ผลต้านออกซิเดชัน ต้านการก่อกลายพันธุ์และต้านความเป็นพิษต่อหน่วยพันธุกรรมของสารสกัดด้วยอะซีโตนและเมทานอลจากถั่วบางชนิด

นางสาวกัลยารัตน์ เครือวัลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ANTIOXIDANT, ANTIMUTAGENIC AND ANTIGENOTOXIC EFFECTS OF THE ACETONE AND METHANOL EXTRACTS FROM SOME LEGUMES

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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Thesis Title	ANTIOXIDANT, ANTIMUTAGENIC AND ANTIGENOTOXIC EFFECTS OF THE ACETONE AND METHANOL EXTRACTS FROM SOME LEGUMES
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Field of Study	Pharmaceutical Chemistry and Natural Products
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กัลยารัตน์ เครือวัลย์ : ผลต้านออกซิเดชัน ต้านการก่อกลายพันธุ์และต้านความเป็นพิษต่อหน่วยพันธุกรรมของสารสกัดด้วยอะซีโตนและเมทานอลจากถั่วบางชนิด.
(ANTIOXIDANT, ANTIMUTAGENIC AND ANTIGENOTOXIC EFFECTS OF THE ACETONE AND METHANOL EXTRACTS FROM SOME LEGUMES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.ลินนา ทองยงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.แก้ว กังสดาลอำไพ, 125 หน้า.

วัตถุประสงค์การศึกษาครั้งนี้คือศึกษาปริมาณสารฟีโนลิคและฟลาโวนอยด์ ฤทธิ์ต้านออกซิเดชัน ฤทธิ์ต้านความเป็นพิษต่อหน่วยพันธุกรรมและฤทธิ์ต้านการก่อกลายพันธุ์ของสารสกัดจากเมล็ดถั่วและเปลือกหุ้มเมล็ดของถั่วดำ ถั่วเขียว ถั่วลิสง ถั่วแดงและถั่วเหลือง โดยปริมาณของสารฟีโนลิคนั้นได้ทำการวิเคราะห์โดยวิธี Folin-Ciocalteu ผลการศึกษาพบว่าสารสกัดเมล็ดถั่วแดงดิบมีปริมาณสารฟีโนลิคสูงสุด (128.5 ± 11.7 มิลลิกรัม GAE/กรัมของสารสกัด) หลังผ่านกระบวนการให้ความร้อนด้วยหม้อนึ่งความดันพบว่าสารสกัดของเมล็ดถั่วและเปลือกหุ้มเมล็ดของถั่วลิสงแสดงปริมาณสารฟีโนลิคสูงสุด (131.2 ± 3.5 และ 498.1 ± 21.8 มิลลิกรัม GAE/ กรัมของสารสกัด ตามลำดับ) และพบสารฟลาโวนอยด์ปริมาณสูงในสารสกัดเมล็ดถั่วดำดิบ (37.5 ± 1.0 มิลลิกรัม CE/ กรัมของสารสกัด) และสารสกัดเปลือกหุ้มเมล็ดดิบของถั่วแดงและถั่วลิสง (328.2 ± 5.7 และ 328.0 ± 12.9 มิลลิกรัม CE/ กรัมของสารสกัด ตามลำดับ) ส่วนฤทธิ์ต้านอนุมูลอิสระทำการศึกษาโดยวิธี DPPH assay และ ferric reducing antioxidant power (FRAP) assay พบว่าสารสกัดเมล็ดถั่วดำแสดงฤทธิ์ในการกำจัดอนุมูลอิสระ DPPH และฤทธิ์ในการรีดิวซ์ (FRAP) สูงสุดคือ 95.2 ± 2.1% และ 423.4 ± 1.7 ไมโครโมลาร์/กรัมของสารสกัด ตามลำดับในถั่วดิบ และ 86.1 ± 1.1% และ 153.9 ± 1.5 ไมโครโมลาร์/กรัมของสารสกัดตามลำดับในถั่วผ่านความร้อน สำหรับสารสกัดเปลือกหุ้มเมล็ดดิบพบว่าถั่วลิสงแสดงฤทธิ์ในการกำจัดอนุมูลอิสระ DPPH ได้สูงสุด 92.3 ± 0.3% รองลงมาคือถั่วแดง (89.7 ± 0.3%) และถั่วดำ (87.1 ± 2.4%) นอกจากนี้สารสกัดเปลือกหุ้มเมล็ดดิบของถั่วลิสงและถั่วแดงยังมีค่า FRAP value สูงสุดคือ 2067.7 ± 112.5 และ 2063.3 ± 58.6 ไมโครโมลาร์/กรัมของสารสกัด ตามลำดับ รองลงมาคือถั่วดำผ่านความร้อน (1495.6 ± 59.4 ไมโครโมลาร์/กรัมของสารสกัด) จะเห็นได้ว่าสารสกัดเปลือกหุ้มเมล็ดของถั่วดำ ถั่วลิสง และถั่วแดง มีปริมาณสารฟีโนลิคและฤทธิ์ต้านอนุมูลอิสระสูง ในขณะที่ถั่วเหลืองต่ำที่สุด

นอกจากนั้นได้ทำการศึกษาฤทธิ์ต้านความเป็นพิษต่อหน่วยพันธุกรรมของสารสกัดจากถั่วโดยใช้วิธี single cell gel electrophoresis (comet) assay และ somatic mutation and recombination test (SMART) ผลการศึกษาในวิธี comet assay พบว่าสารสกัดของเมล็ดถั่วลิสงและถั่วดำที่ผ่านความร้อนแสดงการยับยั้งการแตกหักของดีเอ็นเอที่ถูกเหนี่ยวนำ hydrogen peroxide ได้ 34.7% และ 35.6% ตามลำดับ ส่วนสารสกัดของเปลือกหุ้มเมล็ดของถั่วดำ ถั่วลิสง และถั่วแดง ที่ผ่านความร้อนแสดงผลยับยั้ง ได้ในระดับสูง คือ 63.3%, 63.2% และ 61.8% ตามลำดับ นอกจากนี้การศึกษาด้วยวิธี SMART พบว่าสารสกัดเมล็ดถั่วแดงแสดงฤทธิ์ต้านการก่อกลายพันธุ์ได้สูงสุด (57.2%) รองลงมาคือถั่วลิสง (54.0%) ส่วนสารสกัดเปลือกหุ้มเมล็ดถั่วแสดงฤทธิ์ต้านการก่อกลายพันธุ์ได้เล็กน้อย (6.2- 38.8%) ที่ระดับความเข้มข้นต่ำที่สุด โดยสารสกัดเปลือกหุ้มเมล็ดของถั่วแดงมีฤทธิ์ต้านก่อกลายพันธุ์สูงสุด (38.8%) อย่างไรก็ตามที่ระดับความเข้มข้นสูงพบว่าสารสกัดจากเปลือกหุ้มเมล็ดถั่วทุกตัวอย่างแสดงผลเสริมฤทธิ์การก่อกลายพันธุ์ของ urethane ดังนั้นผลของการศึกษานี้จึงกล่าวได้ว่าฤทธิ์ต้านการก่อกลายพันธุ์หรือฤทธิ์ก่อกลายพันธุ์ร่วมนั้นขึ้นอยู่กับปริมาณของสารฟีโนลิคที่ได้รับ

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ……. ลายมือชื่อนิสิต.....

ปีการศึกษา 2553……. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม…..….....

4976951333: MAJOR PHARMACEUTICAL CHEMISTRY AND NATURAL PRODUCTS
KEYWORDS: ANTIOXIDANT/ ANTIGENOTOXICITY/ ANTIMUTAGENICITY/ COMET ASSAY/ SMART/ HYDROGEN PEROXIDE/ URETHANE

KALYARAT KRUAWAN: ANTIOXIDANT, ANTIMUTAGENIC AND ANTIGENOTOXIC EFFECTS OF THE ACETONE AND METHANOL EXTRACTS FROM SOME LEGUMES. ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., CO-ADVISOR: ASSOC. PROF. KAEW KANGSADALAMPAI, Ph.D., 125 pp.

The objectives of this study were aimed to determine the total phenolic and flavonoid contents, antioxidant activities, antigenotoxicity and antimutagenicity of the extracts of legume seeds and seed coats of black bean, mung bean, peanut, red kidney bean and soybean. The total phenolic content assayed by the Folin–Ciocalteu method. The extract of raw red kidney bean seed exhibited the highest total phenolics (128.5 ± 11.7 mg GAE/g dry extract). After heat treatment (autoclaving), the legume seeds and seed coats extracts of peanut showed the highest total phenolics (131.2 ± 3.5 and 498.1 ± 21.8 mg GAE/g dry extract, respectively). High content of total flavonoid was found in raw seed of black bean (37.5 ± 1.0 mg CE/g dry extract) and raw seed coats of red kidney bean (328.2 ± 5.7 mg CE/g dry extract) and peanut (328.0 ± 12.9 mg CE/g dry extract). Total antioxidant activity measured using DPPH assay and ferric reducing antioxidant power (FRAP) assay. The legume seeds extract of black bean exhibited the highest DPPH scavenging effect and reducing power (FRAP value) with 95.2 ± 2.1% and 423.4 ± 1.7 μM/g dry extract, respectively in raw seeds and 86.1 ± 1.1% and 153.9 ± 1.5 μM/g dry extract, respectively in processed seeds. For raw seed coats, peanut extract displayed the highest scavenging effect with 92.3 ± 0.3% followed by red kidney bean (89.7 ± 0.3%) and black bean (87.1 ± 2.4%). The extracts of raw seed coats of peanut and red kidney bean had high FRAP value (2067.7 ± 112.5 and 2063.3 ± 58.6 μM/g dry extract, respectively), followed by processed black bean (1495.6 ± 59.4 μM/g dry extract). The seed coats extracts of black bean, peanut and red kidney bean had the highest phenolic content and antioxidant activity while soybean extract had the lowest.

This study was also aimed to determine the antigenotoxicity and antimutagenicitiy of legumes extracts by single cell gel electrophoresis (comet) assay and somatic mutation and recombination test (SMART). Processed legume seed extracts of peanut and black bean exhibited the highest inhibition on DNA break induced by hydrogen peroxide (34.7% and 35.6%, repectively) in the Comet assay. Processed seed coats extracts of black bean, peanut and red kidney bean exhibited strong inhibition (63.3%, 63.2% and 61.8%, respectively). By using SMART, legume seeds of red kidney bean showed the highest antimutagenicity (57.2%), followed by peanut (54.0%) in the SMART. Seed coats extracts at the lowest concentration exhibited weak antimutagenic activity (6.2- 38.8%). Seed coats extract of red kidney bean showed the highest antimutagenicity (38.8%). However, at the higher concentrations, the seed coats extracts exhibited synergistic effect on the mutagenicity of urethane. The finding from this study suggested that the antimutagenic/co-mutagenic activity depends upon the levels of phenolics consumed.

Field of Study: Pharmaceutical Chemistry and Natural Products ….. Student's Signature :……..
Academic Year: 2010……. Advisor's Signature :…….……..……
Co-advisor's Signature :…….……..……..

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LIST OF ABBREVATIONS

AU	arbitrary unit
°C	degree Celsius
et al.	*et alia* (and others)
g	gram
GST	Glutathione-*S*-transferase
h	hour
H_2O_2	hydrogen peroxide
MeOH	methanol
mg	milligram
min	minute
ml	millilitre
mM	millimolar
N	normality
$O_2^{\cdot -}$	superoxide radical
ROS	reactive oxygen species
RNS	reactive nitrogen species
SD	standard deviation
SMART	Somatic Mutation and Recombination Test
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

1.1 Background and Significance of the Study

The health and wellness trend is the most influential factor in today's food and beverage industry. The demand for healthy foods is increasing rapidly in many countries. Consumers are recently more health conscious and more concerned about their daily food intake. By considering amounts of saturated fat and cholesterol, World Health Organization has recommended the frequent consumption of legume proteins instead of animal proteins. Legume seeds present one of the most promising alternative protein sources for the nutritional supplementation (Villaluenga *et al*., 2009). Several researches indicated that high legumes consumption is associated with a decreased risk of various types of cancer, such as stomach, pancreas, colon, rectum and breast cancer (Messina *et al*., 1999). Legume seeds, such as black bean [*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griffith], red kidney bean [*Phaseolus vulgaris* L.], mung bean [*Vigna radiata* (L.) Wilzcek], peanut [*Arachis hypogaea* L.] and soybean [*Glycine max* (L.) Merr.] are distributed widely in Thailand. They have been used as food or beverages in daily diet.

The seed coats of some legume seeds (mung bean, peanut and soybean) are low economic value by-products of the legume industry and they are mainly used as animal feed. However, the beneficial-health effect of these by-products can be attributed to micronutrient such as vitamin E, folate, minerals (potassium, magnesium and zinc), fiber and various phenolic compounds present in them. Phenolic compounds have received considerable attention because of their physiological functions, including antioxidant, antigenotoxic and antitumour activities (Kono *et al*., 1995; Saliva *et al*., 1991). Many biological functions such as antigenotoxicity are mediated by the antioxidant property (Velioglu *et al*., 1998). Therefore, the objective of this work was to investigate the antioxidant and antigenotoxic activities of legume seeds and their seed coats of the legume which are usually consumed by Thai people for their possible utilization in functional foods or pharmaceutical supplements and developing value-added products having beneficial-health effects from seed coats.

1.2 Objectives of the Study

The specific objectives of the present study were as followed:

1.2.1 To evaluate total phenolic and flavonoid contents of the extracts of selected legume seeds and seed coats.

1.2.2 To determine the antioxidant activity of the extracts of selected legume seeds and seed coats using DPPH and FRAP assays.

1.2.3 To determine the antigenotoxicity of the extracts of selected legume seeds and seed coats using single cell gel electrophoresis (Comet assay) and somatic mutation and recombination test (SMART).

1.2.4 To determine the effect of heat treatment (autoclaving) of the legumes on total phenolic contents, total flavonoid contents and antioxidant and antigenotoxic activities.

1.3 Benefits of the Study

1.3.1 This study provides the information on the total phenolic content, total flavonoid content, antioxidant activity, antigenotoxic and antimutagenic activities of the selected legume seeds and seed coats.

1.3.2 The information obtained in this study can be used to justify the legume seed that is the most beneficial for consumer.

1.3.3 The results of antioxidant, antigenotoxic and antimutagenic effects of legume seed coats may be used in the development of the value-added products which have beneficial-health effects.

CHAPTER II

LITERATURE REVIEW

2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and peroxyl radical ($ROO^{\cdot}$), are generated in biological systems by aerobic metabolism and also by exogenous sources such as drugs, ultra violet light, ionizing radiation and pollution systems (Briviba and Sies, 1994). Damages mediated by free radicals result in the disruption of membrane fluidity, protein denaturation, lipid peroxidation and oxidative DNA (Kinsella *et al.*, 1993). Many endogenous and exogenous antioxidant defense systems are available in living organisms to limit the levels of ROS and the damage caused by them (Ames *et al*., 1993). These include antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and many non-enzymatic antioxidants such as polyphenols, tocopherols and ascorbic acid to protect the bio molecules such as proteins, lipids and nucleic acids (Anderson and Phillips, 1999; Tavazzi *et al*., 2000). Increasing numbers of research results confirm that injuries due to an excessive production of ROS occur in many common pathological conditions such as aging, cancer, inflammatory states (e.g., rheumatoid arthritis), cataract, atherosceloresis, Parkinson's disease and Alzheimer's disease (Darlington and Stone, 2001). Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken from the diet, both from natural and synthetic origin (Rechner *et al*., 2002). Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, they therefore, appear to be very important in the prevention of many diseases (Halliwell *et al*., 1992). Thus, synthetic antioxidants are widely used in the food industry. However, because of their toxic and carcinogenic effects, their use is being restricted. Thereby, interest in finding natural antioxidants, present in medicinal and dietary plants without undesirable side effects, has increased greatly.

2.2 Antioxidants in Plants

Plants contain a large variety of phytochemicals that possess antioxidant activity. Natural antioxidants occur in all parts of the plant (wood, bark, stems, pods, leaves, fruit, roots, seeds, pollen, and flowers). Typical compounds that exhibit antioxidant activity are phenolic compounds (e.g. phenolic acids, flavonoids, coumarins, lignans, stibenes, tannins), nitrogen compounds (alkaloids, amines, batalains), vitamins and terpenoids (including carotenoids) (Cai *et al*., 2004; Chanwitheesuk *et al*., 2005).

The antioxidant compounds have been demonstrated to protect against oxidation damage by inhibiting or quenching reactive oxygen species. Vitamin C has been proposed, for a long time, as a biological antioxidant. It was found to act as a chain-breaking scavenger for peroxy radicals and to act as a synergist with vitamin E, since vitamin C can donate a hydrogen atom to the vitamin E-derived phenolate radical, thus regenerating its activity. Furthermore, singlet oxygen is very powerfully quenched by carotenoids, especially β-carotene. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox properties of their phenolic hydroxyl groups that allow them to act as reducing agents, hydrogen-donating antioxidants, and oxygen quencher (Rice-Evans *et al*., 1996).

Legumes play an important role in the traditional diets of many regions throughout the world. They are excellent sources of protein, carbohydrate, dietary fiber, lipid, a variety of micronutrients and phytochemicals (Anderson *et al*., 1999; Messina, 1999). However, its nutraceutical value is yet to gain as much attention in the prevention of chronic diseases. The protective effects of dry beans in disease prevention such as cancer may not be entirely associated to dietary fiber, but to phenolic and other non-nutritive compounds (Oomah *et al*., 2006). Polyphenolic compounds from dry beans may possibly act as antioxidants, compounds that have the ability to scavenge free radicals, especially ROS. They can act as chain breakers to stop the propagation of sequential free radical reactions and thereby reduce damage to DNA and membrane. They also have other molecular consequences, including inhibiting generation of ROS, inhibiting metabolic activation of carcinogens, and altering the intercellular redox potential (Halliwell *et al*., 1995).

Three different parts are recognised in the legume seeds: cotyledon, seed coats and embryonic axe, which represent, on average, 89%, 10% and 1%, of the total seed weight, respectively. The cotyledon contains the main reserve substances, basically proteins and carbohydrates. The seed coat, which acts as a protective barrier for the cotyledon, has the highest concentration of phenolic compounds (Duenas *et al*., 2002; Shahidi *et al*., 2001). These naturally occurring phenolic compounds possess antimutagenic and antioxidant activities (Aparicio *et al.*, 2005). However, they must be processed before consumption due to their content of nonnutritive factors, such as trypsin inhibitors, phytic acid and alpha-galactosides (Agustin *et al*., 1989; Vidal-Valverde *et al*., 2002). Because of the presence of many bioactive compounds in legumes with antioxidant activity and the relationship between antioxidants and antimutagenicity, there is a wide interest in the effects of processing on the antioxidant activity and antimutagenicity of legumes.

2.2.1 Phenolic Compounds

Phenolics (hydroxybenzenes) and polyphenolics (containing two or more phenol groups) are ubiquitous in plants. This class of plant metabolites contains more than 8000 known compounds, ranging from simple phenols such as phenol itself through to materials of complex and variable composition such as tannins (Moon *et al*., 2006). More recently, interest has been rekindled with the recognition that many polyphenols, although non-nutrients, show antioxidant, anti-inflammatory, anti-oestrogenic, anti-mutagenic and/or anti-carcinogenic effects, at least in *in vitro* or in animal systems. Several epidemiological studies suggest a correlation between the consumption of foods with a high content of phenolics (such as fruits, vegetables, legumes and wine) with decreasing incidence of diseases, e.g., cancer and cardiovascular disease (Scalbert *et al*., 2005). The importance of the antioxidant constituents in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers as the trend of the future is moving toward functional food with specific health effects (Velioglu *et al*., 1998; Kahkonen *et al*., 1999). The beneficial health-related effects of certain phenols or their potential antioxidant properties, especially when these compounds are present in large quantities in foods, are of importance to consumers.

2.2.2 Flavonoids

Flavonoids are a ubiquitous group of polyphenolic substances which are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers. A great number of plant medicines contain flavonoids, which have been reported by many authors as having antibacterial (Havsteen, 1983) anti-inflammatory (Kim *et al*., 1998), antimutagenic (Edenharder *et al.*, 2001), antiviral (Thomas *et al*., 1988), antineoplastic (Hirano *et al.*, 1994), anti-thrombotic (Lou *et al*., 1989) and vasodilatory actions (Marchand, 2002). Flavonoids may be divided into six different major classes (flavanols, flavanones, flavones, isoflavones, flavonols and anthocyanidins) based on differences in molecular backbone structure. The major classes of flavonoids consist of two fused six-membered rings (an aromatic A-ring and a heterocyclic C ring) connected through a carbon-carbon bridge to an aromatic B-ring. Structure of some phenolic compounds (flavonoid and non-flavonoid compounds) was shown in Table 1 (Ferguson, 2001) and Figure 1. Various classes of flavonoid differ in the level of oxidation and saturation of ring C, while individual compounds within a class differ in the substitution pattern of rings A and B. The differences in the structure and substitution will influence the phenoxyl radical stability and thereby the antioxidant properties of the flavonoids. In addition to antioxidant function, flavonoids may also modulate cell signalling pathways and could have functions relevant for the molecular biology are listed in Table 2.

Table 1. The chemical structures and major food sources of phenolic compounds (Ferguson, 2001)

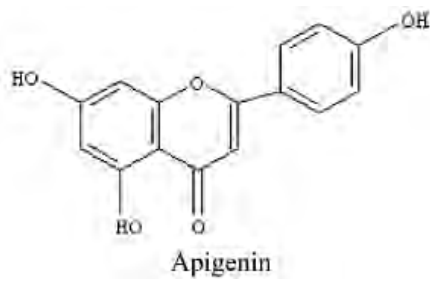
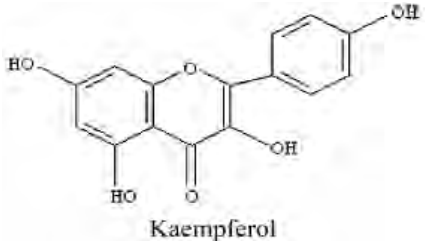
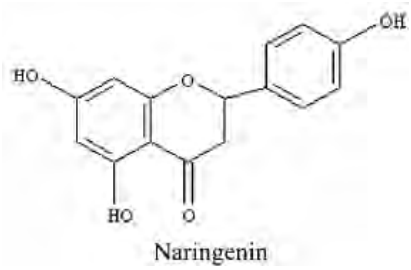
Classes and subclasses	Basic skeleton	Example	Main source
Non-flavonoid compounds			
Phenolic acids	C6–C1	Gallic acid, vanillic acid, syringic acid, tannic acid	Common among higher plants and ferns
Hydroxycinnamic acids	C6–C3	Ferulic acid, *p*-coumaric acid, caffeic acid, sinapic acid	Common in higher plants, often as components of plant cell walls
Coumarins, isocoumarins	C6–C3	Umbelliferone, aesculetin, Scopoletin	
Stilbenes	C6–C2–C6	Resveratrol	Grape skins an especially good source
Anthraquinones	C6–C2–C6		
Lignans, neolignans	$(C6–C3)_2$	Lignans	Common in flaxseeds
Lignins	$(C6\text{-}C3)_n$		Components of certain plant cell walls
Flavonoid compounds			
Flavone			
 Apigenin		Acacetin Apigenin Baicalein Chrysin Diosmetin Luteolin Tangeretin	Parsley, thyme, celery, sweet red peppers, honey, propolis peppers
Flavonol			
 Kaempferol		Galangin Kaempferol Morin Myricetin Quercetin	Onions, kale, broccoli, apples, cherries,

Table 1. The chemical structures and major food sources of phenolic compounds (Ferguson, 2001) (continued)

Classes and subclasses	Basic skeleton	Example	Main source
Flavanone	 Naringenin	Eriodictyol Hesperetin Homoeriodictyol Naringenin	Citrus
Flavanol	 Epicatechin	Catechin Epicatechin Proanthocyanidins	Cocoa, green tea, chocolate, red wine, hawthorn, bilberry, motherwort, and other herbs
Anthocyanin	 Cyanidin	Cyanidins Pigmented compounds	Cherries, grapes, berries, red cabbage
Isoflavone	 Genistein	Biochanin A Genistein Diadzein Equol Formononetin	Red clover, alfalfa, peas, soy and other legumes

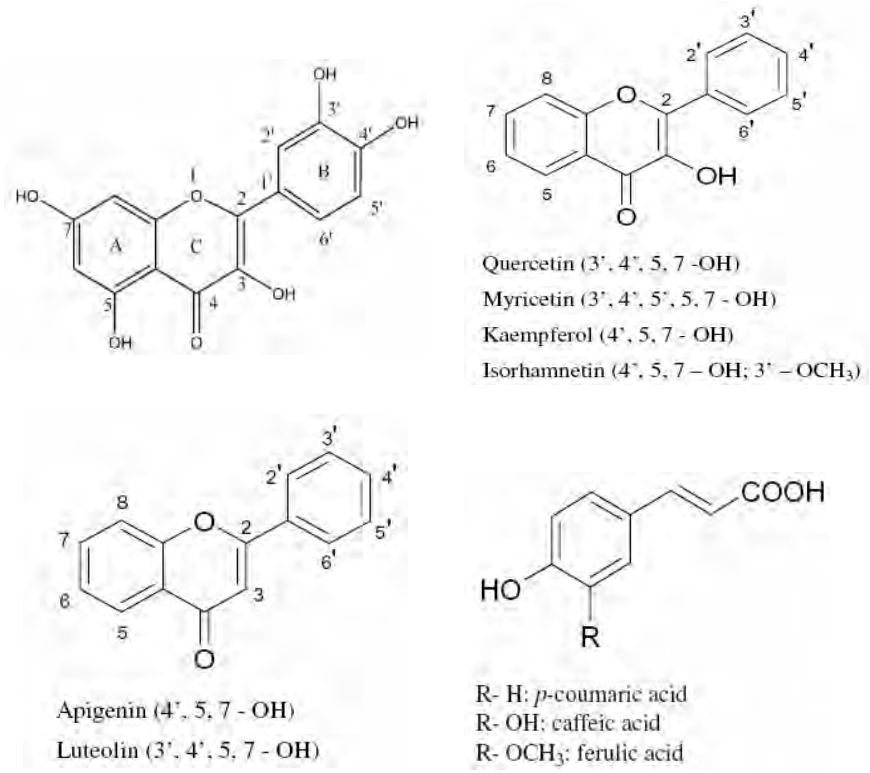


Figure 1. Structures of major phenolic compounds (Wojdyło *et al.*, 2007).

Table 2. Biomolecular activities of flavonoids

- Antioxidative effect: inactivation of oxygen radicals
- Binding of electrophiles
- Induction of protective enzymes: phase 2 enzymes with conjugating activities (GT, GST)
- Apoptosis rate increase
- Cell proliferation inhibition
- Lipid peroxidation inhibition
- Angiogenesis inhibition
- H-Donation (e.g. GSH-peroxidase)
- DNA oxidation inhibition

GT, glucuronosyl transferases; GST, glutathione S-transferases; GSH, glutathione.

(Hoensch and Kirch, 2005).

The potential mechanisms of inhibition of carcinogenesis by flavonoids are demonstrated in Figure 2 (Moon *et al*., 2006). Carcinogenesis is initiated with the transformation of the normal cell into a mutant cell. These cells undergo tumor promotion into benign tumor cells, which progress to malignant cells. Flavonoids can interfere with different steps of this process. Some flavonoids (for example, kaempferol, diosmetin, theaflavin, and biochanin A) can inhibit the metabolic activation of the procarcinogens to their ultimate electrophilic species by phase I enzymes (predominantly CYPs), or their subsequent interaction with DNA. Therefore these agents block tumor initiation (blocking agents). Alternatively, dietary flavonoids (for example, naringenin, quercetin, biochanin A, and prenylchalcones) can stimulate the detoxification of carcinogens by inducing phase II enzymes, leading to their elimination from the body. Flavonoids such as genistein and EGCG suppress the later steps (promotion and progression) of multistage carcinogenesis (suppressing agents) by affecting cell cycle, angiogenesis, invasion, and apoptosis.

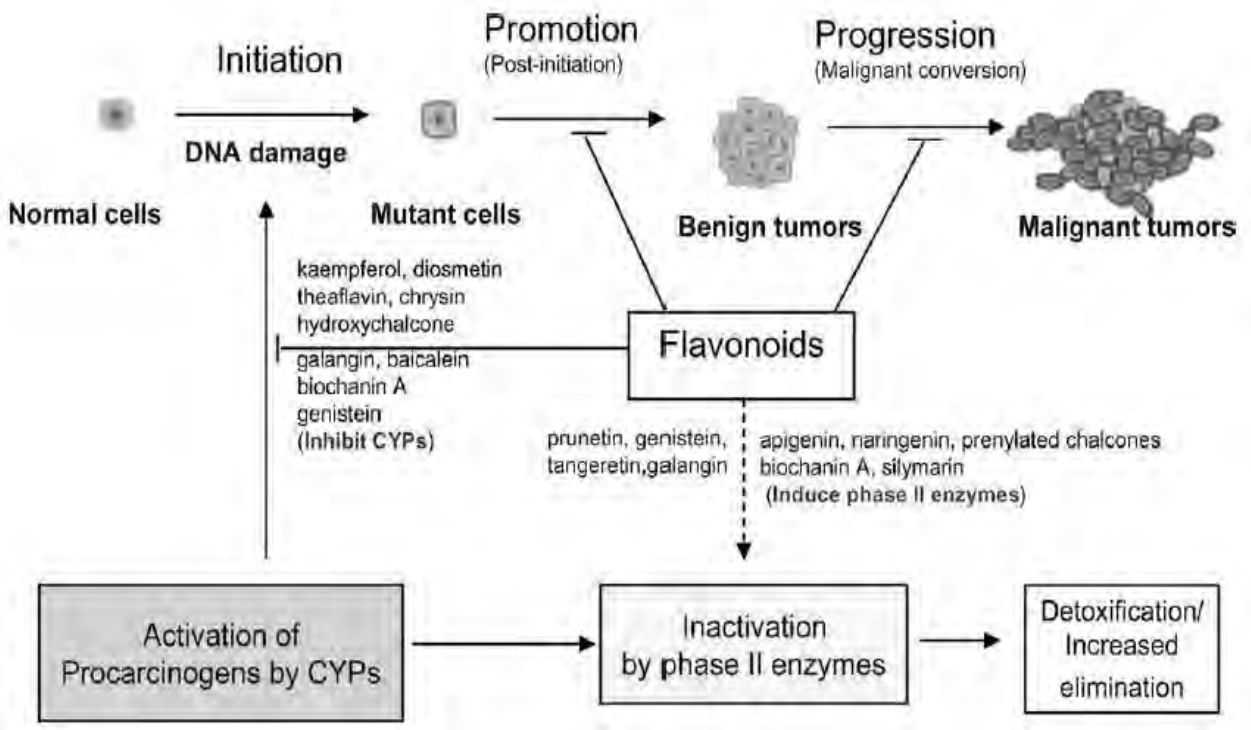


Figure 2. Flavonoids block or suppress multistage carcinogenesis (Moon *et al*., 2006).

2.2.3 **Anthocyanins**

Anthocyanins are flavonoids present in a variety of plants, including beans, and, like other flavonoids, seem to play an important role in preventing human diseases associated with oxidative stress (Duthie *et al*., 2000). The anthocyanidins are aglycons of the anthocyanins. Several studies have suggested that the anthocyanin content and their corresponding antioxidant activity, contribute to the fruits and the vegetables protective effect against degenerative and chronic diseases (Heinonen *et al*., 1998; Record *et al*., 2001). Gasiorowski *et al.* (1997) established that anthocyanins isolated from fruits of *Aronia melacarpa* markedly inhibited the mutagenic activity of benzo[a]pyrene and 2-amino fluorine in the Ames test. In addition, Lazze *et al*. (2003) demonstrated that anthocyanins were effective in reducing DNA damage (single strand breaks and oxidized bases) induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells using the Comet assay. Shih *et al*. (2005) found the ability of anthocyanins to induce phase II detoxifying enzymes in cultured cells. Treatment of rat liver clone 9 cells with 50 µM anthocyanins (Shih *et al.*, 2007) and non-cancerous breast cells with 10-20 µg/ml anthocyanins (Singletary *et al*, 2007) enhanced their antioxidant capacity by activating glutathione-related enzymes (glutathione reductase, glutathione peroxidase, and glutathione S-transferase) as well as the activity of NAD(P)H: quinone reductase.

2.3 Selected Legumes

Legumes are important food crops both economically and nutritionally, and are cultivated and consumed in many countries throughout the world. Many of the micronutrients present in leguminous plants, such as anthocyanins, lecithin, and trypsin inhibitors have been suggested to have protective and therapeutic effects on cancer. These effects make such micronutrients potentially useful in dietary chemopreventive strategies (Lazze *et al*., 2003; Wang and Murphy, 1994).

2.3.1 Black Bean (*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griffith)

Black beans is cultivated and consumed worldwide. López-Reyes *et al*. (2008) showed that administration of 70 mg/kg b.w. of methanol black bean seed extract exhibited strong antifibrotic effects in the CCl_4 chronic liver injury in the animal model. They reduced hepatic fibrosis index by 18% compared to positive controls. Azevedo *et al.* (2003) studied the antimutagenicity of black beans. They found that mice received diets containing 1, 10 or 20% of black beans significantly decreased in the frequency of cyclophosphamide

induced micronucleated polychromatic erythrocytes in bone marrow (approximately 34, 22 and 33% reductions, respectively). Blood peripheral leukocytes also showed statistical significant reductions of DNA damage (tail intensity) in Comet assay when mice treated with cyclophosphamide received diets with 20% black beans (56% reduction). They suggested that anthocyanins, constituent of black beans, might be one of the agents responsible for protection against DNA damage. In addition, anthocyanins protected against DNA damage caused by highly reactive free radicals (Tsuda *et al*., 1994, Lazze *et al*., 2003). Condensed tannins isolated from black beans (0.24–24 μM) did not affect the growth of normal cells, but induced cancer cell (Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cells) death by apoptosis as shown by a concentration-dependent decrease in ATP and cell gross morphology. Metalloproteinases such as MMP-2 and MMP-9, and others played a critical role in the degradation of the basement membrane surrounding the endothelial cells during the initial steps of the angiogenic process (Bawadi *et al*., 2005). Huang *et al*. (1983) demonstrated that delphinidin, which represents 56% of the total anthocyanins in the black beans (Takeoka *et al*., 1997), antagonized the mutagenic activity of a final metabolite of benzylapyrene, *in vitro*.

2.3.2 Red Kidney Bean (*Phaseolus vulgaris* L.)

Red kidney bean is the most widely produced edible legume in Africa, India, Latin America and Mexico (FAO, 1993). This bean usually contains 20–30% protein on a dry basis, and the protein has a good amino acid composition but is low in sulfur-containing amino acids (notably methionine) and tryptophan (Sathe, 2002). The protein of the red kidney bean (phaseolamin), which is known to contain high level of an alpha-amylase inhibitor. It is thought that, by possibly preventing the digestion of complex carbohydrate, this inhibitor may reduce calorie intake, thereby promoting weight loss (Mosca *et al.*, 2008). In addition, the bean produces lower blood glycemic index in humans making it an attractive option for diabetic patients (Viswanathan *et al*., 1989). Rats received diets containing red kidney beans reduced azoxymethane-induced colon cancer in rats (Hangen and Bennink, 2002). In addition, Tsuda *et al.* (1994) found that the anthocyanin pigment from red kidney seed coats may play an important role in the prevention of malonaldehyde (MDA) formation when lipid peroxidation of rabbit erythrocyte membrane was induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH).

2.3.3 Mungbean (*Vigna radiata* (L.) Wilzcek)

Mung bean is an excellent source of vitamins, minerals and protein (27%) with its essential amino acid profile comparable to that of soybean and red kidney bean (Mubarak, 2005). It serves as a health food because of medicinal values such as producing a cooling effect on the human body in the summer. It was also well documented that protein in mung bean exerted both antifungal and antibacterial activity (Wang *et al.*, 2004). Experimental results indicate that mung bean consumption produces small increase in blood glycemic index in humans making it an attractive option for diabetic patients. It was reported to modify glucose and lipid metabolism favorably in rats (Lerer-Metzger *et al.*, 1996). Further, medical research indicates that diabetic patients have significantly accelerated levels of oxidative stress (Mohanty *et al*., 2000) which contributes to most diabetic complications. Hence, food sources with hypoglycemic effect and high antioxidant activity such as mung bean are beneficial for diabetics. Additional, glycation that is the nonenzymatic adduct formation between sugar dicarbonyls and proteins is one key molecular basis of diabetic complications due to hyperglycemia. Experimental results indicate that phytochemicals rich in phenolic antioxidants possess significant *in vitro* antiglycation properties (Lunceford and Gugliucci, 2005). Holman and Turner (1991) found an interesting link between diabetes and L-DOPA mediated dopaminergic functioning in rats. L-DOPA, the precursor of the neurotransmitter dopamine is used in the management of Parkinson disease (Maguire-Zeiss and Federoff, 2003). Research in diabetic rats indicated that hypoglycemic foods with increased L-DOPA content were more effective in managing the disease (Kono and Takada, 1994).

2.3.4 Peanut (*Arachis hypogaea* L.)

Peanut is the fourth most important oilseed in the world and considered a major source of edible oils and protein meals valuable in human nutrition (Nwokolo, 1996) because of its amino acid profile. Peanuts have been identified as a source of phytochemicals such as trans-resveratrol, phytosterols and the isoflavones genistein and daidzein. These biologically active compounds have been reported to have antibacterial, antiviral, anti-inflammatory, anticarcinogenic, estrogenic, and anti-estrogenic properties (Chukwumah *et al.*, 2009). Numerous phytochemical compounds are present in peanuts with potential antioxidant capacity including polyphenolics (Talcott *et al.*, 2005a), tocopherols, and proteins (Bland and Lax, 2000). Seo and Morr (1985) found that *p*-coumaric acid was the predominant compound, accounting for 40–68% of the total

phenolics present. *p*-coumaric acid has been shown to possess significant radical scavenging activities (Rice-Evans *et al.*, 1996). Epidemiological studies suggest that frequent consumption of peanuts may reduce the risk of coronary heart disease (Fraser *et al.*, 1992) and certain types of cancers (Awad *et al.*, 2000). The edible parts of peanuts consist of the kernel and protective skin. The skin has a pink-red color and rich in phenolics and potentially other health promoting compounds. In addition, peanut skins have long been used in China as a traditional Chinese medicine for the treatment of chronic haemorrhage and bronchitis (Jiansu Xin Medical College, 1977)

2.3.5 Soybean (*Glycine max* (L.) Merr.)

Soybean, an important legume, has high protein content with nutritionally balanced amino acid profile. It has various biologically active phytochemicals such as, iso-flavones, genistein, daidzein, coumestrol, phytate, saponins, phytate, lecithin, phytosterols and vitamin E, that provide several health benefits, including protection against oxidative stress (Fritz *et al.*, 2003; Tripathi and Misra, 2005) and anti-carcinogenic properties (Fritz *et al.*, 2003; Mazur *et al.*, 1998). Soybean seeds are a source of vitamin E. This compound is a major biological antioxidant. It quenches free radicals and acts as a terminator of lipid peroxidation, particularly in membranes with high concentrations of unsaturated fatty acids (Burton and Traber, 1990). In addition, soybean and soy products are a particularly abundant source of isoflavones. They contain approximately 0.2–1.6 mg of isoflavones/g dry weight. The principal isoflavones found in soy proteins and soy foods are daidzein, genistein, and glycitein (Ren *et al*., 2001). Isoflavones are diphenolic compounds, which exist in unconjugated (aglycone) or conjugated forms. The aglycone forms are daidzein, genistein, and glycitein (Kurzer and Xu, 1997). Genistein and diadzein, the most significant isoflavones, are heterocyclic phenols that have a structure similar to estrogen (Kaldas and Hugh, 1989). Naturally occurring isoflavones have shown antioxidant activity in different model systems. Their antioxidant properties have been confirmed in the Comet assay. Twenty-four hours supplementation with daidzein and genistein in Jurkat T-cells (2.5-20 μM) and in peripheral blood lymphocytes of healthy subjects (0.01- 2.5 μM) displayed a significantly increased DNA protection from H_2O_2 in both cell types in the Comet assay. In addition, pre-treatment with genistein or equol (a non-steroidal oestrogen metabolised from daidzein) at doses of 0.01- 100 μM significantly protected sperm DNA integrity after H_2O_2-mediated damage (Foti *et al*., 2005). The physiological effects of isoflavones including

possible antioxidant activity, therefore suggesting a role for isoflavones in the prevention of coronary heart disease, endocrine-responsive cancers and male infertility (Sierens *et al*., 2002).

In addition, possible mechanisms of isoflavones that have been studied in animals and humans include enhancement of bile acid excretion, reduced cholesterol metabolism, increased thyroid hormones, and reduced insulin to glucagon ratios (Potter, 1998). Inspite of the recommended therapeutic dose for menopausal women which is 60 mg total isoflavones/day (Brzezinskim *et al*., 1997), a recent panel of world experts determined that the amount of soy isoflavones needed to achieve all of the potential benefits of soy isoflavones, including a reduced risk of breast, prostate and colon cancers (Bajpai *et al.*, 2005), was in the average of 100–160 mg of isoflavones per day (Anderson *et al*., 2000). Also, Jenkins *et al*. (2002) reported that high isoflavone intakes (168 mg/d isoflavones) may decrease the risk of cardiovascular disease by reducing oxidized LDL in men and women. Saponins in soybean have some important biological properties, including hyperlipidemia prevention (Lee *et al*., 2005), anticancer (Sun, 2000), immunoregulation, antioxidation (Yoshiki *et al*., 2001), antimutagenicity (Jun *et al*., 2002) and HIV infection inhibition (Nakashima *et al*., 1989).

2.4 Antioxidant Assays

A broader definition of antioxidant was suggested by Halliwell *et al.* (1995) as “any substance that when present at low concentrations, compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate”. Therefore, according to this definition, not all reductants involved in a chemical reaction are antioxidants; only those compounds which are capable of protecting the biological target meet these criteria. This protection may be based on several mechanisms of action, namely: (i) inhibition of generation and scavenging capacity against ROS/RNS; (ii) reducing capacity; (iii) metal chelating capacity; (iv) activity as antioxidative enzyme; (v) inhibition of oxidative enzymes (Magalhaes *et al*., 2008). The available methodologies for assessing the first two types of action (scavenging capacity and reducing capacity) were evaluated by DPPH assay and FRAP assay, repectively.

2.4.1 Scavenging of 2,2-Diphenyl-1-picrylhydrazyl Radical (DPPH Assay)

DPPH assay has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. The assay determines the stoichiometry for the reaction of DPPH with H-donor for individual substance or the quantity of active OH-groups in complex mixture (Roginsky and Lissi, 2005). This spectrophotometric assay, the purple chromogen stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) is reduced by antioxidant/reducing compounds to the corresponding pale yellow 2,2-diphenyl-1-picrylhydrazine. The method is based on the reduction of DPPH• in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. The absorbance at 520 nm decreased proportionally to the increases of non-radical forms (Ancerewicz *et al*., 1998). The principle of antioxidant (DPPH) assay was shown in Figure 3.

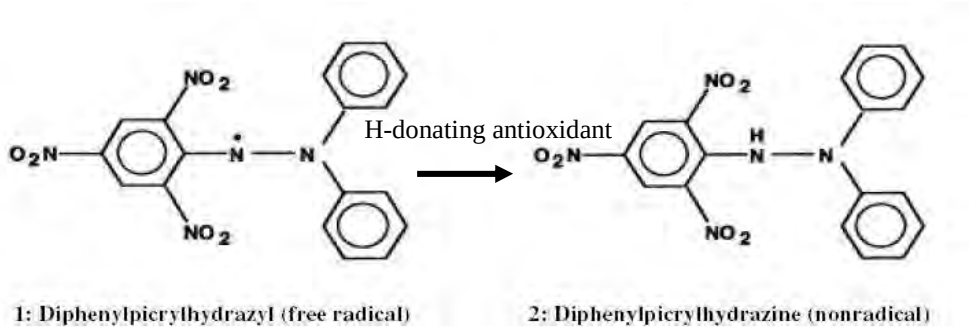


Figure 3. Principle of antioxidant (DPPH) assay

2.4.2 Ferric Reducing Antioxidant Power (FRAP Assay)

The FRAP assay is quick and simple to perform, and reaction is reproducible and linearly related to the molar concentration of the antioxidant(s) present (Benzie *et al*., 1999). This method was initially developed to assay plasma antioxidant capacity, but can be used to measure the antioxidant capacity from a wide range of biological samples and pure compounds to fruits, wines, and animal tissues (Ghiselli *et al*., 1998). The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured ferrous complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ in acidic medium (Benzie and Strain, 1996). The principle of antioxidant (FRAP) assay was shown in Figure 4. FRAP values are calculated by measuring the absorbance increase at 600 nm and relating it to a ferrous ions standard solution. The results were expressed as the combined concentrations of all electron-donating reductants which occur in the samples in a variety of sample plants. As the FRAP assay measures the reducing capacity based upon

reduction of ferric ion, antioxidants that act by radical quenching (H transfer), particularly thiols and carotenoids, will not be determined (Pulido *et al*., 2000; Ou *et al*., 2002).

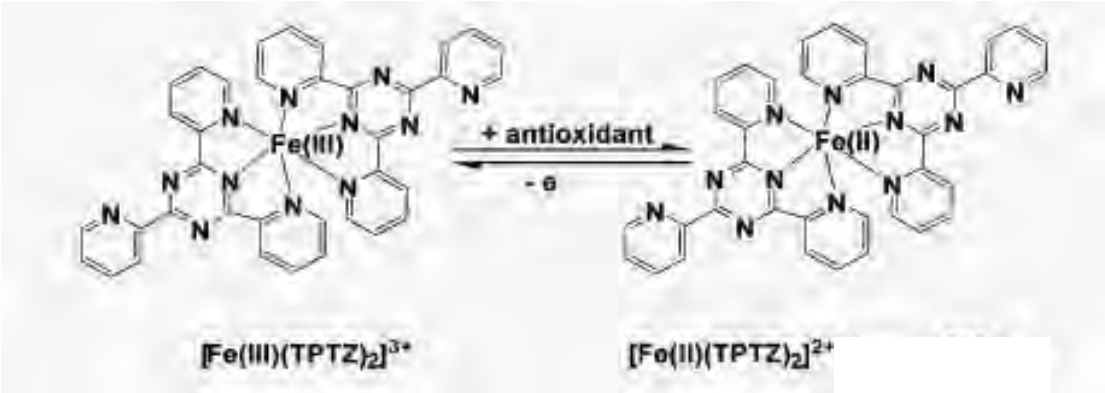


Figure 4. Principle of antioxidant (FRAP) assay

2.4.3 Total Phenolic Content

The exact chemical nature of the Folin–Ciocalteu reagent is not known, but it is accepted that it contains phosphomolybdic/phosphotungstic acid complexes. The Folin–Ciocalteu reagent was used to determine total polyphenol in sample extracts. The chemistry behind the Folin–Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds (oxidizes phenolates) to molybdenum, forming blue complexes that can be detected spectrophotometrically at 750 nm (Singleton and Rossi, 1965). Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents.

2.4.4 Total Flavonoid Content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, which are characterized by a benzo-y-pyrone structure, which is ubiquitous in fruits and vegetables. Total flavonoid can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride which can be monitored spectrophotometrically at 510 nm (Abu Bakar *et al*., 2009).

2.5 Single Cell Gel Electrophoresis (SCGE or Comet Assay)

Among the methods for assessing DNA damage in mammalian cells, the Comet assay in peripheral blood leukocytes has been widely used to elucidate the relationship between diet and carcinogenesis. The Comet assay is simple, rapid, sensitive, and visible. This assay can reflect different types of DNA damage, such as DNA single-or double-strand breaks, or incomplete DNA repairing; and it shows high sensitivity in detecting carcinogens. In addition, this short term experiment has not only been applied to evaluate genotoxic agents, but also to screening of antimutagens and anticarcinogens (Plewa *et al.*, 1998). It is particularly appropriate for studying the low level of damage present in normal human cells, such as peripheral lymphocytes. The cells are embedded in agarose on a microscope slide and lysed with Triton X-100 and 2.5 M NaCl, which remove cytoplasm and most nuclear proteins, but leave the DNA, in supercoiled form. After incubation in alkali, the DNA is electrophoresed at high pH (Collins *et al.*, 2004). DNA damage in Comet assay is visualized at the individual cell level (Figure 5) as an increased migration of genetic material (Comet tail) from the nucleus (Comet head).

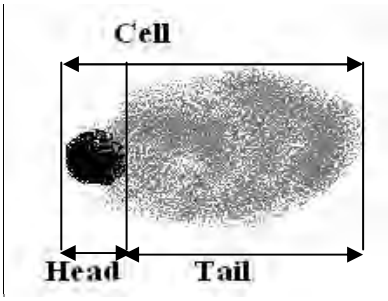


Figure 5. Structure of Comet

2.5.1 Oxidative DNA Damage

H_2O_2 is known to freely diffuse through cell membranes. H_2O_2 is less reactive than its radical counterpart such as the superoxide and the hydroxyl radical, but due to the above mentioned ability to diffuse through membranes, H_2O_2 is a potent cellular oxidant species. The toxicity of H_2O_2 is largely based on its conversion to hydroxyl radical ($^{\cdot}OH$) either through ionizing radiation (as shown in reaction (a) below), by interaction with transition metals such as iron and copper through fenton chemistry (as shown in reaction (b) below), or by interaction with superoxide anion radical through Haber–Weiss reaction (as shown in (c) below). The superoxide radical, through combination of reaction (d) and the Fenton reaction, results in the recycling of transition metal between their oxidized and reduced

states and results in the formation of hydroxyl radical at an appreciable rate (Halliwell *et al*., 2000).

H_2O_2	→	2 ($^{\cdot}OH$)	(a)
$H_2O_2 + Fe^{2+}$ or Cu^{+}	→	Fe^{3+} or $Cu^{2+} + {}^{\cdot}OH + {}^{-}OH$	(b)
$O^{\cdot -}{}_2 + H_2O_2$	→	$O_2 + {}^{\cdot}OH + {}^{-}OH$	(c)
Fe^{3+} or $Cu^{2+} + O^{\cdot -}{}_2$	→	Fe^{2+} or $Cu^{+} + O_2$	(d)

Fenton type reaction is one of the most important metal mediated reaction, where the oxidation of a metal (usually transition metals such as iron (II), copper (I), chromium (III), cobalt (II), nickel (II), vanadium (V)) by H_2O_2 leads to the generation of a hydroxyl radical.

The hydroxyl radical ($^{\cdot}OH$) is the most oxidizing radical generated in the human body. The hydroxyl radical is known to react with DNA and lipid to cause damage and peroxidation, respectively (Aust *et al*., 1985). Attack of $^{\cdot}OH$ radicals on DNA at the sugar residue leads to fragmentation, base loss, and strand breaks with a terminal sugar residue fragment. The $^{\cdot}OH$ radical can also attack DNA bases and produce thymine glycol, 8-hydroxyguanine or 2,6-diamino-4-hydroxy-5-formamidopyrimindine.

2.6 Somatic Mutation and Recombinaiton Test (SMART)

The Somatic Mutation and Recombinaiton Test (SMART) in *Drosophila melanogaster* has been designed to detect genetic damage in a rapid and inexpensive way. It is an *in vivo* system that uses a eukaryotic organism with metabolic machinery similar to that found in mammalian cells (Vogel and Zijlstra, 1987). Several advantages of *Drosophila melanogaster* as a test organism for detection of chemicals with genotoxic activity have been enumerated. The main points are: a short life cycle (10 days at 25°C); easy to detect 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. Also, it is capable of activating enzymatically promutagens and procarcinogens *in vivo* (Sarıkaya and Çakır, 2005).

This assay is based on induced loss of the heterozygosity, which may occur through various mechanisms, such as point mutations, deletions, certain types of chromosome aberrations as well as mitotic recombination and gene conversion (Graf *et al*., 1984). It is

based on the treatment of larvae during the embryogenesis, the imaginal disc cells proliferate mitotically and many genetic events such as point mutation, deletion, somatic recombination and non-disjunction can be determined on the wing of adult flies (Würgler and Vogel, 1986). If a genetic alteration occurs in one cell of the imaginal disc during mitotic proliferation, it will form a clone of mutant cells expressing the phenotype regulated by the specific genetic markers. The use of improved high-bioactivation (HB) strains of *Drosophila melanogaster*, which are characterized by increased cytochrome P450-dependent bioactivation capacity, facilitates the detection of promutagens and procarcinogens of different chemical classes (Graf and Singer, 1989; Graf and Van Schaik, 1992). The SMART assay is also well suited to determine the antimutagenicity of pure chemicals or mixtures (Negishi *et al*., 1989; Graf *et al*., 1989).

2.6.1 Wing Spot Test in *Drosophila*

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 (Graf *et al*., 1984 and 1989; Szabad *et al*., 1983). The two wing hair markers are both located on the left arm of chromosome 3. The appearance of multiple wing hairs (*mwh*, 3-0.0) is a recessive, homozygously viable mutation and produces multiple trichomes per cell instead of the normally unique trichome. The second marker, flare (*flr*^{3}, 3-39.0) is a recessive mutation that produces malformed wing hairs that 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 analysed series, 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). Different types of wing hair mutations are shown in Figure 6.

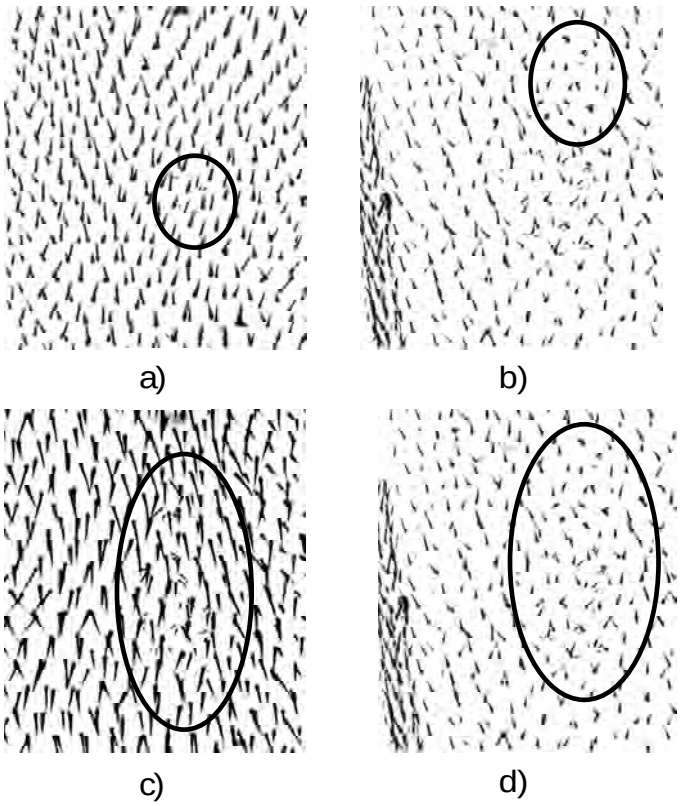


Figure 6. Difference types of wing hair mutation, a) small single spots of *mwh* on wing, b) large single spots of flare on wing, c) large single spots of *mwh* on wing, d) twin spots (By courtesy of Kaew Kangsadalampai).

Several mechanisms lead to genetically marked clones (Figure 7). 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, a second between *mwh* and *flr*^{3}) take place within the same cell, a *flr*^{3} single spot may result. Non disjunctional 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 was 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 recombination event

occurring in the chromosome segment between the two marker genes. In addition, 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, Graf and Frolich, 1991).

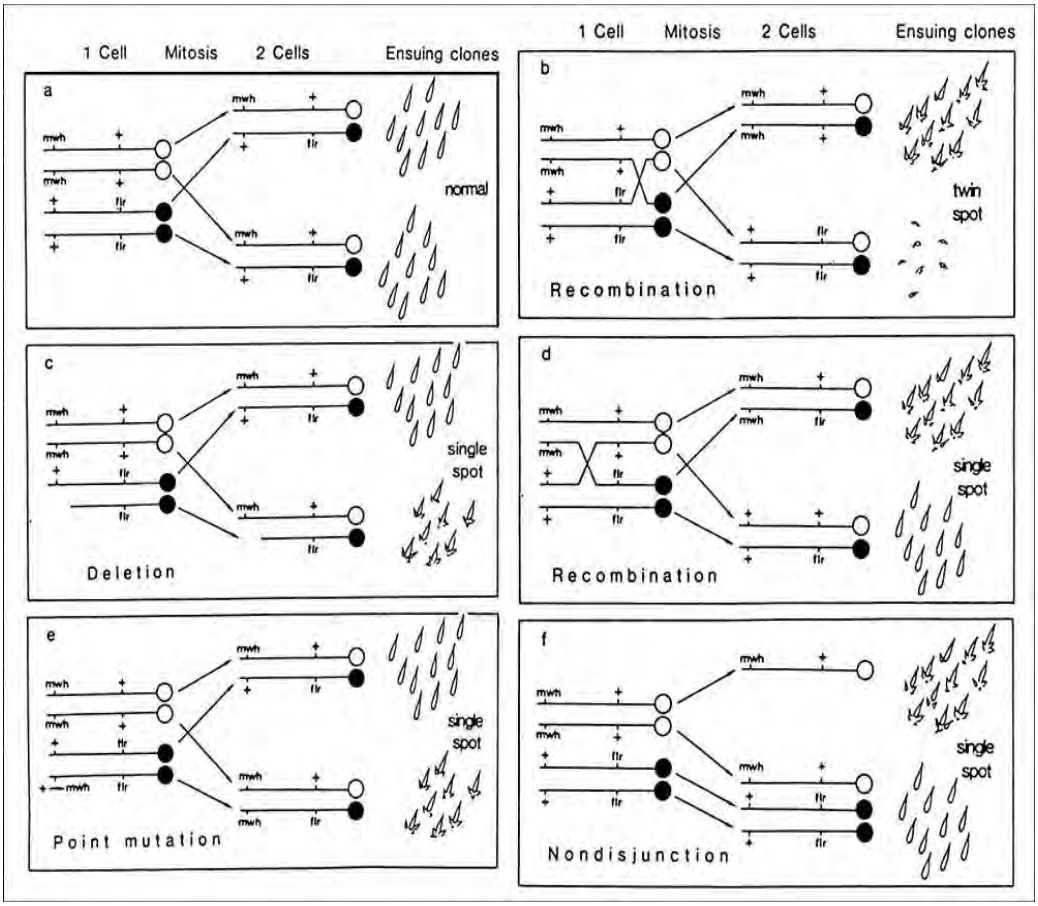


Figure 7. Genetics schemes illustrating various ways of spot formation in the somatic mutation and recombination test with the wing cell markers multiple wing hairs (*mwh*) and flare (*flr*^{3})(a). Twin spots are obtained by recombination proximal to the *flr*^{3} marker (b), while more distal recombination 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 (Graf *et al.*, 1984).

2.6.2 Standard Mutagen for SMART (Urethane)

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, ether, glycerol, chloroform and ethyl ether. The major source of human exposure to urethane is from fermented food products (bread, yogurt and cheese) and alcoholic beverages (white wine and beer) (IARC, 1974; Ough, 1976; Miller and Miller, 1983; Canas *et al*., 1989). Urethane is used as both an animal anesthetic (Kotanidou *et al*., 1996; Norlen *et al*., 2000) and an industrial chemical (Crout, 1976). Furthermore, it was shown to induce genotoxicity in *Drosophila melanogaster* (Zimmerli and Schlatter, 1991). Urethane is generally used as positive standard toxicants in evaluation genotoxicity of the unknown compounds in SMART (Abraham and Graf, 1996).

2.6.2.1 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 Luitz, 1990). This chemical requires metabolic activation to express its mutagenic activity (Frölich and Würgler, 1990). Urethane is metabolized by at least three pathways (Salmon *et al*., 1991; Park *et al*., 1993) as shown in Figure 8. In rodents, more than 90 % of an administered dose of urethane is hydrolyzed to ethanol, ammonia and carbon dioxide by liver microsomal esterases and amidase (Mirvish, 1968; Park *et al*., 1993). This pathway is probably one for detoxification (IARC, 1974). Approximately 0.1% of urethane is reversibly converted by cytochrome P450 subtype 2E1 (CYP2E1) to 2-hydroxyethyl carbamate (Guengerich and Kim, 1991a), a compound that is inactive as a carcinogen (Berenblum *et al*., 1959), to *N*-hydroxyethyl carbamate (Boyland and Nery, 1965; Nery, 1968), a compound that is less carcinogenic than urethane (Mirvish, 1968). Less than 0.5% of urethane is metabolized by CYP2E1 to vinyl carbamate and the metabolite is more potent than its parent compound in its carcinogenicity. Vinyl carbamate, in turn, is converted by epoxidation to the putative ultimate carcinogen vinyl carbamate epoxide (Miller and Miller, 1983; Guengerich and Kim, 1991a; Guengerich *et al*., 1991b). Vinyl carbamate epoxide can covalently bind to DNA, RNA and proteins to form adducts and the initiation of tumorigenesis (Dahl *et al*., 1978; Miller and Miller, 1983; Leithauser *et al*., 1990). The schematic structures of urethane and its metabolites are shown in Figure 9.

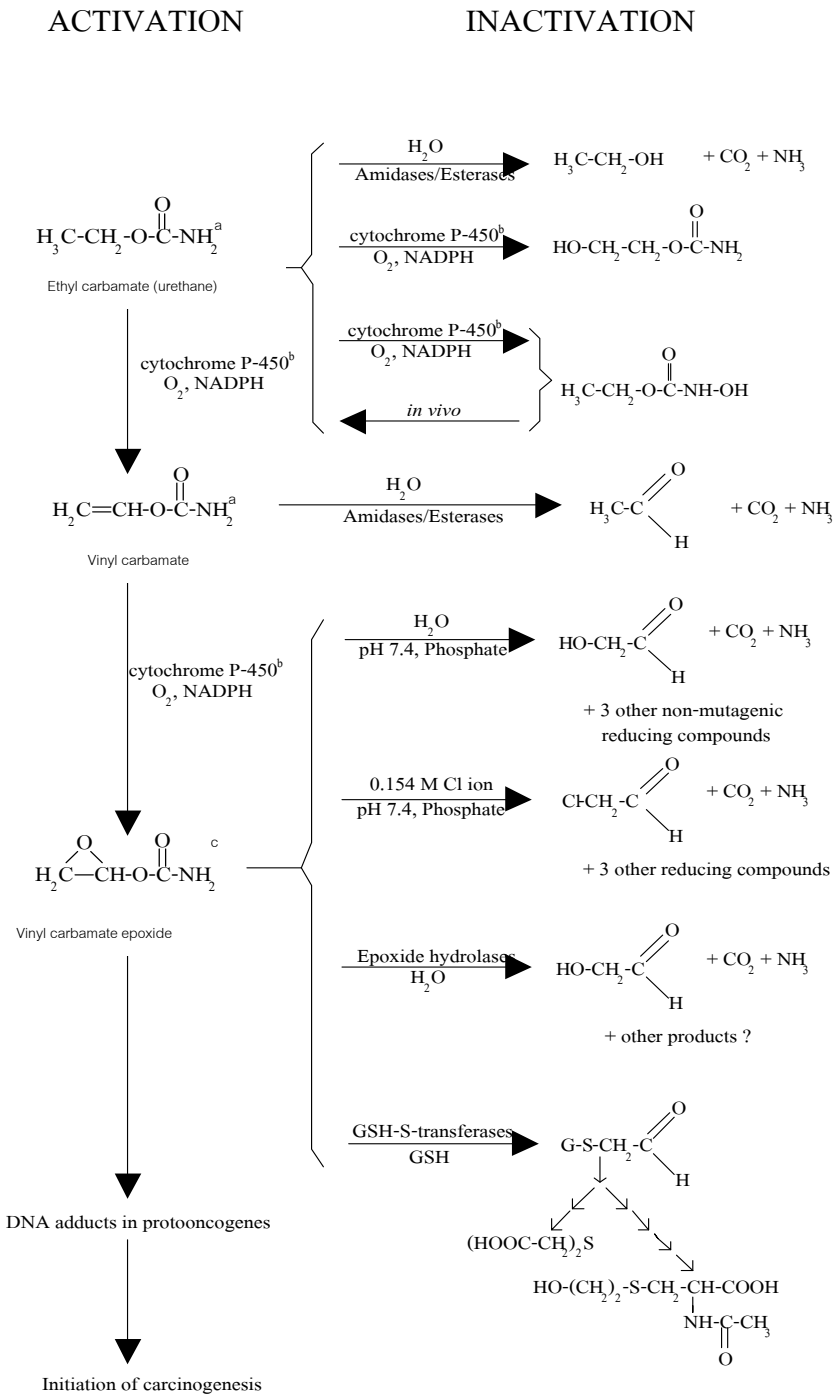


Figure 8. Known and probable activation and inactivation pathways of metabolism of urethane (ethyl carbamate), vinyl carbamate and vinyl carbamate epoxide, (a) Mouse liver microsomes + ethyl carbamate or vinyl carbamate + adenosine 1, N^6-ethenoadenosine, (b) Human liver microsomal cytochrome P450 IIE1, (c) Vinyl carbamate epoxide + adenosine 1, N^6-ethenoadenosine. GSH = glutathione (Park *et al.*, 1993).

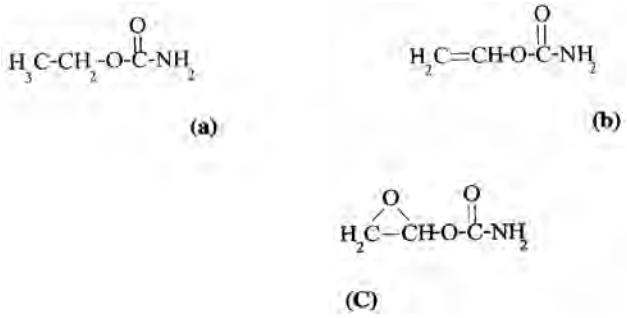


Figure 9. Schematic structures of urethane and its metabolites (a) Urethane (ethyl carbamate); (b) Vinyl carbamate; (c) Vinyl carbamate epoxide (Park *et al*., 1993).

CHAPTER III

MATERIALS AND METHODS

3.1 Sample Preparation

Legume seeds from black bean [*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griffith], red kidney bean (*Phaseolus vulgaris* L.), mung bean [*Vigna radiata* (L.) Wilzcek], peanut [*Arachis hypogaea* L.] and soybean [*Glycine max* (L.) Merr.] were purchased from a supermarket in Bangkok. They were washed and dried at room temperature. The extraction steps of legume seed are shown in Figure 10. Each sample was divided into 2 portions. The first portion was used whereas the second portion was removed the seed coats. For the seed coats removal, legume seeds were soaked in distilled water for 3 h, after that water was drained and the seed coats were removed manually with the aid of a knife.and dried at room temperature. The legume seeds and seed coats were divided into 2 groups; the first group was used as raw samples whereas the second group was processed by autoclaving at 121 ^{o}C for 20 min, and then dried at 60 ^{o}C for 24 h (processed samples). All samples were ground to fine powder using a blender.

For extraction, solvents with increasing polarity were used to increase the effectiveness of the solvents on the extraction of phenolic compounds. Each sample was extracted in two steps. The first step was extracted with 70% acetone followed by a second extraction with 50% methanol. In details, 20 grams of the legume seeds powder (raw legume seeds or processed legume seeds) were firstly extracted with 200 ml of 70% acetone for 24 h at room temperature and then filtered through Whatman No.1 filter paper. The solid residue was re-extracted with 200 ml of 50% methanol, and then followed by a filtration. For seed coats, two grams of the seed coat powder (raw or processed seed coats) were extracted with 40 ml of 70% acetone for 24 h at room temperature and then filtered. The residue was extracted with 40 ml of 50% methanol followed by filtration. The filtered extracts from legume seeds and seed coats were then concentrated in a vacuum evaporator at 40^{o}C. The concentrated extracts were kept in amber bottle and stored in a freezer at -20^{o}C. The concentrated extracts were redissolved in 10% dimethyl sulfoxide (DMSO) to obtain the appropriate concentration for each assay.

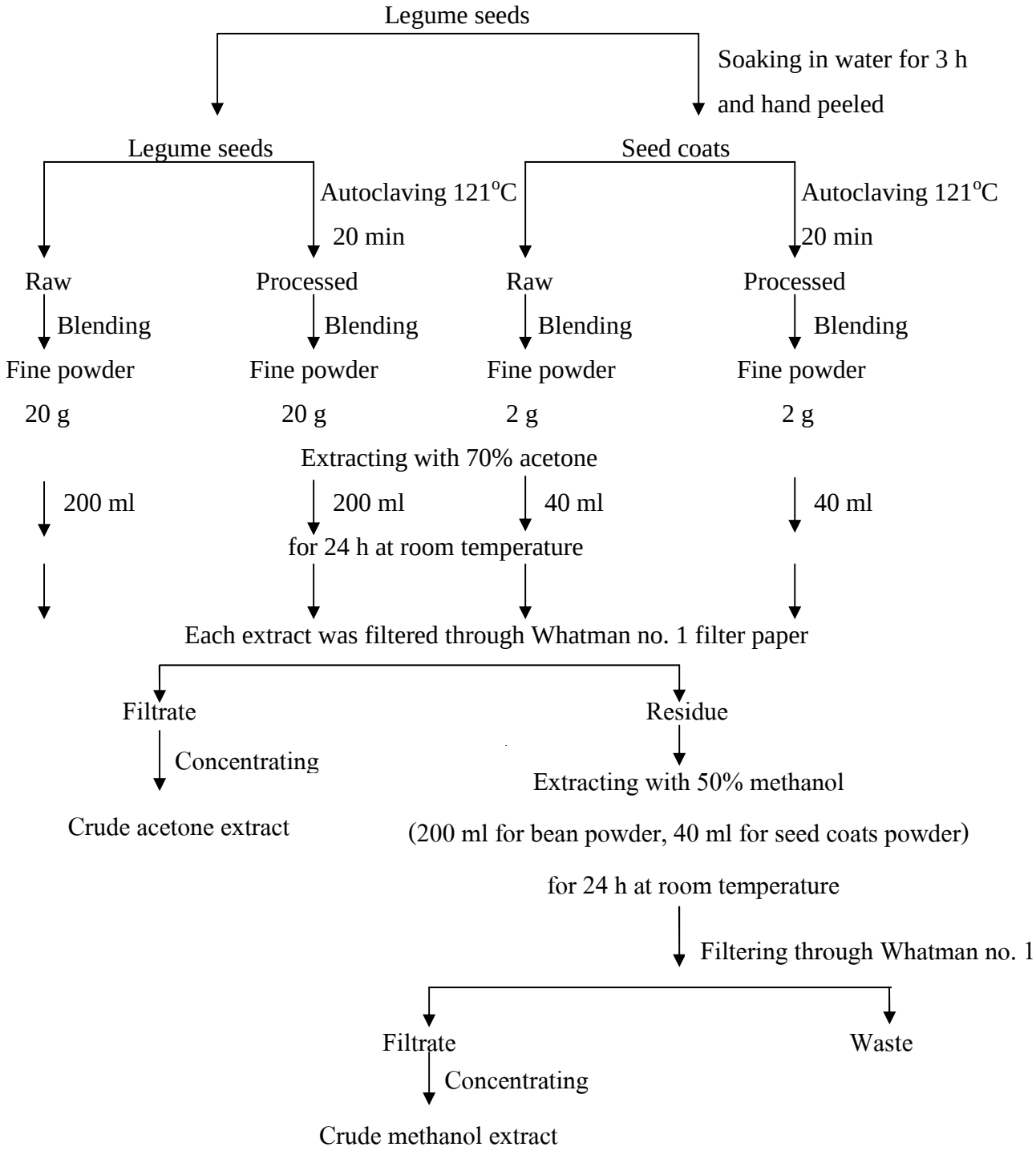


Figure 10. The steps of sample preparation and extraction

3.2 Experimental Design

Overall investigations of biological activities (antioxidant activity and antigenotoxicity) of the extracts of legume seeds and the extracts of seed coats are shown in Figure 11. The antigenotoxicity was evaluated using comet assay (Singh *et al.*, 1988) and somatic mutation and recombination test (SMART) (Graf *et al*, 1984). In addition, antioxidant activity assays namely DPPH assay (Flumoto and Mazza, 2000) and FRAP assay (Griffin and Bhagooli, 2004) were performed. The total polyphenolic content (Amarowicz *et al*., 2004) and total flavonoid content (Jia *et al.*, 1999) were measured using colorimetric assay.

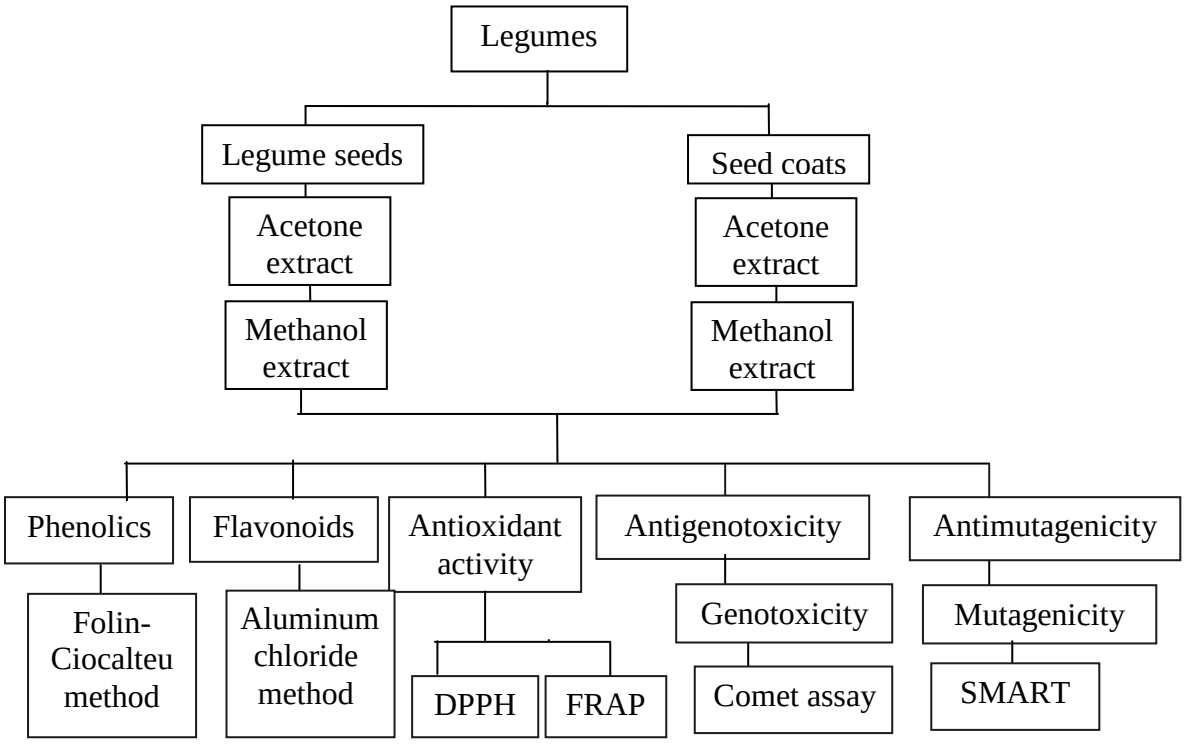


Figure 11. Overall investigations of biological activities (antioxidant activity, antigenotoxicity and antimutagenicity) of the legume extracts

3.3 Antioxidant Activity Assay

3.3.1 Chemicals

TPTZ (2, 4, 6-tripyridyl-s-triazine), ferric chloride hexahydrate, and ferrous sulfate heptahydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylether, Potassium hydroxide, and Sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Propionic acid, DPPH (2, 2′ - diphenyl-1-picrylhydrazyl), Gallic acid and Folin-Ciocalteu reagent were purchased from Fluka Chemika (Buchs, Switzerland). Trolox was purchased from Aldrich Chemical (Milwaukee, WI, Germany). Glacial acetic acid was obtained 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.

3.3.2 Phenolic contents

3.3.2.1 Determination of Total Phenolic Contents

Total phenolic contents of the extracts were determined according to the method described by Amarowicz *et al*. (2004), Swain and Hillis (1959), Naczk and Shahidi (1989). Briefly, 10 µl of each extract was transferred into a 96-well flat bottom microplate (Bibby Sterilin Ltd, UK) containing 160 µl of distilled water. After mixing, 10 µl of Folin–Ciocalteu reagent and 20 µl of a saturated sodium carbonate solution were added. The solution was mixed well and the absorbance was measured at 750 nm after 30 min incubation using microplate reader (Sunrise, Tecan Co., Austria). The readings of extract and reagent blanks were subtracted from the reading of reagent with extract. The total phenolic content was calculated from a calibration curve of gallic acid solutions (ranging from 25 to 800 mg/l), and were expressed as milligram of gallic acid equivalents (GAE) per gram of the extract. All measurements were done in triplicate.

3.3.2.2 Determination of Total Flavonoid Contents

Total flavonoid contents in the extracts were determined using a colorimetric method described by Jia *et al.* (1999) with some modifications. The extract (250 µl) was mixed with 1.25 ml of distilled water and 75 µl of 5% $NaNO_2$ solution in a test tube. After 5 min, 150 µl of 10% $AlCl_3.H_2O$ solution was added and incubated for 6 min. Then, 500 µl of 1 M NaOH and 275 µl of distilled water were added to prepare the mixture. The solution

was mixed well and the absorbance was read at 510 nm. Catechin was used to calculate the standard curve (0.25 –2.5 mM) and the results were expressed as milligram of catechin equivalents (CE) per gram of the extract.

3.3.3 Scavenging of 2, 2′-Diphenyl-1-Picrylhydrazyl Radical (DPPH Assay)

The antioxidant activity of the extract on stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was estimated using the procedure described by Fukumoto and Mazza (2000) with some modifications. The extract was allowed to react with DPPH in order to evaluate the free radical scavenging activity. The activity was monitored by a decrease in an absorbance at 520 nm. An aliquot of 22 µl of the extract or blank reagent (10% DMSO) or standard Trolox (0.04- 1.28 mM in 80% methanol) was added to 200 µl of DPPH in 80% methanol (150 µM) in a 96-well microplate. After incubation at 37^{o}C for 30 min, the absorbance of the solution was read using a microplate reader with a 520 nm filter. The radical scavenging activity was calculated as a percentage of DPPH scavenging activity using the equation (Amarowicz *et al*., 2004):

DPPH scavenging activity (%) = $100 \times [1- (A_E/ A_D)]$

where A_E is the absorbance of the solution containing DPPH and the extract, and A_D is the absorbance of only the DPPH solution.

3.3.4 Ferric Reducing Antioxidant Power (FRAP) Assay

20 µl of each extract or of standard (ferrous sulfate) or of the blank reagent (10% DMSO) was added to each well of a 96-well microplate and run in triplicate. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyls-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio prior to use and heated to 37 ^{O}C in water bath according to the procedure described by Griffin and Bhagooli (2004). The 150 µl of FRAP reagent was added to each well. The change in absorbance from the initial blank was performed after 8 min of incubation using a microplate reader and compared to that of a standard solution. Aqueous solution of known Fe^{2+} concentration (ferrous sulfate 62.5, 125, 250, 500, 1000 µM) was used for calibration. The FRAP values of the extracts were determined using this calibration curve, expressed as µM of ferric reduced per gram of extract. The antioxidant activity was measured by its ability to reduce the Fe^{3+}-TPTZ complex by forming Fe^{2+}-TPTZ and could be monitored by measuring the formation of Perl's Prussian blue at 600 nm.

3.3.5 Statistical Analysis

Results were expressed as mean ± standard deviation. Statistical significance of phenolic content, flavonoid content and antioxidant activity in the extracts of raw samples and processed samples were analysed using student's *t*- test. Differences were considered as a significant value at $P< 0.05$.

3.4 Antigenotoxicity Study

3.4.1 Single Cell Gel Electrophoresis (Comet Assay)

3.4.1.1 Chemicals

Low-melting-point agarose, sodium chloride, EDTA-2Na, trichloroacetic acid, acetic acid, triton X-100, tungstosilicic acid and sodium carbonate were purchased from Merck (Darmstadt, Germany). Tris-HCl, ammonium nitrate, zinc sulphate were purchased from Fluka Chemika (Buchs, Switzerland). Normal melting agarose was purchased from Invitrogen (Aukland, New Zealand). Silver nitrite and formaldehyde were purchased from BDH Chemical Ltd. (Poole, England).

3.4.1.2 Lymphocyte Isolation

Human peripheral blood (7 ml) was obtained from three healthy non-smoking donors (member of the Institute of nutrition, Mahidol University) by venipuncture into heparin-containing vacutainers (BD, Franklin Lakes, NJ). Briefly, blood was diluted 1:1 with phosphate buffer saline (PBS) and carefully layered on the top of lymphocyte separation medium (Isoprep) in a centrifugation tube in a ratio of 1:1. After centrifugation for 30 min at 700 × g (25 ^{o}C), the white layer of lymphocytes at the interface between blood plasma and the Isoprep was carefully transferred into a tube containing RPMI 1640 (Roswell Park Memorial Institute). The lymphocytes were washed twice with RPMI 1640 and centrifuged at 700 × g for 10 min (10 ^{o}C). The cell pellet was resuspended in RPMI 1640 containing no fetal bovine serum. Cell number and viability (Trypan blue exclusion) were determined using a Neubauer Improved Haemacytometer before treatments. In the Comet assay, the final concentration of the cells was adjusted to about 5×10^5 lymphocytes/ml by adding RPMI 1640 medium to the cell suspension. The protocol of this study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.4.1.3 Viability Test

Lymphocyte suspension (800 µl) was mixed with RPMI 1640 medium (190 µl) and different concentrations of the extracts (10 µl) for 30 min at 37^{o}C together with untreated control. The suspension was centrifuged at 800 x g, the lymphocytes were resuspended in RPMI 1640 medium. The cell count was performed on the single cell suspensions according to the cell viability assay (Williams *et al.*, 2004): Trypan blue exclusion procedure. In details, trypan blue solution (10 µl) was added to 10 µl cell suspension in a microcentrifuge tube. The solution was mixed thoroughly and allowed to incubate for 5 min. The 10 µl of solution was loaded onto the hemacytometer by capillary action. Viability results were acquired by cell counts of live and dead cells performed in four squares and averaged. The cell concentration (cell/ml) was calculated (average count per square × dilution factor (2) × conversion factor (10^4)).

3.4.1.4 Comet Assay Procedure

DNA damage in human lymphocytes was analyzed by Comet assay according to Singh *et al.* (1988) with minor modification. The non-cytotoxic concentrations of the extracts (0.2, 1 and 5 µg/ml of legume seed extract or 0.04, 0.2 and 1 µg/ml of seed coat extract) or phosphate buffer saline (PBS) pH 7.4 (10 µl, duplication) were introduced into microcentrifuge tubes; 190 µl of RPMI 1640 medium and 800 µl of lymphocyte suspension were added and incubated at 37 ^{o}C for 30 min in a dark incubator together with untreated control.

To evaluate the protective effect of the extract on H_2O_2 induced DNA damage, 10 µl of the different concentrations of the extract or PBS pH 7.4 were introduced into microcentrifuge tubes; 170 µl of RPMI 1640 medium and 800 µl of lymphocyte suspension were added and incubated at 37 ^{o}C for 25 min in a dark place. The suspension was then treated with 1 mM H_2O_2 (20 µl) for 5 min on ice.

Finally, the lymphocytes were centrifuged at 800x g for 3 min at 4 ^{o}C and used for viability test and in Comet assay. The lymphocytes were resuspended in 100 µl of 0.8% low-melting-point agarose (LMA). This lymphocyte suspension (30 µl) was spread onto a slide that had previously been coated with 1.0% normal melting point agarose (NMA). The slide was covered with a cover-slip and left on ice for 5 min to allow the agarose to solidify. After removing cover-slip, the slide was immersed in freshly prepared cold (4 ^{o}C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris–HCl, pH 10–10.5; 1%

Triton X-100 and 10% DMSO) for at least 1 h. Then, the slide was immersed in freshly prepared alkaline electrophoresis buffer (0.3 M NaOH and 1 mM Na_2ETDA, pH > 13) at 4 ◦C for unwinding (40 min) and then electrophoresed (26 V/ 300 mA, 20 min). The slide was neutralized with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with silver staining. Briefly, the staining protocol was performed as suggested by Garcia *et al*. (2007). Slides were washed twice with distilled water, dried overnight at 37 ^{o}C. Slides were then fixed for 10 min in fixation solution (15% trichloroacetic acid, 5% zinc sulphate heptahydrate, 5% glycerol), washed twice with distilled water, and dried 2.5–3 h at 37 ^{o}C. The dry slides were re-hydrated for 5 min in distilled water, placed back-to-back in a horizontal staining jar, and then stained for 20 min at room temperature (25 ^{o}C), using 100 ml of freshly prepared stain solution comprising 34 ml of vigorously mixed stock solution B (0.05% ammonium nitrate, 0.05% silver nitrate, 0.125% tungstosilicic acid, 0.075% formaldehyde, v/v) and 66 ml of stock solution A (5% sodium carbonate) prepared the same day as the staining. The stained slides were washed 2–3 times with distilled water, and immersed 5 min in a stop solution (acetic acid 1%), washed again, and air-dried. The slides were analyzed using light microscope (Zeiss, KF2). All the above steps were carried out in a yellow light environment to prevent any additional DNA damage.

3.4.1.5 Data evaluation

The evaluation of DNA damage was carried out by the visual score (Noroozi *et al*., 1998). The slides were observed using a microscope. The migration of DNA fragments was determined on 100 randomly selected cells (50 cells from each of duplicate slides) per sample. All experiments were performed two times. Each cell was assigned a score on an arbitrary score of 0– 4 based on perceived Comet tail length migration and relative proportion of DNA in the Comet tail (Figure 12): (a) Class 0, intact nuclei or without tail; (b) Class 1, nuclei with tail less than the diameter of the nucleus; (c) Class 2, tail size varying between one and two times the diameter of the nucleus; (d) Class 3, tail size more than two times the diameter of the nucleus but with the head and tail of the Comet still distinguishable; (e) Class 4, almost all the DNA is in the tail. The total arbitrary unit score for the 100 cells could range from 0 (all cells undamaged: 0 x 100) to 400 (all cells highly damaged: 4 x 100).

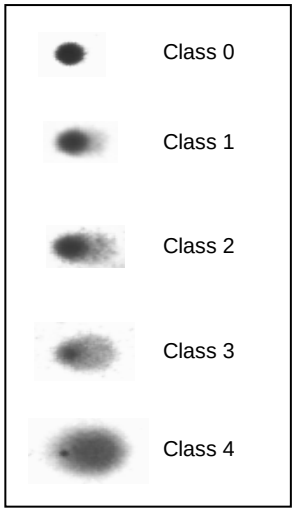


Figure 12. Images of silver stained Comet with various degrees of 5- point scale DNA damage (range: Class 0– 4)

The percentage of inhibition (protective effect of DNA damage) was calculated as following:

$$\text{Percentage of inhibition} = (a - b)/a \times 100$$

Where *a* is the arbitrary unit score induced by H_2O_2 alone and *b* the arbitrary unit score induced by H_2O_2 in the presence of the extract. It is proposed that percentage of inhibition between 20- 40 represented weak inhibition while expression of percent inhibition between 40- 60 and more than 60 are the evidences of moderate and strong inhibition, respectively.

3.4.2 Somatic Mutation and Recombination Test (SMART)

3.4.2.1 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). Other chemicals were of laboratory grade.

3.4.2.2 *Drosophila* Medium

Types of media used in this study are shown in Table 3. The regular medium (negative control) was composed of corn flour (125 g), sugar (100 g), Baker's yeast (50 g) and agar (14 g). The ingredients were mixed and boiled in a beaker containing water 1000

ml until it became sticky as suggested by Robert (1986). Propionic acid was added (5 ml) as a preservative. This medium was used for maintaining the stock of fly culture larvae as well as in preparing larvae for each experiment.

The positive control medium was prepared by substituting 1 ml of 40 mM urethane solution for 1 ml of distilled water in the regular medium. The sample control medium was prepared by substituting 1 ml of the extract of legume seeds or the extract of seed coats (6.25, 12.5, 25 mg/ml) for 1 ml of distilled water in the regular medium. The experimental medium contained each concentration of the extract (1 ml) (describe below) and 40 mM urethane (1 ml); it was used for antimutagenic activity testing of each extract.

Table 3. Composition of media

Component of medium	Regular medium or Negative control	Positive control medium	Experimental medium	Sample control medium
Corn flour	0.25 g	0.25 g	0.25 g	0.25 g
Sugar	0.20 g	0.20 g	0.20 g	0.20 g
Baker's yeast	0.10 g	0.10 g	0.10 g	0.10 g
Agar	0.03 g	0.03 g	0.03 g	0.03 g
Water	**2 ml**	**1 ml**	-	**1 ml**
Urethane(40 mM)	-	**1 ml**	**1 ml**	-
The extract (6.25, 12.5, 25 mg/ml)	-	-	**1 ml**	**1 ml**

3.4.2.3 Survival Study

Virgin *ORR;flr*^{3} females and *mwh* males were mated to obtain 3 days old larvae of improved high bioactivation cross (IHB) on the regular medium. Three days later, a batch of 100 trans-heterozygous (*mwh*+/ +*flr*^{3}) larvae was washed with water and transferred (with the help of a fine artist's brush) to each the sample medium. The larvae were maintained at 25 $\pm$ 1 °C until pupation. After metamorphosis (between days 10- 12 after egg laying), number of surviving adult flies from larvae fed on each medium was recorded and the flies were stored in 70 % ethanol as described by Graf *et al*. (1989).

3.4.2.4 Antimutagenicity Study

The Virgin *ORR; flr*^{3} females and *mwh* males were mated on the regular medium. Six days after mating, 100 of 3-day-old larvae (72 h) were transferred in equal batches to the experimental medium, regular medium (negative control) and positive control medium. The surviving adult flies were collected on days 7- 10 after pupation. Only the insects bearing the marker trans-heterozygous (*mwh*+/+*flr*^{3}) indicated with round wings were stored in 70 % ethanol. Subsequently, the wings were separated from the body using a shape knife and they were lined up on a clean slide. A droplet of Faure's solution as suggested by Graf *et al* (1984) was dropped on the slide then covered by a cover slip. The wings were analyzed under a compound microscope at 400x magnification for the presence of clones of cells showing malformed wing hairs.

3.4.2.5 Data Evaluation

The position of the spots was noted according to the sector of the wing (Figure 13). Different type of spots namely, single spots found either on the multiply wing hairs (*mwh*) or the flare (*flr*^{3}) phenotypes, and twin spots found on 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 wild-type cell rows. Multiply wing hairs (*mwh*) were classified when a wing cell contained three or more hairs instead of one hair per cell as shown in wild-type. Flare wing hairs (*flr*^{3}) 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 5.

The wing spots data was evaluated using the statistical procedure described by Frei and Wurgler (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 as indicated in Figure 14: (1) small single spots of 1 or 2 cells in size, (2) large single spots of 3 or more cells, and (3) twin spots. The estimation of spot frequencies and confidence limits due to mutation 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.

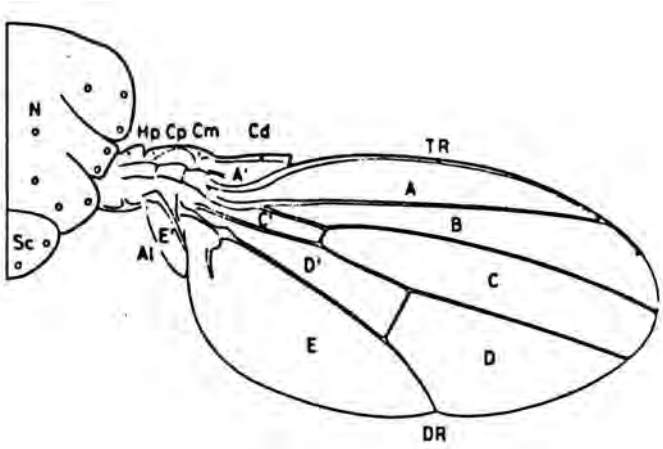


Figure 13. Normal half mesothorax showing the regions A-E of the wing surface scored for spots according to Graf *et al*. (1984).

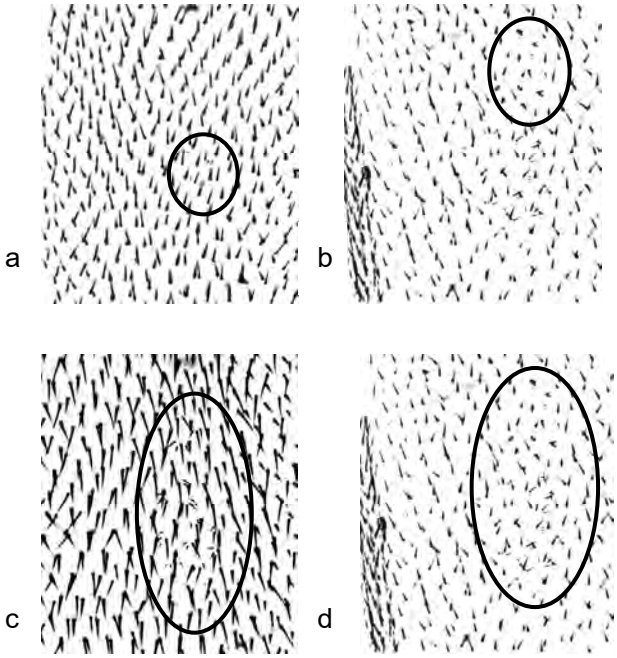


Figure 14. Difference types of wing hair mutation, a) small single spots of *mwh* on wing, b) large single spots of flare on wing, c) large single spots of *mwh* on wing, d) twin spots

The percentage of modification (inhibition or induction) was calculated (Abraham, 1994) as following:

$$\text{Percentage of modification (inhibition or induction)} = (a - b)/a \times 100$$

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

CHAPTER IV

RESULTS

Five legumes namely, black bean [*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griffith], red kidney bean (*Phaseolus vulgaris* L.), mung bean [*Vigna radiata* (L.) Wilzcek], peanut (*Arachis hypogaea* L.) and soybean [*Glycine max* (L.) Merr.] have been used as food or beverages in daily diet. Many leguminous phytochemicals like anthocyanins, lecithin, and trypsin inhibitors have been suggested to have protective and therapeutic effects against cancer, being potentially useful for dietary chemopreventive strategies (Wang and Murphy, 1994; Lazzé *et al*., 2003). In this investigation, legumes were extracted with acetone and methanol; then, they were analysed for their antioxidant activity, genotoxic and antigenotoxic effects.

The percentage yields of dried extracted legume are shown in Table 4. Almost of all legumes extracts, the first extraction with acetone resulted in higher yields than the second extraction with methanol.

Table 4. Percentage yields of dried extracted legume seeds and seed coats

Legumes	Percentage yield (%)							
	Legume seeds				Seed coats			
	Raw		Processed		Raw		Processed	
	Acetone	MeOH	Acetone	MeOH	Acetone	MeOH	Acetone	MeOH
Black bean	8.95	5.98	6.45	7.35	16.96	3.83	9.00	7.25
Mung bean	8.05	8.37	5.86	7.31	9.75	4.20	15.25	4.00
Peanut	6.69	3.13	8.24	2.85	13.35	4.28	21.00	8.50
Red kidney bean	8.66	6.60	7.77	7.42	12.63	2.93	9.25	7.75
Soybean	10.08	5.25	8.84	7.55	11.96	9.16	9.75	6.00

4.1 Total Phenolic and Total Flavonoid Contents

Total phenolic and total flavonoid contents of extracted legume seeds are shown in Tables 5 and 6, respectively. The total phenolic content of each legume seed extract was determined from the regression equation of calibration curve ($y = 0.0011x + 0.0756$) and expressed as gallic acid equivalents (mg GAE/g dry extract). As a result, most of phenolic compounds were collected after the first extraction with 70% acetone (Table 5). Percentage of total phenolic content found within the first extraction was 55.1-80.3% in raw legume seeds and 74.0-86.9% in processed legume seeds.

In this study, raw red kidney bean exhibited the highest total phenolic content (128.5 ± 11.7 mg GAE/g dry extract). After heat treatment, the processed legume seeds extracted from mung bean and red kidney bean were shown to have lower levels of total phenolic content than that of the raw extracts (Figure 15). In contrast, extracts of processed legume seeds from black bean and peanut displayed higher level of total phenolic content than that of the raw samples (Figure 15). The processed legume seeds of peanut displayed the highest phenolic content (131.2 ± 3.5 mg GAE/g dry extract) whereas the processed soybean seed showed the lowest phenolic content (40.3 ± 5.4 mg GAE/g dry extract).

Table 5. Effect of heat treatment on total phenolic content of legume seed extracts and percentage of total phenolic content of legume seeds found within the first and the second extraction

Legume seeds	Phenolic content (mg GAE/g dry extract)[1]	
	Raw	Processed
First extraction with 70% acetone		
Black bean	53.4 ± 14.1 [a] (67.9%)	87.6 ± 2.1 [b] (81.6%)
Mung bean	87.1 ± 2.1 [a] (80.2%)	65.9 ± 3.3 [b] (79.2%)
Peanut	66.2 ± 2.5 [a] (64.7%)	97.1 ± 0.8 [b] (74.0%)
Red kidney bean	103.2 ± 6.2 [a] (80.3%)	58.5 ± 5.0 [b] (76.0%)
Soybean	28.8 ± 1.4 [a] (55.1%)	35.0 ± 2.1 [a] (86.9%)
Second extraction with 50% methanol		
Black bean	25.3 ± 1.2 [a] (32.1%)	19.7 ± 1.2 [b] (18.4%)
Mung bean	21.5 ± 2.5 [a] (19.8%)	17.3 ± 0.8 [a] (20.8%)
Peanut	36.2 ± 0.8 [a] (35.3%)	34.1 ± 1.7 [a] (26.0%)
Red kidney bean	25.3 ± 2.1 [a] (19.7%)	18.5 ± 0.0 [b] (24.0%)
Soybean	23.5 ± 2.2 [a] (44.9%)	5.3 ± 1.7 [b] (13.1%)
Total [2]		
Black bean	78.7 ± 21.6 [a] (100%)	107.3 ± 4.7 [b] (100%)
Mung bean	108.6 ± 6.5 [a] (100%)	83.2 ± 5.8 [b] (100%)
Peanut	102.4 ± 4.7 [a] (100%)	131.2 ± 3.5 [b] (100%)
Red kidney bean	128.5 ± 11.7 [a] (100%)	77.0 ± 7.1 [b] (100%)
Soybean	52.3 ± 5.1 [a] (100%)	40.3 ± 5.4 [a] (100%)

Values are presented as mean ± S.D. of triplicate determinations. Values in the same rows followed by different letters (a, b) are significantly different at $P < 0.05$.

[1] Total phenolic contents based a standard curve generated by 25-800 mg/l of gallic acid ($y = 0.0011x + 0.0756$; $R^2 = 0.991$)

[2] Total values are sum of average measurement for acetone and methanol extracts.

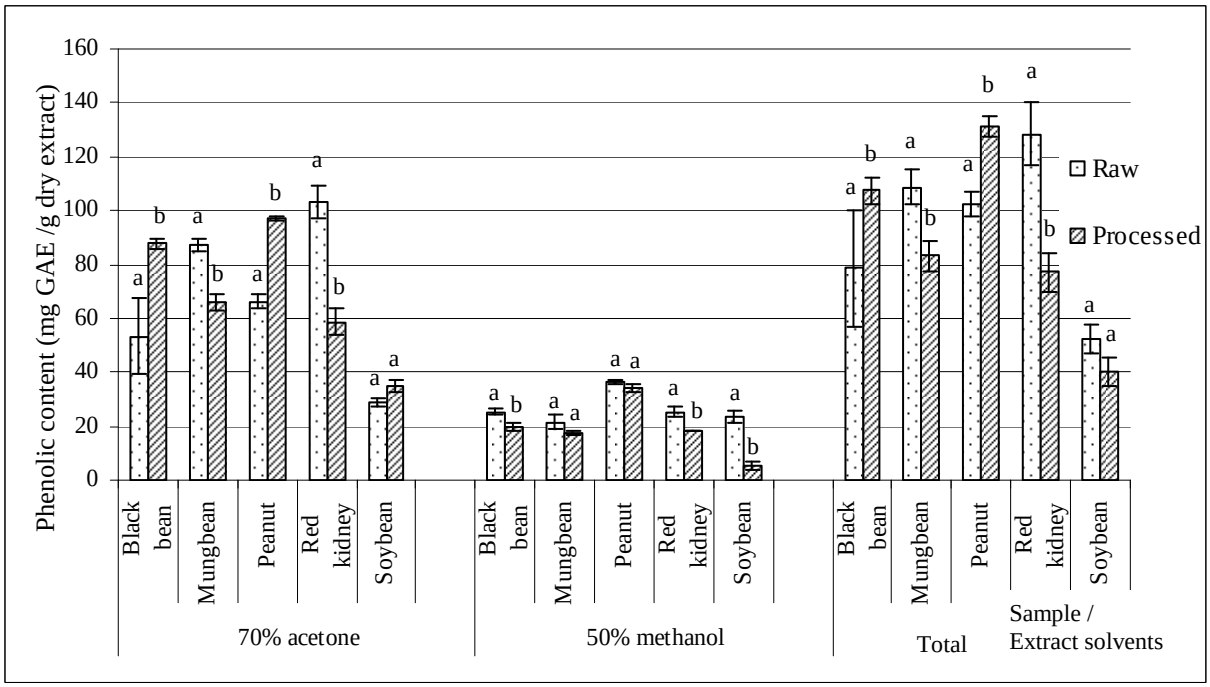


Figure 15. Effect of heat treatment on total phenolic content of the legume seed extracts. Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Total values are sum of average measurement for acetone and methanol extracts. Different letters (a, b) show significantly difference at $P< 0.05$.

The flavonoid content of the legume seed extracts of was determined from the linear regression equation of the standard curve, catechin ($y = 0.0037x - 0.0034$) that was expressed as catechin equivalents (mg CE/g dry extract). Table 6 shows that most of the flavonoid compounds were detected after the first extraction with 70% acetone.

The total flavonoid contents of the raw legume seed extracts contained about 4 – 37 mg CE/g dry extract and could be put in order as black bean> red kidney bean> mung bean> peanut> soybean. After heat treatment, only legume seed extract of peanut showed to increase level of total flavonoid content (Figure 16).

Table 6. Effect of heat treatment on total flavonoid content of legume seed extracts and percentage of total flavonoid content of legume seeds found within the first and the second extraction

Legume seeds	Flavonoid content (mg CE/g dry extract) [1]	
	Raw	Processed
First extraction with 70% acetone		
Black bean	35.0 ± 0.7 [a] (93.2%)	33.2 ± 0.2 [a] (95.1%)
Mung bean	20.4 ± 0.4 [a] (91.1%)	17.8 ± 0.6 [b] (91.8%)
Peanut	14.8 ± 0.2 [a] (86.7%)	23.2 ± 0.6 [b] (95.7%)
Red kidney bean	28.6 ± 0.6 [a] (90.7%)	25.5 ± 0.4 [b] (91.4%)
Soybean	2.4 ± 0.2 [a] (53.0%)	4.3 ± 0.6 [b] (66.8%)
Second extraction with 50% methanol		
Black bean	2.5 ± 0.0 [a] (6.8%)	1.7 ± 0.0 [b] (4.9%)
Mung bean	2.0 ± 0.0 [a] (8.9%)	1.6 ± 0.2 [a] (8.2%)
Peanut	2.3 ± 0.0 [a] (13.3%)	1.0 ± 0.2 [b] (4.3%)
Red kidney bean	2.9 ± 0.2 [a] (9.3%)	2.4 ± 0.2 [a] (8.6%)
Soybean	2.1 ± 0.2 [a] (47.0%)	2.1 ± 0.2 [a] (33.2%)
Total [2]		
Black bean	37.5 ± 1.0 [a] (100%)	34.9 ± 0.3 [a] (100%)
Mung bean	22.4 ± 0.6 [a] (100%)	19.4 ± 1.1 [a] (100%)
Peanut	17.1 ± 0.3 [a] (100%)	24.2 ± 1.1 [b] (100%)
Red kidney bean	31.5 ± 1.1 [a] (100%)	27.9 ± 0.8 [a] (100%)
Soybean	4.5 ± 0.6 [a] (100%)	6.4 ± 1.1 [a] (100%)

Values are presented as mean ± S.D. of triplicate determinations. Values in the same rows followed by different letters (a, b) are significantly different at $P < 0.05$.

[1] Total flavonoid contents based a standard curve generated by 12.5-200 mg/l of catechin ($y = 0.0037x + 0.0034$; $R^2 = 0.9993$)

[2] Total values are sum of average measurement for acetone and methanol extracts.

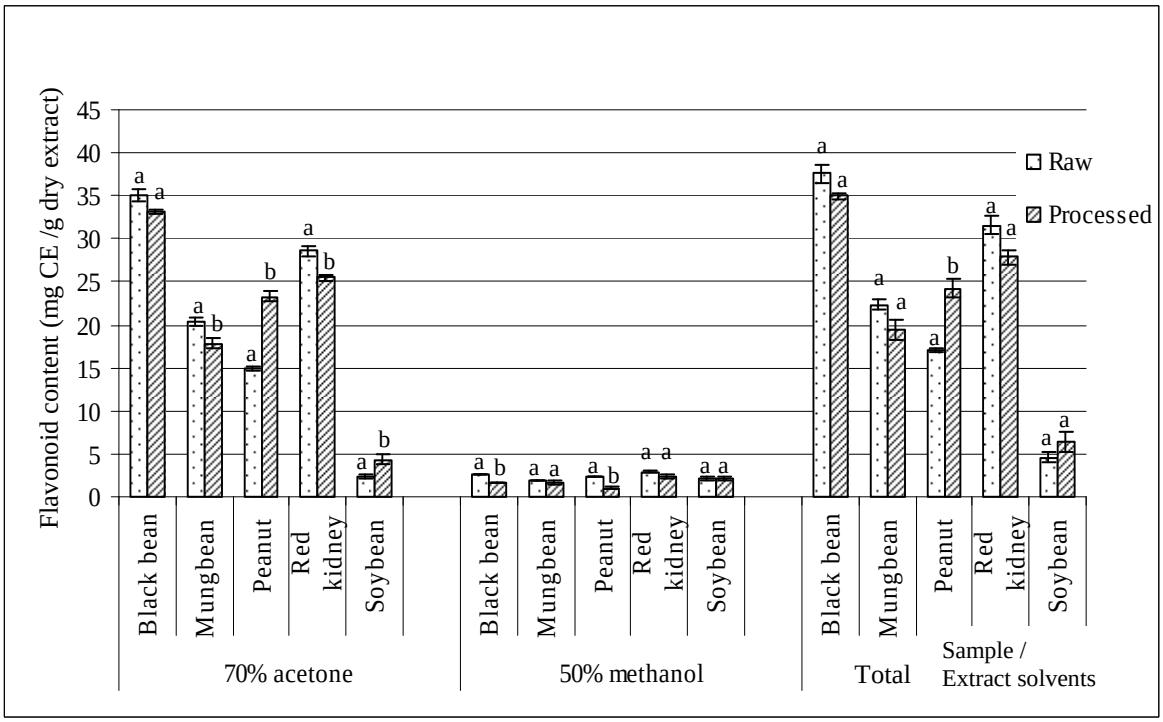


Figure 16. Effect of heat treatment on total flavonoid content of the legume seed extracts. Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Total values are sum of average measurement for acetone and methanol extracts. Different letters (a, b) show significant difference at $P< 0.05$.

In the case of seed coats, the phenolic content was determined from linear regression equation of calibration curve ($y = 0.0016x + 0.0942$). Likewise, most of the phenolic compounds were detected with the first extraction using 70% acetone (Table 7).

According to sample extracts, the raw seed coat of peanut and red kidney bean displayed high content of total phenolics (438.7 ± 34.8 and 429.1 ± 32.0 mg GAE/g dry extract, respectively) (Figure 17). Interestingly, heat treatment showed the increase phenolic content of all tested seed coats. The processed seed coats of black bean, peanut and red kidney bean contained high level of phenolic content (> 450 mg GAE/g). The lowest phenolic content of both raw and processed seed coat was observed in soybean (43.0 ± 2.3 and 52.8 ± 4.1 mg GAE/g dry extract, respectively).

Table 7. Effect of heat treatment on total phenolic content of seed coat extracts and percentage of total phenolic content of seed coats found within the first and the second extraction

Seed coats	Phenolic content (mg GAE/g dry extract)[1]	
	Raw	Processed
First extraction with 70% acetone		
Black bean	212.9 ± 7.9 [a] (66.6%)	289.0 ± 13.6 [b] (59.7%)
Mung bean	128.4 ± 5.9 [a] (62.1%)	158.4 ± 9.8 [b] (57.9%)
Peanut	331.8 ± 15.6 [a] (75.6%)	355.0 ± 9.6 [a] (71.3%)
Red kidney bean	335.9 ± 19.8 [a] (78.3%)	339.0 ± 15.5 [a] (73.4%)
Soybean	32.6 ± 1.2 [a] (75.8%)	41.5 ± 1.0 [b] (78.6%)
Second extraction with 50% methanol		
Black bean	106.6 ± 3.8 [a] (33.4%)	194.9 ± 19.8 [b] (40.3%)
Mung bean	78.2 ± 9.6 [a] (37.9%)	115.1 ± 7.3 [b] (42.1%)
Peanut	106.9 ± 9.0 [a] (24.4%)	143.1 ± 5.8 [b] (28.7%)
Red kidney bean	93.2 ± 2.8 [a] (21.7%)	123.1 ± 10.3 [b] (26.6%)
Soybean	10.4 ± 0.4 [a] (24.2%)	11.3 ± 1.9 [a] (21.4%)
Total [2]		
Black bean	319.5 ± 16.5 [a] (100%)	483.9 ± 47.2 [b] (100%)
Mung bean	206.6 ± 21.9 [a] (100%)	273.5 ± 24.2 [b] (100%)
Peanut	438.7 ± 34.8 [a] (100%)	498.1 ± 21.8 [a] (100%)
Red kidney bean	429.1 ± 32.0 [a] (100%)	462.1 ± 36.5 [a] (100%)
Soybean	43.0 ± 2.3 [a] (100%)	52.8 ± 4.1 [a] (100%)

Values are presented as mean ± S.D. of triplicate determinations. Values in the same rows followed by different letters (a, b) are significantly different at $P < 0.05$.

[1] Total phenol contents based a standard curve generated by 25-800 mg/l of gallic acid ($y = 0.0016x + 0.0942$; $R^2 = 0.988$)

[2] Total values are sum of average measurement for acetone and methanol extracts.

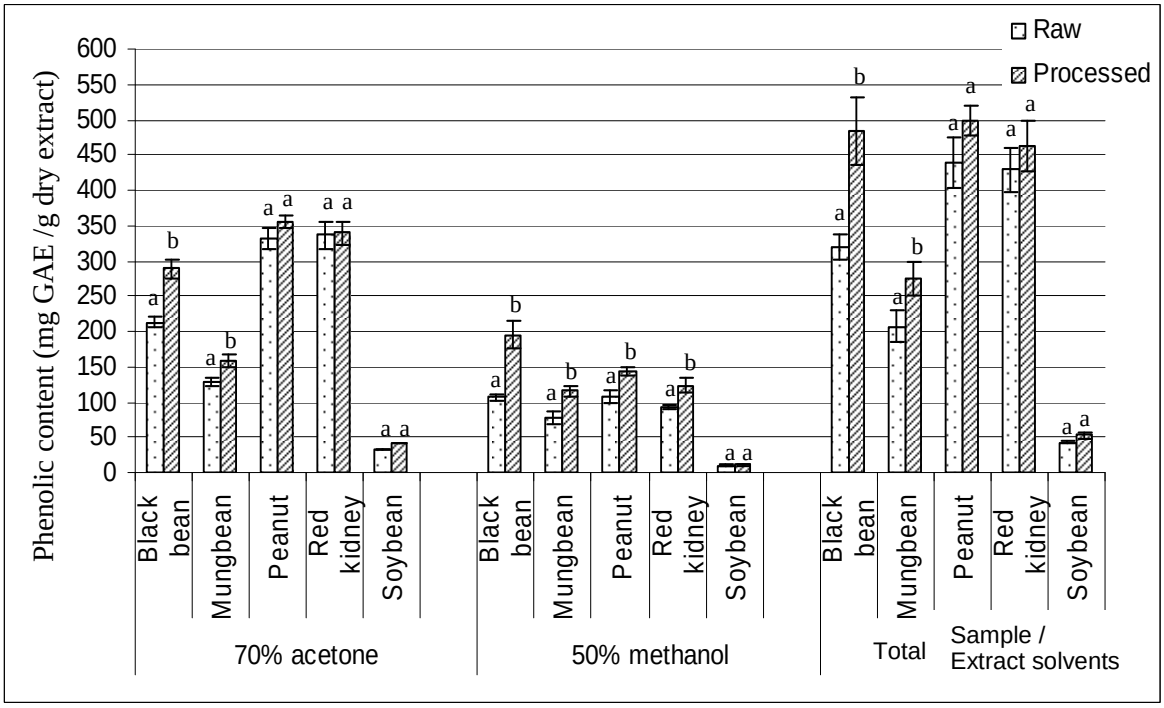


Figure 17. Effect of heat treatment on total phenolic content of the seed coat extracts. Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Total values are sum of average measurement for acetone and methanol extracts. Different letters (a, b) show significant difference at $P< 0.05$.

The total flavonoid content of the seed coat extracts was determined using the regression equation of catechin ($y = 0.0032x + 0.0023$). The raw seed coats of peanut and red kidney bean showed high content of total flavonoids (328.0 ± 12.9 and 328.2 ± 5.7 mg CE/g dry extract, respectively). The lowest flavonoid content was observed in soybean (5.9 ± 1.8 mg CE/g dry extract) (Figure 18). Likewise, heat treatment decreased flavonoid content of all tested seed coat except that of black bean.

Table 8. Effect of heat treatment on total flavonoid content of seed coat extracts and percentage of total flavonoid content of seed coats found within the first and the second extraction

Seed coats	Flavonoid content (mg CE/g dry extract) [1]	
	Raw	Processed
First extraction with 70% acetone		
Black bean	64.6 ± 1.0 [a] (73.5%)	102.4 ± 1.3 [b] (62.1%)
Mung bean	63.5 ± 4.6 [a] (41.4%)	53.5 ± 3.3 [a] (66.1%)
Peanut	234.5 ± 4.9 [a] (71.5%)	130.5 ± 0.9 [b] (71.6%)
Red kidney bean	269.7 ± 2.9 [a] (82.2%)	156.7 ± 6.2 [b] (78.7%)
Soybean	2.7 ± 0.2 [a] (45.8%)	4.9 ± 0.0 [b] (96.1%)
Second extraction with 50% methanol		
Black bean	23.3 ± 2.7 [a] (26.5%)	62.5 ± 3.8 [b] (37.9%)
Mung bean	89.7 ± 4.6 [a] (58.6%)	27.4 ± 1.8 [b] (33.9%)
Peanut	93.5 ± 4.2 [a] (28.5%)	51.7 ± 1.3 [b] (28.4%)
Red kidney bean	58.5 ± 1.1 [a] (17.8%)	42.4 ± 0.4 [b] (21.3%)
Soybean	3.2 ± 1.1 [a] (54.2%)	0.2 ± 0.4 [b] (3.9%)
Total [2]		
Black bean	87.9 ± 5.2 [a] (100%)	164.9 ± 7.2 [b] (100%)
Mung bean	153.2 ± 13.0 [a] (100%)	80.9 ± 7.2 [b] (100%)
Peanut	328.0 ± 12.9 [a] (100%)	182.2 ± 3.1 [b] (100%)
Red kidney bean	328.2 ± 5.7 [a] (100%)	199.1 ± 9.3 [b] (100%)
Soybean	5.9 ± 1.8 [a] (100%)	5.1 ± 0.6 [a] (100%)

Values are presented as mean ± S.D. of triplicate determinations. Values in the same rows followed by different letters (a, b) are significantly different at $P < 0.05$.

[1] Total flavonoid contents based a standard curve generated by 12.5-200 mg/l of catechin ($y = 0.0032x + 0.0023$; $R^2 = 0.9999$)

[2] Total values are sum of average measurement for acetone and methanol extracts.

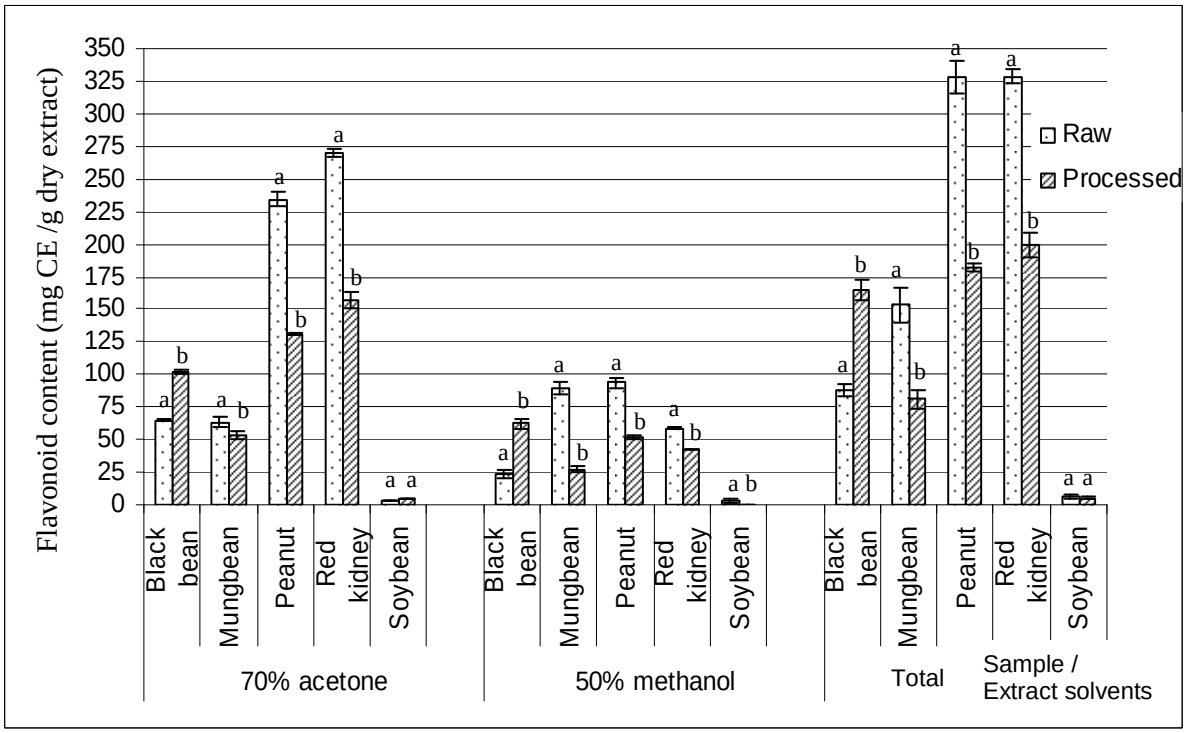


Figure 18. Effect of heat treatment on total flavonoid content of the seed coat extracts. Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Total values are sum of average measurement for acetone and methanol extracts. Different letters (a, b) show significant difference at $P< 0.05$.

4.2 Antioxidant Activity

4.2.1 Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl Radical (DPPH Assay)

This study screened antioxidant activity of the first solvent extracts (70% acetone) and the second solvent extracts (50% methanol) of legume seeds and seed coats by DPPH scavenging effect.

The extraction of raw legume seeds from black bean, mung bean and red kidney bean using acetone showed high level of DPPH scavenging activity (>80%) whereas the low levels of DPPH scavenging activity (≤ 25%) were observed in soybean and in methanol extracts of all tested legume seeds (Table 9 and Figure 19). The processed legume seeds revealed lower DPPH scavenging activity than that of raw legume seeds, which is correlated with their total phenolic content (Figure 15).

Table 9. Percentage of DPPH scavenging activity of the legume seed extracts (1 mg/ml)

Legume seeds	% Scavenging effect	
	Raw	Processed
First extraction with 70% acetone		
Black bean	95.2 ± 2.1 [a]	86.1 ± 1.1 [b]
Mung bean	92.6 ± 1.4 [a]	70.6 ± 5.2 [b]
Peanut	66.5 ± 2.5 [a]	66.3 ± 4.6 [a]
Red kidney bean	85.2 ± 7.7 [a]	58.0 ± 7.0 [b]
Soybean	18.9 ± 2.4 [a]	15.5 ± 3.1 [a]
Second extraction with 50% methanol		
Black bean	25.5 ± 2.9 [a]	8.4 ± 1.3 [b]
Mung bean	9.9 ± 1.6 [a]	3.7 ± 0.5 [b]
Peanut	15.7 ± 1.4 [a]	8.5 ± 1.1 [b]
Red kidney bean	15.5 ± 1.8 [a]	5.3 ± 1.5 [b]
Soybean	4.2 ± 0.7 [a]	7.0 ± 0.9 [a]

DPPH free radical scavenging activity was expressed as % DPPH scavenging activity per 1 g of extract. Values are presented as mean ± S.D. of triplicate determinations. Values in the same rows followed by different letters (a, b) are significantly different at P< 0.05.

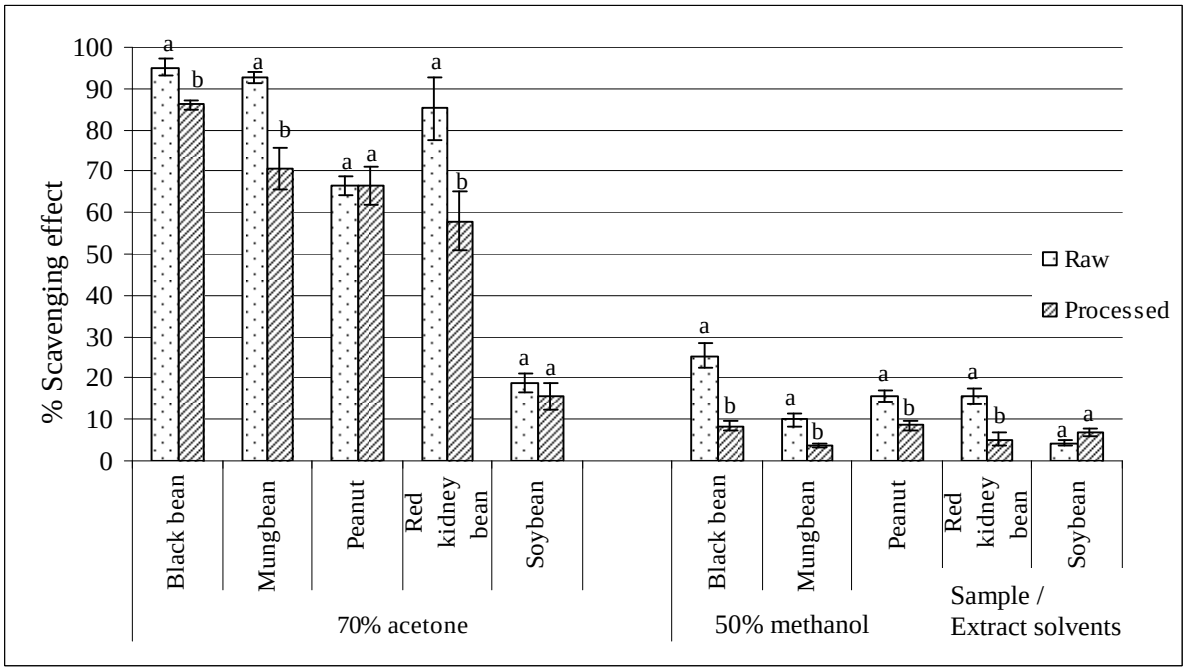


Figure 19. Percentage of DPPH scavenging activity of the legume seed extracts (1 mg/ml). Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Different letters (a, b) show significant difference at P< 0.05.

Raw seed coat extracted by both solvents displayed high level of DPPH scavenging effect (>80%), however extracts of soybean and black bean by 50% methanol showed low level of DPPH scavenging effect (<25%) (Table10). The extract of peanut seed coat showed the highest level of DPPH scavenging activity (92.3 ± 0.3%) whereas the lowest level of DPPH scavenging activity was observed in soybean (0%).

After heat treatment, all extracts of seed coats, except those of black bean and soybean exhibited lower scavenging activity than those of raw seed coats (Figure 20).

Table 10. Percentage of DPPH scavenging activity of the seed coat extracts (1 mg/ml)

Seed coat	% Scavenging effect	
	Raw	Processed
First extraction with 70% acetone		
Black bean	87.1 ± 2.4 [a]	88.8 ± 0.7 [a]
Mung bean	86.6 ± 4.8 [a]	82.5 ± 2.1 [b]
Peanut	92.3 ± 0.3 [a]	88.2 ± 0.4 [b]
Red kidney bean	89.7 ± 0.3 [a]	87.9 ± 0.1 [a]
Soybean	0.9 ± 0.5 [a]	17.5 ± 2.2 [b]
Second extraction with 50% methanol		
Black bean	39.9 ± 1.3 [a]	78.4 ± 7.2 [b]
Mung bean	88.4 ± 1.0 [a]	66.5 ± 3.9 [b]
Peanut	90.0 ± 1.1 [a]	73.0 ± 7.7 [b]
Red kidney bean	87.4 ± 0.8 [a]	72.0 ± 3.0 [b]
Soybean	0 [a]	0 [a]

DPPH free radical scavenging activity was expressed as % DPPH scavenging activity in 1 g of extract. Values are presented in mean ± S.D. (n = 3). Values in the same rows followed by different letters (a, b) are significantly different at $P < 0.05$.

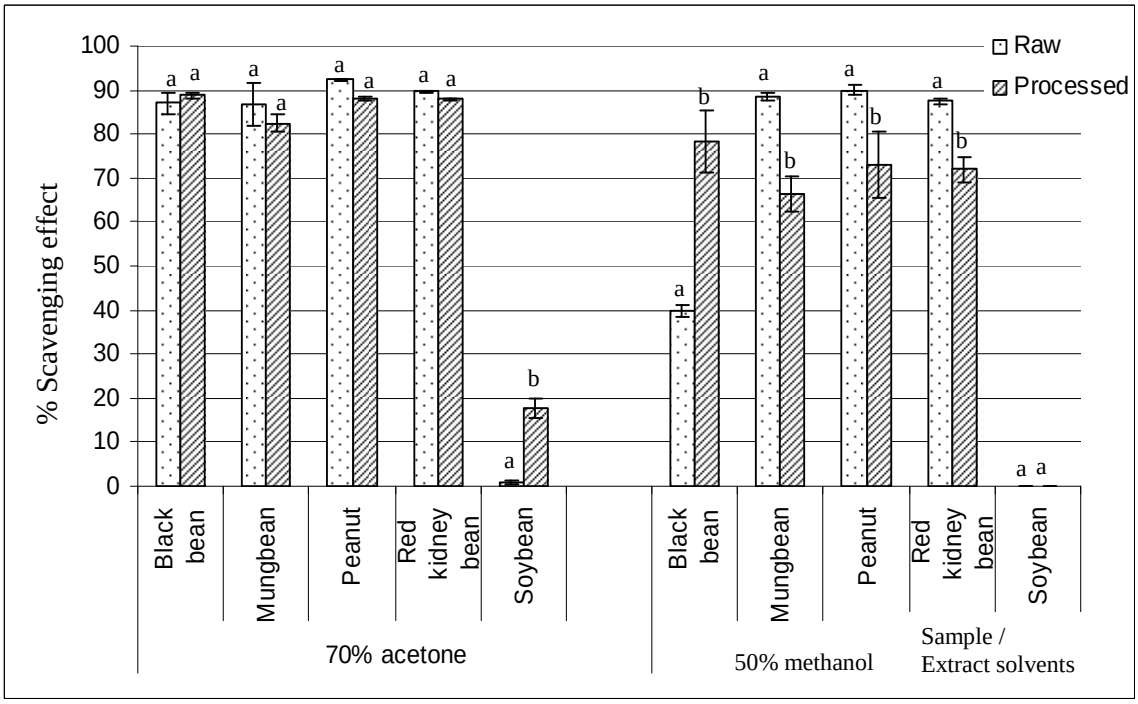


Figure 20. Percentage of DPPH scavenging activity of the seed coat extracts (1 mg/ml). Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Different letters (a, b) show significant difference at $P< 0.05$.

4.2.2 Ferric Reducing Antioxidant Power (FRAP Assay)

FRAP assay was used to measure the antioxidant activity of the extracts from their ability to reduce TPTZ-Fe (II) to TPTZ-Fe (III) complex. The results were expressed as μM ferric reduction to ferrous per 1 g dry extract. The reducing ability (FRAP value) of the legume seed extracts is shown in Table 11. The reducing powers for acetone extracts of raw legume seeds, acetone extracts of processed legume seeds, methanol extracts of raw legume seeds and methanol extracts of processed legume seeds were in the range of 58.1 ± 5.6 to 923.1 ± 26.1 μM/g dry extract, 73.1 ± 5.6 to 553.8 ± 15.6 μM/g dry extract, 21.6 ± 5.6 to 69.9 ± 9.6 μM/g dry extract and 6.3 ± 0.5 to 30.9 ±1.0 μM/g dry extract, respectively.

Acetone extract of raw black bean seeds had the highest reducing power (923.1 ± 26.1 μM/g dry extract). All of processed legume seed extracts by acetone, except those of peanut and soybean, showed to have lower ferric reducing effect than that of the raw legume seed extracts (Figure 21).

Table 11. FRAP value (ferric reducing power) of the legume seed extracts

Legume seeds	FRAP value [1] (μM/g dry extract)	
	Raw	Processed
First extraction with 70% acetone		
Black bean	923.1 ± 26.1 [a]	553.8 ± 15.6 [b]
Mung bean	474.9 ± 14.6 [a]	333.1 ± 13.6 [b]
Peanut	225.6 ± 11.5 [a]	270.2 ± 11.5 [b]
Red kidney bean	398.4 ± 15.7 [a]	259.5 ± 9.1 [b]
Soybean	58.1 ± 5.6 [a]	73.1 ± 5.6 [b]
Second extraction with 50% methanol		
Black bean	69.9 ± 9.6 [a]	29.9 ± 1.0 [b]
Mung bean	21.6 ± 5.6 [a]	6.3 ± 0.5 [b]
Peanut	40.9 ± 3.0 [a]	30.9 ± 1.0 [b]
Red kidney bean	24.1 ± 5.6 [a]	20.6 ± 1.0 [b]
Soybean	23.4 ± 1.5 [a]	14.9 ± 2.0 [b]

[1] FRAP value based a standard curve generated by 62.5- 1000 μM of ferrous sulfate ($y = 0.001x + 0.0124$; $R^2 = 0.9993$). FRAP was expressed as μM ferric reduction to ferrous per 1 g of extract. Values are presented in mean ± S.D. (n = 3). Values in the same rows followed by different letters (a, b) are significantly different at $P < 0.05$.

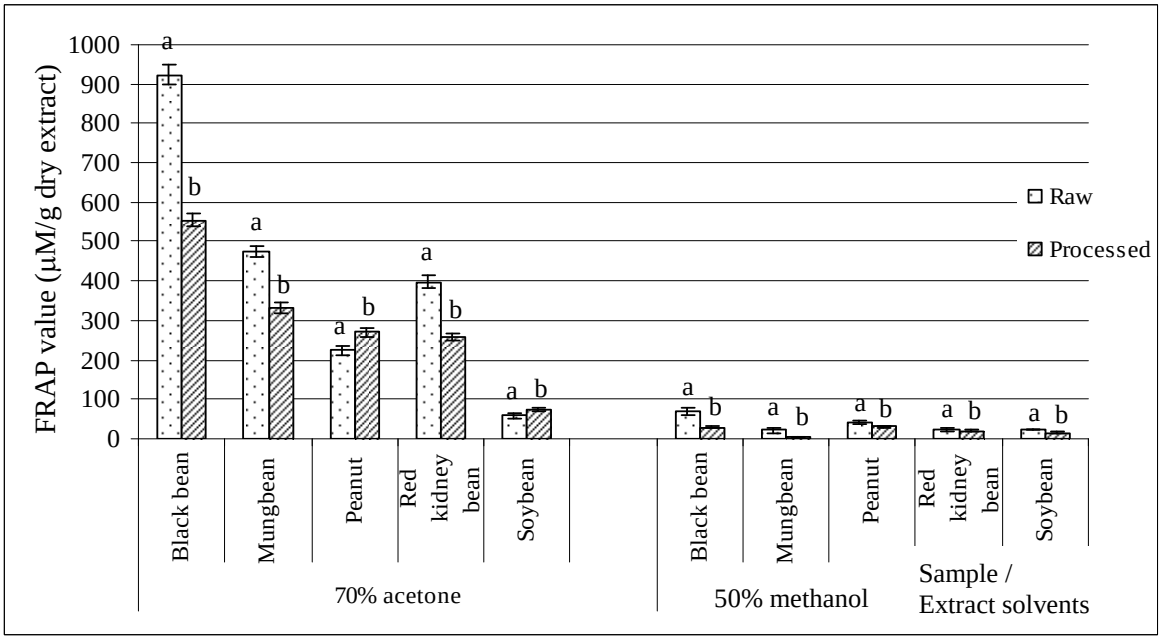


Figure 21. FRAP value (ferric reducing power) of the legume seed extracts. Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Different letters (a, b) show significant difference at $P < 0.05$.

The reducing power (FRAP value) of the seed coat extracts is presented in Table 12. The acetone extracts of raw peanut and red kidney bean had high FRAP value (2067.7 ± 112.5 and 2063.3 ± 58.6 μM/g dry extract, respectively), followed by processed black bean (1495.6 ± 59.4 μM) and processed red kidney bean (1077.9 ± 24.4 μM/g dry extract). Raw soybean displayed the lowest reducing ability (36.4 ± 2.9 μM/g dry extract). For methanol extracts, the FRAP value of raw mung bean showed the highest reducing power (1515.6 ± 15.7 μM/g dry extract) followed by raw peanut (1269.0 ± 42.8 μM/g dry extract). The raw soybean had the lowest reducing power (17.7 ± 2.9 μM/g dry extract). All extracts of processed seed coats, except those of black bean and soybean, exhibited lower ferric reducing effect than that of the extracts of raw seed coats (Figure 22).

Table 12. FRAP value (ferric reducing power) of the seed coat extracts

Seed coat	FRAP value [1] (μM/g dry extract)	
	Raw	Processed
First extraction with 70% acetone		
Black bean	739.5 ± 67.5[a]	1495.6 ± 59.4 [b]
Mung bean	768.7 ± 35.1 [a]	953.3 ± 12.1 [b]
Peanut	2067.7 ± 112.5 [a]	928.23 ± 49.2 [b]
Red kidney bean	2063.3 ± 58.6 [a]	1077.9 ± 24.4 [b]
Soybean	36.4 ± 2.9 [a]	68.2 ± 1.9 [b]
Second extraction with 50% methanol		
Black bean	346.4 ± 15.1 [a]	729.5 ± 34.2 [b]
Mung bean	1515.6 ± 15.7 [a]	357.2 ± 23.5 [b]
Peanut	1269.0 ± 42.8 [a]	706.6 ± 7.6 [b]
Red kidney bean	707.9 ± 54.4 [a]	489.7 ± 12.9 [b]
Soybean	17.7 ± 2.9 [a]	23.3 ± 1.8 [a]

[1] FRAP value based a standard curve generated by 62.5- 1000 μM of ferrous sulfate (y = 0.0013x + 0.0243; R^2 = 0.9993). FRAP was expressed as μM ferric reduction to ferrous per 1 g of extract.

Values are presented in mean ± S.D. (n = 3). Values in the same rows followed by different letters (a, b) are significantly different at P< 0.05.

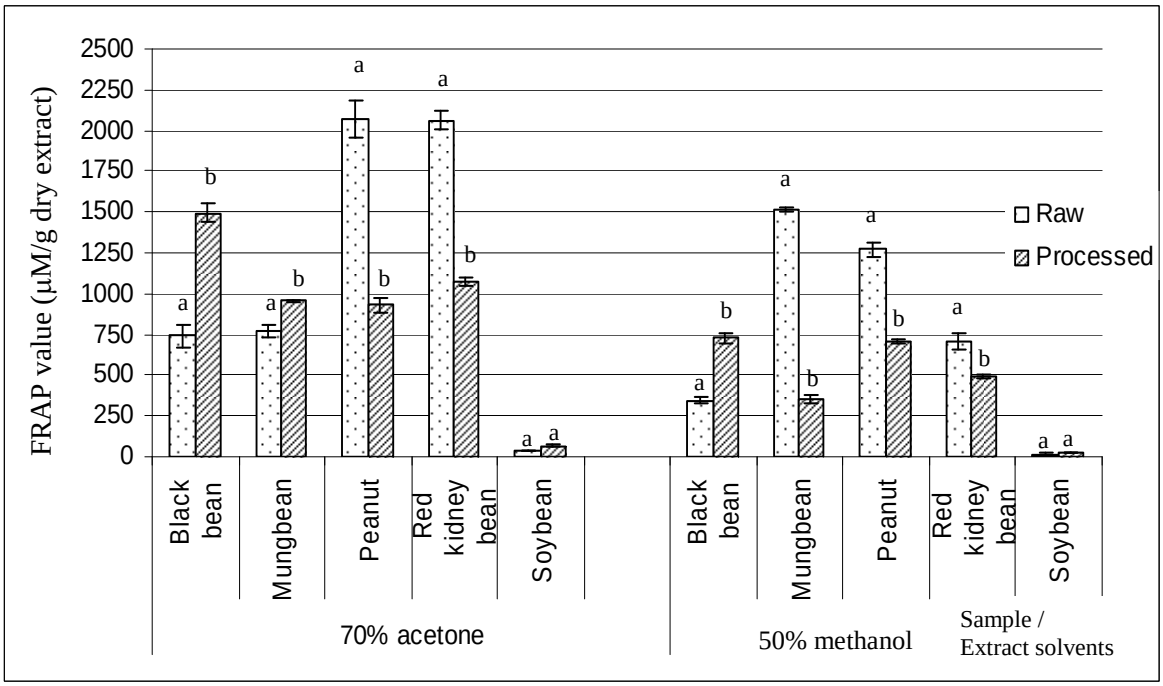


Figure 22. FRAP value (ferric reducing power) of the seed coat extracts. Bar graph indicates mean of triplicate data. Error bar represents standard deviation. Different letters (a, b) show significant difference at $P< 0.05$.

Subsequently, the results indicated that the seed coat extracts of peanut and red kidney bean displayed outstanding antioxidant property (scavenging DPPH effect and ferric reducing effect) compared to the extracts of other seed coats. In addition, the processed seed coat of black bean exhibited much higher total phenolic content and antioxidant activity than those of the raw seed coat of black bean.

In addition, all of the raw legume seed extracts, except peanut, showed to have higher antioxidant activity than that of the processed legume seed extracts. The processed black bean had the highest antioxidant activity, followed by mung bean and peanut.

4.3 Single Cell Gel Electrophoresis (Comet Assay)

4.3.1 Cytotoxicity

The trypan blue exclusion assay was used to determine the viability of the lymphocytes after treatment with different concentrations (1 - 50 μg/ml) of the legume seed and seed coat extracts at 37 ^{o}C for 30 min. The cell viability was observed to be lesser than 90% when cells treated with high concentrations of the legume seed extracts (≥ 10 μg/ml) and the seed coat extracts (≥ 5 μg/ml). Therefore, the appropriate concentrations of the legume seed extracts (0.2, 1 and 5 μg/ml) and the seed coat extracts (0.04, 0.2 and 1 μg/ml) were used for further study.

4.3.2 Effect of the Extracts of Legumes on DNA Damage in Human Lymphocytes

The Comet assay is the most frequently used assays for routine screening of potential genotoxic agents. The degree of DNA damage (strand breaks) was expressed as arbitrary unit score (0; all undamaged to 400; all maximally damaged). Tables 13 and 14 show the DNA damage of human lymphocytes treated with different concentrations of the legume extracts at 37 ^{o}C for 30 min. The results showed that lymphocytes exposed to the extracts at the cytotoxic concentrations (% viability < 90%) of legume seeds (≥ 10 μg/ml) or seed coats (≥ 5 μg/ml) induced DNA damage when compared to the arbitrary unit score of DNA damage (arbitrary units) of the negative control (water).

Table 13. DNA damage (arbitrary unit score) of the legume seed extracts in human lymphocytes using Comet assay

Sample	Conc. (μg/ml)	Arbitrary unit score (0- 400)			
		First extraction with acetone		Second extraction with methanol	
		Raw	Processed	Raw	Processed
Water	-	9 ± 3.5	11 ± 0.7	8 ± 0.7	8 ± 2.8
H_2O_2 (20 μM)	-	343 ± 38.2	328 ± 33.9	287 ± 23.3	323 ± 29.0
Black bean	1	8 ± 2.8	10 ± 0.0	7 ± 1.4	8 ± 1.4
	5	9 ± 1.4	12 ± 0.7	8 ± 2.1	9 ± 4.2
	10	8 ± 1.4	20 ± 2.8 *	8 ± 0.7	9 ± 2.8
	25	10 ± 2.1	25 ± 4.2 *	8 ± 1.4	11 ± 1.4
	50	18 ± 2.8 *	37 ± 5.7 *	10 ± 1.4	15 ± 3.5 *
Mung bean	1	7 ± 0.7	12 ± 0.7	7 ± 0.0	9 ± 2.8
	5	9 ± 3.5	11 ± 2.8	7 ± 0.7	11 ± 4.2
	10	10 ± 2.8	15 ± 3.5*	9 ± 1.4	19 ± 7.1*
	25	9 ± 2.8	21 ± 2.8*	10 ± 1.4	17 ± 4.9*
	50	14 ± 2.1*	36 ± 5.7*	12 ± 0.7	19 ± 5.7*
Peanut	1	8 ± 4.2	9 ± 0.7	9 ± 1.4	7 ± 1.4
	5	7 ± 1.4	8 ± 1.4	8 ± 0.7	8 ± 1.4
	10	8 ± 0.7	11 ± 2.8	8 ± 1.4	8 ± 0.0
	25	9 ± 0.7	10 ± 1.4	9 ± 0.7	13 ± 2.1
	50	11 ± 1.4	13 ± 3.5	11 ± 3.5	31 ± 4.9*
Red kidney bean	1	7 ± 1.4	8 ± 1.4	8 ± 2.8	11 ± 2.8
	5	9 ± 0.7	7 ± 0.7	9 ± 2.1	10 ± 2.8
	10	10 ± 0.7	10 ± 1.4	10 ± 1.4	5 ± 3.5*
	25	8 ± 1.4	10 ± 0.7	14 ± 4.2	21 ± 2.8*
	50	16 ± 4.2*	14 ± 2.8*	17 ± 2.8*	23 ± 4.2*
Soybean	1	8 ± 1.4	9 ± 2.8	9 ± 2.1	9 ± 1.4
	5	9 ± 0.7	10 ± 2.1	8 ± 1.4	10 ± 2.8
	10	8 ± 1.4	12 ± 4.2	9 ± 0.7	25 ± 4.9*
	25	7 ± 1.4	11 ± 2.8	10 ± 1.4	23 ± 4.2*
	50	9 ± 0.7	16 ±4.2*	11 ± 2.1	31 ± 7.1*

The lymphocytes were incubated with the legume seed extracts at 37 ^{o}C for 30 min. The results are expressed as arbitrary unit score. Data represent mean ± S.D. of two independent experiments.

* Cytotoxic concentrations (% viability < 90%)

Table 14. DNA damage (arbitrary unit score) of the seed coat extracts in human lymphocytes using Comet assay

Sample	Conc. (μg/ml)	Arbitrary unit score (0- 400)			
		First extraction with acetone		Second extraction with methanol	
		Raw	Processed	Raw	Processed
Water	-	8 ± 0.7	10 ± 1.4	8 ± 1.4	11 ± 2.8
H_2O_2 (20 μM)	-	265 ± 31.1	271 ± 32.5	246 ± 26.9	244 ± 28.3
Black bean	1	8 ± 1.4	9 ± 2.1	7 ± 1.4	9 ± 0.7
	5	7 ± 1.4	10 ± 1.4	9 ± 2.1	8 ± 1.4
	10	10 ± 0.7	12 ± 4.2	10 ± 2.8	10 ± 5.7
	25	14 ± 4.2*	35 ± 6.4*	10 ± 2.1	12 ± 5.7
	50	21 ± 3.5*	39 ± 5.7*	19 ± 3.5*	29 ± 7.8*
Mung bean	1	7 ± 1.4	10 ± 2.8	7 ± 1.4	9 ± 2.8
	5	10 ± 2.8	12 ± 3.5	7 ± 2.8	11 ± 4.2
	10	13 ± 3.5*	33 ± 6.4*	8 ± 1.4	14 ± 8.5*
	25	15 ± 1.4*	35 ± 4.2*	9 ± 2.8	15 ± 4.9*
	50	25 ± 3.5*	40 ± 3.5*	9 ± 0.7	34 ± 7.1*
Peanut	1	10 ± 0.7	9 ± 0.0	7 ± 1.4	11 ± 3.5
	5	9 ± 1.4	11 ± 2.8	8 ± 2.8	10 ± 5.7
	10	12 ± 2.1	10 ± 2.8	13 ± 4.9	11 ± 2.8
	25	12 ± 1.4	11 ± 1.4	11 ± 1.4	13 ± 4.2
	50	13 ± 2.8	13 ± 2.8	17 ± 4.2*	35 ± 4.9*
Red kidney bean	1	7 ± 0.7	10 ± 2.1	8 ± 0.7	12 ± 4.2
	5	8 ± 1.4	11 ± 2.1	8 ± 1.4	16 ± 6.4*
	10	9 ± 1.4	10 ± 1.4	11 ± 2.8	27 ± 7.1*
	25	8 ± 1.4	11 ± 2.8	20 ± 4.2*	38 ± 7.8*
	50	12 ± 0.7*	15 ± 4.2*	21 ± 7.8*	34 ± 8.5*
Soybean	1	9 ± 0.7	9 ± 2.8	8 ± 1.4	12 ± 3.5
	5	8 ± 0.0	11 ± 2.8	7 ± 1.4	23 ± 5.7*
	10	8 ± 1.4	18 ± 4.9*	9 ± 0.7	30 ± 7.8*
	25	9 ± 1.4	33 ± 7.8*	16 ± 4.2*	33 ± 7.1*
	50	16 ± 4.2*	35 ± 5.7*	13 ± 1.4*	39 ± 6.4*

The lymphocytes were incubated with the seed coat extracts at 37 ^{o}C for 30 min. The results are expressed as arbitrary unit score. Data represent mean ± S.D. of two independent experiments.

* Cytotoxic concentrations (% viability < 90%)

4.3.3 Protective Effect of the Extracts of Legumes on DNA Damage Induced by H_2O_2 in Human Lymphocytes

The ability of the legume seed extracts to protect against the DNA damage induced by H_2O_2 in human lymphocytes is shown in Tables 15 and 16. The processed legume seed extracts showed higher inhibition on DNA break than that of the raw legume seed extracts (Figure 23). For the first extraction with 70% acetone, raw and processed legume seeds at the highest concentration (5 μg/ml) showed weak inhibition on DNA break (8.1- 36.4%). Acetone extracts of processed black bean exhibited the highest inhibition (36.4%). On the other hand, almost of raw and processed legume seeds extracted by 50% methanol showed to have negligible inhibition (<20%) when testing at the highest concentration (5 μg/ml).

Table 15. The effects of the raw legume seed extracts on DNA damage (arbitrary unit score) induced by H_2O_2 in human lymphocytes using Comet assay

Sample	Conc. (μg/ml)	Raw legume seeds extracts			
		First extraction with acetone		Second extraction with methanol	
		AU [1]	%Inhibition	AU [1]	%Inhibition
Water	-	14 ± 2.1		23 ± 1.4	
H_2O_2 (20 μM)	-	309 ± 32.5		275 ± 28.3	
Black bean	0. 2	263 ± 18.4	14.9	277 ± 17.0	0.0
	1	226 ± 19.8	26.9	273 ± 26.9	0.7
	5	234 ± 25.5	24.3	267 ± 17.0	2.9
Mung bean	0. 2	297 ± 21.2	3.9	275 ± 16.3	0.0
	1	280 ± 24.0	9.4	275 ± 21.2	0.0
	5	278 ± 18.4	10.0	292 ± 26.9	0.0
Peanut	0. 2	258 ± 35.4	16.5	253 ± 32.5	8.0
	1	245 ± 13.4	20.7	264 ± 27.6	4.0
	5	215 ± 33.9	30.4	239 ± 33.9	13.1
Red kidney bean	0. 2	288 ± 31.1	6.8	250 ± 19.8	9.1
	1	281 ± 33.9	9.1	247 ± 28.3	10.2
	5	284 ± 14.1	8.1	196 ± 21.2	28.7
Soybean	0. 2	286 ± 24.7	7.4	240 ± 33.2	12.7
	1	263 ± 32.5	14.9	238 ± 36.8	13.5
	5	253 ± 22.6	18.1	273 ± 22.6	0.7

The lymphocytes were incubated with the legume extracts at 37 ^{o}C for 25 min before exposure to H_2O_2 (5 min on ice). The results are expressed as arbitrary unit score and percentage of inhibition. Arbitrary unit score showed mean ± S.D. of two independent experiments.

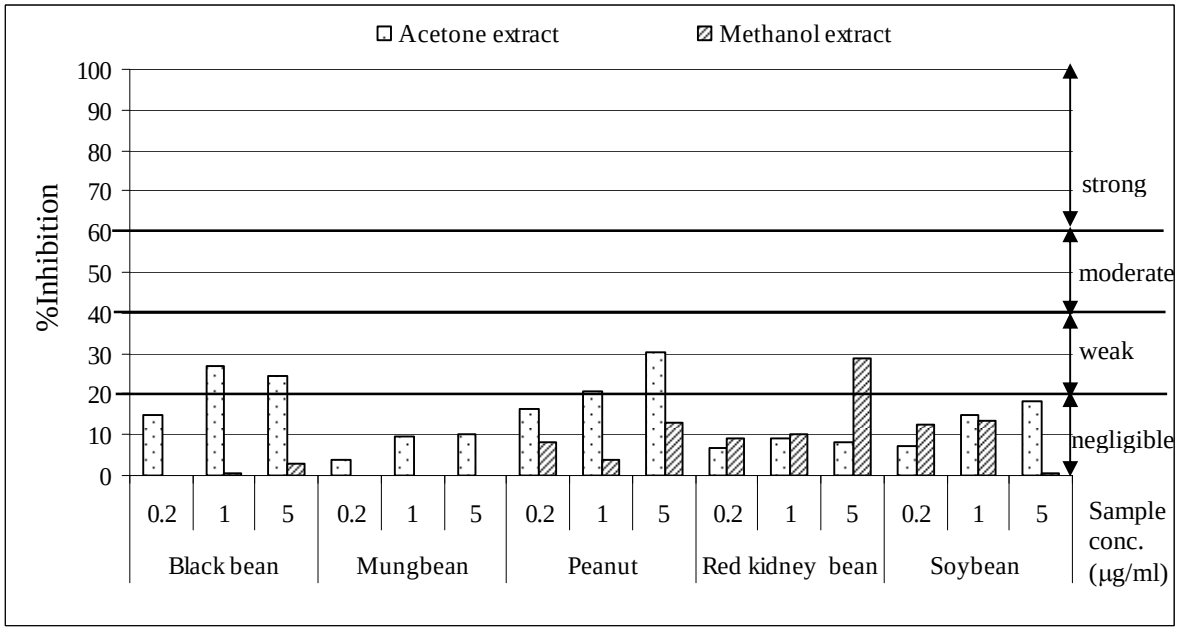
[1] AU = arbitrary unit score

Table 16. The effects of the processed legume seed extracts on DNA damage (arbitrary unit score) induced by H_2O_2 in human lymphocytes using Comet assay

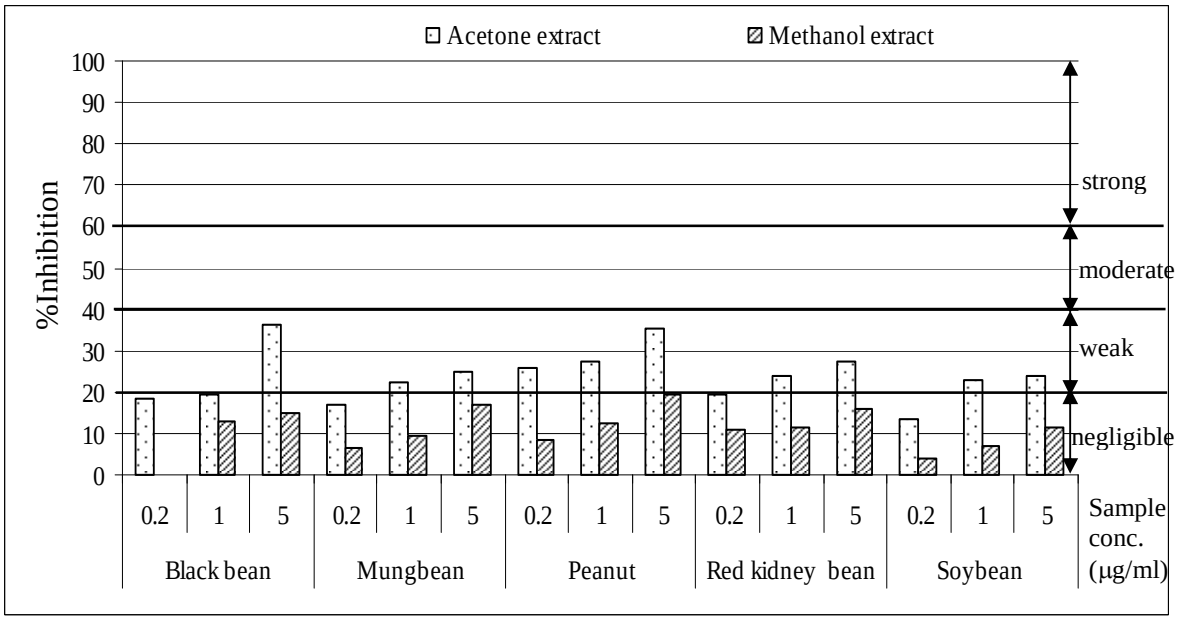
Sample	Conc. (μg/ml)	Processed legume seeds extracts			
		First extraction with acetone		Second extraction with methanol	
		AU [1]	%Inhibition	AU [1]	%Inhibition
Water	-	10 ± 2.1		6 ± 1.4	
H_2O_2 (20 μM)	-	338 ± 18.4		334 ± 25.5	
Black bean	0. 2	276 ± 35.4	18.3	344 ± 17.7	0.0
	1	272 ± 33.9	19.5	291 ± 5.7	12.9
	5	215 ± 28.3	36.4	285 ± 24.0	14.7
Mung bean	0. 2	281 ± 17.7	16.9	312 ± 22.6	6.6
	1	262 ± 17.0	22.5	302 ± 23.3	9.6
	5	253 ± 11.3	25.1	277 ± 21.2	17.1
Peanut	0. 2	250 ± 22.6	26.0	305 ± 17.0	8.7
	1	246 ± 25.5	27.2	293 ± 21.2	12.3
	5	218 ± 28.3	35.5	270 ± 22.6	19.2
Red kidney bean	0. 2	273 ± 26.9	19.2	298 ± 12.7	10.8
	1	257 ± 29.7	24.0	296 ± 31.1	11.4
	5	245 ± 28.3	27.5	281 ± 36.8	15.9
Soybean	0. 2	293 ± 24.0	13.3	321 ± 18.4	3.9
	1	261 ± 24.7	22.8	310 ± 24.0	7.2
	5	257 ± 20.5	24.0	296 ± 12.7	11.4

The lymphocytes were incubated with the legume extracts at 37 ^{o}C for 25 min before exposure to H_2O_2 (5 min on ice). The results are expressed as arbitrary unit score and percentage of inhibition. Arbitrary unit score showed mean ± S.D. of two independent experiments.

[1] AU = arbitrary unit score



(A)



(B)

Figure 23. Percentage of inhibition provided by different concentrations of the extracts of raw legume seeds (A) and processed legume seeds (B) on DNA damage induced by H_2O_2 in human lymphocytes using Comet assay. The cells were incubated with the legumes extracts 25 min before exposure to H_2O_2 (5 min on ice).

The ability of the seed coat extracts to protect against the DNA damage induced by H_2O_2 in human lymphocytes is shown in Tables 17 and 18. At the highest concentration (1 μg/ml), the first extraction with 70% acetone of raw seed coats showed weak to moderate inhibition on DNA break (31.7- 53.7%) while the second extraction with 50% methanol of raw seed coats showed weak inhibition on DNA break (24.9- 38.8%) (Figure 24). The maximum inhibitory effect of DNA damage was observed for exposure to the processed seed coat extracts (43.0- 63.5%).

The acetone extracts of processed seed coats of black bean, peanut and soybean showed greater than 60% of inhibitory effect. On the other hand, methanol extracts of processed seed coats of black bean and red kidney bean showed to have more than 59% inhibitory effect (Figure 24).

This result indicated that the DNA strand breaks induced by 20 μM H_2O_2 in human lymphocytes are inhibited by the legume seed extracts and the seed coat extracts.

Table 17. The effects of the raw seed coat extracts on DNA damage (arbitrary unit score) induced by H_2O_2 in human lymphocytes using Comet assay

Sample	Conc. (μg/ml)	Raw seed coats extracts			
		First extraction with acetone		Second extraction with methanol	
		AU	%Inhibition	AU	%Inhibition
Water	-	14 ± 2.1		10 ± 1.4	
H_2O_2 (20 μM)	-	287 ± 37.5		281 ± 33.9	
Black bean	0.04	149 ± 25.5	48.1	201 ± 8.5	28.5
	0.2	165 ± 21.2	42.5	199 ± 11.3	29.2
	1	133 ± 26.2*	53.7	172 ± 15.6	38.8
Mung bean	0.04	206 ± 18.4	28.2	255 ± 19.8	9.3
	0.2	201 ± 21.2	30.0	251 ± 22.6	10.7
	1	196 ± 14.8	31.7	211 ± 18.4	24.9
Peanut	0.04	208 ± 30.4	27.5	242 ± 26.9	13.9
	0.2	218 ± 11.3	24.0	223 ± 31.1	20.6
	1	174 ± 18.4	39.4	195 ± 15.6	30.6
Red kidney bean	0.04	174 ± 26.9	39.4	227 ± 21.2	19.2
	0.2	150 ± 18.4*	47.7	209 ± 32.5	25.6
	1	139 ± 14.1*	51.6	174 ± 7.1*	38.1
Soybean	0.04	204 ± 29.0	28.9	213 ± 26.9	24.2
	0.2	162 ± 36.8	43.6	197 ± 17.0	29.9
	1	182 ± 33.2	36.6	190 ± 19.1	32.4

The lymphocytes were incubated with the seed coat extracts at 37 ^{o}C for 25 min before exposure to H_2O_2 (5 min on ice). The results are expressed as arbitrary unit score and percentage of inhibition. Arbitrary unit score showed mean ± S.D. of two independent experiments.

* p<0.05 refers to differences between hydrogen peroxide-treated lymphocytes preincubated with or without sample extracts.

AU = arbitrary unit score

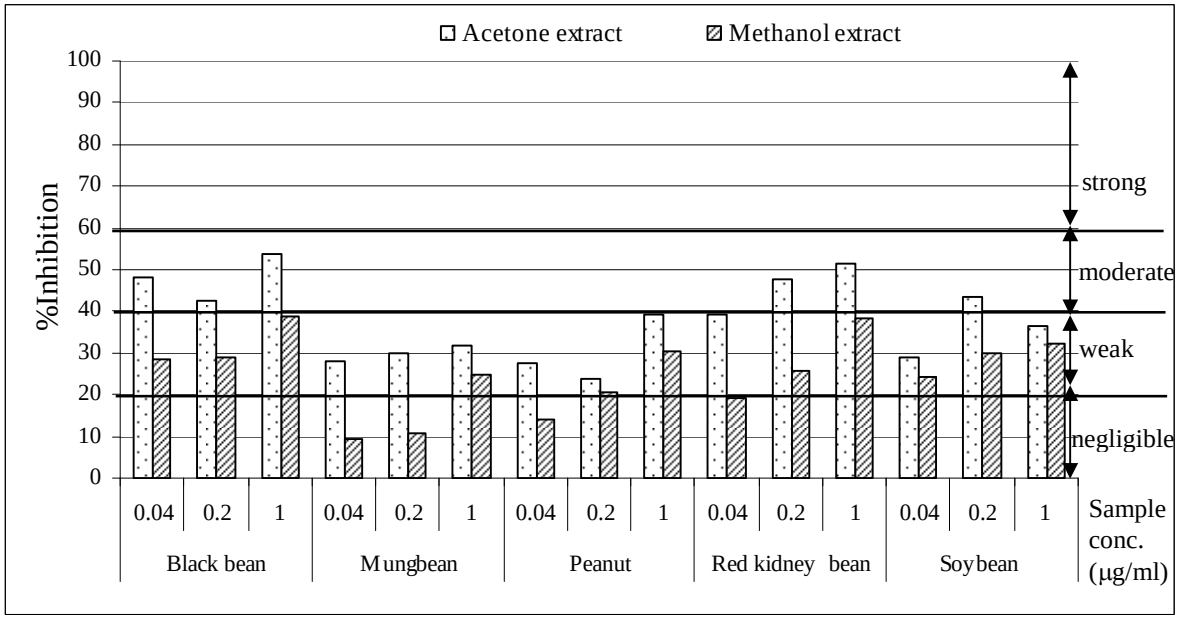
Table 18. The effects of the processed seed coat extracts on DNA damage (arbitrary unit score) induced by H_2O_2 in human lymphocytes using Comet assay

Sample	Conc. (μg/ml)	Processed seed coats extracts			
		First extraction with acetone		Second extraction with methanol	
		AU	%Inhibition	AU	%Inhibition
Water	-	14 ± 2.1		17 ± 1.4	
H_2O_2 (20 μM)	-	251 ± 35.4		323 ± 22.6	
Black bean	0.04	127 ± 17.0*	49.4	195 ± 19.8*	39.6
	0.2	119 ± 8.5*	52.6	183 ± 11.3*	43.3
	1	92 ± 15.6*	63.3	130 ± 18.4*	59.8
Mung bean	0.04	198 ± 38.2	21.1	192 ± 25.5*	40.6
	0.2	180 ± 19.8	28.3	193 ± 32.5*	40.2
	1	117 ± 21.2*	53.4	175 ± 26.9*	45.8
Peanut	0.04	139 ± 14.1	57.0	163 ± 33.9*	35.1
	0.2	145 ± 15.6	55.1	135 ± 26.9*	46.2
	1	118 ± 29.7	63.5	119 ± 36.8*	52.6
Red kidney bean	0.04	146 ± 14.8	54.8	166 ± 18.4*	33.9
	0.2	166 ± 12.7	48.6	123 ± 22.6*	51.0
	1	184 ± 18.4	43.0	96 ± 15.6**	61.8
Soybean	0.04	177 ± 19.1	45.2	209 ± 24.0*	16.7
	0.2	118 ± 11.3*	63.5	163 ± 21.2*	35.1
	1	124 ± 15.6*	61.6	134 ± 28.3*	46.6

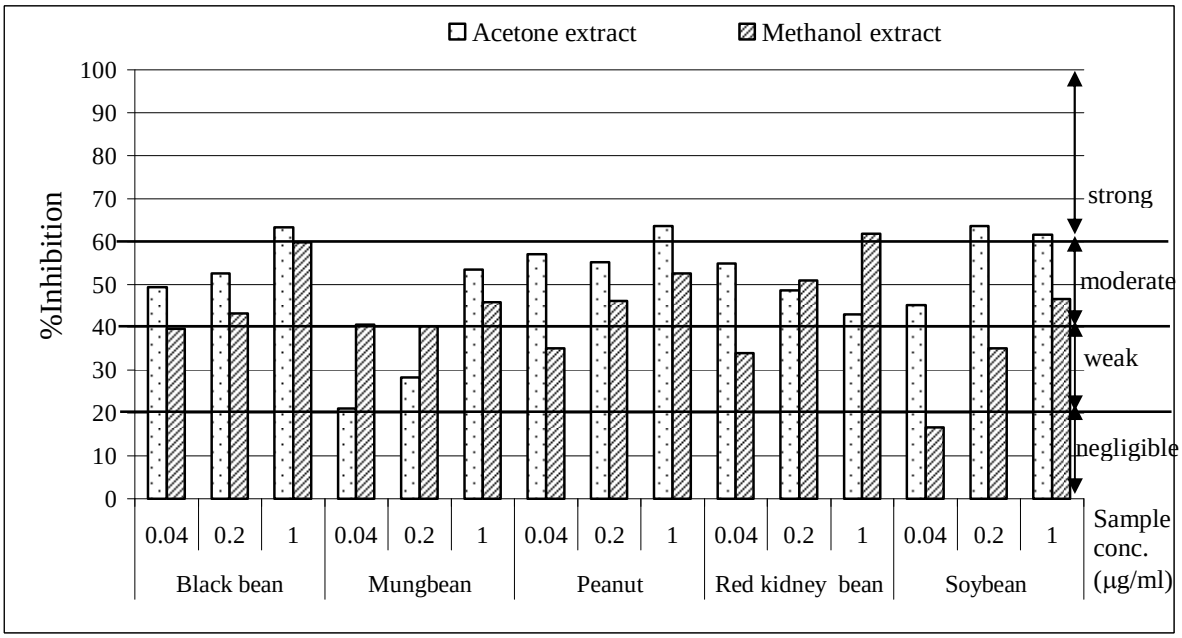
The lymphocytes were incubated with the seed coat extracts at 37 ^oC for 25 min before exposure to H_2O_2 (5 min on ice). The results are expressed as arbitrary unit score and percentage of inhibition. Arbitrary unit score showed mean ± S.D. of two independent experiments.

* $p<0.05$ and ** $p<0.01$ refer to differences between hydrogen peroxide-treated lymphocytes preincubated with or without sample extracts.

AU = arbitrary unit score



(A)



(B)

Figure 24. Percentage of inhibition provided by different concentrations of the extracts of raw seed coats (A) and processed seed coats (B) on DNA damage induced by H_2O_2 in human lymphocytes using Comet assay. The cells were incubated with the extracts of seed coats 25 min before exposure to H_2O_2 (5 min on ice).

4.4 Somatic Mutation and Recombination Test (SMART)

4.4.1 Percentage of Surviving Adult Flies and Mutagenicity of the Extracts of Legumes

Table 19 shows the number of surviving adult flies obtained from the larvae brought up on each sample medium containing acetone extracts or methanol extracts of legume seeds and seed coats, negative control medium and positive control medium. The percentages of surviving adult flies brought up on all experimental medium are higher than 50%. The results indicated that all concentrations used were non-toxic for further study.

The data (Tables 20-23) indicated that the legume seed extracts were not 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. At the highest concentration (25 mg/tube), all extracts of seed coats, except of those of acetone extract of mung bean and methanol extracts of red kidney bean and soybean, were not mutagenic.

Table 19. The percentage of survival adult flies fed on control and sample medium containing the extracts of legumes (mg per tube)

Sample	Amount of extract (mg/tube)	Urethane (mM)	Percent of surviving flies (%)			
			Legumes seeds		Seed coats	
			First extraction with acetone	Second extraction with methanol	First extraction with acetone	Second extraction with methanol
Water	-	-	98	86	98	97
Urethane	-	20	96	87	83	80
Black bean	6.25	-	89	94	85	75
	12.5	-	98	91	76	85
	25	-	94	83	73	83
	6.25	20	89	87	87	79
	12.5	20	85	74	87	72
	25	20	84	80	85	58

Table 19. The percentage of survival adult flies fed on control and sample medium containing the extracts of legumes (mg per tube) (continued)

Sample	Amount of extract (mg/tube)	Urethane (mM)	Percent of surviving flies (%)			
			Legumes seeds		Seed coats	
			First extraction with acetone	Second extraction with methanol	First extraction with acetone	Second extraction with methanol
Mung bean	6.25	-	94	91	90	84
	12.5	-	84	88	96	84
	25	-	88	84	91	78
	6.25	20	74	79	91	81
	12.5	20	84	85	91	95
	25	20	76	66	99	81
Peanut	6.25	-	98	94	97	86
	12.5	-	99	86	93	87
	25	-	98	78	90	88
	6.25	20	80	75	96	95
	12.5	20	83	98	93	92
	25	20	75	99	84	89
Red kidney	6.25	-	96	94	73	70
Bean	12.5	-	89	85	78	77
	25	-	89	80	87	76
	6.25	20	81	97	86	84
	12.5	20	80	90	97	78
	25	20	86	84	72	70
Soybean	6.25	-	90	87	91	85
	12.5	-	99	77	90	82
	25	-	97	75	80	81
	6.25	20	81	94	80	85
	12.5	20	85	84	69	76
	25	20	78	86	63	76

Table 20. Wing spots induction of the first extraction with acetone of legume seeds

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
1	Water	Negative	-	40	0.050(2)	0.025(1)	0.025(1)i	0.100(4)
	Urethane	Positive	-	40	11.45 (458)+	2.55(102)+	1.525(61)+	15.525(621)+
	Black bean	Sample	6.25	40	0.100(4)i	0	0	0.100(4)i
			12.5	40	0.100(4)i	0.025(1)i	0	0.125(5)i
			25	40	0.175(7)i	0	0	0.175(7)i
	Mung bean	Sample	6.25	40	0.050(2)i	0.025(1)i	0.025(1)i	0.100(4)i
			12.5	40	0.150(6)i	0.025(1)i	0.025(1)i	0.200(8)i
			25	40	0.150(6)i	0	0.025(1)i	0.175(7)i
	Peanut	Sample	6.25	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			12.5	40	0.125(5)i	0.075(3)i	0	0.200(8)i
			25	40	0.125(5)i	0.025(1)i	0.050(2)i	0.200(8)i
	Red kidney	Sample	6.25	40	0.075(3)i	0	0	0.075(3)i
	Bean		12.5	40	0.075(3)i	0	0	0.075(3)i
			25	40	0.200(8)i	0	0.025(1)i	0.225(9)i
	Soybean	Sample	6.25	40	0.050(2)i	0	0.025(1)i	0.075(3)i
			12.5	40	0.050(2)i	0.025(1)i	0.025(1)i	0.100(4)i
			25	40	0.100(4)i	0	0.025(1)i	0.125(5)i

Table 20. Wing spots induction of the first extraction with acetone of legume seeds (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
2	Water	Negative	-	40	0.050(2)	0.025(1)	0	0.075(3)
	Urethane	Positive	-	40	6.475 (259)+	2.85(114)+	1.8(72)+	11.125(445)+
	Black bean	Sample	6.25	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			12.5	40	0.125(5)i	0.025(1)i	0	0.150(6)i
			25	40	0.150(6)i	0	0.050(2)i	0.200(8)i
	Mung bean	Sample	6.25	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			12.5	40	0.125(5)i	0	0.025(1)i	0.150(6)i
			25	40	0.150(6)i	0	0.025(1)i	0.175(7)i
	Peanut	Sample	6.25	40	0.050(2)i	0.025(1)i	0	0.075(3)i
			12.5	40	0.100(4)i	0.050(2)i	0	0.150(6)i
			25	40	0.100(4)i	0.050(2)i	0.025(1)i	0.175(7)i
	Red kidney	Sample	6.25	40	0.050(2)i	0	0.025(1)i	0.075(3)i
	Bean		12.5	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			25	40	0.125(5)i	0.050(2)i	0.025(1)i	0.200(8)i
	Soybean	Sample	6.25	40	0.050(2)i	0.025(1)i	0	0.075(3)i
			12.5	40	0.050(2)i	0.025(1)i	0.025(1)i	0.100(4)i
			25	40	0.075(3)i	0	0.025(1)i	0.100(4)i

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with negative control:

+ = positive; - = negative; i= inconclusive; Propability level $\alpha=\beta=0.05$. One side statistical tests.

Table 21. Wing spots induction of the second extraction with methanol of legume seeds

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
1	Water	Negative	-	40	0.050(2)	0	0.050(2)	0.100(4)
	Urethane	Positive	-	40	7.725(309)+	3.250(130)+	1.525(61)+	12.500(500)+
	Black bean	Sample	6.25	40	0.050(2)i	0	0.025(1)i	0.075(3)i
			12.5	40	0.175(7)i	0.025(1)i	0	0.200(8)i
			25	40	0.150(6)i	0.025(1)i	0.025(1)i	0.200(8)i
	Mung bean	Sample	6.25	40	0.100(4)i	0.025(1)i	0	0.125(5)i
			12.5	40	0.150(6)i	0.050(2)i	0	0.200(8)i
			25	40	0.275(11)+	0	0	0.275(11)i
	Peanut	Sample	6.25	40	0.075(3)i	0.025(1)i	0.025(1)i	0.125(5)i
			12.5	40	0.100(4)i	0	0.025(1)i	0.125(5)i
			25	40	0.125(5)i	0.050(2)i	0	0.175(7)i
	Red kidney	Sample	6.25	40	0.150(6)i	0	0.025(1)i	0.175(7)i
	Bean		12.5	40	0.100(4)i	0.025(1)i	0.025(1)i	0.150(6)i
			25	40	0.100(4)i	0	0.025(1)i	0.125(5)i
	Soybean	Sample	6.25	40	0.125(5)i	0	0.025(1)i	0.150(6)i
			12.5	40	0.175(7)i	0	0.050(2)i	0.225(9)i
			25	40	0.100(4)i	0	0.025(1)i	0.125(5)i

Table 21. Wing spots induction of the second extraction with methanol of legume seeds (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
2	Water	Negative	-	40	0.075(3)	0	0	0.075(3)i
	Urethane	Positive	-	40	6.950(278)+	4.900(196)+	2.500(100)+	14.350(574)+
	Black bean	Sample	6.25	40	0.050(2)i	0.025(1)i	0	0.075(3)i
			12.5	40	0.050(2)i	0	0	0.050(2)i
			25	40	0.200(8)i	0.050(2)i	0.025(1)i	0.275(11)i
	Mung bean	Sample	6.25	40	0.125(5)i	0.025(1)i	0.025(1)i	0.175(7)i
			12.5	40	0.150(6)i	0.025(1)i	0.050(2)i	0.225(9)i
			25	40	0.100(4)i	0.050(2)i	0.025(1)i	0.175(7)i
	Peanut	Sample	6.25	40	0.100(4)i	0.050(2)i	0	0.150(6)i
			12.5	40	0.100(4)i	0.025(1)i	0.025(1)i	0.150(6)i
			25	40	0.150(6)i	0	0	0.150(6)i
	Red kidney	Sample	6.25	40	0.150(6)i	0	0.050(2)i	0.200(8)i
	Bean		12.5	40	0.150(6)i	0.025(1)i	0.025(1)i	0.175(7)i
			25	40	0.175(7)i	0	0	0.175(7)i
	Soybean	Sample	6.25	40	0.100(4)i	0.025(1)i	0	0.125(5)i
			12.5	40	0.100(4)i	0	0.025(1)i	0.125(5)i
			25	40	0.125(5)i	0	0	0.125(5)i

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

Table 22. Wing spots induction of the first extraction with acetone of seed coats

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
1	Water	Negative	-	40	0.075(3)	0.025(1)	0	0.100(4)
	Urethane	Positive	-	40	6.80(272)+	1.625(65)+	4.650(186)+	13.075(523)+
	Black bean	Sample	6.25	40	0.075(3)i	0.075(3)i	0.025(1)i	0.175(7)i
			12.5	40	0.125(5)i	0.050(2)i	0	0.175(7)i
			25	40	0.150(6)i	0.050(2)i	0	0.200(8)i
	Mung bean	Sample	6.25	40	0.050(2)i	0	0	0.050(2)i
			12.5	40	0.175(7)i	0.025(1)i	0	0.200(8)i
			25	40	0.25(10)+	0.050(2)i	0	0.3(12)+
	Peanut	Sample	6.25	40	0.075(3)i	0	0	0.075(3)i
			12.5	40	0.075(3)i	0.050(2)i	0	0.125(5)i
			25	40	0.125(5)i	0.025(1)i	0	0.150(6)i
	Red kidney	Sample	6.25	40	0.025(1)i	0.050(2)i	0.025(1)i	0.100(4)i
	Bean		12.5	40	0.125(5)i	0.025(1)i	0	0.150(6)i
			25	40	0.150(6)i	0.025(1)i	0	0.175(7)i
	Soybean	Sample	6.25	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			12.5	40	0.150(6)i	0.075(3)i	0	0.225(9)i
			25	40	0.100(4)i	0.025(1)i	0.025(1)i	0.150(6)i

Table 22. Wing spots induction of the first extraction with acetone of seed coats (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
2	Water	Negative	-	40	0.075(3)	0.025(1)	0	0.100(4)
	Urethane	Positive	-	40	7.275(291)+	3.075(123)+	1.525(61)	11.875 (475)+
	Black bean	Sample	6.25	40	0.100(4)i	0.025(1)i	0	0.125(5)i
			12.5	40	0.125(5)i	0.025(1)i	0.025(1)i	0.175(7)i
			25	40	0.175(7)i	0.025(1)i	0	0.200(8)i
	Mung bean	Sample	6.25	40	0.075(3)i	0	0.025(1)i	0.100(4)i
			12.5	40	0.125(5)i	0	0	0.200(8)i
			25	40	0.200(8)i	0.050(2)i	0	0.25(10)i
	Peanut	Sample	6.25	40	0.050(2)i	0.025(1)i	0	0.075(3)i
			12.5	40	0.075(3)i	0.050(2)i	0	0.125(5)i
			25	40	0.125(5)i	0.050(2)i	0	0.175(7)i
	Red kidney	Sample	6.25	40	0.050(2)i	0.025(1)i	0.025(1)i	0.100(4)i
	Bean		12.5	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			25	40	0.125(5)i	0.025(1)i		0.150(6)i
	Soybean	Sample	6.25	40	0.075(3)i	0.025(1)i	0	0.100(4)i
			12.5	40	0.125(5)i	0.050(2)i	0	0.175(7)i
			25	40	0.150(6)i	0.025(1)i	0	0.175(7)i

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

Table 23. Wing spots induction of the second extraction with methanol of seed coats

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
1	Water	Negative	-	40	0.050(2)	0.025(1)	0	0.075(3)
	Urethane	Positive	-	40	10.275(411)+	4.65(186)+	1.825(73)+	16.75(670)+
	Black bean	Sample	6.25	40	0.050(2)i	0.050(2)i	0.025(1)i	0.125(5)i
			12.5	40	0.175(7)i	0.025(1)i	0.025(1)i	0.225(9)i
			25	40	0.175(7)i	0	0.050(2)i	0.225(9)i
	Mung bean	Sample	6.25	40	0.200(8)i	0	0	0.200(8)i
			12.5	40	0.175(7)i	0.050(2)i	0	0.225(9)i
			25	40	0.125(5)i	0.050(2)i	0.050(2)i	0.225(9)i
	Peanut	Sample	6.25	40	0.175(7)i	0	0	0.175(7)i
			12.5	40	0.100(4)i	0.025(1)i	0.025(1)i	0.150(6)i
			25	40	0.200(8)i	0	0.025(1)i	0.225(9)i
	Red kidney	Sample	6.25	40	0.100(4)i	0	0	0.100(4)i
	Bean		12.5	40	0.075(3)i	0.050(2)i	0.025(1)i	0.150(6)i
			25	40	0.225(9)+	0.025(1)i	0.025(1)i	0.275(11)+
	Soybean	Sample	6.25	40	0.125(5)i	0	0.025(1)i	0.150(6)i
			12.5	40	0.150(6)i	0.025(1)i	0	0.175(7)i
			25	40	0.4(16)i	0.025(1)i	0.025(1)i	0.45(18)+

Table 23. Wing spots induction of the second extraction with methanol of seed coats (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)			
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)
2	Water	Negative	-	40	0.050(2)	0.050(2)	0	0.100(4)
	Urethane	Positive	-	40	7.025(281)+	4.85(194)+	1.775(71)+	13.650(546)+
	Black bean	Sample	6.25	40	0.075(3)i	0.050(2)i	0.025(1)i	0.150(6)i
			12.5	40	0.150(6)i	0.025(1)i	0	0.175(7)i
			25	40	0.175(7)i	0.025(1)i	0.025(1)i	0.225(9)i
	Mung bean	Sample	6.25	40	0.150(6)i	0.025(1)i	0	0.175(7)i
			12.5	40	0.125(5)i	0.050(2)i	0.050(2)i	0.225(9)i
			25	40	0.150(6)i	0.050(2)i	0.050(2)i	0.25(10)i
	Peanut	Sample	6.25	40	0.125(5)i	0.025(1)i	0	0.150(6)i
			12.5	40	0.125(5)i	0.025(1)i	0.025(1)i	0.175(7)i
			25	40	0.200(8)i	0	0.025(1)i	0.225(9)i
	Red kidney	Sample	6.25	40	0.125(5)i	0	0.025(1)i	0.150(6)i
	Bean		12.5	40	0.150(6)i	0.025(1)i	0.025(1)i	0.200(8)i
			25	40	0.200(8)i	0.025(1)i	0	0.225(9)i
	Soybean	Sample	6.25	40	0.100(4)i	0.050(2)i	0	0.150(6)i
			12.5	40	0.200(8)i	0.025(1)i	0	0.225(9)i
			25	40	0.275(11)+	0.050(2)i	0.025(1)i	0.35(14)+

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

4.4.2 Antimutagenicity of the Extracts of Legumes in SMART

Tables 24- 27 show the percentage of inhibition of the legume seed and seed coat extracts on mutagenicity induced by urethane in somatic mutation and recombination test.

Co-administration of the legume seed extracts with urethane reduced the mutagenic effects of 20 mM urethane. Increasing antimutagenic effect was evidently shown when the concentration of extract was increased (Figures 25 and 26).

The antimutagenicity of legume seeds extracted by acetone at the highest concentration (25 mg/tube) displayed moderate activities (40.6-57.2%) in trials 1 and 2. In trial 1, it was found that red kidney bean showed the highest antimutagenicity (57.2%), followed by peanut (54.0%). In trial 2, peanut exhibited the highest antimutagenicity (53.1%), followed by black bean (52.4%). Soybean possessed the lowest antimutagenicity in trial 1 (44.4%) and trial 2 (40.6%).

At the highest concentration (25 mg/tube), the antimutagenicity of legume seeds extracted by methanol exhibited weak to moderate activities (36.2-48.2%) in trials 1 and 2. In trial 1, it was found that black bean exhibited the highest antimutagenicity (48.2%), followed by soybean (44.7%). In trial 2, black bean also showed the highest antimutagenicity (45.3%), followed by soybean (40.5%). Red kidney bean possessed the lowest antimutagenicity in trial 1 (37.7%) and trial 2 (36.2%).

In the case of seed coats, the antimutagenicity of seed coats extracted by acetone at the lowest concentration (6.25 mg/tube) exhibited weak antimutagenic activity (6.2-38.8%) in trials 1 and 2. In trial 2, red kidney bean showed the highest antimutagenicity (38.8%), followed by soybean (37.3%). The antimutagenicity of seed coats extracted by methanol showed negligible activity. Moreover, at the higher concentrations (12.5 and 25 mg/tube), the extracts of all seed coats, except that of soybean, exhibited synergistic effect on the mutagenicity of urethane (Figures 27 and 28). Interestingly, the extracts of seed coats showed high phenolic content but had synergistic effect on the mutagenicity of urethane.

Table 24. Antimutagenicity (%inhibition) of the first extraction with acetone of legume seeds

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				%Inhibition
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water	Negative	-	40	0.075(3)	0.025(1)	0	0.100(4)	
	Urethane	Positive	-	40	10.125(405)+	6.925(277)+	2.525(101)+	19.575(783)+	
	Black bean	Sample	6.25	40	7.475(299)+	3.05(122)+	1.6(64)+	12.125(485)+	38.1
			12.5	40	6.25(250)+	2.775(111)+	1.3(52)+	10.325(413)+	47.3
			25	40	4.525(181)+	3.625(145)+	1.675(67)+	9.825(393)+	49.8
	Mung bean	Sample	6.25	40	8(320)+	2.125(85)+	1.35(54)+	11.475(459)+	41.4
			12.5	40	5.6(224)+	2.725(109)+	1.05(42)+	9.375(375)+	52.1
			25	40	5.275(211)+	3(120)+	1.5(60)+	9.775(391)+	50.1
	Peanut	Sample	6.25	40	6.05(242)+	2.75(110)+	1.375(55)+	10.175(407)+	48.0
			12.5	40	6.675(267)+	2.25(90)+	0.85(34)+	9.775(391)+	50.1
			25	40	5.025(201)+	2.575(103)+	1.4(56)+	9(360)+	54.0
	Red kidney	Sample	6.25	40	8.175(327)+	4.2(168)+	1.575(63)+	13.95(558)+	28.7
	Bean		12.5	40	4.3(172)+	3.625(145)+	1.625(65)+	9.55(382)+	51.2
			25	40	4.85(194)+	2.175(87)+	1.35(54)+	8.375(335)+	57.2
	Soybean	Sample	6.25	40	8.95(358)+	3.925(157)+	2.125(85)+	15(600)+	23.4
			12.5	40	7.25(290)+	4.175(167)+	1.85(74)+	13.275(531)+	32.2
			25	40	5.8(232)+	3.275(131)+	1.8(72)+	10.875(435)+	44.4

Table 24. Antimutagenicity (%inhibition) of the first extraction with acetone of legume seeds (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				%Inhibition
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
2	Water	Negative	-	40	0.175(7)	0.025(1)i	0	0.200(8)i	
	Urethane	Positive	-	40	9.45(378)+	4.45(178)+	2.475(99)+	16.375(655)+	
	Black bean	Sample	6.25	40	8.225(329)+	1.65(66)+	1.5(60)+	11.375(455)+	30.5
			12.5	40	4.65(186)+	2.075(83)+	1.425(57)+	8.15(326)+	50.2
			25	40	5.325(213)+	1.425(57)+	1.05(42)+	7.8(312)+	52.4
	Mung bean	Sample	6.25	40	5.65(226)+	2.9(116)+	1.9(76)+	10.45(418)+	36.2
			12.5	40	4.3(172)+	2.575(103)+	2.3(92)+	9.175(367)+	44.0
			25	40	3.95(158)+	2.475(99)+	1.375(55)+	7.8(312)+	52.4
	Peanut	Sample	6.25	40	5.8(232)+	2.225(89)+	1.775(71)+	9.8(392)+	40.2
			12.5	40	5.05(202)+	2.3(92)+	1.45(58)+	8.8(352)+	46.3
			25	40	3.85(154)+	2.225(89)+	1.6(64)+	7.675(307)+	53.1
	Red kidney	Sample	6.25	40	7.1(284)+	2.875(115)+	1.65(66)+	11.625(465)+	29.0
	Bean		12.5	40	5.575(223)+	2.375(95)+	1.4(56)+	9.35(374)+	42.9
			25	40	5.525(221)+	1.65(66)+	0.825(33)+	8(320)+	51.1
	Soybean	Sample	12.5	40	8.225(329)+	2.9(116)+	2.225(89)+	13.35(534)+	18.5
			25	40	6.95(278)+	2.1(84)+	0.975(39)+	10.025(401)+	38.8
			6.25	40	6.9(276)+	1.8(72)+	1.025(41)+	9.725(389)+	40.6

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with negative control:

+ = positive; - = negative; i= inconclusive; Propability level $\alpha=\beta=0.05$. One side statistical tests.

Table 25. Antimutagenicity (%inhibition) of the second extraction with methanol of legume seeds

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				% Inhibition
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water	Negative	-	40	0.150(6)	0	0	0.150(6)	
	Urethane	Positive	-	40	10.8(432)+	3.8(152)+	1.85(74)+	16.45(658)+	
	Black bean	Sample	6.25	40	5.8(232)+	3.25(130)+	1.825(73)+	10.875(435)+	33.9
			12.5	40	5.575(223)+	2.6(104)+	1.1(44)+	9.275(371)+	43.6
			25	40	4.175(167)+	3(120)+	1.35(54)+	8.525(341)+	48.2
	Mung bean	Sample	6.25	40	6.15(246)+	3.675(147)+	2.175(87)+	12(480)+	27.1
			12.5	40	5.5(220)+	3.1(124)+	1.65(66)+	10.25(410)+	37.7
			25	40	5.05(202)+	3.55(142)+	1.25(50)+	9.85(394)+	40.1
	Peanut	Sample	6.25	40	6.075(243)+	4.15(166)+	1.9(76)+	12.125(485)+	26.3
			12.5	40	7.775(311)+	2.575(103)+	1.35(54)+	11.7(468)+	28.9
			25	40	6.15(246)+	2.75(110)+	1.175(47)+	10.075(403)+	38.8
	Red kidney	Sample	6.25	40	7.575(303)+	3.725(149)+	2.025(81)+	13.325(533)+	19.0
	Bean		12.5	40	6(240)+	2.5(100)+	2(80)+	10.5(420)+	36.2
			25	40	6.4(256)+	2.8(112)+	1.05(42)+	10.25(410)+	37.7
	Soybean	Sample	6.25	40	9.7(388)+	3(120)+	1.375(55)+	14.075(563)+	14.4
			12.5	40	6.875(275)+	2.8(112)+	1.925(77)+	11.6(464)+	29.5
			25	40	5.1(204)+	2.45(98)+	1.55(62)+	9.1(364)+	44.7

Table 25. Antimutagenicity (%inhibition) of the second extraction with methanol of legume seeds (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				% Inhibition
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
2	Water	Negative	-	40	0.150(6)	0.025(1)	0	0.175(7)	
	Urethane	Positive	-	40	9.55(382)+	3.675(147)+	1.95(78)+	15.175(607)+	
	Black bean	Sample	6.25	40	6.2(248)+	3.05(122)+	1.425(57)+	10.675(427)+	29.7
			12.5	40	5.775(231)+	2.375(95)+	1.175(47)+	9.325(373)+	38.6
			25	40	5.175(207)+	1.4(56)+	1.725(69)+	8.3(332)+	45.3
	Mung bean	Sample	6.25	40	6.425(257)+	3.3(132)+	1.675(67)+	11.4(456)+	24.9
			12.5	40	5.475(219)+	2.85(114)+	1.45(58)+	9.775(391)+	35.6
			25	40	5.025(201)+	2.45(98)+	1.65(66)+	9.125(365)+	39.9
	Peanut	Sample	6.25	40	6.45(258)+	3.175(127)+	1.95(78)+	11.575(463)+	23.7
			12.5	40	5.875(235)+	2.7(108)+	1.725(69)+	10.3(412)+	32.1
			25	40	4.7(188)+	2.6(104)+	1.775(71)+	9.075(363)+	40.2
	Red kidney	Sample	6.25	40	6.875(275)+	3.125(125)+	1.85(74)+	11.85(474)+	21.9
	Bean		12.5	40	6.1(244)+	2.75(110)+	2.225(89)+	11.075(443)+	27.0
			25	40	5.3(212)+	2.575(103)+	1.8(72)+	9.675(387)+	36.2
	Soybean	Sample	6.25	40	7.175(287)+	3.8(152)+	2.35(94)+	13.325(533)+	12.2
			12.5	40	6.225(249)+	3.125(125)+	1.675(67)+	11.025(441)+	27.3
			25	40	3.35(134)+	3.65(146)+	2.025(81)+	9.025(361)+	40.5

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with negative control:

+ = positive; - = negative; i= inconclusive; Propability level $\alpha=\beta=0.05$. One side statistical tests.

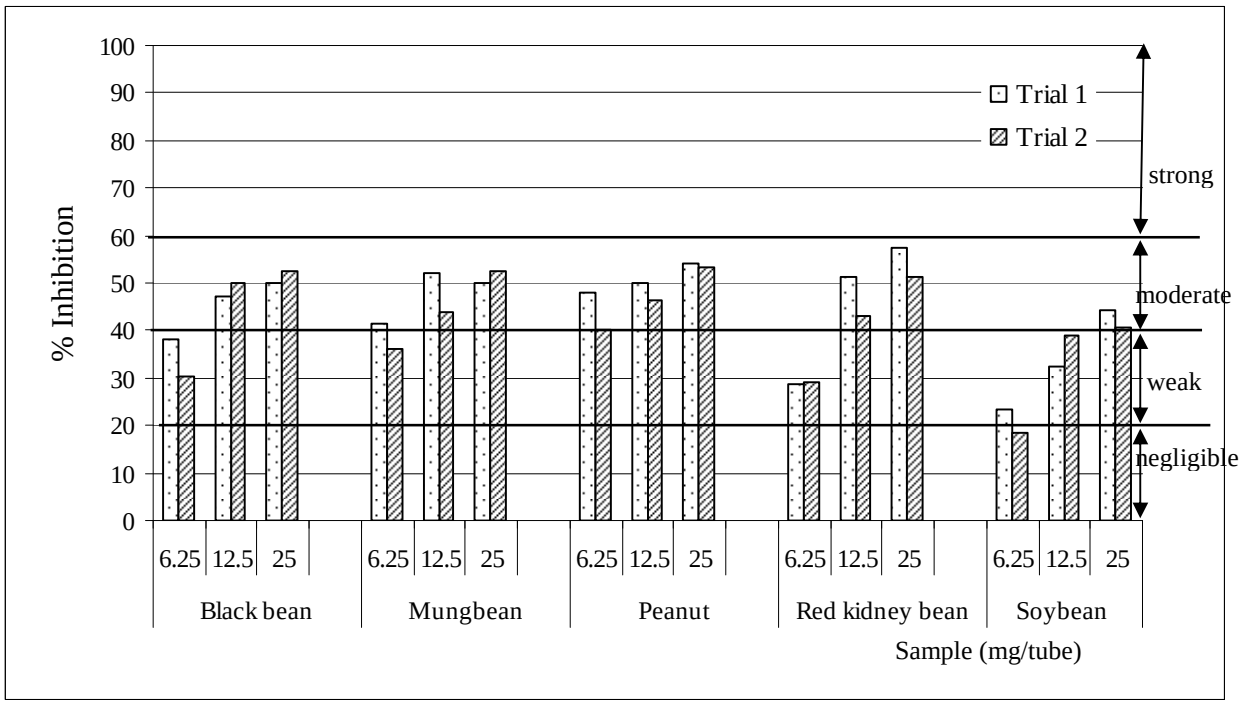


Figure 25. Percentage of inhibition of the first extraction with acetone of legume seeds

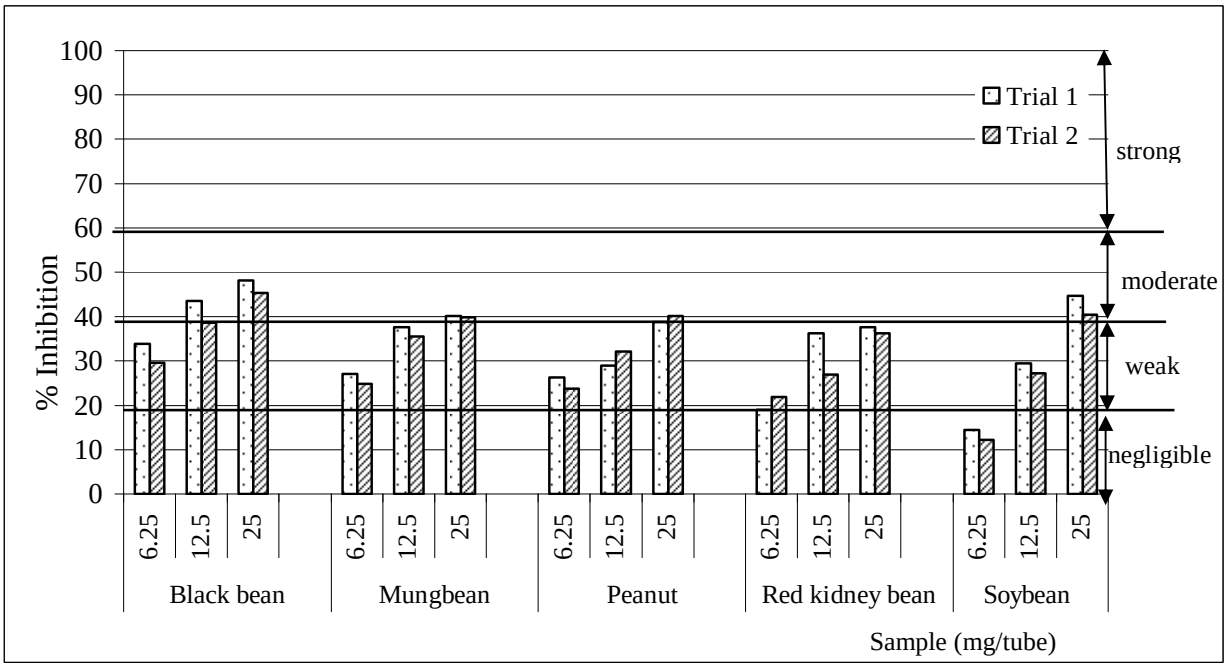


Figure 26. Percentage of inhibition of the second extraction with methanol of legume seeds

Table 26. Percentage of modification (inhibition or induction) of the first extraction with acetone of seed coats

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				%inhibition or induction (-)
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water	Negative	-	40	0.125(5)	0.05(2)	0.025(1)	0.2(8)	
	Urethane	Positive	-	40	4.575(183)+	4.6(184)+	1.275(51)+	10.45(418)+	
	Black bean	Sample	6.25	40	4.45(178)+	2.45(98)+	1.4(56)+	8.3(332)+	20.6
			12.5	40	4.125(165)+	2.225(89)+	1.775(71)+	8.125(325)+	22.2
			25	40	6.35(254)+	3.55(142)+	1.6(64)+	11.5(460)+	-10.0
	Mung bean	Sample	6.25	40	4.625(185)+	2.25(90)+	1.55(62)+	8.425(337)+	19.4
			12.5	40	4.425(177)+	3.925(157)+	1.85(74)+	10.2(408)+	2.4
			25	40	5.8(232)+	4.55(182)+	2.3(92)+	12.65(506)+	-21.1
	Peanut	Sample	6.25	40	4.1(164)+	3.2(128)+	1.55(62)+	8.85(354)+	15.3
			12.5	40	5.75(230)+	3.15(126)+	1.95(78)+	10.85(434)+	-3.8
			25	40	5.4(216)+	4.95(198)+	1.8(72)+	12.15(486)+	-16.3
	Red kidney	Sample	6.25	40	4.95(198)+	2.7(108)+	1.45(58)+	9.1(364)+	12.9
	Bean		12.5	40	6.4(256)+	2.7(126)+	1.45(78)+	11.5(460)+	-10.0
			25	40	6.6(264)+	3.45(138)+	2.15(86)+	12.2(488)+	-16.7
	Soybean	Sample	6.25	40	3.575(143)+	2.275(91)+	0.9(36)+	6.75(270)+	35.4
			12.5	40	7.1(284)+	2.7(108)+	1.875(75)+	11.675(467)+	-11.7
			25	40	7.7(308)+	3.3(132)+	1.95(78)+	12.95(518)+	-23.9

Table 26. Percentage of modification (inhibition or induction) of the first extraction with acetone of seed coats (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				%inhibition or induction (-)
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
2	Water	Negative	-	40	0.15(6)	0.025(1)	0.025(1)	0.2(8)	
	Urethane	Positive	-	40	6.55(262)+	2.275(91)+	0.9(36)+	9.725(389)+	
	Black bean	Sample	6.25	40	5.05(202)+	2.125(85)+	0.725(29)+	7.9(316)+	18.8
			12.5	40	5.025(201)+	1.55(62)+	0.825(33)+	7.4(296)+	23.9
			25	40	7.85(314)+	2.6(104)+	1.225(49)+	11.675(467)+	-20.1
	Mung bean	Sample	6.25	40	4.95(198)+	2.325(93)+	1.15(46)+	8.425(337)+	13.4
			12.5	40	6.475(259)+	1.95(78)+	0.925(37)+	9.35(374)+	3.9
			25	40	7.35(294)+	2.525(101)+	1.45(58)+	11.325(453)+	-16.5
	Peanut	Sample	6.25	40	5.9(236)+	2.025(81)+	1.2(48)+	9.125(365)+	6.2
			12.5	40	8.3(332)+	2.3(92)+	1.3(52)+	11.9(476)+	-22.4
			25	40	9.025(361)+	2.575(103)+	1.25(50)+	12.85(514)+	-32.1
	Red kidney	Sample	6.25	40	4.225(169)+	1.075(43)+	0.65(26)+	5.95(238)+	38.8
	Bean		12.5	40	5.775(231)+	2.85(114)+	1.9(76)+	10.525(421)+	-8.2
			25	40	6.475(259)+	3.5(140)+	2.05(82)+	12.025(481)+	-23.7
	Soybean	Sample	6.25	40	3.2(128)+	1.95(78)+	0.95(38)+	6.1(244)+	37.3
			12.5	40	5.45(218)+	2.7(108)+	2.425(97)+	10.575(423)+	-8.7
			25	40	5.6(224)+	3.975(159)+	1.95(78)+	11.525(461)+	-18.5

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with negative control:

+ = positive; - = negative; i= inconclusive; Propability level $\alpha=\beta=0.05$. One side statistical tests.

Table 27. Percentage of modification (inhibition or induction) of the second extraction with methanol of seed coats

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				%inhibition or induction (-)
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
1	Water	Negative	-	40	0.125(5)	0.05(2)	0.025(1)	0.2(8)	
	Urethane	Positive	-	40	4.575(183)+	4.6(184)+	1.275(51)+	10.45(418)+	
	Black bean	Sample	6.25	40	4.225(169)+	3.45(138)+	1.65(66)+	9.325(373)+	10.8
			12.5	40	5.85(234)+	3.95(158)+	2.85(114)+	12.65(506)+	-21.1
			25	40	6.95(278)+	4.5(180)+	2.65(106)+	14.1(564)+	-34.9
	Mung bean	Sample	6.25	40	5.225(209)+	4.05(162)+	1.7(68)+	10.975(439)+	-5.0
			12.5	40	7.625(305)+	3.1(124)+	1.275(51)+	12(480)+	-14.8
			25	40	5.675(227)+	4.075(163)+	1.725(69)+	13.2(528)+	-26.3
	Peanut	Sample	6.25	40	4.75(190)+	2.4(96)+	1.05(42)+	8.2(328)+	21.5
			12.5	40	6.45(258)+	3.5(140)+	1.7(68)+	11.65(466)+	-11.5
			25	40	7.35(294)+	3.3(132)+	1.9(76)+	12.55(502)+	-20.1
	Red kidney	Sample	6.25	40	4.9(196)+	2.75(110)+	1.5(60)+	9.15(366)+	12.4
	Bean		12.5	40	5.8(232)+	3.7(148)+	1.35(54)+	10.85(434)+	-3.8
			25	40	5.15(206)+	5.2(208)+	1.7(68)+	12.05(482)+	-15.3
	Soybean	Sample	6.25	40	4.875(195)+	3.675(147)+	1.325(53)+	9.875(395)+	5.5
			12.5	40	4.275(171)+	3.575(143)+	1.625(65)+	9.475(379)+	9.3
			25	40	4.05(162)+	3.25(130)+	1.8(72)+	9.1(364)+	12.9

Table 27. Percentage of modification (inhibition or induction) of the second extraction with methanol of seed coats (continued)

Trial	Sample	Type of media	Amount of extract (mg/tube)	No. of wings	Spots per wing[a] (no. of spots)				%inhibition or induction (-)
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m=2)	
2	Water	Negative	-	40	0.175(7)	0.075(3)	0(0)	0.25(10)	
	Urethane	Positive	-	40	5.875(235) +	6.225(249) +	2.6(104) +	14.7(588) +	
	Black bean	Sample	6.25	40	6.4(256) +	4.625(185) +	2.15(86) +	13.175(527) +	10.4
			12.5	40	8.3(332) +	6.05(242) +	2.925(117) +	17.275(691) +	-17.5
			25	40	8.975(359) +	6.775(271) +	3.425(137) +	19.175(767) +	-30.4
	Mung bean	Sample	6.25	40	7.475(299) +	5.375(215) +	2.45(98) +	15.3(612) +	-4.1
			12.5	40	7.85(314) +	5.35(214) +	3.05(122) +	16.25(650) +	-10.5
			25	40	8.725(349) +	5.85(234) +	3.325(133) +	17.9(716) +	-21.8
	Peanut	Sample	6.25	40	5.175(207) +	4.475(179) +	2.55(102) +	12.2(488) +	17.0
			12.5	40	8.4(336) +	4.925(197) +	2.425(97) +	15.75(630) +	-7.1
			25	40	8.8(352) +	5.525(221) +	2.525(101) +	16.85(674) +	-14.6
	Red kidney	Sample	6.25	40	5.025(201) +	5.95(238) +	2.025(81) +	13(520) +	11.6
	Bean		12.5	40	6.175(247) +	6.325(253) +	2.575(103) +	15.075(603) +	-2.6
			25	40	9.05(362) +	5.25(210) +	2.425(97) +	16.725(669) +	-13.8
	Soybean	Sample	6.25	40	5.3(212) +	5.5(220) +	3.2(128) +	14(560) +	4.8
			12.5	40	6(240) +	5.125(205) +	2.1(84) +	13.225(529) +	10.0
			25	40	3.675(147) +	5.425(217) +	2.7(108) +	11.8(472) +	19.7

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with negative control:

+ = positive; - = negative; i= inconclusive; Propability level $\alpha=\beta=0.05$. One side statistical tests.

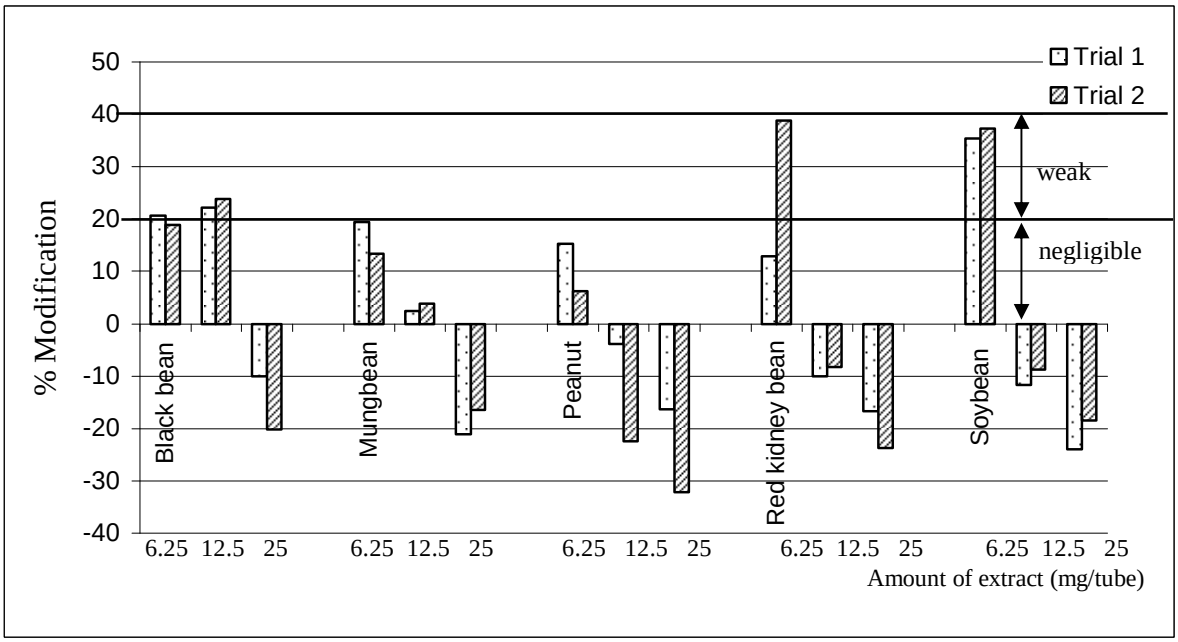


Figure 27. Percentage of modification (inhibition or induction) of the first extraction with acetone of seed coats

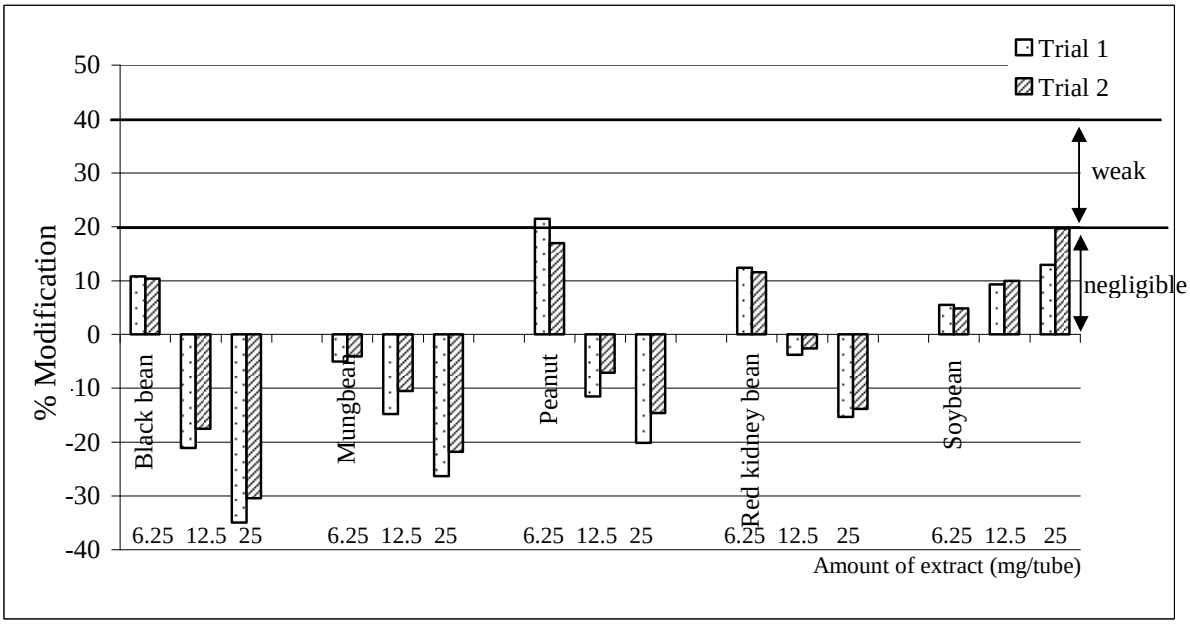


Figure 28. Percentage of modification (inhibition or induction) of the second extraction with methanol of seed coats

CHAPTER V

DISCUSSION

There are some evidences suggesting that free radicals cause oxidative damage to DNA, proteins, and lipids and contribute significantly to aging and degenerative diseases such as cancer, immune system decline, brain dysfunction (e.g. Alzheimer's disease), and cataracts (Droge, 2002). Antioxidants are compounds that have the ability to scavenge reactive oxygen species. They can act as chain breakers to stop the propagation of sequential free radical reactions and thereby reduce damage to DNA and membrane. Phenolic antioxidants represent an important group of bioactive compounds in foods (Formica and Regelson, 1995; Kahkonen *et al*., 1999). They also act as protective factors against oxidative damage (Castillo *et al*., 2000; Kikuzaki *et al*., 2002) and have antimutagenic activity (Standley *et al*., 2001).

5.1 Total Phenolic and Total Flavonoid Contents

In this study, two-step extraction was performed using 70% acetone for the first extraction and 50% methanol as the second extraction. As a result, most of the phenolic compounds including flavonoids were extracted with 70% acetone. The second extraction with 50% methanol could increase the extractability of phenolic compounds. According to a previous study, methanol has been proven to be the best solvent for low molecular weight components, such as (+)-catechin, (-)-epicatechin and epigallocatechin, while 70% acetone is the most potent solvent for proanthocyanidins including polymers from grape seeds (Kallithraka *et al*., 1995; Prior and Gu, 2005).

The total phenolic content in the extracts was determined by the Folin–Ciocalteu method. This method roughly indicates numbers of phenolic hydroxyl groups in the extracts (Singleton and Rossi, 1965). This study found that the extracts of seed coats contained more phenolic content and antioxidant activity than those of legume seeds. According to Desphande *et al*. (1982) and Gonzalez de Mejıa *et al*. (1999), phenolic compounds are mainly located in the seed coats. In general, seed coats may play an important role in chemical protection from oxidative damage by possessing endogenous antioxidants such as phenolic compounds to protect inner materials.

In the present study, the relationship between seed coats color and phenolic contents was observed. The darker color of the seed coat extracts, such as black bean, peanut and red kidney bean had greater phenolic contents (> 450 mg GAE/g dry extract) than the lighter color of the seed coat extracts such as soybean. In support of this observation, Boateng *et al.* (2008) found that beans with darker seed coats (red kidney bean and pinto bean) had significantly ($p< 0.05$) higher level of total phenolics compared to those with lighter seed coats (black-eyed pea and soybean). Takahashi *et al.* (2005) reported that black soybean contained higher polyphenolic content than yellow soybean. Feenstra (1960) indicated that the seed coats color was determined by the amount of phenolic compounds such as flavonol glycosides, condensed tannins (procyanidins) and anthocyanidins.

Flavonoids are the most widely distributed group of phenolic compounds in legumes. This study found that the acetone extracts of raw seed coats of red kidney bean and peanut contained very high level of flavonoid contents. Beninger and Hosfield (2003) found the high anthocyanin contents in the red kidney beans. Lou *et al*. (2001) reported there were eight flavonoids were separated from the water soluble fraction of peanut skins.

The difference between total phenolic content and total flavonoid content of the extracts of legumes indicated that the major compounds of the phenolics are non-flavonoid compounds (phenolic acids i.e. gallic acid, ellagic acid, ferulic acid etc.).

5.2 The Effect of Heat Treatment on Total Phenolic Content

Legumes are excellent sources of protein, carbohydrates, dietary fiber, lipids, a variety of micronutrients and phytochemicals. However, they must be processed before consumption due to their nonnutritive compounds, such as trypsin inhibitors and phytic acid (Agustin *et al*., 1989; Vidal-Valverde *et al*., 2002). After heat treatment (autoclaving), most of extracts of legume seeds, except those of mungbean, red kidney bean and soybean were shown to have higher level of total phenolic content. On the other hand, all of extracts of legume seeds, except that of peanut had unchanged levels of total flavonoid contents (Figure 16). In addition, the extracts of processed seed coats exhibited higher level of phenolic content than that of the extracts of raw seed coats. Thus, the cooking processes can change the physical characteristics and chemical composition of legumes. Previous studies performed on different cooked vegetables showed that the total phenolic content and antioxidant capacity could be either higher or lower in comparison to the fresh samples (Ismail *et al*., 2004; Lombard *et al*., 2005; Turkmen *et al*., 2005). Food processing, like

cutting of the vegetable tissue including exposure to high temperature, could lead to cellular disruption and disassociation of some phenolic compounds from cellular structures such as lignin and polysaccharides (Bernhart and Schlich, 2005). Moreover, the alteration in their chemical composition could make them more extractable, causing them to be more readily detected in the supernatant of the extractable polyphenols (Cohen *et al*., 2001). Additionally, during heating, maillard reaction products might contribute to the increase of phenolic like complex that contributed to higher absorbance reading (Yu *et al*., 2005).

Likewise, heat treatment could also affect the level of total flavonoid content. The result confirmed that the total flavonoid content of the processed seed coat (mung bean, peanut and red kidney bean) were significantly lower than that of the extracts of raw samples. Soong and Barlow (2004) previously explained that the heat treatment could possibly affect the stability of some flavonoid compounds due to chemical and enzymatic decomposition. Prior and Gu (2005) found that proanthocyanidins (flavonoids) in fresh plums and grapes were degraded during the drying processing.

5.3 Antioxidant Activity of Extracts of Legumes

In this study, the antioxidant activity of the legumes extracts was investigated using DPPH free radical scavenging assay and FRAP (ferric reducing antioxidant power) assay. The mechanisms of action of DPPH and FRAP are different, i.e. scavenging of DPPH radicals by providing hydrogen atoms or by electron donation in the DPPH assay and reduction of ferric ion in the FRAP assay.

The legume seeds extract from black bean had the highest activity in scavenging of DPPH radicals. According to Huang *et al*. (1983), they found high anthocyanins content in the black beans. Anthocyanins have been reported to contribute greatly to the antioxidant properties. The acetone extract of mung bean also displayed high activity in scavenging of DPPH radicals. Duh *et al*. (1999) found that the extract of mung bean exhibited antioxidant activity by inhibiting lipid peroxidation in a liposome model system.

In the case of seed coats, the extracts of black bean, peanut and red kidney bean, which contain high phenolic contents, have a strong antioxidant activity. Phenolics have been reported to have higher antioxidant activities, as compared to the common antioxidants, vitamin C and vitamin E in the *in vitro* system (Rice-Evans *et al*., 1997; Scott *et al*., 1993). The results of this study showed that the extraction of peanut seed coats by both solvents at 1 mg/ml had very high values of DPPH scavenging activity (90.0%).

Resveratrol, a phytochemical found in grape seed and wine, was also found in peanut skin in much higher concentration than that in peanut kernels (Sanders *et al*., 2000). From previous study, Fremont (2000) found that resveratrol had free radical scavenging capacity. An earlier study by Lou *et al*. (2004) showed that the proanthocyanidins in peanut skins had free radical-scavenging effects, which could protect the seed fatty residue from oxidation. Moreover, Talcott *et al*. (2005b) found that free *p*-coumaric acid, *p*-coumaric acid ester, *p*-hydroxybenzoic acid, and tryptophan were the predominant soluble polyphenolic that likely contributed to the antioxidant capacity of peanuts.

Although the methanol extracts of seed coat showed high DPPH scavenging effect, lower phenolic content was also observed. The phenolic content in the extracts might be high enough to scavenge DPPH radical in the reaction. Furthermore, it is likely that other phytochemicals other than phenolics in the extracts may potentially play a role in DPPH scavenging effect. In addition, results from FRAP assay suggested that the sample extracts containing high phenolic contents had excellent reducing power and could reduce ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex. This result confirmed the result reported by Rice-Evans *et al*. (1997) that phenolic compounds exhibited redox properties, (i.e. act as reducing agents, hydrogen donators and singlet oxygen quenchers). The redox potential of phenolic phytochemicals plays a crucial role in determining the antioxidant properties.

To study effect of heat treatment, legumes were autoclaved at 121 ^{o}C for 20 min. The processed legume seeds revealed lower antioxidant activities (lower scavenging effect and reducing power) than those of the raw legume seeds, which is correlated with their total phenolic content. For seed coats, the results suggested that the decrease of antioxidant activities of the extracts was correlated with the decrease in flavonoid content rather than the increase of total phenolic content. The increase of total phenolic content might be due to the increase of non-flavonoid compounds from the breakdown of cellular constituents. Baderschneider and Winterhalter (2001) found that non-flavonoid phenolic compounds such as hydroxybenzoics and hydroxycinnamics of lentils and peas were attributed less antioxidant activity than flavonoids. Interestingly, heat treated legume seeds and seed coats still retained high antioxidant activity. This finding indicated that the cooking of legume seeds before consumption or seed coat by-product of legume industry, which commonly removed by roasting, could represent an inexpensive source of natural antioxidants.

5.4 Antigenotoxicity of the Extracts of Legumes

5.4.1 Protective Effect of the Extracts of Legumes on DNA Damage Induced by H_2O_2 in Comet Assay

The exposure of lymphocytes to the extracts at the cytotoxic concentrations of legume seeds (≥ 10 μg/ml) or seed coats (≥ 5 μg/ml) induced DNA damage as compared to the scores of DNA damage (arbitrary units) of the negative control (water). It was possible to see a relationship between cytotoxicity and genotoxicity determined in the comet assay. Other authors had already postulated the importance of performing comet assay at non-cytotoxic concentrations for a better evaluation of DNA damage (Hartman and Speit, 1997).

To determine antigenotoxicity, this study used 20 μM H_2O_2 to induce DNA damage in human lymphocytes. Although H_2O_2 is a relatively stable oxidant, it is believed to cause DNA strand breaks after conversion to the hydroxyl radical by a Fenton reaction, as shown in the following: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$. This may result in DNA instability, mutagenesis and ultimately carcinogenesis (Mello Filho *et al*., 1984; Meneghini, 1988; Schraufstatter *et al*., 1988; Halliwell and Aruoma, 1991). Fe is a common chemical element of cells, and is essential for organisms as a co-factor in oxygen transport.

However, the DNA damage in human lymphocytes induced by H_2O_2 was decreased by pre-treating the cells with the extracts of legumes. At the concentration of 1 μg/ml, the extracts of processed seed coats which contain high phenolic contents showed higher inhibitory effect of DNA damage than those of the raw seed coat extracts. Several works have reported that phenolics exhibited antioxidant activity and antigenotoxic effect by attributing to scavenging of reactive oxygen species. Ruch *et al*. (1984) proposed that H_2O_2 might be scavenged by phenolics, which could donate electrons to H_2O_2 and neutralize H_2O_2 to water. Greenrod and Fenech (2003) reported that phenolic components of wine, such as catechin or caffeic acid were protective against DNA damage and cytotoxic effects of H_2O_2 in plasma or blood. Lopes *et al*. (1999) and Chen *et al.* (2001) showed that some phenolics (tannic acid and its related compounds) inhibit hydroxyl radical formation from Fenton reaction by complexing ferrous ions. Moreover, several reports found some phenolic compounds in legumes such as anthocyanins in black beans (Tsuda *et al*., 1994; Lazze *et al*., 2003) or resveratrol (Fremont, 2000) and procyanidins (Karchesy and Hemingway, 1988; Lou *et al*., 1999) in peanut. These phenolics might be the compounds responsible for inhibition of DNA damage in human lymphocytes induced by H_2O_2.

Although total phenolic content of the extract of seed coat of soybean was very low, the inhibitory effect of the extract on DNA damage induced by H_2O_2 showed high potency. This result demonstrated that non-phenolic compounds in soybean seed coats could neutralize H_2O_2 or other free radicals formed in the system. In addition, Maillard reaction products might be generated during the heat treatment. According to Yilmaz and Toledo (2005), during heat treatment, the brown color of peanut increases due primarily to sugar–amino acid reactions with subsequent production of melanoidins. Maillard reaction products especially melanoidins, possess antioxidant capacity through scavenging oxygen radicals (Yilmaz and Toledo, 2005).

5.4.2 Antimutagenicity of the extracts in SMART

Antioxidants are known to have an inhibitory effect on genotoxic action of several known mutagens. It is also well known that antimutagenic effects are often specific to certain classes of mutagen and/or certain test systems (Stich and Rosin, 1984). In the present study, urethane, a well known genotoxic carcinogen was used for inducing mutations. It is found in very small quantities in several fermented foods and beverages such as stone-fruit brandies, sherries and table wines (Schlatter and Luitz, 1990).

The result from this study indicated that the extracts of legume seeds had the protective effects against *in vivo* induction of somatic mutation and mitotic recombination by urethane in dose-response manner. The antimutagenicity of legume seeds extracted by acetone showed moderate activities (40.6-57.2%) at the highest concentration. These findings would be of interest to know whether there is involvement of possible mechanisms of antimutagenicity. As urethane was co-administered with the extracts of legume seeds, complex formation between constituents of the extracts and urethane or its metabolites was a possible mechanism leading to detoxification. In addition, the presence of phenolics in the extracts may be responsible for mutagenicity of urethane. Prochaska and Talahay (1988) reported that polyphenols may induce phase II detoxification enzymes such as glutathione transferase (GST) that will enhance the excretion of mutagens. Polyphenols may also inhibit specific cytochrome P450s (CYPs), which in turn leads to protect against mutagenesis by decreasing the metabolic activation of urethane (Abraham and Graf, 1996). According to Huang *et al*., 1983, some polyphenols (tannins and catechins) could inhibit activities of cytochrome P-450-dependent monooxygenase.

Approximately 0.1% of urethane was reported to be able to convert into N-hydroxyurethane (Boyland and Nery, 1965; Nery, 1968). N-hydroxyurethane is then hydrolysed by esterase to generate hydroxylamine and exert its carcinogenic effect in multiple organs via generating O_2^- and $NO^{\cdot}$ to cause oxidation and depurination of DNA (Sakano *et al*., 2002). Thus, it is possible that antioxidant activity of the extracts of legume seeds might scavenge O_2^- and $NO^{\cdot}$ in urethane metabolism. Ferguson *et al*. (2004) suggested that antioxidant could scavenge free radicals and prevent their interactions with cellular DNA. On the other hand, co-administration of the extracts of seed coats at the higher concentrations (12.5 and 25 mg/tube) with urethane increased the mutagenic effects of urethane. It is quite possible that the extracts of seed coats which have an abundance of phenolic compounds (flavonoids) exhibit prooxidant activity. The phenolic compounds can both behave as antioxidants and prooxidants depending on their concentration. Moreover, Virgilio and co-worker (2004) found that uptake of complex plant-derived extracts may modulate the genotoxicity of one flavonoid by the anti-genotoxic capacity of other flavonoids.

Many flavonoids induce genetic damage in a variety of prokaryotic and eukaryotic systems (Stopper *et al*., 2005; Silva *et al*., 2000; Boos and Stopper, 2000; Yamashita and Kawanishi, 2000). A number of polyphenols, including quercetin, can bind to DNA (Alvi *et al*., 1986) and this direct interaction may be an important mechanism of mutagenicity. Several classes of plant derived antioxidant polyphenols also exhibit oxidative DNA damage particularly in the presence of transition metal ions such as copper. Tannic acid (TA) produced a weak positive response in SMART. In the presence of Cu (II), tannic acid causes DNA degradation through generation of reactive oxygen species such as hydroxyl radicals (Cunha *et al*., 1994). Ahma *et al*. (2005) found that DNA damage by resveratrol–Cu(II) occurs by both Haber Weiss reaction (a) and Fenton reaction (b).

(a) $O2^{\cdot -} + H_2O_2 = O_2 + OH^{\cdot} + OH^-$ (Haber Weiss)

(b) $H_2O_2 + Cu\ (I) = {}^{\cdot}OH + OH^- + Cu\ (II)$ (Fenton reaction).

Superoxide is formed by polyphenols by reducing molecular oxygen. The addition of a second electron to the superoxide anion gives peroxide ion. At neutral pH, the peroxide ion protonates to form H_2O_2. Thus, H_2O_2 can immediately take part in both Haber Weiss and Fenton-type $OH\cdot$ formation and DNA cleavage reaction. Further, Cu(II) can be reduced to Cu(I) by resveratrol and it is the re-oxidation of Cu(I) to Cu(II) in the ternary complex which gives rise to $OH\cdot$ (Rahman *et al*., 1990).

These finding suggested that antioxidant and antigenotoxic potential of phenolic compounds and other phytochemicals of legume seeds and their seed coats may play an important role in reducing the risks of diseases associated with oxidative DNA damage including cancer. However, the antioxidants/ pro-oxidants activity or anti-mutagenic/co-mutagenic activity largely depends upon the amount of phenolics (Christine and Smith, 2000).

CHAPTER VI

CONCLUSION

The data obtained from antioxidant assay methods (DPPH scavenging activity and FRAP assay) revealed that the degree of antioxidant activity of the extracts may be attributed to the extent of phenolics content. The extracts of seed coats, especially peanut, black bean and red kidney bean, have higher ferric reducing power and DPPH scavenging activity than the mung bean and soybean due to the existence of high amount of phenolic compounds such as proanthocyanidins and flavonol which could be contributing to the antioxidant activity.

The present investigation suggested that none of the extracts of samples at non-cytotoxic concentrations was genotoxin in comet assay. All the extracts of seed coats decreased the DNA fragmentation in human lymphocytes induced by H_2O_2. High content of phenolic compounds in processed seed coats of black bean, peanut and red kidney bean exhibited strong inhibition of DNA fragmentation. Working with the *Drosophila*, the extracts of legume seeds contained lower content of phenolics than the extracts of seed coats, showed as appropriate phenolics content for inhibiting mutagenicity of urethane in SMART. Phenolics in the extracts may induce phase II detoxification enzymes that will enhance the excretion of mutagens. In addition, polyphenols may also inhibit specific cytochrome P450s (CYPs), which in turn leads to protect against mutagenesis by decreasing the metabolic activation of urethane**.** However, the data also show that higher concentration of the seed coat extracts exhibited synergistic effect on the mutagenicity of urethane. Under certain conditions, e.g. a high content of phenolics in the presence of redox-active metals (copper, iron) may behave as pro-oxidants. Based on this study results, intake of concentrated phenolics should not be supported, but food and beverage containing high amount of phenolics remains an important contribution to health benefit.

Heating is used to remove seed coats in the legume manufacture. This study found that heat processing could increase the content of phenolic compounds due to dissociation of some phenolic compounds from cell wall. By-products of legume manufacture in Thailand consist of mung bean, peanut and soybean. In these seed coats by-products, the results indicated that the extracts of seed coats of mung bean and peanut showed high

antioxidant activity, whereas the high inhibition on DNA break induced by H_2O_2 was observed in the extracts of seed coats of peanut and soybean. Therefore, all these by-products could provide inexpensive source of natural antioxidants and antigenotoxic compounds.

In addition, the legume seeds must be processed before consumption. Heating has slightly deleterious effect on antioxidant activity. The antioxidant activity of the extracts of processed legume seeds was in the order of black bean> mung bean = peanut> red kidney bean> soybean. Moreover, all tested legume seeds exhibited moderate inhibition on mutagenicity induced by urethane and showed weak inhibition on DNA break induced by H_2O_2.

Therfore, the results indicate that intake of legume-derived phenolics and other phytochemical components in our daily foods and berverage may reduce oxidative damage and protect against mutagenicity of some mutagens. Seed coats of mung bean, peanut and soybean which are the by-products of bean manufacturing in Thailand may develop value-added products with beneficial-health effects.

Further Study

The results obtained from this study encourage further investigation to identify and quantify phenolic compounds in seed coats. Their antioxidant activities in biological systems are needed for better understanding of their mechanism of action. Furthermore, if seed coats phenolics are used as dietary supplements, the safety and potential toxic effects have to be distinguished in detail and extensively studied.

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APPENDICES

APPENDIX A

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

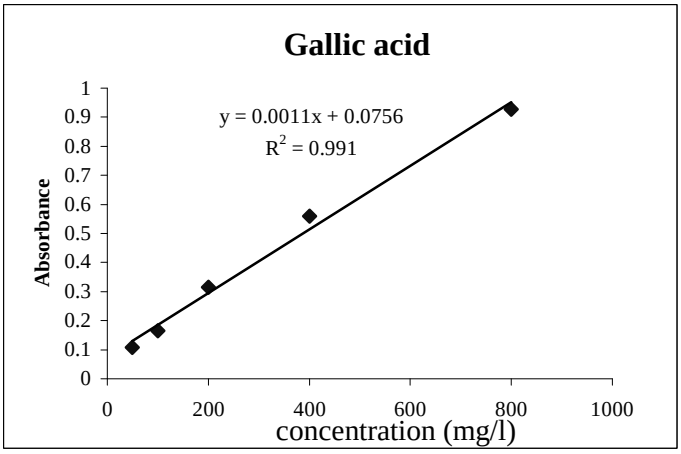
1. Folin-Ciocalteu reagent
2. Saturated sodium carbonate solution
3. 800 mg/l Gallic acid

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

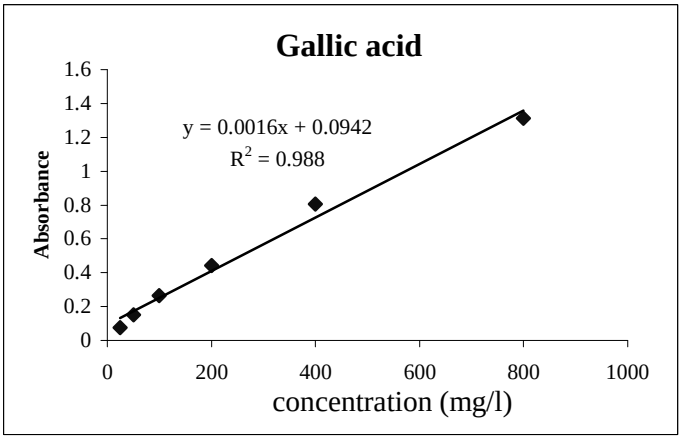
Standard curve for total phenolic and total flavonoid contents and FRAP assay

1. Total phenolic content

1.1. Standard curve for legume seeds

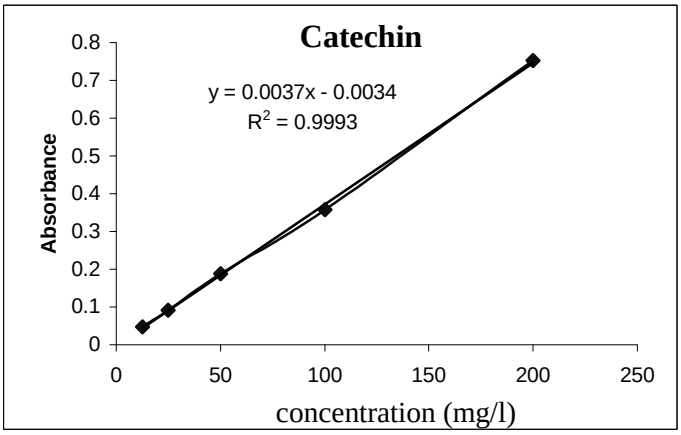


1.2. Standard curve for seed coat

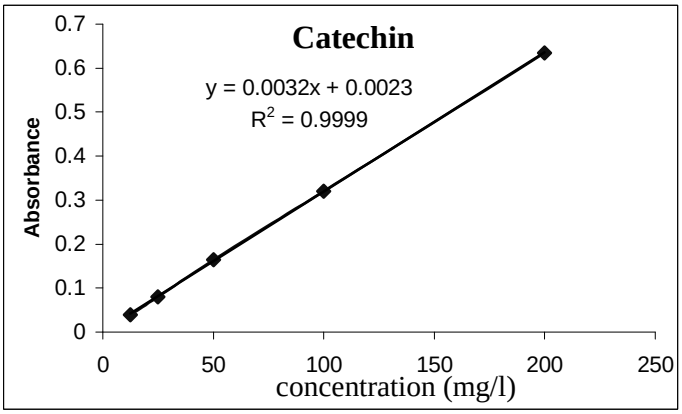


2. Total flavonoid content

2.1. Standard curve for legume seeds

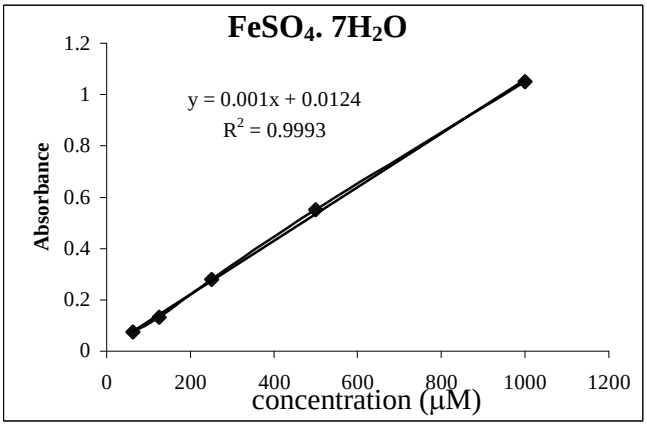


2.2. Standard curve for seed coat

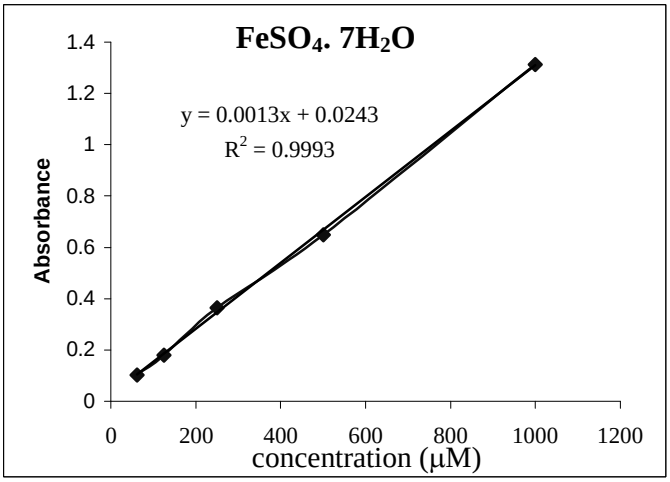


3. **FRAP assay**

3.1. Standard curve for legume seeds



3.2. Standard curve for seed coat



APPENDIX B

PREPARATION OF REAGENTS FOR COMET ASSAY

1. Preparation of slides

1.1 Prepare 1.0% Normal melting agarose (NMA 500 mg per 50 ml in PBS)

1.2 Prepare 0.8% Low melting agarose (LMA 240 mg per 30 ml in PBS)

Microwave until near boiling and the agarose dissolves. While NMA agarose is hot, dip slides up and wipe underside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried. Store the slides at room temperature until needed. The slides generally prepare the day before use.

2. Lysing Solution: (equivalent to 2 liters in the final preparation with the addition of 1% Triton X-100 and 10% DMSO)

Ingredients per 2000 ml:

2.5 M NaCl (292.2 g)

100 mM $Na_2EDTA.2H_2O$ (74.4 g)

10 mM Trizma base (2.4 g)

3. Electrophoresis Buffer (300 mM NaOH / 1 mM EDTA):

Prepare from stock solutions:

10 N NaOH (200 g/500 ml distilled water)

200 mM EDTA (14.89 g/200 ml distilled water, pH 10)

Store both at room temperature. For 1X Buffer (made fresh before each electrophoresis run): per liter, add 30 ml NaOH and 5.0 ml EDTA, adjust volume to 1000 ml, mix well. Prior to use, measure the pH of the buffer to ensure >13.

4. Neutralization Buffer

0.4 M Tris

48.5 g of Tris added to 800 ml distilled water, adjust pH to 7.5 with concentrated (>10 M) HCl: adjust volume to 1000 ml with distilled water, store at room temperature.

5. Fixation solution

15% trichloroacetic acid, 5% zinc sulphate heptahydrate, 5% glycerol

6. Staining solution

Freshly prepared in the dark

100 ml of stain solution composed by 34 ml of vigorously mixed stock solution B and 66 ml of stock solution A

Stock solution A: 5% sodium carbonate

Stock solution B: 0.05% ammonium nitrate

0.05% silver nitrate

0.125% tungstosilicic acid

0.075% formaldehyde

The solution B was prepared from convenient stocks solutions of ammonium nitrate, silver nitrate and tungstosilicic acid. These stocks solutions can be kept at 4 ^{o}C for several weeks.

7. Stop Solution

1% acetic acid solution

APPENDIX C
STATISTICAL CONSIDERATION

The wing spots data was evaluated using the statistical procedure described by Frei and Wurgler (1988). 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_1 and p_u one-sidedly at the level α, and q_u and p_1 also one-sidedly at the level β. In this way and in agreement with the foregoing section, a confidence limit $m_1 > 1$ led to rejection of H_o, while a confidence limit $m_u < m$ led to rejection of H_A.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

$$q_u n = (1-p_1) n$$

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.

BIOGRAPHY

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