CHAPTER 1

Introduction

1.1 Statement of the problem

Cancer-causing death is the prominent global problem and has been concerned about its prospective outcome. In Asian countries, the incidence of liver cancer is the third and sixth ranks in male and female, respectively (Ferlay, 2010). The risk factors of liver cancer include viral hepatitis B and C infections, alcohol consumption, smoking as well as ingestion of aflatoxin B_1 contaminated food. Recently, liver operation, transplantation, radiation and chemotherapeutic drugs are used for treatment of liver cancer. However, these provide some disadvantages such as side effects, high cost and specificity of use. Due to cancer is a preventable disease, the preventive strategies including vaccination, life style adjustment and chemoprevention using natural products have been concerned.

The consumption of fruits and vegetable containing chemopreventive agents has been achieved in cancer prevention. Intake of cruciferous vegetables such as broccoli, cauliflower and cabbage, is able to lower risk of bladder, prostate and lung cancer (Michaud, 1999; Cohen, 2000; London, 2000). High consumption of tomatoes or tomato products can prevent prostate cancer (Chen, 2013). Likewise, blueberries have potential to reduce liver cancer (Weiguang, 2006). The action of these cancer preventive natural products could work through scavenging reactive oxygen species in cells, induction of detoxifying system, inhibition of cell mutation and proliferation or induction of cancer cell death (Pratheeshkumar, 2012; Doraia and Aggarwal, 2014).

Rice (*Oryza sativa* var. *indica*) is a staple food in Asia including Thailand. Pigmented rice contains higher phytochemical contents and some biological activities than non-colored rice (Chakuton, 2012; Deng, 2013). The rich-anthocyanins in purple rice have been reported about various health benefits such as antioxidant activity, plasma lipid-lowering properties, anti-allergic activity, anti-cancer cell invasion ability and

immunomodulating activity (Ichikawa, 2001; Chen, 2006; Choi, 2007; Zawistowski, 2009).

The previous reports found that purple rice variety Kum Phayao shows the strongest antimutagenicity against several mutagens in *Salmonella* mutation assay and anticarcinogenic enzyme-inducing properties in Hepa1c1c7 cells as well as cytotoxic on human hepatocellular HepG2 cells when compared to other purple rice varieties including Kum Doisaket and Kum Nan (Chewonarin, 2012). To cast light on anticlastogenicity of purple rice bran variety Phayao in animal model, rat liver micronucleus test was performed. Furthermore, the inhibitory mechanism of purple rice bran involving phase I and II xenobiotic metabolizing enzymes was examined.

1.2 Literature reviews

1.2.1 Mechanism of chemical-induced carcinogenesis

Environmental factors including diet, occupational exposure, infection and pollution are the predominant causes of cancer. The chemical-induced carcinogenesis is composed of at least three stages which are termed as initiation, promotion and progression stages. The biochemical and morphological changes in each stage are caused by either genetic or epigenetic alteration (Weston and Harris, 2000). The process of carcinogenesis is depicted in Figure 1.

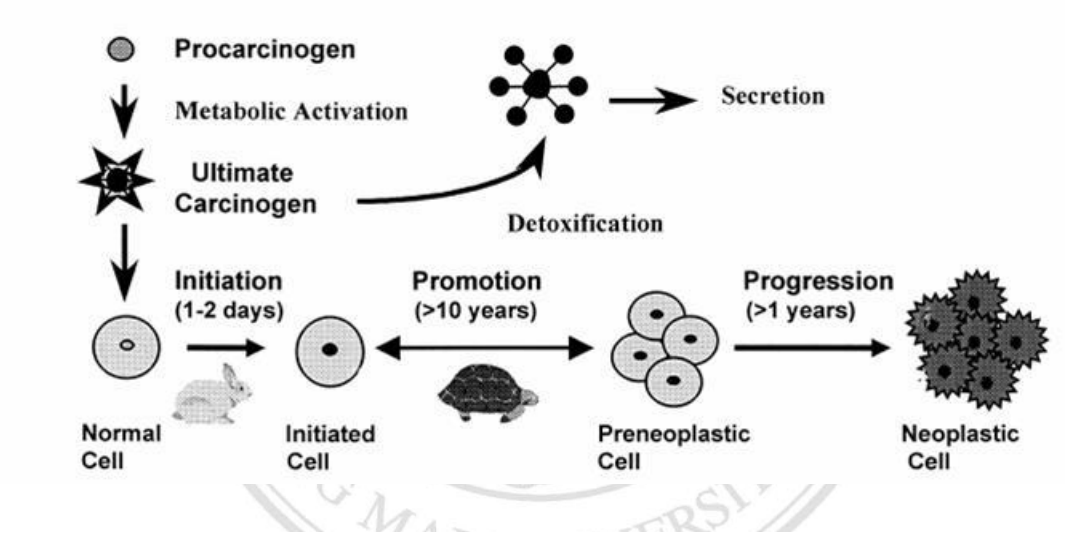

Figure 1. Multistage carcinogenesis (Surh, 1999)

The initiation stage is an irreversible process and occurs when DNA in normal cell fails from repairing system after potential carcinogen exposure. DNA damaging agent can covalently bind to DNA in normal cell establishing DNA adduct. If cell containing DNA mutation divides before DNA repairing systems can act, this normal cell will be transformed to the initiated cell. The genetic modifications in this step include mutations in genes that control cell proliferation, cell death and DNA repair such as proto-oncogenes and tumor suppressor genes (Oliveira, 2007). The substances that can interact with genetic materials through the metabolic or non-metabolic enzyme activation causing of DNA mutation or chromosomal damage are termed genotoxic carcinogens (Figure 2).

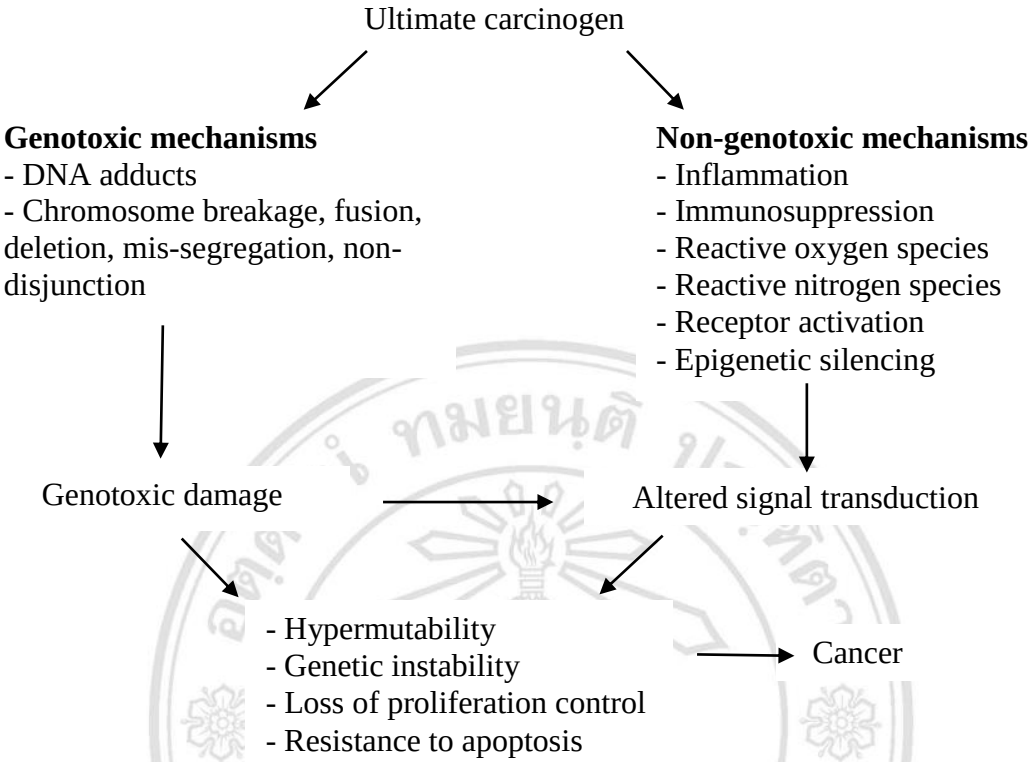

Figure 2. Mechanism of carcinogens (adapted from Oliveira, 2007)

The second stage is tumor promotion. The initiated cells are selectively clonal expanded by carcinogens or themselves if the initiators are complete carcinogens. The uncontrolled proliferation leads to form preneoplastic lesions and benign tumors, sequentially. This stage is reversible which is involved in either enhancement or repression of gene expression leading to induction of cell proliferation and inhibition of apoptosis. The non-genotoxic carcinogens play a role in this step. They do not directly react with DNA, but they can enhance tumor development by modulate cell proliferation, gene expression or signal transduction (Luch, 2005; Poirier, 2012).

The progression stage is the step that benign tumors are transformed to be malignant tumors. This stage is acquired through genetic and epigenetic alterations. It is classified by irreversibility, genetic instability, faster growth, invasion and metastasis to other organs.

1.2.2 Mutagenesis and carcinogenesis

The mutagenic response of genetic alteration from chemical carcinogens might lead to a permanent change in structure or amount of the genetic materials of cells. These changes are able to impact on a gene segment or whole chromosomes. The modification of single gene due to DNA bases change, point mutation, with addition, deletion or rearrangement in a small-scale change is called point mutation. The large-scale changes are shown as numerical (aneuploidy) or structural (clastogenicity) change in the portion of chromosome including translocation, deletion and duplication. The point mutation or chromosomal aberration of proto-oncogene, a proform of oncogene, can cause overexpression of growth-stimulating ability of oncogenes. While the mutation of tumor suppressor genes by gene inactivation raise the proliferation of mutated cells. These genetic alterations in somatic cells can produce genetic instability and development of cancer during clonal expansion (Klaassen, 2013).

1.2.3 Short-term mutagenicity tests

In order to assess the genotoxic potential of foreign compounds, the screening genetic or mutagenic tests have been developed. The combination of tests has been used to identify different end-points of genetic damage including gene mutation, clastogenicity and aneuploidy (Eastmond, 2009).

The Ames test is an abundant method to detect DNA mutation by chemical inducing. This test is used to evaluate frameshift or base pair substitution in the strain of bacteria *Salmonella typhimurium* which require essential amino acid, histidine, for growth. Generally, mutant bacteria with defect on synthesis of histidine (His^-) are unable to growth in a histidine-free medium. The substance as a mutagen will reverse His^- gene to His^+ gene that can grow in medium and form as colonies. This assay is rapid and convenient *in vitro* test but unable to detect other types of mutation.

The chromosomal aberration assay is required to test substance-causing DNA break that leads to several genetic diseases. This method is able to detect in either *in vitro* or *in vivo* models using defective cell score measurement. It is also a method used to assess chromosomal change in a number or structure such as translocations, deletions or inversions.

The micronucleus assay is a toxicological screening test using *in vitro* and *in vivo* clastogenicity or structural chromosome change. Micronucleus is a small chromosome fragment during metaphase/anaphase of mitosis. These acentric chromosomal fragments are indicator of DNA damage stimulated by clastogens. However, the whole lost chromosome from the failure of chromosome segregation induced by aneugens is detectable using micronucleus assay (Figure 3). The increase of micronucleus formation indicates large chromosome damage. For *in vitro* test, an exogenous source of metabolic activation is required, but cannot entirely mimic *in vivo* conditions. This technique is widely used in several tissues such as bone marrow, red blood cells, liver, intestine, skin, spleen and lung. Nonetheless, the xenobiotic metabolism is not involved in all those tissues which some carcinogens might show a negative result in this assay such as diethylnitrosamine. As the liver is major organ for xenobiotic metabolism, thus, it is an appropriate organ for investigating clastogenicity of chemicals in animals using micronucleus as an end-point marker.

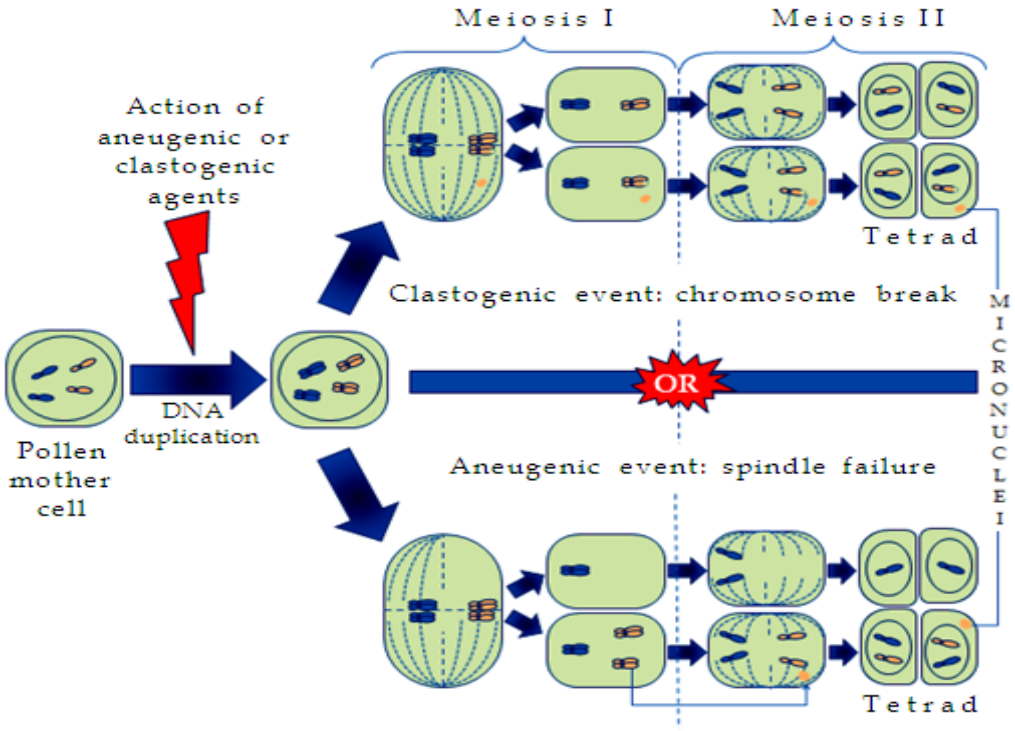

Figure 3. Formation of liver micronucleus (Meireles, 2011)

1.2.4 Xenobiotic metabolism

Xenobiotic metabolism is a major system for the biotransformation, elimination or detoxification of endogenous or exogenous xenobiotics and then led to excretion them from the body (Xu, 2005) by altering lipophilic compounds to be hydrophilic compounds (Dekant, 2009). Xenobiotic metabolism occurs in several organs including liver, a major site of synthesis of biologically functional proteins (Rose and Hodgson, 2004), and extrahepatic tissues (e.g. intestine, brain, kidney, placenta, lung and skin) (Pavek and Dvorak, 2013). Xenobiotics are commonly lipophilic compounds. They introduce to the body via absorption into blood circulation and distribution to tissues and organs (Pandit and Soltis, 2011). The primary recognition event, xenobiotic compounds can bind to ligand-binding proteins, xenosensors, as part of a nuclear receptor such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR). They subsequently regulate the specific transcriptional target genes controlling the expression of phase I, II and III metabolizing enzymes (Dunn, 2012). The major of phase I enzymes or modifying enzymes are oxidation, reduction, and hydrolysis. They aim to transform their substrates to be reactive or polar compounds which are inactive forms or reactive and toxic metabolites. Then phase II enzymes conjugate phase I products with small polar molecules to increase polarity. The modified xenobiotics are exported through cell membrane by phase III enzymes or xenotransporters (Figure 4) (Lee, 2011).

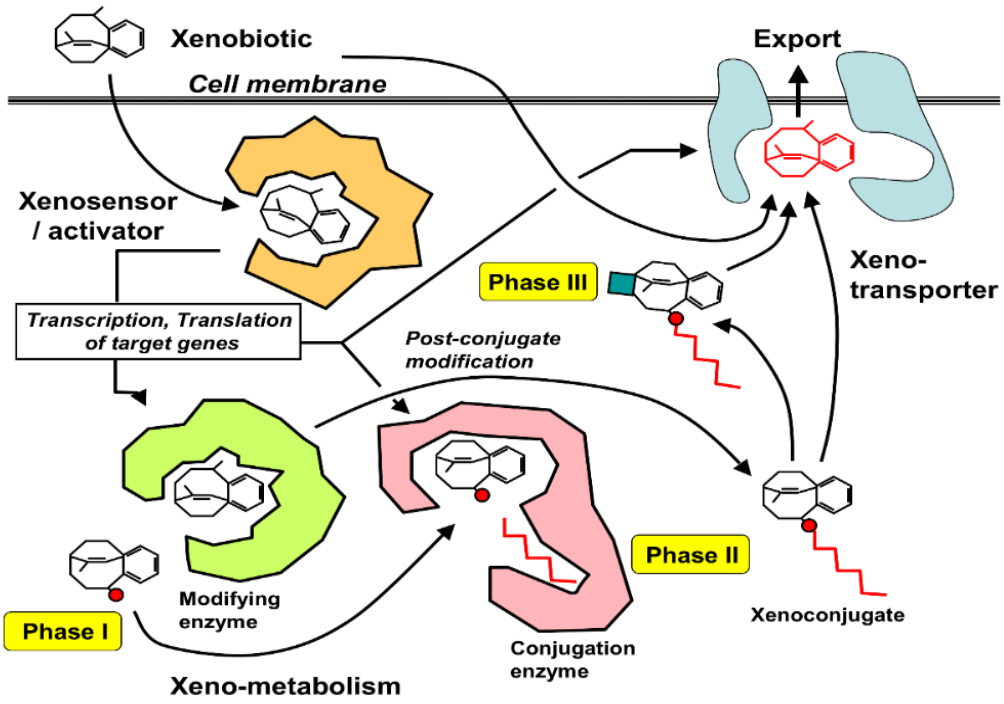

Figure 4. Xenobiotic metabolism (Dunn, 2012)

1.2.4.1 Phase I xenobiotic metabolizing enzymes

The characterization of phase I enzymes is presented as introduction of a functional group (-OH, $-NH_2$, or -SH) within a molecule to increase the hydrophilicity and called functionalization. These enzyme families contain two main groups; cytochrome P450 and non-cytochrome P450 enzymes.

Cytochrome P450

Cytochrome P450 enzymes, heme-containing proteins, are located in the inner membrane of endoplasmic reticulum or mitochondria of cells, mainly in hepatocytes. Their roles are involved in metabolism of endogenous and exogenous substances. The reactions of cytochrome P450 (CYP) include monooxygenation, epoxylation, dealkylation and hydroxylation. Commonly, the catalytic cycle can be summarized as;

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$$

This reaction required energy in form of NADPH from the electron donor, NADPH-cytochrome P450 reductase (CPR). After binding of substrates (drug, RH) to heme (Fe^{3+}) of CYP, the conformation change is occurred and induces electron transfer from NAD(P)H through FAD and FMN-coenzymes resulting to the reduction of Fe^{3+} ion. Then, oxygen molecule binds to Fe^{2+} and the second electron from CPR, or cytochrome b5 is transferred to produce negatively charge peroxy group. The peroxy group is rapidly protonated with release one molecule of water and form iron (V)-oxo species. The RH reacts with iron (V)-oxo species, releasing the hydroxylated product and the enzyme returns to original state (Figure 5).

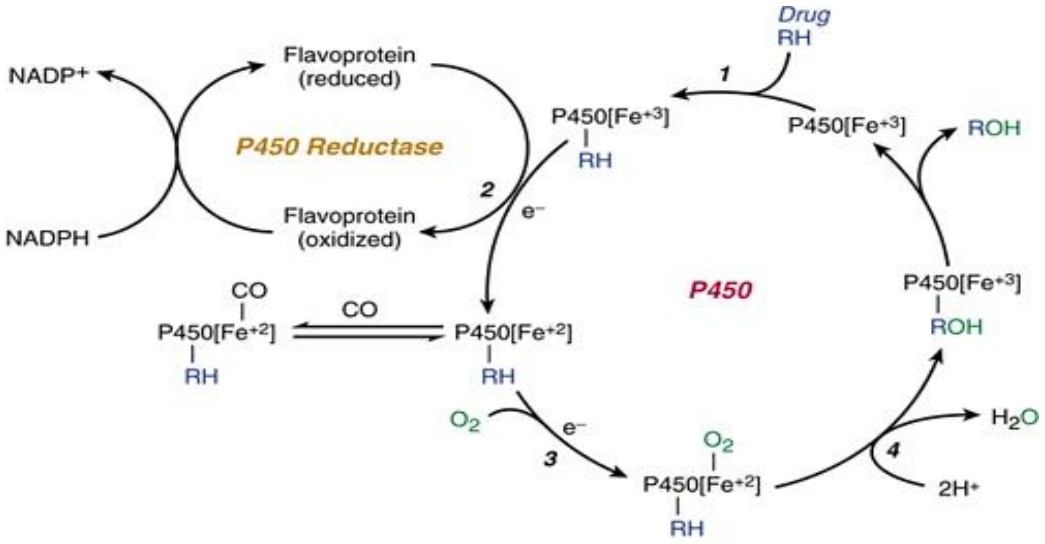

Figure 5. Catalytic cycle of cytochrome P450 (Singh, 2007)

Mainly human and rat phase I enzyme families consist of CYP1, CYP2, and CYP3 which involve in the xenobiotic biotransformation (Nedelcheva and Gut, 1994). CYP1 enzymes generally metabolize endo- and exogenous substrates such as polycyclic aromatic hydrocarbons (PAHs), estradiol, benzo[a]pyrene and aflatoxin B_1. The major isozymes are well-known as CYP1A1, CYP1A2 and CYP1B1. CYP1A2 is an important enzyme in coffee metabolism and involves in many cancer activations such as lung, breast and ovarian cancer (Kotsopoulos, 2007; Osawa, 2007).

CYP2 family is a key enzyme in several metabolisms of drugs and toxins. Major human hepatic CYP2 enzymes are CYP2A6, CYP2B6 and CYP2E1. The oxidative reaction of CYP2A6 is showed in the metabolism of nicotine in tobacco and be inducible by phenobarbital and rifampicin (Messina, 1997).

For CYP3 enzymes, the abundant subfamily is CYP3A exhibits various biological activities against toxins. The CYP3A4 plays an important role in several drug interactions as CYP3A4 inducers; rifampicin, carbamazepine and rifabutin. Notably, some substances act as CYP3A4 inhibitors, like grapefruit juice, cyclosporine or ketoconazole, which decrease catalytic capacity of CYP3A4 in drug metabolism (Ogu, 2000).

Non-cytochrome P450 (CYP)

Non-cytochrome P450 enzymes are located in cytoplasm, mitochondria and membrane of smooth endoplasmic reticulum. Oxidation is the main reaction of these enzymes. They are non-inducible or less inducible than CYPs.

Flavin-containing monooxygenases (FMOs) are important non-cytochrome P450 enzymes which are FAD containing flavoprotein. They increase solubility of insoluble nucleophilic compounds and excretion. Their reaction requires NADPH as cofactor and contains the property of heat labile.

Monoamine oxidases (MAOs) are flavin-containing amine oxidoreductases that catalyze the oxidative deamination of monoamines. They are mostly found at the outer membrane of mitochondria.

Aldehyde dehydrogenase (ADH) is a polymorphic enzyme that catalyze the dehydrogenation of aldehydes to carboxylic acids in the metabolism of drugs and xenobiotics such as ethanol (Penner, 2012).

1.2.3.2 Phase II xenobiotic metabolizing enzymes

The functionalized phase I metabolite or parent xenobiotics are conjugated with a polar endogenous molecule (e.g. glucuronic acid, sulfate, glutathione) to form easily excretable compounds by transferase reaction of phase II enzymes, called biotransformation.

UDP-glucuronyltransferase

UDP-glucuronyltransferases are major phase II metabolizing enzymes. They are able to transform the lipophilic moieties such as steroids, bilirubin and drugs to be water-soluble metabolites. Its reaction is glucuronidation involved the transfer of glucuronic acid group of uridine 5'-diphospho-glucuronic acid (UDPGA) to functional group of xenobiotic substrate and produce β-D-glucopyranosiduronic acids or glucuronide derivatives, which are more polar and more easily excreted. The main biological functions of reactions are against some toxic dietary carcinogens and represent key factors in the homeostasis of endogenous elements such as bilirubin, steroid and thyroid hormones (Figure 6).

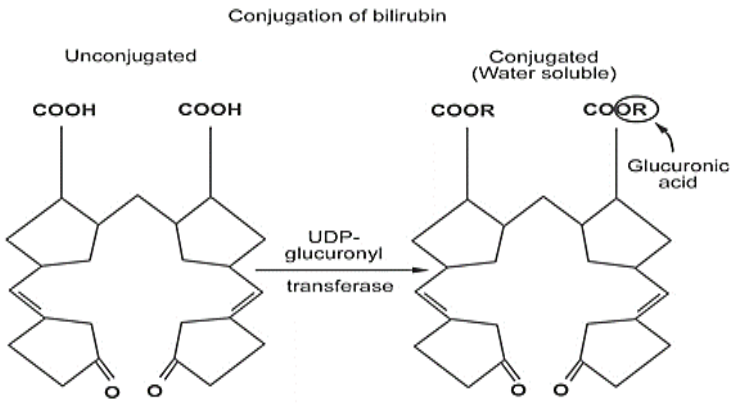

Figure 6. UDP-glucuronyltransferase reaction of bilirubin (Chhabra, 2012)

Sulfotransferase

Sulfotransferases are large enzyme groups of phase II detoxification by transfering sulfate from a donor (3'-phosphoadenosine-5'-phosphosulfate; PAPS) to acceptor (3'-phosphoadenosine-5'-phosphate; PAP) (Figure 7). They are widespread distributed in many organs e.g. liver, lung, kidney, brain and skin. This reaction, sulfonation, is able to increase the solubility of compounds and excretion, but it can also activate some substances to form active metabolites such as steroidal drug cyproterone acetate and tamoxifen (Werner, 1996; Kim, 2005). The predominant subfamilies in human are SULT1A1 and SULT2A1 (Nowell, 2006).

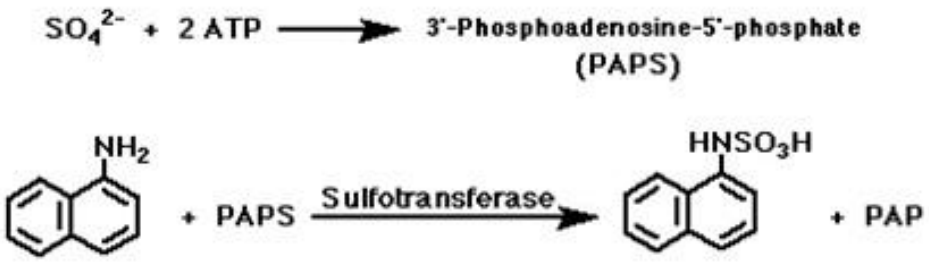

Figure 7. Reaction of sulfotransferase (Guillemette, 2003)

Glutathione *S*-transferase

Glutathione *S*-transferases are phase II detoxification enzymes which catalyse the conjugation of glutathione (GSH) to electrophilic compounds via interaction between xenobiotic (RX) and the catalytic residue of GSH, thiol group. The thioether bonding of electrophiles and GSH almost produce the less reactive than the parental compound. The conjugative ability of GST can eliminate harmful electrophilic molecules from the cell and increase the solubility of hydrophobic xenobiotics to easy removal (Figure 8).

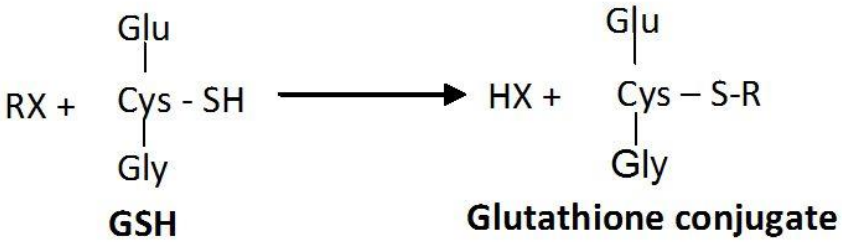

Figure 8. Reaction of glutathione *S*-transferase (Simic, 2009)

1.2.5 Toxicity of aflatoxin B_1

Aflatoxin B_1 (AFB_1) is a secondary metabolite produced by *Aspergillus flavus* and *Aspergillus paraciticus* that contaminate in naturally food such as peanuts, corn, rice, dried fruits and other grains. The International Agency for Research on Cancer (IARC) classified AFB_1 as group 1 genotoxic carcinogen. It is the most potent hepatotoxic agent containing mutagenicity, teratogenicity, carcinogenicity and immune suppression in human and animals (Hamid, 2013). AFB_1 is primarily metabolized in the liver by CYP 1A2 and 3A4 to produce either carcinogenic metabolites (AFB_1 8, 9-epoxide; AFBO) or less mutagenic forms (AFM_1, AFQ1 and AFP1). The AFBO can be either toxified or detoxified by various fates; excreted form through conjugation with glutathione-*S* transferases (GST), toxic and/or carcinogenic forms occurred when AFBO interact with genetic materials including proteins and DNA. Severely, the formation of DNA adduct with N7-guanine leads to gene mutation on codon 249 of the p53 tumor suppressor gene by G to T transversion (Figure 9). These mutations mostly are found in hepatocellular carcinoma patients (Murphy, 2006; Moudgil, 2013).

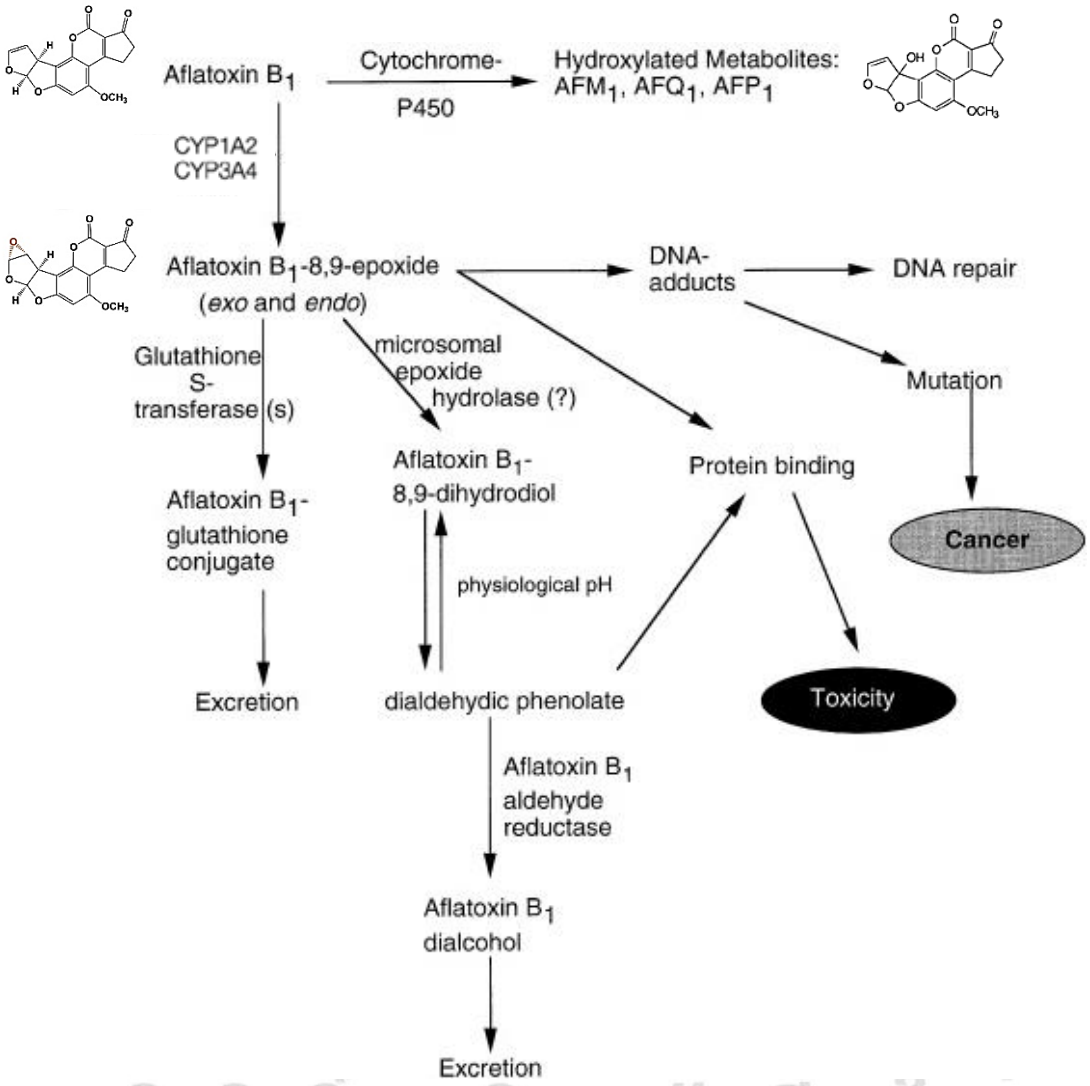

Figure 9. Metabolism and toxicity of AFB_1 (Bammler, 2000)

1.2.6 Phytochemicals and chemoprevention

The strategy of cancer chemoprevention is defined as the consumption of synthetic or natural agents to prevent or suppress the carcinogenesis. Since the usage of natural dietary compounds or phytochemicals is abundantly found in fruits and vegetables and be the harmless and protective way to reduce the risk of chronic diseases. Several biological substances have been identified as chemopreventive agents such as resveratrol, curcumin, sulforaphane and gingerol (Russo, 2005). Their abilities were performed from specific color, aroma and flavor through anticarcinogenicity, antimutagenicity or cell death enhancement or stimulating carcinogen removal process. The prominent phytochemicals in plants are classified depending on their bioactive structures as following (Saxena, 2013) (Figure 10);

Phenolic compounds are the largest class and widely spread in plants. The structure contains one or more aromatic rings with hydroxyl group (-OH). The dietary phenolic compounds contain numerous well-known compounds e.g. flavonoids, phenolic acids, stilbenes, coumarins and tannins. Phenolic acids are one third of phenolics. They are divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids which are conjugated with other chemicals. Flavonoids are two thirds of dietary phenolics consist of two aromatic rings (A and B) and linked to C ring by 3 carbons (Figure 11). Due to the variation of heterocyclic C ring, it is able to be classified as flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids.

The predominant flavonoid in colored plants is anthocyanins which are derived from anthocyanidins. They were widely distributed in red or purple pigmented vegetables and fruits. Aglycone is commonly structure of anthocyanins consisting of aromatic rings without sugar moiety. The most common forms were found as cyanidin-3-glucoside and peonidin-3-glucoside (Figure 11).

The important roles of phenolic compounds include the function against pathogens in plant and also as chemopreventive agents in human such as antioxidant, anti-inflammation, antiatherosclerosis, antibacterial infection, anticarcinogen and antimutagens (Huang, 2009). Anthocyanins from grape can reduce inflammation-

related atherosclerosis (Kuntz, 2015). Purple sweet potato anthocyanins protect amyloid-beta peptide injury in Alzheimer's disease through the inhibition of oxidative damage (Ye, 2010). Anthocyanins from *Hibiscus sabdariffa* reduce liver lesions including inflammation by prevention of oxidative damage (Wang, 2000). In addition, anthocyanins in black rice suppress CAL 27 cell metastasis by reduction of MMP-2, MMP-9, and NF-κB p65 expression (Fan, 2015).

Carotenoids are natural pigments in terpenoid family containing a polyene hydrocarbon chain terminated by rings and/or oxygen atoms. The most widely carotenoids are found, include β-carotene and lycopene from carrots and tomatoes, respectively. These compounds present as lipophilic by their long unsaturated aliphatic chains as some fatty acids in their structures and can react with free radicals.

Alkaloids are heterocyclic nitrogen-containing compounds. They can be produced by organisms such as bacteria, fungi, plants and animals. The example of alkaloids comprises indoles, quinine and vinblastine. In plants, the high amounts of these compounds are determined in leaves, fruits or seeds, root or bark. However, it might probably be found as different alkaloid types in the same plants. Their functional benefits are antihypertension, antiarrhythmic effect, antimalarial activity and anticancer actions.

Organosulfur compounds are mineral sulfur containing phytochemicals and have one or more carbon-sulfur bonds. Their specific characterizations are shown as pungent flavors and aromas. These dietary compounds are mostly found in cruciferous vegetables, such as garlic, broccoli, cabbage and onion, as forms of isothiocyanates, indoles and allylic sulfur compounds.

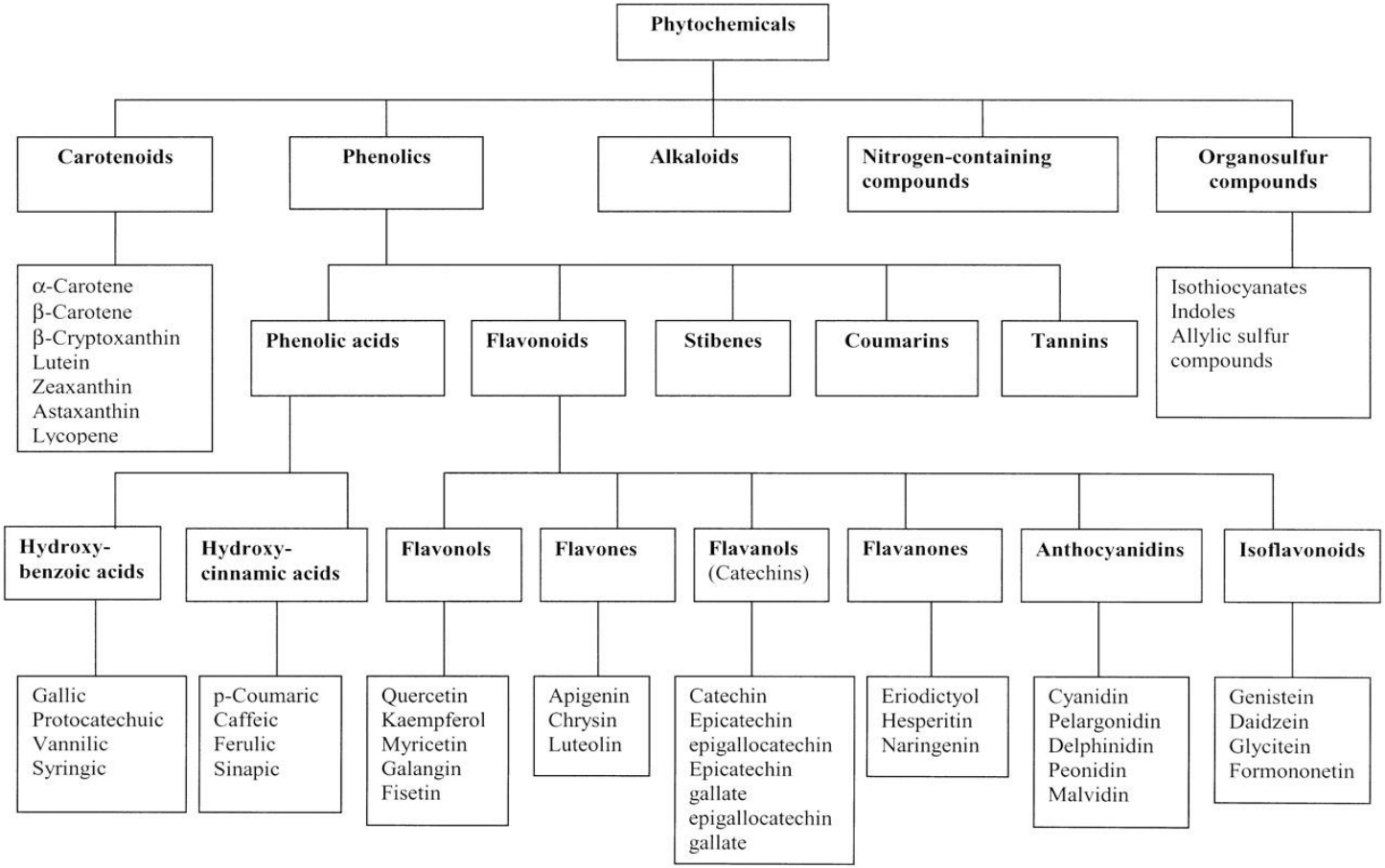

Figure 10. Classification of dietary phytochemicals (Liu, 2004)

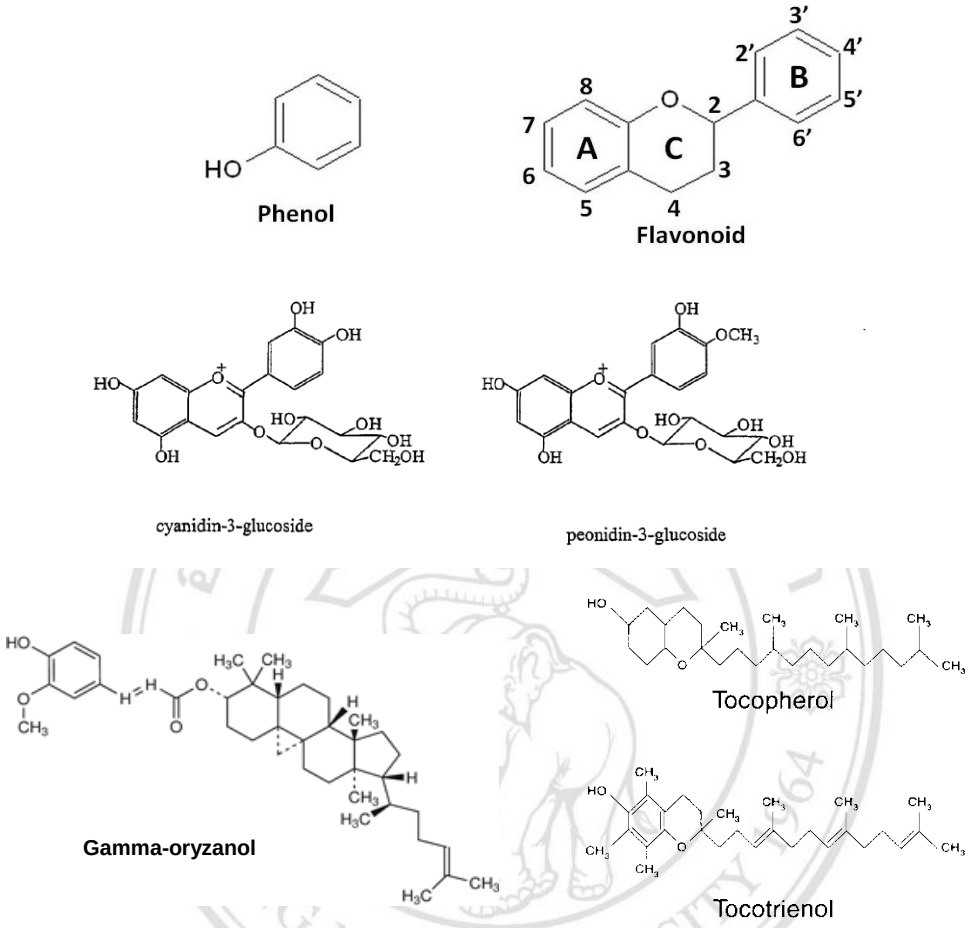

Figure 11. Structures of phytochemical found in purple rice

1.2.7 Modes of action of cancer chemopreventive agents

The phytochemical chemopreventive agents are segregated to two groups relating with their mechanistic actions (Figure 12) (Wattenberg, 1985; Ugbogu, 2013).

Blocking agents are carcinogenic preventive substances. The three mechanistic actions of them include inhibit the metabolic stimulation of carcinogens to form ultimate substances, enhance carcinogenic removal by increase detoxifying manner and scavenge the reactive forms of carcinogens. Many previous reports demonstrated that the enzyme inducers might have different cancer preventive mechanisms. Indole-3-carbinol is able to induce both phases I and II enzymes whereas curcumin presents as phase I suppressor and phase II inducer (Manson, 2000).

Suppressing agents are cancer-inhibiting compounds, which affect promotion and progression phases. The roles of these compounds have been reported through control of cell proliferation, differentiation and apoptosis. Phenols, isothiocyanates, retinoids and carotenoids are most studied on their cancer suppressor activity. β-carotene exhibits a suppressive effect on DMBA- induced mammary neoplasia (Wattenberg, 1985).

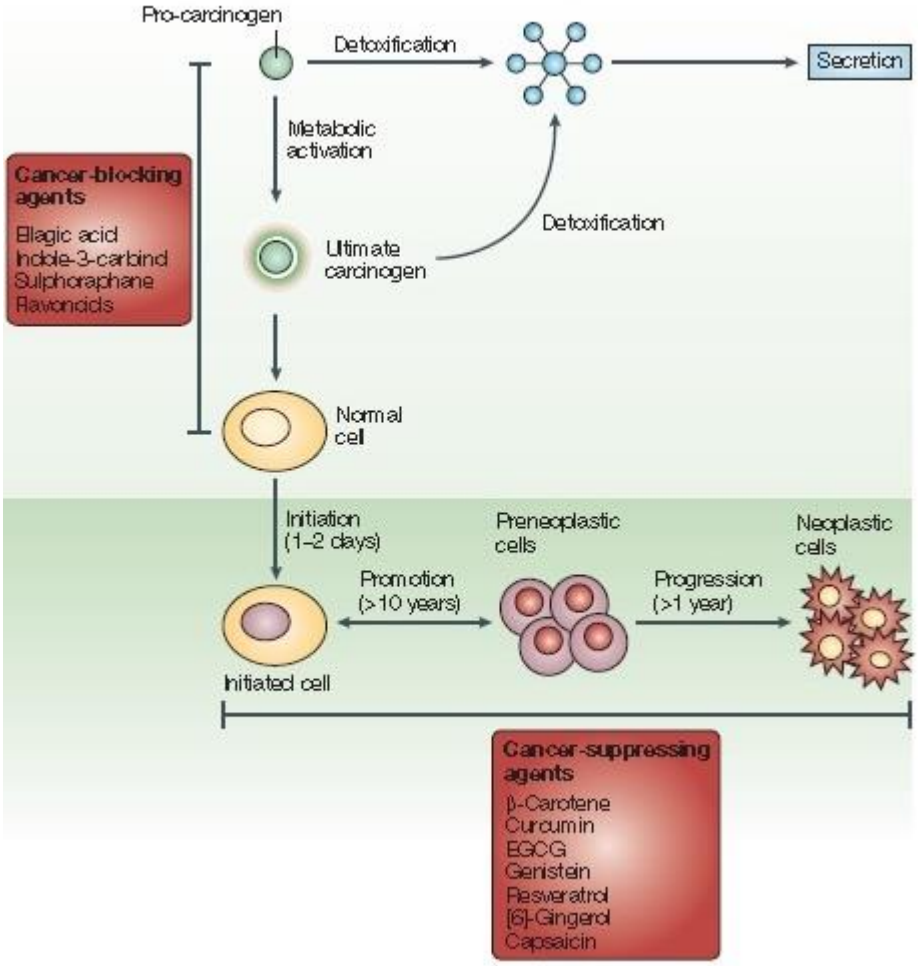

Figure 12. Roles of phytochemical chemopreventive agents (Surh, 2003)

1.2.8 Purple rice bran

For Asian, rice is a widespread main food, which is a source of several nutritive ingredients e.g. carbohydrate, protein, fats, vitamins, minerals and fibers. Especially, the pigmented rice is now interesting for consumption and study with the special properties of its specific color. For example, γ-oryzanol in brown rice prevents obesity and diabetes (Kozuka, 2013), phenolic compounds of black rice contain antioxidative activity (Zhang, 2015), anthocyanins from purple rice (Figure 14) protect against light-induced retinal damage (Tanaka, 2011) and inhibit VEGF-induced angiogenesis (Tanaka, 2012). Their constituents and biological functions of pigmented rice have been reported including protecting cell injury by scavenge reactive oxygen species, inhibiting lipid peroxidation, anti-inflammatory and anti-tumor properties (Baek, 2015).

Rice bran, an outer layer of rice, is a nourish part including proteins, lipids, fiber, oil, minerals and vitamins (Figure 13). It is a valuable source of biological phytochemicals such as γ-oryzanol, tocopherols and tocotrienols. The health-promoting properties of rice bran are demonstrated as ROS scavenging effect, anti-inflammation, cholesterol-lowering effect and anticancer activity. Gamma-oryzanol is a mixture of ferulate esters of phytosterols and triterpene alcohols. The three major constituents are cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate (Figure 11). Furthermore, tocotrienols and tocopherols which are members of vitamin E are normally found in rice bran. The core structure of vitamin E contains a chroman ring with hydroxyl group and difference of double bonds on 12-carbon aliphatic side chain. Tocotrienols contain 3 double bonds, while tocopherols do not consist of double bonds (Figure 11). Outstandingly, the pigmented rice brans are interesting due to the contents of numerous bioactive compounds and their biological activities. Likewise purple rice bran, it contains high amount of phenolics and flavonoids (anthocyanins). Hydrophilic and lipophilic antioxidants could prevent the progression of cancer through inhibition of angiogenesis (Jang, 2009; Tanaka, 2012). Thai purple rice bran is slightly investigated and exhibits some protective effects such as a potent antioxidant, immunomodulation and cytotoxic on cancer cell (Punyatong, 2008;

Chakuton, 2012; Saenjum, 2012; Phetpornpaisan, 2014). However, the different cultivars provide various amount and types of bioactive compounds. Previous reports demontrate that Kum Phayao exhibits higher potent anticarcinogenic effect in Hepa1c1c7 cells than Kum Doisaket and Kum Nan (Chewonarin, 2012). Furthermore, the purple rice bran contains higher potential of antimutagenicity against some mutagenic agents in bacterial mutation assay than variant Doisaket and Nan.

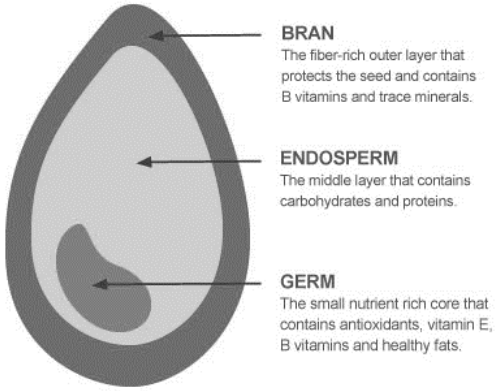

Figure 13. The components of rice grain

Figure 14. Purple rice

1.3 Objectives

1. To investigate effect of purple rice bran extracts on rat liver micronucleus formation
2. To evaluate effect of purple rice bran extracts on rat liver detoxifying enzymes
3. To assess effect of purple rice bran extracts on AFB_1-initiated hepatocarcinogenesis by rat liver micronucleus assay
4. To explain the possibly inhibitory mechanism of purple rice bran extracts on AFB_1 metabolism in rats