the consumers since none was mutagenic. The supporting data were performed in toxicological investigation of aqueous-methanolic extract of the calyces of roselle in Wistar albino rats (Akindahunsi and Olaleye, 2003) which indicated that the extract appeared safe up to the level of 180 mg/kg per day. As well as the annatto color from *Bixa orellana* L. which considered not genotoxic, based mainly on *in vitro* screening data (Sasaki *et al*., 1980; Haveland-Smith, 1981; Ishidate *et al*., 1984; Fujita *et al*., 1988). Other color extracts namely, beetroot and sappan have no toxicology studies as they have been widely used for decades (Kapadia *et al*., 2003; Lim *et al*., 2006) and no adverse effect has been reported.

5.2 Antimutagenicity of Samples

Urethane is metabolically activated by cytochrome P-450 enzyme system (Schlatter and Lutz, 1990) to be vinyl epoxide, the carcinogenic active metabolite (Dahl *et al*., 1978), which is further detoxified with glutathione-*S*-transferase (GST) conjugation (Kemper *et al*., 1995). The aim of co-administration study was to elucidate whether each natural color extract and its colored dessert could inhibit the mutagenicity of urethane. If number of spots per wing reduces, it is postulated that the sample may act as a scavenger of urethane or as an inducer of glutathione-*S*-transferase (GST) or an inhibitor of cytochrome P-450 system. To clarify such hypothesis, the pre-feeding types 1 and 2 were performed in this study. Contacting only on the early stage of larval period that was supposed to induce or inhibit the activity of enzyme in biotransformation system was designed as pre-feeding type 1 study. While continuous feeding of sample to the larvae in type 2 study allowed them to contact the possible inducer or inhibitor of enzymes in each sample for the whole

period of larval stage. In case that the reduction of urethane induced spots per wing in the co-administration was nearly the same as of the result of pre-feeding type 2 study and the inhibition of both studies were stronger than that of the pre-feeding type 1, it seemed that samples could scavenge urethane and/or induced the enzymatic system to detoxify urethane.

The results indicated that all color extracts (at the concentrations tested) could reduce the mutagenicity of urethane. The antimutagenicity potential of the extracts except that from annatto color in co-administration and pre-feeding type 2 were strong to moderate but weak to negligible effects in pre-feeding type 1. This indicated that the colors might scavenge urethane and continuous feeding of these colors might activate the detoxifying enzymes (phase II) and/or inhibit the activating enzymes (phase I) of urethane. This protective outcome could be the results of more than one mechanism and the antimutagenicity could be the total effect of all components in each color extract.

Gasiorowski *et al*. (1997), Galati and O'Brien (2004), and Moon *et al*. (2006) suggested that the level of phase II detoxifying enzymes such as glutathione-*S*-transferase (GST), quinone reductase (QR), and UDP-glucuronyltransferase (UGT) were induced by flavonoids and it was one of the mechanisms responsible for the chemopreventive activity of the compounds. Chemically, anthocyanins, widely distributed in plants including the calyx of roselle, are flavonoids. Anthocyanins not only possess antioxidant ability (Pool-Zobel *et al*., 1999; Tsuda *et al*., 2000) but also mediate other physiological functions related to cancer suppression (Kamei *et al*., 1995; Meiers *et al*., 2001; Nagase *et al*., 1998). Anthocyanins, depending on the concentrations ingested, confer protection to DNA by preventing radical hydroxyl attack. In these reactions, cyanidin-DNA co-pigmentation is suggested as a possible defense mechanism against oxidative damage of DNA. When an anthocyanin complexes with DNA, it is no longer vulnerable to nucleophilic attack by an OH· group (Sarma and Sharma, 1999). As well as beetroot extract, rich in betalain pigment, has demonstrated chemopreventive properties against both lung and skin cancers (Kapadia *et al*., 1996). Wettasinghe *et al* (2002) founded that beetroot pigment had the property in free-radical scavenging and inducing quinone reductase (a phase II enzyme important as a marker of the initiation stage of carcinogenesis) in murine hepatoma cells *in vitro*. The antioxidative property of these pigments will be discussed below.

Introducing these colors to steamed starchy desserts, the ones containing roselle and beetroot colors kept their moderate to strong antimutagenicity activity in both co-administration and two types of pre-feeding experiment whereas the others, namely sappan and annatto were weak to negligible activity. It indicated that antimutagenicity activity of color extracted from roselle and beetroot are more stable to moist heat than those from sappan or annatto color. Literature review showed that anthocyanins and betalains exhibit greater stability under acidic conditions. But they will readily convert to colorless derivatives and subsequently to brown pigments under normal processing and storage conditions. A number of factors influence anthocyanins and betalains stability, including pH, heat-humidity, light, oxygen, enzymes, as well as the presence of ascorbic acid, sugars, sulfur dioxide or sulfite salts, metal ions and copigments (Francis, 1989; Jackman *et al*., 1987). Gradinaru *et al*. (2003) indicated that, despite color fading, the breakdown products of anthocyanins still exhibit significant antiradical power. These result suggested that anthocyanins from roselle calyx presented in steamed starchy dessert may continue to provide their antioxidative effects even after some color loss had occurred during processing and storage. This outcome maybe the reason why the steamed desserts containing roselle color, even though beetroot color; could preserve the antimutagenicity activity.

5.3 Antioxidant Activity, Total Phenolic Contents, and Antimutagenicity

The present study has revealed that each color extract and its colored dessert (except original dessert and annatto dessert) had antioxidative activity and certain amount of phenolic compounds. Various antioxidants including anthocyanins, quercetin, *L*-ascorbic acid and protocatechuic acid are in the calyx of roselle (Hirunpanich, *et al*., 2005). Several flavonoids and polyphenols are the phytochemicals isolated from heartwood of sappan (Badami *et al*., 2003). Nagai *et al*. (1986) found 4-*O*-methylsappanol and protosappanin A while Nagai and Nagumo (1986) isolated protosappanin B, protosappanin E and Miyahara *et al*. (1986) identified brazilein, caesalpin J. The other antioxidant of sappan is brazilin (Kim *et al*., 1997). These compounds might reduce of the mutagenicity of urethane. Stich *et al*. (1982) suggested that some antioxidants had an inhibitory effect on genotoxic action of several known mutagens possibly by decreasing the level of cytochrome P-450.

The correlation between total phenolic contents and antioxidant potential (either DPPH assay or FRAP assay) is observed in the present study. The correlation

coefficient (R^2) obtained from linear regression between antioxidant activity expressed as TEAC and total phenolic contents of the sample extract is 0.933, whereas that from the regression between antioxidant activity expressed as FRAP value and total phenolic contents is 0.9134. It suggests that phenolic compounds are one of the dominant antioxidant components in selected color extracts. These data are in accordance with others, who have shown that high total phenolic contents increased the antioxidant activity and there was a direct relationship between antioxidant activity and total phenolic contents in selected herbs, vegetables, and fruits (Velioglu *et al*., 1998; Holasova *et al*., 2002). Phenolic compounds had a major contribution to antioxidant activity (Velioglu *et al*., 1998; Zheng and Wang, 2001; Sun *et al*., 2002). Many typical compounds that exhibit antioxidant activity are phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Cai *et al*., 2004; Chanwitheesuk *et al*., 2005). Also, it can be concluded that the antioxidant activity of the color extracts was not limited to phenolic compounds. Activity may come from the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins as well (Javanmardi *et al*., 2003).

It is proposed that antioxidant activity and certain amount of phenolic compounds of each color extract might reduce the formation of ultimate metabolites of urethane responsible for inducing wing spots detected in this investigation. It was documented that *N*-hydroxyurethane, a urethane metabolite (Boyland and Nery, 1965; Nery, 1968), was hydrolyzed by esterase to generate hydroxylamine and exerted its mutagenic effect in multiple organs via generating $O_2^{\cdot -}$ and $NO^{\cdot}$ to cause oxidation and depurination of DNA (Sakano *et al*., 2002). Thus, it was possible that antioxidant activity from these natural colors may reduce $O_2^{\cdot -}$ and/or $NO^{\cdot}$ in urethane metabolism. Antioxidants have been suggested to scavenge free radicals, and prevent their interactions with cellular DNA (Ferguson *et al*., 2004).

It has been clearly shown in the present study that all selected natural colors and their respective natural colored desserts had antioxidant activity except that of annatto dessert. It might be possible that the water-soluble extract from annatto seed in this experiment not only contained small amount of polyphenolic compounds but also they were labile after extraction and/or during heat processing. Annatto is the main

source of pigments, namely bixin and norbixin that are carotenoids. Water-soluble annatto extracts are prepared by alkaline hydrolysis whereby bixin (oil-soluble) is converted to norbixin (water-soluble) (Kiokias and Gordon, 2003). There are few reports on the antioxidant activity of annatto carotenoids. Haila *et al*. (1996) reported that bixin strongly inhibited the autoxidation of rapeseed oil triglycerides although other carotenoids such as lutein or lycopene had no antioxidant effect. There is only one report on the antioxidant activity of norbixin studied by Kiokias and Gordon (2003) who reported that norbixin inhibited the oxidative deterioration of methanol extract of virgin olive oil assessed in both bulk olive oil and oil-in-water emulsions.

The results of this study indicated that all selected natural colors and their natural colored desserts were safe and had some health benefits to consumers because they could counteract the mutagenicity of urethane, except annatto dessert that the antimutagenic substances were labile in the moist heat. The protective effects of each color may be due to the presence of antimutagenic components. However, further chemical identification in these natural colors will be required to reveal the possible compounds that respond to antimutagenicity. Furthermore, the stability of each color added to different types of processed food should be studied in order to ensure the functional property of phytochemicals. The results of this investigation suggest that these natural colors should be introduced as ingredients in health food in order to protect the consumer from some mutagens that have similar bio-characteristic to urethane.

CHAPTER VI
CONCLUSION

Four natural red colors namely, Krajeab-daeng (*Hibiscus sabdariffa* L.), Beetroot (*Beta vulgaris* L.), Fang (*Caesalpinia sappan* L.), and Kam-sad (*Bixa orellana* L.) were selected to study for their antimutagenicity in two forms; the color extract and the steamed starchy dessert model containing each color, using somatic mutation and recombination test (SMART). The results showed that neither the color extracts nor their colored steamed starchy desserts was mutagenic. All color extracts exhibited antimutagenicity in both co-administration and pre-feeding studies. Introducing these colors to the steamed starchy dessert, annatto dessert was the only one that seemed to have no antimutagenicity in both studies. It proposed that all of these extracted colors might scavenge urethane, induce the enzymatic system to detoxify urethane (phase II) and/or inhibit some enzymes of cytochrome P-450 system that might had their responsibility in activation of urethane (phase I). In an attempt to introduce these colors to any food, the cooking method should be considered to maintain the health benefit of these pigments. In addition, these selected natural colors had phenolic compounds and antioxidant activities (determined by using DPPH and FRAP assays) that might be responsible for antigenotoxic action of the mutagen. Further chemical identification in the tested color extracts should be investigated to reveal the exact compounds that respond for antimutagenicity. The results of this investigation suggested that these natural colors should be promoted as food colors or functional foods to optimize the health benefit for consumers.

REFERENCES

THAI

กระยาทิพย์ เรือนใจ. 2549. ปั้นแต่งขนมไทย. พิมพ์ครั้งที่ 1. กรุงเทพมหานคร: ต้นธรรม.

บุศรารัตน์ สายเชื้อ. 2545. แอนโทไซยานินจากกระเจี๊ยบแดง *Hibiscus sabdariffa* L. เพื่อใช้เป็นสีผสมอาหาร. วิทยานิพนธ์ปริญญามหาบัณฑิต สาขาเทคโนโลยีทางอาหาร ภาควิชาเทคโนโลยีทางอาหาร คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย.

พเยาว์ เหมือนวงษ์ญาติ. 2530. สีธรรมชาติและสีสังเคราะห์. พิมพ์ครั้งที่ 13. เชียงใหม่: ศูนย์พิมพ์คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่.

เพียง อุดมเกียรติกูล. 2533. การเตรียมสีผสมอาหารชนิดผงจากธรรมชาติโดยวิธีสเปรย์ดราย. วิทยานิพนธ์ปริญญามหาบัณฑิต สาขาอาหารเคมี ภาควิชาอาหารเคมี คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย.

วีระชัย ธารมณีวงศ์ และคณะ. 2532. การสกัดสีจากแก่นฝาง. โครงการการเรียนการสอนเพื่อเสริมประสบการณ์ ภาควิชาอาหารเคมี คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย.

ENGLISH

Abraham, S.K. 1991. Inhibitory effects of coffee on the genotoxicity of carcinogens in mice. Mutat. Res. 262(2): 109-114.

Abraham, S.K. 1994. Antigenotoxicity of coffee in the *Drosophila* assay for somatic mutation and recombination. Mutagenesis 9(4): 383-386.

Abraham, S.K., and Graf, U. 1996. Protection by coffee against somatic genotoxicity in *Drosophila*: role of bioactivation capacity. Food Chem. Toxicol. 34: 1-14.

Abraham S.K., Singh S.P., and Kesavan P.C. 1998. *In vivo* antigenotoxic effects of dietary agents and beverages co-administered with urethane: assessment of the role of glutathione-*S*-transferase activity. Mutat. Res. 413: 103-110.

Agarwal, M., Srivastava, V.K., Saxena, K.K., and Kumar, A. 2006. Hepatoprotective activity of *Beta vulgaris* against CCl_4-induced hepatic injury in rats. Fitoterapia 77: 91–93.

Agner, A.R., Bazo, A.P., Ribeiro, L.R., and Salvadori, D.M.F. 2005. DNA damage and aberrant crypt foci as putative biomarkers to evaluate the chemopreventive effect of annatto (*Bixa orellana* L.) in rat colon carcinogenesis. Mutat. Res. 582: 146-154.

Akindahunsi, A.A., and Olaleye, M.T. 2003. Toxicological investigation of aqueous-methanolic extract of the calyces of *Hibiscus sabdariffa* L. J. Ethnopharmacol. 89: 161-164.

Alian, A., El-Ashwah, E., and Eid, N. 1983. Antimicrobial properties of some Egyptian non-alcoholic beverages with special reference to tamarind. Egypt J. Food Sci. 11(1-2): 109-114.

Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, P., Barl, B., and Weil, J.A. 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem. 84: 551-562.

Badami, S., Moorkoth, S., Rai, S.R., Kannan, E., and Bhojraj, S. 2003. Antioxidant activity of *Caesalpinia sappan* heartwood. Biol. Pharm. Bull. 26(11): 1534-1537.

Baron, J., Cole, B. F., Mott, L., Haile, R., Grau, M., Church, T. R. *et al*. 2003. Neoplastic and antineoplastic effects of β-carotene on colorectal adenoma recurrence: results of a randomized trial. J. Natl. Cancer 95: 717-722.

Becker, H.J. 1976. Mitotic recombination. In: Ashburner, M., and Novitski, E. (eds.), The genetics and biology of *Drosophila* volume 1, pp. 1019-2087. London: Academic Press.

Benzie, I.F.F., and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239: 70–76.

Böhm, H. and Rink, E. 1988. Cell culture and somatic cell genetics of plants. 1st ed. New York: Academic Press.

Bokern, M., Heuer, S., Wray, V., Witte, L., Macek, T., Vanek, T. *et al*. 1991. Ferulic acid conjugates from cell cultures of *Beta vulgaris*. Phytochem. 30: 3261-3265.

Boyland, E., and Nery, R. 1965. The metabolism of urethane and related compounds. Biochem. J. 94: 198-208.

Bridle, P., and Timberlake, C. F. 1997. Anthocyanins as natural food colour-selected aspects. Food. Chem. 58(1-2): 103-109.

Butz, D., Edenharder, R., Fister, H., and Tauscher, B. 1997. The influence of high pressure processing on antimutagenic activities of fruit and vegetable juice. Food Res. Int. 30(3-4): 287-291.

Cai, Y., Luo, Q., Sun, M., and Corke, H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74: 2157-2184.

Carlson, G.P. 1994. The effect of inducers and inhibitors of urethane metabolism on its *in vitro* and *in vivo* metabolism in rats. Cancer Lett. 87(2): 145-150.

Cederberg, H., and Ramel, C. 1989. Modifications of the effect of bleomycin in the somatic mutation and recombination test in *Drosophila melanogaster*. Mutat. Res. 214(1): 69-80.

Chang, Y-C, Huang, H-P, Hsu, J-D, Yang, S-F, and Wang, C-J. 2005. *Hibiscus* anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. Toxicol. Appli. Pharmacol. 205: 201-212.

Chankhaao. 1983. Precious drugs from medicinal plants Waan Yaa. 1st ed. Bangkok: Cheewin Press.

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

Charleux, J. L. 1996. Beta-carotene, vitamin C, and vitamin E: the protective micronutrients. Nutr. Rev. 54(11): S109-S114.

Chewonarin, T., Kinouchi, T., Kataoka, K., Arimochi, H., Kuwahara, T., Vinitketkumnuen, U. *et al*. 1999. Effects of roselle (*Hibiscus sabdariffa* Linn.), a Thai medicinal plant, on the mutagenicity of various known mutagens in *Salmonella typhimurium* and on formation of aberrant crypt foci induced by the colon carcinogens azoxymethane and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in F344 rats. Food Chem. Toxicol. 37: 591-601.

Choi, S.Y., Yang, K.M., Jeon, S.D., Kim, J.H., Khil, L.Y., Chang, T.S., *et al*. 1997. Brazilin modulates immune function mainly by augmenting T cell activity in halothane administered mice. Planta Med. 63: 405-408.

Counsell, J. N., Jeffries, G. S., and Knewstubb, C. J. 1979. Some other natural colors and their applications. In: Counsell, J. N., and Dunastable, J. A. (eds.), Natural colors for foods and other uses, pp. 122-151. London: Applied Science.

Dahl, G.A., Miller, J.A., and Miller, E.C. 1978. Vinyl carbamate as a promutagen and a more carcinogenic analog of ethyl carbamate. Cancer Res. 38: 3793-3804.

De Flora, S., Astengo, M., Serra, D., and Bennicelli, C. 1986a. Inhibition of urethane-induced lung tumors in mice by dietary N-acetylcysteine. Cancer Lett. 32(3):

235-241.

De Flora, S., Rossi, G.A., and De Flora, A. 1986b. Metabolic, desmutagenic and anticarcinogenic effects of *N*-acetylcysteine. Respiration 1: 43-49.

Delgado-Rodriguez, A., Ortiz-Marttelo, R., Villalobos-Pietrini, R., Gomez-Arroyo, S., and Graf, U. 1999. Genotoxicity of organic extracts of airborne particles in somatic cells of *Drosophila melanogaster*. Chemosphere 39(1): 33-43.

Delgado-Vargas, F., Jimenez, A., and Paredes-Lopez, O. 2000. Natural pigments: carotenoids, anthocyanins, and betalains - characteristics, biosynthesis, processing, and stability. Crit. Rev. Food Sci. Nutr. 40: 173-289.

DiMascio, P., Devasagayam, T.P.A., Kaiser, S., and Sies, H. 1990. Carotenoids, tocopherols and thioles as biological singlet molecular oxygen quenchers. Biochem. Soc. Transact. 18: 1054-1056.

Duthie, G., Duthie, S., and Kyle, J. 2000. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutr. Res. Rev. 13: 79-106.

El-Shayeb, N.M.A., and Mabrouk, S.S. 1984. Utilization of some edible and medicinal plants to inhibits to inhibit aflatoxin formation. Nutr. Rep. Int. 29(2): 273-282.

Escribano, J., Pedreno, M. A., Garcia-Carmona, F., and Munoz, R. 1998. Characterization of the antiradical activity of betalains from *Beta vulgaris* L. roots. Phytochem. Anal. 9: 124-127.

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

Fernandes, A.C.S., Almeida, C.A., Albano, F., Laranja, G.A.T., Felzenszwalb, I., Lage, C.L.S. *et al*. 2002. Norbixin ingestion did not induce any detectable DNA breakage in liver and kidney but caused a considerable impairment in plasma glucose levels of rats and mice. J. Nutr. Biochem.13: 411-420.

Ferreira, E.B., Hulme, A.N., McNab, H., and Anita, Q.A. 2004. The natural constituents of historical textile dyes. Chem. Soc. Rev. 33, 329-336.

Field, K.J., and Lang, C.M. 1988.Hazards of urethane (ethyl carbamate): a review of the literature. Lab. Anim. 22: 255-262.

Francis, F. J. 1989. Food colorants: anthocyanins. Crit. Rev. Food Sci. Nutr. 28: 273-314.

Frei, H., and Würgler, F.E. 1988. Statistical methods to decide whether mutagenicity

test data from *Drosophila* assay indicate a positive, negative or inconclusive result. Mutat. Res. 203: 297-308.

Frölich, A., and Würgler, F.E. 1988. Validation of an efficient *in vivo* genotoxicity test with somatic cells of *Drosophila*. Mutat. Res. 203: 211-217.

Frölich, A., Würgler, F.E. 1989. New tester strains with improved bioactivation capacity for the *Drosophila* wing spot test. Mutat. Res. 216: 179-187.

Frölich, A., and Würgler, F.E. 1990a. Genotoxicity of ethyl carbamate in the *Drosophila* wing spot test: dependence on genotype-controlled metabolic capacity. Mutat. Res. 244(3): 201-208.

Frölich, A., and Würgler, F.E. 1990b. *Drosophila* wing-spot test: improved detectability of genotoxicity of polycyclic aromatic hydrocarbons. Mutat. Res. 234(2): 71-80.

Frölich, A., and Würgler, F.E. 1991 The "High Bioactivation" cross for the SMART assay with the wing *Drosophila* .Inf. Serv. 70: 246-247.

Fuhrman, B., Elis, A., and Aviram, M. 1997. Hypocholesterolemic effect of lycopene and β-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. Biochem. Bioph. Res. 233: 658–662.

Fujita, H., Nakano, M., and Sasaki, M. 1988. Mutagenicity test of food additives with *Salmonella typhimurium* TA97 and TA102. Kenkyu Nenpo-Tokyo-Toritsu Elsei Kenkynsho 39: 343-350.

Fukumoto, L.R., and Mazza, G. 2000. Assessing antioxidant and pro-oxidant activities of phenolic compounds. J. Agric. Food Chem. 48: 3597-3604.

Galati, G., and O'Brien, P.J. 2004. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. In: Catherine Rice-Evans (ed.), Serial Review: Flavonoids and isoflavones (phytoestrogens): Absorption, Metabolism, and Bioactivity. J. Free Radic. Biol. Med. 37(3): 287-303.

Gasiorowski, K., Szyba, K., Brokosa, B., Kołaczyńska, B., Jankowiak-Włodarczyk, M., and Oszmiański, J. 1997. Antimutagenic activity of anthocyanins isolated from *Aronia melanocarpa* fruits. Cancer Lett. 119: 37-46.

Goodman, G., Schaeffer, S., Omenn, G., Chen, C., and King, I. 2003. The association between lung and prostate cancer risk, and serum micronutrients: results and

lessons learned from b-carotene and retinol efficacy trial. Cancer Epidem. Biomark. Preven. 12: 518-526.

Goodwin, T. W. 1980. Biochemistry of the Carotenoids. Vol. 1: Plants.2nd ed., New York: Chapman and Hall.

Goodwin, T. W. 1992. Biosynthesis of carotenoids: an overview. Method. Enzymol. 214: 330-340.

Gordon, H. T., and Bauernfeind, J. C. 1982. Carotenoids as food colorants. Crit. Rev. Food Sci. Nutr. 18: 59-97.

Gradinaru, G., Biliaderis, C.G., Kallithrakac, S., Kefalas, P., and Garcia-Viguera, C. 2003. Thermal stability of *Hibiscus sabdariffa* L. anthocyanins in solution and in solid state: effects of copigmentation and glass transition. Food Chem. 83: 423-436.

Graf, U., Würgler, F.E., Katz, A.I., Frei, H., Juon, H., Hall, C.B. *et al*. 1984. Somatic mutation and recombination test in *Drosophila melanogaster.* Environ. Mutagen 6: 153-188.

Graf, U., and Singer, D. 1989. Somatic mutation and recombination test in *Drosophila melanogaster* (wing spot test): effects of extracts of airborne particulate matters from fire-exposed and non fire-exposed building ventilation filters. Chemosphere 19: 1094-1097.

Graf, U., Frei, H., Kägi, A., Katz, A.J., and Würgler, F.E. 1989. Thirty compounds tested in the *Drosophila* wing spot test. Mutat. Res. 222: 359-373.

Graf, U., and van Schaik, N. 1992. Improved high bioactivation cross for the wing somatic mutation and recombination test in *Drosophila melanogaster*. Mutat. Res. 271: 59-67.

Graf, U., Heo, O.S., and Olvera Ramirez, O. 1992a. The genotoxicity of chromium (VI) oxide in the wing spot test of *Drosophila melanogaster* is over 90% due to mitotic recombination. Mutat. Res. 266: 197-203.

Graf, U., Wild, D., and Würgler, F.E. 1992b. Genotoxicity of amine 2-amino-3-methylimidazo-4,5-quinoline (IQ) and related compounds in *Drosophila*. Mutagenesis 7(2): 145-149.

Griffin, S.P., and Bhagooli, R. 2004. Measuring antioxidant potential in corals using the FRAP assay. J. Exp. Marine Bio. Ecol. 302: 201-211.

Guengerich, F.P., and Kim, D.H. 1991. Enzymatic oxidation of ethyl carbamate and

its role as an intermediate in the formation of 1, N6-ethenoadenosine. Chem. Res. Toxicol. 4(4): 413-421.

Guengerich, F.P., Kim, D.H., and Iwasaki, M. 1991. Role of human cytochrome P-450 2E1 in the oxidation of many low molecular weight cancer suspects. Chem. Res. Toxicol. 4(2): 168-179.

Guzman, R.J., Espinosa, J., and Graf, U. 1998. Analysis of the *in vivo* nitrosation capacity of the larvae used in the wing somatic mutation and recombination test of *Drosophila melanogaster*. Mutat. Res. 412(1): 69-81.

Haila, K. M., Lievonen, S. M., and Heinonen, M. I. 1996. Effects of lutein, lycopene, annatto, and α-tocopherol on autoxidation of triglycerides. J. Agri. Food Chem. 44: 2096-2100.

Hallagan, J.B., Allen, D.C., and Borzelleca, J.F. 1995. The safety and regulatory status of food, drug and cosmetic colour additives exempt from certification. Food Chem. Toxicol. 33: 515-528.

Halliwell, B., Aeschbach, R., Loliger, J., and Aruoma, O.I. 1995. The characterization of antioxidants. Food Chem. Toxicol. 33: 601- 617.

Harborne, J. B. 1988. The flavonoids: recent advances. In: Goodwin, T. W. (ed.), Plant Pigments, pp. 298-343. London: Academic Press.

Harborne, J. B., and Grayer, R. J. 1988. The anthocyanins. In: Harborne, J.B. (ed.), The flavonoids, pp. 1-20. London: Chapman and Hall Ltd.

Hari, R. K., Patel, T. R., and Martin, A. M. 1994. An overview of pigment production in biological systems: functions, biosynthesis, and applications in food industry. Food Rev. Int. 10(1): 49-70.

Harntrikanon, N. 2006. Effect of two Thai desserts made from fermented rice powder containing natural food colors derived from *Monascus* spp. on urethane mutagenicity in *Drosophila melnogaster* and their antioxidant activity. M.S. Thesis in Food and Nutrition Toxicology. Faculty of Graduate Studies. Mahidol University.

Haveland-Smith, R.B. 1981. Evaluation of the genotoxicity of some natural food colours using bacterial assays. Mutat. Res. 91: 285-290.

Havsteen, B. 2002. The biochemistry and medical significance of the flavonoids. Pharma. Therapeut. 96: 67-202.

Hayatsu, H., Inada, N., Kakutani, T., Arimoto, S., Negishi, T., Mori, K. *et al*. 1992.

Suppression of genotoxicity of carcinogens by (-)epigallocatechin gallate. Prev. Med. 21(3): 370-376.

Herbach, K.M., Stintzing, F.C., and Carle, R. 2006. Betalain stability and degradation-structural and chromatic aspects. J. food Sci. 71: 41-50.

Hirunpanich, V., Utaipat, A., Morales, N.P., Bunyapraphatsara, N., Sato, H., Herunsalee, A. *et al*. 2005. Antioxidant effects of aqueous extracts from dried calyx of *Hibiscus sabdariffa* L. (Roselle) *in vitro* using rat low-density lipoprotein (LDL). Biol. Pharm. Bull. 28(3): 481-484.

Holasova, M., Fiedlerova, V., Smecinova, H., Orsak, M., Lachman, J., and Vavreinova, S. 2002. Buckwheat-the source of antioxidant activity in functional foods. Food Res. Int. 35: 207-211.

Horvathova, K., Vachalkova, A., and Novotny, L. 2001. Flavonoids as chemoprotective agents in civilization diseases. Neoplasma 48: 435-441.

Hou, D-X, Tong, X., Terahara, N., Luo, D., and Fujii, M. 2005. Delphinidin 3-sambubioside, a *Hibiscus* anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. Arch. Biochem. Biophys. 440: 101-109.

IARC. 1974. Vinyl chloride: IARC monographs on the evaluation of the carcinogenic risks of chemicals to humans, some anti-thyroid and related substances, nitrofurans and industrial chemicals. IARC Lyon 7: 291-305.

Ishidate, M., Sofuni, K., Yoshikawa, K., Hayashi, M., Nohmi, M., and Matsuoka, A. 1984. Primary mutagenicity screening of food additives currently used in Japan. Food Chem. Toxicol. 22: 623-636.

Jackman, R. L., Yada, R. Y., Tung, M. A., and Speers, R. A. 1987. Anthocyanins as food colorants-a review. J. Food Biochem. 11: 201-247.

Jain, C. K., Agarwal, S., and Rao, A. V. 1999. The effect of dietary lycopene on bioavailability, tissue distribution, and *in vivo* antioxidant properties and colonic preneoplasia in rats. Nutr. Res. 19: 1383-1391.

Javanmardi, J., Stushnoff, C., Locke, E., and Vivanco, J.M. 2003. Antioxidant activity and total phenolic content of *Iranian Ocimum* accessions. Food Chem. 83: 547-550.

JECFA. 1982. Evaluation of certain food additives and contaminants. In: Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives.

Technical Report Series No.683.

Kamei, H., Kojima, T., Hasegawa, M., Koide, T., Umeda, T., Yukawa, T. *et al*. 1995. Suppression of tumor cell growth by anthocyanins *in vitro*. Cancer Invest. 13, 590-594.

Kang, S., Seeram, N., Nair, M., and Bourquin, L. 2003. Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. Cancer Lett. 194: 13-19.

Kapadia, G., Tokuda, H., Konoshima, T., and Nishino, H. 1996. Chemoprevention of lung and skin cancer by *Beta vulgaris* (beet) root extract. Cancer Lett. 100: 211-214.

Kapadia, G.J., Azuine, M.A., Sridhar, R., Okuda, Y., Tsuruta, A., Ichiishi, E *et al*. 2003. Chemoprevention of DMBA-induced UV-B promoted, NOR-1-induced TPA promoted skin carcinogenesis, and DEN-induced phenobarbital promoted liver tumors in mice by extract of beetroot. Pharmacol. Res. 47: 141-148.

Katz, A.J. 1989. Sodium thiosulfate inhibits cisplatin-induced mutagenesis in somatic tissue of *Drosophila*. Environ. Mol. Mutagen 13(2): 97-99.

Kemper, R.A., Myers, S.R., and Hurst, H.E. 1995. Detoxification of vinyl carbamate epoxide by glutathione: evidence for participation of glutathione-*S*-transferase in metabolism of ethyl carbamate. Toxicol. Appl. Pharmacol. 135: 110-118.

Khachik, F., Carvalho, L., Bernstein, P., Muir, G., Zhao, D., and Katz, N. 2002. Chemistry, distribution, and metabolism of tomato carotenoids and their impact on human health. Exp. Biol. Med. 227: 845-851.

Kim, D. S., Baek, N. I., Oh, S. R., Jung, K. Y., Lee, I. S., and Lee, H. K. 1997. NMR assignment of brazilin. Phytochem. 46: 177-178.

Kim, E.C., Hwang, Y.S., Lee, H.J., Lee, S.K., Park, M.H., Jeon, B.H. *et al*. 2005. *Caesalpinia sappan* induces cell death by increasing the expression of p53 and p21WAF1/CIP1 in head and neck cancer cells. Am. J. Clin. Med. 33(3): 405-414.

Kiokias, S., and Gordon, M.H. 2003. Antioxidant properties of annatto carotenoids. Food Chem. 83: 523-529.

Klamt, F., Dal-Pizzol, F., Bernard, E.A., and Moreira, J.C. 2003. Enhanced UV-mediated free radical generation; DNA and mitochondrial damage caused by

retinol supplementation. Photochem. Photobiol. Sci. 2(8): 856-860.

Kläui, H. 1979. Carotenoids and their applications. In: Counsell, J. N. and Dunastable, J. A. (eds.), Natural Colours for Food and Other Uses, pp. 91-122. London: Applied Science.

Kobayashi, N., Schmidt, J., Nimtz, M., Wray, V., and Schliemann, W. 2000. Betalains from Christmas cactus. Phytochem. 54: 419-426.

Koes, R. E. K., Quattrocchio, F., and Mol, J. N. M. 1994. The flavonoid biosynthetic pathway in plants: function and evolution. Bio. Essays 16(2): 123-132.

Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K., and Nagao, A. 2001. Carotenoids affect proliferation of human prostate cancer cells. J. Nutr. 131: 3303-3306.

Kovary, K., Louvain, T.S., Silva, M.C.C., Albano, F., Pires, B.B.M., Laranja, G.A.T. *et al*. 2001. Biochemical behaviour of norbixin during *in vitro* DNA damage induced by reactive oxygen species. Br. J. Nutr. 85: 431-440.

Kucuk, O., Sarkar, F., Djuric, Z., Sakr, W., Pollak, M., Khachik, F. *et al*. 2002. Effects of lycopene supplementation in patients with localized prostate cancer. Exp. Biol. Med. 227: 881-885.

Kurata, N., Hurst, H.E., Benz, F.W., Kemper, R.A., and Waddell, W.J. 1991. Studies on inhibition and induction of metabolism of ethyl carbamate by acetone and related compounds. Evidence for metabolism by cytochromes P-450. Drug Metab. Dispos. 19(2): 388-393.

Leathers, R., Davin, C., and Zrÿd, J. 1992. Betalain producing cell cultures of *Beta vulgaris* L. (red beet). *In Vitro* Cell Dev. Biol. 28: 39-45.

Lee, S.H., Won, H.S., Khil, L.Y., Moon, C.H., Chung, J.H., and Moon, C.K. 1994. Effects of brazilin on glucose metabolism in epididymal adipocytes from streptozotocin induced diabetic rats. J. Appl. Pharmacol. 2: 65-70.

Lehmann, M., Franco, A., de Souza Prudente Vilar, K., Lukza Reguly, M., and de Andrade, H.H. 2003. Doxorubicin and two of its analogues are preferential inducers of homologous recombination compared with mutational events in somatic cells of *Drosophila melanogaster*. Mutat. Res. 539(1-2): 167-175.

Leithauser, M.T., Liem, A., Stewart, B.C., Miller, E.C., and Miller, J.A. 1990. 1, *N*6-ethenoadenosine formation, mutagenicity and murine tumor induction as indicators of the generation of an electrophilic epoxide metabolite of the

closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. Carcinogenesis 11(3): 463-473.

Lieber, C.S., Baraona, E., Leo, M.A., and Garro, A. 1987. Metabolism and metabolic effects of ethanol, including interaction with drugs, carcinogens and nutrition. Mutat. Res. 186(3): 201-233.

Lila, M.A. 2004. Plant pigments and human health. In: Plant pigments and their manipulation, 248-274. California: Davis.

Lim, M-Y, Jeon, J-H, Jeong, E-Y, Lee, C-H, and Lee, H-S. 2006. Antimicrobial activity of 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* toward intestinal bacteria. Food Chem. 87: 213-219.

Lindsley, D.L., and Zimm, G.G. 1992. The genome of *Drosophila melanogaster*. San Diego (CA): Academic Press.

Liu, C., Lian, F., Smith, D., Russell, R., and Wang, X. 2003. Lycopene supplementation inhibits lung squamous metaplasia and induces apoptosis via upregulating insulin-like growth factor-binding protein3 in cigarette smoke-exposed ferrets. Cancer Res. 63: 3138-3144.

Mabry, T. J., and Dreiding, A. S. 1968. The betalaines. In: Mabry, R., Alston, E., and Runeckles, V. C. (eds.), Recent Advances in Phytochemistry, Vol.1., pp. 145-160. New York: Appleton Century Crofts.

Matsumoto, H., Inaba, H., Kishi, M., Tominanga, S., Hirayama, M., and Tusda, T. 2001. Orally administrated delphinidin-3-rutinoside and cyaniding-3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J. Agr. Food Chem. 49: 1546-1551.

Meiers, S., Kemeny, M., Weyand, U., Gastpar, R., von Angerer, E., and Marko, D., 2001. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. J. Agric. Food Chem. 49: 958-962.

Miller, J.A., and Miller, E.C. 1983. The metabolic activation and nucleic acid adducts of naturally occurring carcinogens: recent results with ethyl carbamate and the spice flavors safrole and estragole. Br. J. Cancer 48(1): 1-15.

Mirvish, S.S. 1968. The carcinogenic action and metabolism of urethane and *N*-hydroxy urethane. Adv. Cancer Res. 11: 1-42.

Miyahara, K., Kawasaki, T., Kinojo, J. E., Shimokawa, T., Yamahara, J., and Yamasaki, M. 1986. The X-ray analysis of caesalpin J from sappan lignum.

Chem. Pharm. Bull. 34: 4166-4169.

Mok, M.S., Jeon, S.D., Yang, K.M., So, D.S., and Moon, C.K. 1998. Effects of brazilin on induction of immunological tolerance by sheep red blood cells in C57BL/6 female mice. Arch. Pharm. Res. 21: 769-773.

Moon, C.K., Chung, J.H., Lee, Y.M., Lee, S.H., Wang, G.S., Park, K.S. *et al*. 1988. Effects of brazilin on erythrocyte deformability and its related biochemical factors in streptozotocin induced diabetic rats. Arch. Pharm. Res. 11: 149-154.

Moon, C.K., Lee, S.H., Lee, M.O., and Kim, S.G. 1993. Effects of brazilin on glucose oxidation, lipogenesis and therein involved enzymes in adipose tissues from diabetic KK-mice. Life Sci. 53: 1291-1297.

Moon, Y.J., Wang, X., and Morris, M.E. 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism: Mini-review. Toxicol. *In Vitro* 20: 187-210.

Morton, J. 1989. Information within the book review on Potter's New Cyclopedia of Botanical Drugs and Preparations. Econ. Bot. 43: 280-281.

Naasani, I., Oh-hashi, F., Oh-hara, T., Feng, W., Johnston, J., Chan, K. *et al*. 2003. Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells *in vitro* and *in vivo*. Cancer Res. 63: 824-830.

Naczk, M., and Shahidi, F. 1989. The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola. Food Chem. 31(2): 159-164.

Nagai, M., and Nagumo, S. 1986. Protosappanin B, a new dibenzoxocin derivative from sappan lignum (*Caesalpinia sappan* L.). Heterocycles 24: 601-606.

Nagai, M., Nagumo, S., Lee, S. M., Eguchi, I., and Kawai, K. I. 1986. Protosappanin A, a novel biphenyl compound from sappan lignum. Chem. Pharm. Bull. 34: 1-6.

Nagase, H., Sasaki, K., Kito, H., Haga, A., and Sata, T. 1998. Inhibitory effect of delphinidin from *Solanum melongena* on human fibrosarcoma HT-1080 invasiveness *in vitro*. Planta Med. 64, 216-219.

Negishi, T., Arimoto, S., Nishizaki, C., and Hayatsu, H. 1989. Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-l-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2). Carcinogenesis 10(1): 145-149.

Negishi, T., Shiotani, T., Fujikawa, K., and Hayatsu, H. 1991. The genotoxicities of

N-nitrosamines in *Drosophila melanogaster in vivo*: the correlation of mutagenicity in the wing spot test with the DNA damages detected by the DNA-repair test. Mutat. Res. 252(2): 119-128.

Negishi, T., Nakano, H., Kitamura, A., Itome, C., Shiotani, T., and Hayatsu, H. 1994. Inhibition activity of chlorophyllin on the genotoxicity of carcinogens in *Drosophila*. Cancer Lett. 83: 157-164.

Nery R. 1968. Some aspects of the metabolism of urethane and *N*-hydroxyurethane in rodents. Biochem. J. 106: 1-13.

Nielsen, I. L., Dragsted, L. O., Ravn-Haren, G., Freese, R., and Rasmussen, S. E. 2003. Absorption and excretion of black currant anthocyanins in humans and Watanabe heritable hyperlipidemic rabbits. J. Agr. Food Chem. 51: 2813-2820.

Nyberg, F., Hous, S., Pershagen, G., and Lambert, B. 2003. Dietary fruit and vegetables protect against somatic mutation *in vivo*, but low or high intake of carotenoids does not. Carcinogenesis 24: 689-696.

Oak, M., Chataigneau, M., Keravis, T., Chataigneau, T., Beretz, A., Andriantsitohaina, R. *et al*. 2003. Red wine polyphenolic compounds inhibit vascular endothelial growth factor expression in vascular smooth muscle cells by preventing the activation of the p38 mitogenactivated protein kinase pathway. Arterioscl. Throm. Vas. Biol. 23: 1001-1007.

Oh, S.R., Kim, D.S., Lee, I.S., Jung, K.Y., Lee, J.J., and Lee, H.K. 1998. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. Planta Med. 64: 456-458.

Oliveira, F.C., Edwards, G.M., Velozo, S., and Nesbitt, M. 2002. Vibrational spectroscopic study of brazilin and brazilein, the main constituents of brazil wood from Brazil. Vib. Spectrosc. 28: 243-249.

Olson, J. A. 1993. Molecular actions of carotenoids. In: Canfield, L. M., Krinsky, N. I., and Dunastable, J. A. (eds.), Carotenoids in Human Health, pp. 156-166. New York: Annals of the New York Academy of Sciences.

Olvera, O., Zimmering, S., Arceo, C., and Cruces, M. 1993. The protective effects of chlorophyllin in treatment with chromium(VI) oxide in somatic cells of *Drosophila*. Mutat. Res. 301(3): 201-204.

Olvera, O., Zimmering, S., Arceo, C., Guzman, J., and de la Rosa, M.E. 1995.

Evidence for the protective effect of ascorbic acid (vitamin C) in treatment with gamma-rays and chromium (VI) oxide (CrO_3) in somatic cells of *Drosophila*. Mutat. Res. 346(1): 19-21.

Park, K.K., Liem, A., Stewart, B.C., and Miller, J.A. 1993. Vinyl carbamate epoxide, a major strong electrophilic, mutagenic and carcinogenic metabolite of vinyl carbamate and ethyl carbamate (urethane). Carcinogenesis 14: 441-450.

Parker, R. S. 1996. Absorption, metabolism, and transport of carotenoids, FASEB J. 10: 542-551.

Perez-Chiesa, Y., and Rodriguez, A. 1993. Absence of mutagenicity of benzo[*c*]phenanthridine alkaloids in somatic cells of *Drosophila melanogaster*: comparison with 7,12-dimethylbenz[*a*]anthracene and chrysene. Mutat. Res. 298(4): 277-283.

Phanrattanamala, S. 2004. Effect of hot water extracts from some herbs on urethane induced somatic mutation and recombination in *Drosophila melanogaster*. M.S. Thesis in Food and Nutrition Toxicology. Faculty of Graduate Studies. Mahidol University.

Piatelli, M. 1976. Betalains. In: Goodwin, T. W. (ed.), Chemistry and biochemistry of plant pigments, pp. 560-596. New York: Academic Press.

Piatelli, M. 1981. The betalains: structure, biosynthesis and chemical taxonomy. In: Conn, E. E., (ed.), The biochemistry of plants: a comprehensive treatise, 557-575. New York: Academic Press.

Pietta, P., Simonetti, P., and Mauri, P. 1988. Antioxidant activity of selected medicinal plants. J. Agric. Food Chem. 46: 4487-4490.

Platz, E. A., Clinton, S., Erdman, J. W. Jr., Ferruzzi, M. G., Willett, M. G., and Giovannucci, E. L. 2003. Variations in plasma lycopene and specific isomers over time in a cohort of U.S. men. J. Nutr. 133: 1930-1936.

Pool-Zobel, B.L., Bub, A., Schroder, N., and Rechkemmer, G. 1999. Anthocyanins are potent antioxidants in model systems but do not reduce endogenous oxidative DNA damage in human colon cells. Eur. J. Nutr. 38: 227-234.

Prajapati, N.D., Purohit, S.S., Sharma, A.K., and Kumar, T.A. 2003. A handbook of medicinal plants. India: Agorbios.

Preston, H.D., and Richard, M.D. 1980. Extraction and chemistry of annatto. Food Chem. 5: 47-56.

Pszczola, D. E. 1998. Natural colors: pigments of imagination. Food Technol. 52(6): 70-76.

Radlwimmer, F. B., and Yokoyama, S. 1997. Cloning and expression of the red visual pigment of goat *(Capra hircus*), Gene 198: 211-215.

Rahden-Staron, I. 2002. The inhibitory effect of the fungicides captan and captafol on eukaryotic topoisomerases *in vitro* and lack of recombinagenic activity in the wing spot test of *Drosophila melanogaster*. Mutat. Res. 518: 205-213.

Robert, D.B. 1986. Drosophila: a practical approach. Oxford: IRL Press.

Rodriguez-Amaya, D.B. 1997. Carotenoids and Food Preparation: The Retention of Provitamin A Carotenoids in Prepared, Processed, and Stored Foods. In: Rodriguez-Amaya, D.B., Enhancing the carotenoid levels of foods through agriculture and food technology. Internet Paper for Food, Nutrition and Health Theme. Food Africa, Internet Forum 31 March – 11 April 2003.

Romert, L., Magnusson, J., and Ramel, C. 1990. The importance of glutathione and glutathione transferase for somatic mutations in *Drosophila melanogaster* induced *in vivo* by 1,2-dichloroethane. Carcinogenesis 11(8): 1399-1402.

Sakano, K., Oikawa, S., Hiraku, Y., and Kawanishi, S. 2002. Metabolism of carcinogenic urethane to nitric oxide is involved in oxidative and damage. Free Radic. Biol. Med. 33: 703-714.

Santana-Rios, G., Orner, G.A., Amantana, A., Provost, C., Wu, S.Y., and Dashwood, R.H. 2001. Potent antimutagenic activity of white tea in comparison with green tea in the *Salmonella* assay. Mutat. Res. 495(1-2): 61-74.

Sarma, A. D., and Sharma, R. 1999. Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. Phytochem. 52: 1313-1318.

Sasaki, M., Sugimura, K., Yoshida, M., and Abe, S. 1980. Cytogenetic effects of 60 chemicals on cultured human and Chinese hamster cells. Senshokutai 20: 574-584.

Sato, T., Nishino, H., Nagase, H., Niikawa, M., and Kito, H. 1995. The effect of bio-antimutagens on chlorambucil and methotrexate using the wing spot test in *Drosophila melanogaster*. Jpn. J. Toxicol. Environ. Health 41: 452-457.

Schlatter, J., and Lutz, W.K. 1990. The carcinogenic potential of ethyl carbamate (urethane): risk assessment at human dietary exposure levels. Food Chem. Toxicol. 28(3): 205-211.

Schwartz, S. J. 1983. Chemical alterations of betacyanin and chlorophyll pigments during processing and a toxicological investigation of betalain pigments. Diss. Abst. Intern. 43(11): 3524-3525.

Schwartz, S. J., von Elbe, J. H., Pariza, M. W., Goldsworthy, T., and Pitot, H. C. 1983. Inability of red beet betalain pigments to initiate or promote hepatocarcinogenesis. Food Chem. Toxicol. 21(5): 531-535.

Serraino, I., Dugo, L., Dugo, P., Mondello, L., Mazzon, E., Dugo, G. *et al*. 2003. Protective effects of cyanidin-3-*O*-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. Life Sci. 73: 1097-1114.

Shanmuganayagam, D., Beahm, M., Osman, H., Krueger, C., Reed, J., and Folts, J. 2002. Grape seed and grape skin extracts elicit a greater antiplatelet effect when used in combination than when used individually in dogs and humans. J. Nutr. 132: 3592-3598.

Siefer, E., Rettura, G., and Levenson, S. H. 1981. Carotenoids and cell-mediated immune responses. In: Charalambous, G. and Inglett, G. (eds.), The Quality of Foods and Beverages. Vol. 2: Chemistry and Technology, 335-347. New York: Academic Press.

Sies, H., and Stahl, W. 2003. Non-nutritive bioactive constituents of plants: lycopene, lutein, and zeaxanthin. Int. J. Vit. Nutr. Res. 73: 95-100.

Snodderly, D. M. 1995. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. Am. J. Clin. Nutr. 62S: 1448S-1461S.

Stich, H.F., Rosin, M.P., and Bryson, L. 1982. Inhibition of mutagenicity of a model nitrosation reaction by naturally occurring phenolics, coffee and tea. Mutat. Res. 95(2-3): 119-128.

Stoewsand, G.S., Anderson, J.L., and Munson, L. 1991. Inhibition by wine of tumorigenesis induced by ethyl carbamate (urethane) in mice. Food Chem. Toxicol. 29(5): 291-295.

Stookey, L.L. 1970. Ferrozine: a new spectophotometric reagent for iron. Anal. Chem. 42: 779-783.

Strack, D., Steglich, W., and Wray, V. 1993. Betalains, In: Methods in Plant Biochemistry, 421-450. Orlando: Academic Press.

Sujamnong, P. 1981. Thai-Chinese medical text book, folkloric medicines, herbal medicines and traditional medicines in Thailand. Bangkok: Praewitaya.

Sun, J., Chu, Y.F., Wu, X.Z., and Liu, R.H. 2002. Antioxidant and antiproliferative activities of common fruits. J. Agric. Food Chem. 50(25): 7449-7454.

Swain, T., and Hillis, W.E. 1959. The phenolic constituents of *Prunus domestica*: The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10: 63-68.

Taylor-Mayne, S. 1996. Beta-carotene, carotenoids, and disease prevention in humans. FASEB J**.** 10: 609-701.

Teplizky, S. R., Kiefer, T. L., Cheng, Q., Dwivedi, P. D., Moroz, K., Myers, L. *et al*. 2001. Chemoprevention of NMU-induced rat mammary carcinoma with the combination of melatonin and 9-*cis*-retinoic acid. Cancer Lett. 168: 155-163.

Tiburi, M., Reguly, M.L., Schwartsmann, G., Cunha, K.S., Lehmann, M., and Rodrigues de Andrade, H.H. 2002. Comparative genotoxic effect of vincristine, vinblastine, and vinorelbine in somatic cells of *Drosophila melanogaster*. Mutat. Res. 519: 141-149.

Toniolo, P., van Kappel., A., Akhmedkhanov, A., Ferrari, P., Kato, I., Shore, R. *et al*. 2001. Serum carotenoids and breast cancer. Am. J. Epidemiol. 153: 1142-1147.

Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S., and Osawa, T. 1996. Inhibition of lipid peroxidatin and the active oxygen radical scavenging effect of anthocyanins pigments isolated from *Phaseolus vulgaris* L., Biochem. Pharmacol. 52: 1033-1039.

Tsuda, T., Horio, F., and Osawa, T. 2000. The role of anthocyanins as an antioxidant under oxidative stress in rats. Biofactors 13, 133-139.

Turkmen, N., Poyrazoglu, E.S., Sari, F., and Velioglu, Y.S. 2006. Studied effects of cooking methods on chlorophylls, pheophytins and colour of selected green vegetables: Original article. Int. J. Food Sci. Tech. 41: 281-288.

USDA (United States Department of Agriculture). 2006. The PLANTS Database (Database)., National Plant Data Center. Baton Rouge. Louisiana.

van den Berg, R., Haenen, M.M., van den Berg, H., and Bast, A. 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem. 66:

511-517.

van Schaik, N. and Graf, U. 1991. Genotoxicity evaluation of five tricyclic antidepressants in the wing somatic mutation and recombination test in *Drosophila melanogaster*. Mutat. Res. 260: 99-104.

Velioglu, Y.S., Mazza, G., Gao, L., and Oomah, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. 46: 4113-4117.

Vereltzis, K. P., and Buck, E. M. 1984. Color stability and sensory attributes of chicken frankfurters made with betalains and potassium sorbate versus sodium nitrite. J. Food Protect. 47: 41-45.

Vereltzis, K. P., Buck, E. M., and Labbe, R. G. 1984. Effectiveness of a betalains/potassium sorbate system versus sodium nitrite for color development and control of total aerobes, *Clostridium perfringens* and *Clostridium sporogenes* in chicken frankfurters. J. Food Protect. 47, 532-536.

Vogel, E.W., and Zijlstra, J.A. 1987. Mechanistic and methodological aspect of chemically induced somatic mutation and recombination in *Drosophila melanogaster*. Mutat. Res. 182(5): 243-264.

von Elbe, J.H., and Schwartz, S.J. 1981. Absence of mutagenic activity and a short-term toxicity study of beet pigments as food colorants. Arch. Toxicol. 49 (1): 93-98.

Waddell, W.J., Marlowe, C., and Pierce, W.M. 1987. Inhibition of the localization of urethane in mouse tissues by ethanol. Food Chem. Toxicol. 25(7): 527-531.

Wang, X., Liu, C., Bronson, R., Smith, D., Krinsky, N., and Russell, R. 1999. Retinoid signaling and activator protein-1 expression in ferrets given β-carotene supplements and exposed to tobacco smoke. J. Natl. Cancer. 91: 60–66.

Wattenberg, L. W. 1996. Chemoprevention of cancer. Preventive Med 25: 44-45.

Wettasinghe, M., Bolling, B., Plhak, L., Xiao, H., and Parkin, K. 2002. Phase II enzyme-inducing and antioxidant activities of beetroot (*Beta vulgaris* L.) extracts from phenotypes of different pigmentation. J. Agr. Food Chem. 50: 6704-6709.

Wetwitayaklung, P., Phaechamud, T., and Keokitichai, S. 2005. The Antioxidant activity of *Caesalpinia sappan* L. heartwood in various ages. Naresuan Uni. J.

13(2): 43-52.

Wiart, C. 2002. Medicinal Plants of South East Asia. 2nd ed. Malaysia : Prentice Hall.

Wong, D. W. S. 1989. Colorants. In: Mechanism and Theory in Food Chemistry, 147-187. Westport, CT .AVI.

Würgler, F.E., and Vogel, E.W. 1986. *In vivo* mutagenicity testing using somatic cells of *Drosophila melanogaster*. In: De Serres, F.J. (ed.), Chemical mutagens, principles and methods for their detection, pp. 1-72. New York: Plenum Press.

Würgler, F.E., Graf, U., and Frolich, A. 1991. *Drosophila* somatic mutation and recombination tests. In: Suttajit, M. and Rojanapo, W. (eds.), Environmental mutagens, carcinogens and Teratogens: principles and short-term assays, pp. 118-136. Proceedings of the Second Southeast Asian Workshop on Short-Term Assays for Detecting Environmental Mutagens, Carcinogens and Teratogens; February 6-17; Bangkok and Chiang Mai, Thailand: Star Press.

Xu, H.X., and Lee, S.F. 2004. The antibacterial principle of *Caesalpina sappan*. Phytother. Res. 18:647-651.

Yamamoto, T., Pierce, W.M., Hurst, H.E., Chen, D., and Waddell, W.J. 1988. Inhibition of the metabolism of urethane by ethanol. Drug Metab. Dispos. 16(3): 355-358.

Yamamoto, T., Pierce, W.M., Hurst, H.E., Chen, D., and Waddell, W.J. 1990. Ethyl carbamate metabolism: *in vivo* inhibitors and *in vitro* enzymatic systems. Drug Metab. Dispos. 18(3): 276-280.

Yang, K.M., Jeon, S.D., So, D.S., and Moon, C.K. 2000. Brazilin augments cellular immunity in multiple low dose streptozotocin (MLDSTZ) induced type I diabetic mice. Arch. Pharm. Res. 23:626-632.

Ye, M., Xie, W.D., Lei, F., Meng, Z., Zhao, Y.N., Su, H. *et al*. 2006. Brazilein, an important immunosuppressive component from *Caesalpinia sappan* L. Int. Imm. Pharmacol. 6: 426-432.

Yingming, P., Ying, L., Hengshan, W., and Min, L. 2004. Antioxidant activities of several Chinese medicine herbs. Food. Chem. 88: 347-350.

You, E-J, Khil, L-Y, Kwak, W-J, Won, H-S, Chae, S-H, Lee, B-H *et al*. 2005. Effects of brazilin on the production of fructose-2,6-bisphosphate in rat hepatocytes. J. Ethnopharmacol. 102: 53-57.

Zhang, L.X., Cooney, R.V., and Bertram, J.S. 1991.Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C_3H_{10}-T1-2 cells. Carcinogenesis 12: 2109-2114.

Zheng, W., and Wang, S.Y. 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49(11): 5165-5170.

Zimmering, S., Olvera, O., Hernandez, E., Cruces, M.P., Arceo, C., and Pimental, E. 1990. Evidence for a radioprotective effect of chlorophyllin in *Drosophila*. Mutat. Res. 245(1): 47-49.

Zimmerli, B., and Schlatter, J. 1991. Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. Mutat. Res. 259: 325-350.

APPENDICES

APPENDIX A
PREPARATION OF STEAMED STARCHY DESSERT

Ingredients

1. Arrowroot flour 15 g
2. Tapioca flour 5 g
3. Rice flour 1.25 g
4. Sugar 20 g
5. Coconut milk 10.5 g
6. Water 40 ml
7. Various amount of each natural color powder

Directions

1. Boil the sugar with some water in a pot, simmer until the syrup become thick and clear.
2. Add the coconut milk in the pot, stir frequently until cooked. Leave to warm.
3. Put all kind of flour into the bowl and mix well. Set aside.
4. Slowly add the coconut milk syrup to the flour and mix thoroughly.
5. Mix the color with the flour (except the original dessert, no color added). Set aside.
6. Set a steamer and heat a tray.
7. Pour the mixture into a tray and steam. Close the lid and steam for about 15 min, remove from heat and left to cool off.

Tip

Do not open the lid while steaming because the cooler air will cause to lose good appearance.

APPENDIX B
STATISTICAL CONSIDERATION

In experiments 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 indicated 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_l and p_u one-sidedly at the level α, and q_u and p_l also one-sidedly at the level β. In this way and in agreement with the foregoing section, a confidence limit $m_l > 1$ 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_l) for the proportion of spots in the experimental series

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

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

$$f_{t,l} = q_l n / N_c$$

This was the following complementarily, namely that the lower confidence limit for the number of spots in the experimental series ($q_l n$) 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.,

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

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 C
PREPARATION OF REAGENTS FOR ANTIOXIDANT ASSAY

DPPH Reagent:

Chemicals

1. 150 μM DPPH$^{\bullet}$ (2,2′-diphenyl-1-picrylhydrazl) in 80% Methanol
2. 1.28 mM Trolox in 80% Methanol

   Standard Trolox was run in triplicate using several concentrations. (1.28, 0.64, 0.32, 0.16, 0.08 mM)

FRAP Reagent:

Chemicals

1. 300 mM Acetate buffer (pH 3.6)

   (3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) plus 16 ml glacial acetic acid and made up to 1 L with distilled water.)
2. 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl
3. 20 mM $FeCl_3.6H_2O$

   Mixing the reagent from 1-3 before use and heated to 37 °C

   300 mM Acetate buffer: 10 mM TPTZ solution: 20 mM $FeCl_3.6H_2O$ (ratio 10:1:1)
4. 1000 μM $FeSO_4.7H_2O$

   Standard $FeSO_4.7H_2O$ was run in triplicate using several concentrations. (1000, 500, 250, 125 and 62.5 μM)

Phenolics Reagent:

Chemicals

1. Folin-Ciocalteu reagent
2. Saturated sodium carbonate solution
3. 800 mg/l Gallic acid

   Standard Gallic acid was run in triplicate using several concentrations. (800, 400, 200, 100, 50, and 25 mg/l)

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