องค์ประกอบ ความเสถียร และฤทธิ์ทางชีวภาพของแอนโทไซยานิน และสารประกอบฟีนอลิกจากข้าวสีม่วงของไทย

นางสาวรัสรินทร์ ฉัตรทองพิศุทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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COMPOSITION, STABILITY AND BIOACTIVITY OF ANTHOCYANINS AND PHENOLIC COMPOUNDS FROM THAI DARK PURPLE RICE

Rassarin Chatthongpisut

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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COMPOSITION, STABILITY AND BIOACTIVITY OF ANTHOCYANINS AND PHENOLIC COMPOUNDS FROM THAI DARK PURPLE RICE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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รัสรินทร์ ฉัตรทองพิสุทธิ์ : องค์ประกอบ ความเสถียร และฤทธิ์ทางชีวภาพของแอนโทไซยานินและสารประกอบฟีนอลิกจากข้าวสีม่วงของไทย (COMPOSITION, STABILITY AND BIOACTIVITY OF ANTHOCYANINS AND PHENOLIC COMPOUNDS FROM THAI DARK PURPLE RICE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 156 หน้า.

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาระบุชนิดของของสารแอนโทไซยานิน และสารประกอบฟีนอลิกในแต่ละส่วนของเมล็ดข้าวมะลินิล คือ ส่วนของรำ ข้าวกล้อง และข้าวขัด และศึกษาความเสถียรต่อความร้อนของสารแอนโทไซยานินและกรดฟีนอลิกฤทธิ์ต้านออกซิเดชัน และประเมินฤทธิ์ต้านการเจริญเติบโตต่อเซลล์มะเร็งลำไส้ใหญ่ นอกจากนี้เพื่อศึกษาชีวภาพความพร้อมของการนำไปใช้ (bioaccessibility) ของสารแอนโทไซยานินจากข้าวกล้องสุกโดยการจำลองสภาวะการย่อยของร่างกาย

จากผลการทดลองพบว่ารำของข้าวมะลินิลเป็นบริเวณหลักที่พบสารแอนโทไซยานินจากการระบุชนิดด้วยเทคนิคลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี (LC-MS/MS) สารแอนโทไซยานินหลักที่พบ คือ ไซยานิดีน-3-กลูโคไซด์ (cyanidin-3-glucoside, cy-3-glu) และพีโอนิดีน-3-กลูโคไซด์ (peonidin-3-glucoside, pn-3-glu) ส่วนสารประกอบฟีนอลิกหลักที่ได้จากการสกัดด้วยสารสกัดเมทานอลปรับกรด คือกรดโปรโตคาทีคูอิก (protocatechuic acid, PCA) และกรดวานิลิก (vanillic acid, VA) จากการศึกษาความเสถียรต่อความร้อนของสารแอนโทไซยานินของข้าวกล้อง พบว่าภายใต้การให้ความร้อนด้วยน้ำร้อนในช่วงอุณหภูมิ 60-90 องศาเซลเซียส เกิดการสลายตัวของสารแอนโทไซยานินและการลดลงของฤทธิ์ต้านออกซิเดชันมากกว่าการให้ความร้อนด้วยลมร้อนที่อุณหภูมิเดียวกัน และพบว่าการสลายตัวด้วยความร้อนของสารไซยานิดีน-3-กลูโคไซด์และโอนิดีน-3-กลูโคไซด์ ส่งผลให้ปริมาณกรดโปรโตคาทีคูอิกและกรดวานิลิกเพิ่มขึ้น

กรดฟีนอลิกที่ตรวจพบในข้าวกล้องสุก ได้แก่ กรดโปรโตคาทีคูอิกและกรดวานิลิก ซึ่งเป็นกรดฟีนอลิกอิสระ (free phenolic acids) และกรดเฟอรูลิก (ferulic acid, FA) และกรดพาราคูมาริก (p-Coumaric acid, p-Cou) ซึ่งเป็นกรดฟีนอลิกยึดเหนี่ยว (bound phenolic acids) วิธีการหุงข้าวไมโครเวฟเป็นวิธีการหุงข้าวที่ส่งผลให้มีการสูญเสียแอนโทไซยานินสารฟีนอลิกสูงสุด คือ 65% และ 47.8% ตามลำดับ และพบการลดลงของฤทธิ์ต้านออกซิเดชันสูงที่สุด (p<0.05) การลดลงของไซยานิดีน-3-กลูโคไซด์มีความสัมพันธ์กับการเกิดของกรดโปรโตคาทีคูอิก

จากการประเมินฤทธิ์ต้านการเจริญเติบโตของสารสกัดจากข้าวดิบและข้าวกล้องสุกที่ผ่านการสกัดด้วยเมทานอลต่อเซลล์มะเร็งลำไส้ใหญ่พบว่าสารสกัดจากข้าวดิบและข้าวกล้องสุกที่ผ่านการหุงโดยใช้หม้อนึ่งความดันไอมีฤทธิ์ยับยั้งการเจริญเติบโตต่อเซลล์มะเร็งลำไส้ใหญ่ดีที่สุด โดย

ความเข้มข้นของสารที่ทำให้เซลล์มะเร็งลดลงร้อยละ 50 (IC_{50}) เท่ากับ 12.63 และ 16.11 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ

จากการศึกษาชีวภาพความพร้อมของการนำไปใช้ของแอนโทไซยานินจากข้าวกล้องสุกที่ผ่านการหุงด้วยหม้อหุงข้าวไฟฟ้าพบว่าชีวภาพความพร้อมของการนำไปใช้ของแอนโทไซยานินเพิ่มขึ้นหลังจากการย่อยในปาก กระเพาะอาหาร และลำไส้เล็ก สภาวะแวดล้อมของการย่อยและเอนไซม์ที่ทำหน้าที่ย่อยอาหารเป็นปัจจัยสำคัญในการส่งเสริมการปลดปล่อยสารฟีนอลิกจากโครงสร้างของข้าวกล้องสุก ในขณะที่การปลดปล่อยสารแอนโทไซยานินเกิดจากสภาวะแวดล้อมในระบบย่อยอาหารเป็นหลัก ในขั้นตอนสุดท้ายหลังจากการย่อยอาหารในลำไส้เล็กพบการปลดปล่อยของสารฟีนอลิกและแอนโทไซยานิน เท่ากับ 62.5% และ 10.67% ตามลำดับ จากการประเมินฤทธิ์ต้านการเจริญเติบโตของสารสกัดแอนโทไซยานินจากข้าวกล้องหุงสุกต่อเซลล์มะเร็งลำไส้ใหญ่ พบว่าสารสกัดแอนโทไซยานินสามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่ HCT116 และ HT-29 โดย IC_{50} เท่ากับ 37.20 และ 37.19 มิลลิกรัมต่อมิลลิลิตร ตามลำดับหลังจากการบ่มเป็นระยะเวลา 72 ชั่วโมง งานวิจัยนี้แสดงให้เห็นว่าข้าวมะลินิลสุรินทร์ ซึ่งประกอบด้วยสารออกฤทธิ์ทางชีวภาพ ได้แก่ แอนโทไซยานินและกรดฟีนอลิก มีศักยภาพและมีบทบาทที่สำคัญในการยับยั้งการเจริญเติบโตของมะเร็งลำไส้ใหญ่

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

RASSARIN CHATTHONGPISUT : COMPOSITION, STABILITY AND BIOACTIVITY OF ANTHOCYANINS AND PHENOLIC COMPOUNDS FROM THAI DARK PURPLE RICE. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 156 pp.

PIGMENTED RICE/ANTIOXIDANT ACTIVITY/THERMAL STABILITY/ BIOACCESSIBILITY/ANTIPROLIFERATIVE ACTIVITY

The objectives of this study were to identify anthocyanins and phenolic compounds in various fractions, including bran, whole rice, and milled rice, of Thai Mali Nil Surin rice (*Oryza sativa* L., MNS). In addition, thermal stability of anthocyanins and phenolic acids as well as antioxidant activity and antiproliferative activity of cooked rice against human colon cancer cells were investigated. Bioaccessibility of anthocyanins of cooked Thai dark purple rice *in vitro* digestion was also evaluated.

Anthocyanins and phenolic compounds were mainly located in the bran fraction of 2 cultivars, MNS2 and MNS6. Predominant anthocyanins were cyanidin-3-glucoside (cy-3-glu) and peonidin-3-glucoside (pn-3-glu), while protocatechuic acid (PCA) and vanillic acid (VA) were major phenolic compounds extracted by acidified methanol. Degradation of anthocyanins and a decrease of antioxidant activity of husk-removed rice occurred to a greater extent in water heating than in hot-air heating at any of the studied temperatures of 60-90°C. Thermal degradation of cy-3-glu and pn-3-glu resulted in the formation of PCA and VA.

PCA and VA are major free phenolic acids, while ferulic acid (FA) and p-coumaric acid (p-Cou) are major bound phenolic acids of cooked dark purple rice.

Based on cooking methods, microwave heating resulted in the greatest loss of 65% anthocyanins and 47.8% phenolics as well as free radical scavenging activity and reducing power (p<0.05). A decrease of cy-3-glu was in concomitant with an increase of PCA. Methanolic extract of raw rice and rice cooked by autoclave showed the highest inhibition of Caco-2 cell proliferation with IC_{50} of 12.63 and 16.11 µg/mL, respectively.

Bioaccessibility of phenolics and anthocyanins of MNS6 cooked by a rice cooker increased after oral, gastric and pancreatic digestion. Phenolics were released from the matrices of cooked dark purple rice by the action of digestive enzymes and environments, whereas anthocyanins were mostly released by the digestive environments. At the end of intestinal phase, 62.50% of phenolics and 10.67% of anthocyanins were released from the cooked rice matrix. Anthocyanin extract significantly inhibited proliferation of HCT116 and HT-29 colon cancer cell lines *in vitro* with IC_{50} of 37.20 and 37.19 µg/mL, respectively, after 72 h of incubation. This study indicated that anthocyanins and phenolic acids contained in Mali Nil Surin rice could be a potential source of bioactive compounds which plays an important role in chemoprevention of human colon cancer.

School of Food Technology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

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CHAPTER I

INTRODUCTION

1.1 Introduction

Rice, *Oryza sativa* L., is the most important cereal crop and the staple food source being consumed by over half of the world's population (Hu, Zawistowski, Ling, and Kits, 2003). A worldwide paddy rice production in 2012 was about 719 million metric tons (MMT) (FAOSTAT, 2014). Thailand is the fifth largest rice producer in the world with paddy rice production of 38.9 MMT and Thailand is also one of the largest rice exporters of the world with the export quantity of 6.6 MMT in the value of 133,839 million baths (4084 million USD) in 2013 (Office of Agriculture Economics, Ministry of Agriculture and Cooperatives, 2014). In Thailand, pigmented rice is another rice variety that is grown in the North and Northeastern regions. Its production is rather limited due to its low yield and pest intolerance (Ministry of Agriculture and Cooperatives, 2014). In the past, brown rice, wild rice and pigmented rice, such as dark red, dark purple, dark blue, red brown and black purple grains are not widely consumed in Thailand due to their taste and texture although pigmented rice is a rich source of phytochemicals such as anthocyanins and phenolic compounds.

Food provides not only essentials nutrients needed for life but also other bioactive compounds for health and disease prevention. Bioactive compounds in plant-derived foods are defined as "phytochemicals". Oxidative stress can cause oxidative damage to large biomolecules, such as proteins, DNA, and lipids, resulting in

an increased risk for cruel diseases such as some cancers, cardiovascular diseases and type II diabetes (Liu, 2007; Yawadio, Tanimori, and Morita, 2007; Yokoyama, 2004). Therefore, to prevent or slow down the oxidative stress induced by free radicals, sufficient amounts of antioxidants need to be consumed. Fruits, vegetables, and grains contain a wide variety of antioxidant compounds which may help protect cellular systems from oxidative damage and lower the risk of chronic diseases. The most studied phytochemicals are phenolics that are secondary metabolites and are major compounds in pigmented rice.

Phenolic compounds, in particular phenolic acids, are usually found in outer layer of the grain especially in pigmented rice. The basic chemical forms of natural phenolic acids can be subdivided into two classes, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acid derivatives include p-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acids. They can also be found in the form of sugar derivatives and organic acids in plant foods. Generally, the hydroxycinnamic acids and their derivatives are usually found in plant-derived foods and they are exclusively derived from p-coumaric, caffeic, ferulic, and sinapic acids. Ferulic acid and its derivatives are the most abundant hydroxycinnamic acids found in cereal grains. Pigmented rice contains a wide range of phenolic acids and flavonoids, mainly anthocyanins (Zhou, Robards, Helliwelland, and Blanchard, 2004; Tian, Nakamura, and Kayahara, 2004; Zhu, Cai, Bao, and Corke, 2010). Predominant phenolic acids in rice are ferulic acid, p-coumaric acid and diferulate which are not present in significant quantities in fruits and vegetables (Adom and Liu, 2002). However, the level of these compounds in rice was affected by various factors, such as UV-B tolerance (Caasi-Lit, Whitecross, Nayudu, and Tanner, 1997) and storage time or condition (Tsugita, Ohta, and Kato, 1983; Zhou et al., 2004). In addition, each part of rice grain contained varied

amount of phenolic compounds (Butsat and Siriamornpun, 2009). Phenolic compounds found in white rice are plant cell wall components in the form of free, soluble conjugated, and insoluble bound to polysaacharides containing glucose, arabinose, xylose, galactose, rhamnose, and mannose (Tian, Nakamura, Cui, and Kayahara, 2005; Zhu et al., 2010). These phenolic compounds have been reported to have antioxidant (Baublis, Lu, Clydesdale, and Decker, 2000; Miller, Rigelhof, Marquart, Prakash, and Kanter, 2000), antimutagenic (Ferguson, Fong, Pearson, Ralph, and Harris, 2003), and anticancer (Williams, Williams, and Weisburger, 1999) activities.

Flavonoids are a group of phenolics which are ubiquitous in plants (Shahidi and Ho, 2005). Flavonoids are the most frequently found in nature as conjugates in glycosylated or esterified forms but can occur as aglycones as a result of food processing. Anthocyanins are the main flavonoids found in pigmented rice grain. They are glycoside of anthocyanidins with glucose, galactose, arabinose, rhamnose, xylose, and fructose (Choia, Jeonga, and Lee, 2007; Hosseinian and Beta, 2007; Hosseinian, Li, and Beta, 2008; Mazza, Cacace, and Kay, 2004). In pigmented rice, anthocyanins are located in the pericarp and aleurone layers of the seed. Thus, bran has the highest content of anthocyanins (Abdel-Aal, Young, and Rabalski, 2006). Besides their coloring effects, anthocyanins and anthocyanidins (aglycone of anthocyanins) show antioxidant properties. They also prevent lipid oxidation in human low-density lipoproteins (LDL) *in vitro* and liposome (Satué-Gracia, Heinonen, and Frankel, 1997), In addition, cytotoxicity of anthocyanins against human monocytic leukemia cells (Hyun and Chung, 2004) and SKHep-1 human hepatocellular carcinoma (Chen et al., 2006). Thailand has a great number of varieties of pigmented rice. A great deal of interest in pigmented rice in particular black rice and dark purple rice is given to its characteristics and antioxidant activity due to phenolic compounds and anthocyanins.

Phenolic compounds of white rice have been widely studied. However, phenolic compounds and anthocyanins of Thai pigmented rice grains as related to antioxidant activities have not been well characterized.

Naturally, phenolic compounds and anthocyanins are highly instable (Giusti and Wrolstad, 2003). Their stability is affected by several factors, such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins, and metal ions (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodíguez, and Galán-Vidal, 2009; Rein, 2005). To achieve the maximum benefits, it is critical to understand distribution of bioactive compounds (Fares, Platani, Baiano, and Menga, 2010). Temperature is an important factor causing degradation of anthocyanins. The contents of anthocyanins and phenolic acids, thus, change upon cooking. Pressure cooking resulted in 79.8% loss of cyanidin-3-glucoside in black rice (Hiemori, Koh, and Mitchell, 2009). In addition, degradation of cyanidin-3-glucoside occurred in concomitantly with the formation of protocatechuic acid. Changes of both cyanidin-3-glucoside to protocatechuic acid content affected antioxidant activity and bioactivities of the black rice. The method of rice cooking would also affect degradation of anthocyanins of black rice in different manner. Stability of anthocyanins of Thai pigmented rice as affected by cooking methods has not been fully realized.

Bioactive compounds in foods must be available in the target tissue in order to exert their biological properties. Biological properties of dietary polyphenols depend on their absorption in the gut and their bioavailability (Saura-Calixto, Serrano, and Goňi, 2007). The bioavailability of a dietary compound is dependent upon bioaccessibility, digestive stability and the efficiency of its transepithelial passage. Bioaccessibility is defined as the amount of food constituent that is released from the

solid food matrix and present in the gut (Chandrasekara and Shahidi, 2012). It is widely accepted that only polyphenols released from the food matrix by the actions of chemical environments, digestive enzymes and bacterial microflora (large intestine) are bioavailable. Bioaccessibility and bioavailability of different polyphenols vary quantitatively and qualitatively with dietary source (Manach, Williamson, Morand, Scalbert, and Rémésy, 2005; Tagliazucchi, Verzelloni, Bertolini, and Conte, 2010). In addition, the bioaccessibility depends on initial concentration of polyphenols in the food matrix, the composition of the matrix and host-related factors, such as enzyme concentrations (Bouayed, Deußer, Hoffmann, and Bohn, 2012). In human, the digestive process starts in the mouth where the initial degradation of polysaccharides and triglycerides during mastication, under the effect of salivary α-amylase (Hinsberger and Sandhu, 2004; Pederson, Bardow, Jensen, and Nauntofte, 2002). Subsequently, the food bolus is subjected to gastro-intestinal digestion, where digestive enzymes of the stomach and the small intestine secreted from liver/biliary system and pancreas. Finally, food bolus is passed to the large intestine and colonic bacterial fermentation occurs. The final stage plays a key role in the release of nutrients and non-nutrients, making them available for absorption through the gut barrier, especially in the proximal intestine (Biehler and Bohn, 2010; Hinsberger and Sandhu, 2004; Saura-Calixto et al., 2007). *In vitro* methods are useful to study the stability of polyphenols under gastro-intestinal conditions and the release from food matrices. Numerous studies reported the effect of *in vitro* gastro-intestinal digestion on the stability of pure phenolic compounds and anthocyanins from beverages and solid food matrices (McDougall et al., 2005; Tagliazucchi et al., 2010). Different rice cooking methods including electric rice cooking, microwave heating, or sterilization, would have different degradative effect on anthocyanins and phenolic compounds, which, in

turn, resulted in the difference of bioaccessibility and bioavailability. Bioaccessibility of anthocyanins from cooked dark purple rice is important since only the compounds released from the food matrix are potentially bioavailable, exerting their beneficial biological effects. Therefore, bioaccessibility of Thai dark purple rice cooked by various methods should be investigated so that its health benefits would be pointed out.

Colon cancer, also known as colorectal cancer, is the fourth and fifth leading causes of cancer deaths in males and females in Thailand in 2012, respectively (Srisukho, Srivatanakul, and Sumitsawan, 2012). The incidence rate of colon cancer is increasing in Thailand at the present. Colon cancer develops in the cells lining the inside of the colon and/or rectum. Diet is the most important exogenous factors so far identified in the etiology of colon cancer (Tomatis, 1990). Vegetables and fruits, dietary fiber, micronutrients, and phytochemicals appear to be protective against general cancer at the regular consumption (Hakimuddin, Paliyath, and Meckling, 2006). Foods rich in polyphenols have been shown to have cytoprotective and cytotoxic effect on various human cancer cells, such as human colon cancer cells, hepatic cancer cells and leukaemia (Chen et al., 2006; Hyun and Chung, 2004; Netzel et al., 2007; Pan, Lin, Lin, and Chen, 2007; Ugartondo, Mitjans, Touriño, Torres, and Vinardell, 2007). Olsson, Gustavsson, Andersson, Nilsson, and Duan (2004) reported that berries extract decreased the proliferation of colon cancer cells HT-29 and the effect was concentration-dependent. Anthocyanins-rich extracts (AREs) from grape, bilberry, and chokeberry suppressed HT-29 cell proliferation by 50% at concentrations of 25-75 mg of cyanidin-3-glucoside equiv/mL. They suggested that antioxidants such as vitamin C, carotenoids and anthocyanins may play an important role for inhibition of colon cancer cell proliferation *in vivo* by synergistic effects. AREs from blueberries effectively inhibit the growth of Caco-2 cells (Yi, Fischer, Krewer, and Akoh, 2005).

Furthermore, Jing, Bomser, Schwartz, He, Magnuson, and Giusti (2008) found that all AREs from various food sources suppressed HT-29 cell growth to varied degree as follows: purple corn (GI50~14 µg of cyanidin-3-glucoside equiv/mL) > chokeberry and bilberry > purple carrot and grape > radish and elderberry (GI50 > 100 µg of cyanidin-3-glucoside equiv/mL). It also reported that extracted phenolic compounds inhibited the growth of HT-29 cells to a lesser extent than the extracted anthocyanin (chokeberry). Thus anthocyanins are believed to be primary antiproliferative components. Recently, Hui et al. (2010) reported that ARE from Chinese raw black rice reduced the viability of breast cancer cells MCF-7 *in vitro* and *in vivo* by inducing apoptosis and suppressing angiogenesis. Anticancer activity from pigmented rice is usually based on raw rice, which is not the form of consumption. The effect of anthocyanins of cooked dark purple rice on antiproliferative effect against cancer cells has not been thoroughly studied.

1.2 Research objectives

The objectives of this research were:

1. To identify anthocyanins and phenolic compounds in various fractions of Thai dark purple rice and to investigate its thermal stability.

2. To investigate thermal stability of anthocyanins and phenolic acids, and antioxidant activity of Thai dark purple rice cooked by various methods.

3. To investigate bioaccessibility of anthocyanins of cooked Thai dark purple rice *in vitro*, and to evaluate antiproliferative activity of anthocyanin extract of cooked dark purple rice sample against human colon cancer cells.

1.3 Research hypotheses

Individual anthocyanins and phenolic compounds vary among various fraction of rice grain, the highest content of anthocyanins and phenolic compounds is found in bran, followed by whole rice and endosperm. Hot air and hot water heating have different effect on the stability of anthocyanin and phenolic compounds of Thai dark purple rice. Various cooking methods, namely electric rice cooking, microwave heating, and sterilization affect the content of anthocyanin and phenolic compounds as well as their antioxidant activity. In addition, the extract from rice cooked by various methods may have different inhibitory effect on colon cancer cell proliferation. The content of anthocyanins released from cooked dark purple rice varies during the phase of mouth and gastro-intestinal digestion. Determination of such contents would lead to the proper estimation of bioavailability of anthocyanins in cooked rice.

1.4 Scope of the study

Anthocyanins and phenolic compounds in various fractions of 2 cultivars of non-glutinous Thai dark purple rice grains were identified and quantitatively determined. In addition, antioxidant activities of these fractions were investigated. Stability of anthocyanins of Thai dark purple rice and their antioxidant activity under hot air and hot water heating at various temperatures were investigated. In addition, the changes of anthocyanins and phenolic compounds of Thai dark purple rice cooked by various cooking devices, namely an electric rice cooker, autoclave and microwave oven were investigated. Antiproliferative effect of methanolic extracts of rice cooked by 3 methods against human colon cancer cells was evaluated. Bioaccessibility of

anthocyanins from cooked dark purple rice based on *in vitro* model of mouth and gastro-intestinal digestion was investigated.

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CHAPTER II

LITERATURE REVIEWS

2.1 Pigmented rice

2.1.1 General information

Pigmented rice is a cereal grain containing pigments. It is widely consumed in many countries of Asia, such as Korea and China. The endosperm of rice kernels typically coated with firmly adhering bran (pigment pericarp) that is difficult to remove on milling without causing excessive breakage. Pigmented rice, such as dark red, dark purple, dark blue, black, and purple black grains, contains high content of anthocyanins (Figure 2.1).

Figure 2.1 Pigmented rice in Thailand, Mali Nil Surin 6 (dark purple rice).

From: Surin Rice Research Center, 2009.

It was reported that rice having dark color of pericarp layer has an important nutrition, minerals and non-micronutrients, such as dietary fiber, polyphenol content and antioxidants higher than rice having light-colored pericarp layer (white, yellow, and light red) (Choi, Jeong, and Lee, 2007; Kong and Lee, 2010). The major polyphenols in pigmented rice are anthocyanins which are considered as antioxidant (Ryu, Park and Ho, 1998; Ichikawa et al., 2001; Abdel-Aal, Young, and Rabalski, 2006; Hiemori, Koh, and Mitchell, 2009). The predominant phenolic compounds in black rice and pigmented brown rice is ferulic acid (Yawadio, Tanimori, and Morita, 2007).

At present, consumers are interested in pigmented rice worldwide due to its unique nutritional value and antioxidant properties. There were numerous works in pigmented rice on anthocyanins, phenolic compounds, antioxidants, and nutraceuticals properties in the world but limit data was known for pigmented rice in Thailand (Cho, Paik, Yoon, and Hahn, 1996; Ryu et al., 1998; Ichikawa et al., 2001; Hu, Zawistowski, Ling, Kitts, 2003; Hyun and Chung, 2004; Abdel-Aal et al., 2006; Chen, Kuo, Chiang, Chiou, Hsieh, and Chu, 2006; Nam, Choi, Kang, Koh, Kozukue, Friedman, 2006; Yawadio et al., 2007; Hiemori et al., 2009; Shen, Jin, Xiao, Lu, and Bao, 2009; Zawistowski, Kopec, and Kitts, 2009; Kong and Lee, 2010; Tananuwong and Tewaruth, 2010; Zhu, Cai, Bao, and Corke, 2010). These results found that Japanese and Korean pigmented rice contained predominant anthocyanins: cyanidin-3-glucoside and peonidin-3-glucoside, similar to Canadian and American pigmented rice but some individual anthocyanins are different. Japanese and Korean pigmented rice contains cyanidin-3-rhamnoside, cyanidin-3,5-diglucoside, and malvidin-3-galactoside, while cyanidin diglucosides and a cyanidin rutinoside has been found in Canadian and American pigmented rice. Anthocyanin composition in pigmented rice may affect

antioxidant activity and nutraceutical properties because individual anthocyanins have the difference antioxidant activity.

2.1.2 Mali Nil Surin rice

Aroma pigmented rice, Mali Nil Surin rice (*Oryza sativa* L.), obtained from screening of pure native pigmented rice variety, Mali Dam No.53 (seed coat color is black) which is sensitive to photoperiod. Mail Nil Surin rice is a one of 2 varieties obtained from Surin Rice Research Center in 2005. The seed coat of Mali Nil Surin rice is dark purple. The yield under organic production, some properties of 2 varieties are presented in Table 2.1.

Table 2.1 Properties of Mali Nil Surin rice variety.

Variety	Yield (kg/rai)	amylose (%)	Aroma	Plant length (cm)	Ear of paddy/clumb
Mali Nil Surin No.2	369	13.50	+	163	9
Mali Nil Surin No.6	224	13.22	+	161	7

From: Surin Rice Research Center, 2008.

Mali Nil Surin seed rice has a long grain and low amylose content. Amylose content of starch reveals the appearance and texture of rice and also affects the cooking and eating qualities. Therefore, Mali Nil Surin rice is soft, tender, fluffy and good aroma when cooked and is known as Esan's excellent aromatic non-glutinous pigmented rice. The color of seed coat of Mali Nil Surin rice shows that it is rich of anthocyanins like other pigmented cereals such as, purple corn, blue corn and black rice. Anthocyanins exhibit antioxidant activities. The consumption of

anthocyanin-rich foods can promote health and reduce the risk of chronic diseases, such as cancer and cardiovascular disease. Therefore, Mali Nil Surin rice is known as special quality rice.

2.2 Anthocyanins

2.2.1 Chemistry of anthocyanins

Anthocyanins (in Greek *anthos* means flower, and *kyanos* means blue) are phytochemical compounds and are the most important plant pigments visible to the human eye (Kong, Chia, Goh, Chai, and Brouillard, 2003). They are responsible for red, purple, and blue color of numerous flowers, fruits, vegetables and cereal grains (Hiemori et al., 2009; Ngo and Zhao, 2009). They are a group of water-soluble natural colorants that belong to the widespread class of phenolic compounds and family of flavonoids (Kong et al., 2003; Hu et al., 2003). Anthocyanins are glycosides and acylglycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (Figure 2.2). The differences among individual anthocyanins related to the number of hydroxyl groups, the position of the attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule (Table 2.2).

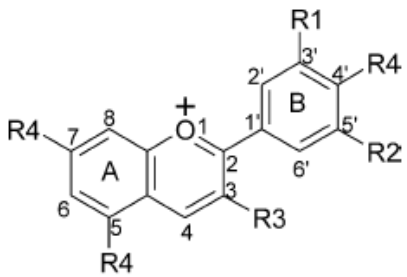


Figure 2.2 Flavylium cation. R_1 and R_2 are H, OH, or OCH_3; R_3 is a glycosyl or H; and R_4 is OH or a glycosyl.

From: Kong et al., 2003.

The most commonly occurring anthocyanin aglycones in higher plants are cyanidin (Cy), peonidin (Pn), malvidin (Mv), petunidin (Pt), delphinidin (Dp), and pelargonidin (Pg). The distribution of the six most common anthocyanidins in plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). Anthocyanins have been recognized as health-enhancing substances due to their antioxidant activity (Abdel-Aal et al., 2006; Nam et al., 2006; Philpott, Gould, Lim, and Ferguson, 2006; Satue-Gracia, Heinonen, and Frankel, 1997), anti-inflammatory (Tsuda, Horio, and Osawa, 2002), anti-atherosclerosis (Hiemori et al., 2009; Xia et al., 2006), anticancer (Zhao, Giusti, Malik, Moyer, Magnuson, 2004; Hyun and Chung, 2004; Kamei et al., 1995), hyperlipidemia (Hiemori et al., 2009, Guo, Ling, Wang, Liu, Hu, and Xia, 2007; Kwon et al., 2007), and hypoglycemic effects (Tsuda, Horio, Uchida, Aoki, and Osawa, 2003). Therefore, they gain interest from the food industry as functional colorants or functional food ingredients (Hiemori et al., 2009; Abdel-Aal et al., 2006).

Table 2.2 Naturally occurring anthocyanidins.

		Substitution pattern							
Name	Abbreviation	3	5	6	7	3′	4′	5′	Color
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H	Orange
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	6OHCy	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	Lt	H	OH	H	OH	OH	OH	H	Orange
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	5-MCy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	Orange
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidin	Rs	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	Tr	H	OH	H	OH	OH	OH	OH	Red

Me = methyl group (-CH_3).

From: Kong et al., 2003.

Cyanidin-3-glucoside, cyanidin-3-rhamnoside, cyanidin-3-rutinoside, cyanidin-3,5-diglucoside, peonidin-3-glucoside, and malvidin-3-galactoside have been found in Japanese, Korean, Canadian, and American pigmented rice varieties (Cho et al., 1996; Ryu et al., 1998; Abdel-Aal et al., 2006; Yawadio et al., 2007; Hiemori et al., 2009). A survey of these pigmented rice varieties (*Oryza sativa* L. *indica* and *Oryza sativa* L. *japonica*) demonstrated that cyanidin-3-glucoside and peonidin-3-glucoside are predominant ones. Thus, these results showed that some individual anthocyanins such as cyanidin-3-rhamnoside, cyanidin-3-rutinoside and malvidin-3-galactoside, may be found in some varieties of pigmented rice (Abdel-Aal et al., 2006; Cho et al., 1996).

Cho et al. (1996) determined the chemical structure of the purified anthocyanins from a pigmented rice cultivar in Korea. The experiments were performed using Amberlite XAD-7 column and preparative paper chromatography for the purification anthocyanins and using UV/Vis and NMR spectroscopy for the determination of individual anthocyanins. They found that major anthocyanins of a Korean pigmented rice cultivar (Suwon 415) were cyanidin-3-*O*-*β*-D-glucopyranoside. Ryu et al. (1998) isolated and characterized anthocyanins pigment in 10 newly bred black rice varieties of Korea and China using High Performance Liquid Chromatography (HPLC). They extracted anthocyanins using 0.5% trifluoroacetic acid (TFA) in 95% ethanol overnight and crude anthocyanins were purified by gel filtration and Amberlite XAD-7 column to remove sugars, amino acids, organic acids, low molecular phenols, and polymerized dark brown pigments. Major anthocyanins of pigmented rice were identified as cyanidin-3-glucoside, peonidin-3-glucoside with cyanidin-3-glucoside being the predominant one of 80-100%. Other anthocyanins found with a very low amount are cyanidin-3-rhamnoside, cyanidin-3-diglucoside and

malvidin-3-glucoside. Abdel-Aal et al. (2006) characterized anthocyanin composition of pigmented rice; red rice and black rice, from Canada. They found that cyanidin-3-glucoside and peonidin-3-glucoside were major anthocyanins in black and red rice. Cyanidin-3-glucoside was the most abundant anthocyanins in black rice and red rice, accounting for 88.2 and 67% of the total anthocyanins, respectively. Peonidin-3-glucoside was lower in black and red rice. These studies demonstrated that pigmented rice from various places around the world contain cyanidin-3-glucoside and peonidin-3-glucoside as major anthocyanins.

2.2.2 Extraction and purification

The extraction of phytochemicals from plant materials is the first step in the utilization of bioactive compounds in the qualitative and quantitative analysis and preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products. The form of plant materials is an important and affects the extraction efficiency. However, phenolic compounds can be extracted from fresh, frozen or dried samples. Before extraction, plant samples are usually treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. To retain high levels of phenolic content in plant samples, freeze-drying is more suitable than air-drying (Abascal, Ganora, and Yarnell, 2005; Dai and Mumper, 2010). The solvent extraction has been the most common method for extraction. Anthocyanins are polar compounds, thus the most common solvents used in the extraction are aqueous mixtures of ethanol, methanol or acetone (Kähkönen, Hopia, and Heinonen, 2001). The extraction of anthocyanins from grape pulp with methanol is 20% more effective than with ethanol, and 73% more effective than with only water (Metvier, Francis, and Clydesdale, 1980). Adjustment to low pH is found to enhance the efficiency of the anthocyanin extraction. Therefore, acidified methanol or ethanol

is the most common solvent (Cacace and Mazza, 2003; Castañeda-Ovando, Pacheco-Hernández, Páez- Hernández, Rodríguez, Galán-Vidal, 2009; Kapasakalidis, Rastall, and Gordon, 2006). The solvent destroys the cell membranes, simultaneously dissolves the anthocyanins and stabilizes them (Naczk and Shahidi, 2004). Nevertheless, ethanol is preferred to use in food industry due to the methanol toxicity. Extraction period is another factor that should consider for the extraction of anthocyanins. The time for the extraction usually varied from 1 min to 24 h has been reported. Longer extraction time increases the chance of oxidation of phenolics unless reducing agents are added (Khanna, Viswanatham, Krishnan, and Sanwai, 1968).

In acidified solvent extractions, strong acid media should be avoided because the acid may change the native form of anthocyanins, the glycoside bonds of 3-monoside anthocyanins could be destroyed, and acylated anthocyanins might be degraded by hydrolysis reaction (Kapasakalidis et al., 2006). Besides the acidified methanol and ethanol extraction, there are also reports with other extractants. Cacace and Mazza (2003) proposed to use sulfured water for the extraction of anthocyanins from black currents. The maximum amount of extracted anthocyanins from berries at SO_2 level of 1000-1200 ppm at 30-35°C and a solvent to berry ratio of 19:1 (v/w). Awika, Rooney, and Waniska (2005) used 0.1% HCl in methanol and 70% aqueous acetone to extract anthocyanins from black sorghum. They found that aqueous acetone extracts produced very low peaks at the 480 nm wavelength used for anthocyanin detection difference from acidified methanol extracts. This phenomenon occurs in aqueous acetone extract because the anthocyanin molecules undergo significant structural modification. Pyranoanthocyanins were products from oxidation of anthocyanins mediated by acetone (Lu and Foo, 2001). It could be concluded that

acidified methanol resulted in significantly higher values for total anthocyanins than aqueous acetone (Lee, Finn, and Wrolstad, 2004).

Pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE) are advanced methods for anthocyanin extraction. PLE also known under the trade name of accelerated solvent extraction (ASE), is a relatively new technology for the extraction of anthocyanins under high temperature and pressure. Ju and Howard (2003) indicated that PLE (80-100°C) with acidified water was an effective in extracting anthocyanins from red grape skins as the acidified 60% methanol. MAE is a process utilizing microwave energy to facilitate partition analytes from the sample matrix into the solvent. The main advantage of this technique is the reduction of extraction time and solvent volume as compared to the conventional extraction techniques (Eskilsson and Bjorklund, 2000). Liazid, Guerrero, Cantos, Palma, and Barroso (2011) reported that anthocyanins can be extracted from grapes using MAE in 5 min, at 100°C and 40% methanol in water. However, the compounds found in the low concentration, lower than 7% for glucosides (the main components) and lower than 9% for the acyl derivatives when compared to the solid-liquid maceration classical methods. Before selection the method, it is very important to assume that anthocyanins do not degrade under the proposed PLE and MAE conditions.

Solid-phase extraction (SPE) on C18 cartridges or Sephadex is commonly used for the intial purification of the crude anthocyanins extracts (Donner, Gao, and Mazza, 1997; Jing, Bomser, Schwartz, He, Magnuson, and Giusti, 2008). The anthocyanins are bound strongly to adsorbents through their unsubstituted hydroxyl groups and are separated subsequently from other compounds by increasing the polarity with different solvents (da Costa, Horton, and Margolis, 2000).

2.2.3 Analysis and identification

Anthocyanins are soluble in polar solvents, and they are normally extracted from plant materials using methanol that contains small amounts of hydrochloric acid or formic acid. The acid prevents the degradation of non-acylated anthocyanins pigments. The simplest assay for the quantification of anthocyanins as a group is based on the measurement of absorption at a wavelength between 490 nm and 550 nm, where all anthocyanins show a maximum absorption. This band is far from the absorption bands of other phenolics, which have spectral maximum in the UV range (Fuleki and Francis, 1968).

In general, traditional spectrophotometric assay is simple and fast screening method to quantify classes of phenolic compounds in the crude extract. Anthocyanins from various fruits and vegetables which they are rich in sugar and other small organic acids use the pH differential method for the determination of total anthocyanin content. This method takes the advantage of the structural transformations of anthocyanin chromophore as a function of pH. By this method the absorption of the sample is measured at pH 1 (anthocyanins as colored oxonium salts) as well as at pH 4.5 (anthocyanins as colorless hemiketals). Recently, Abdel-Aal and Hucl (1999) developed a simple, rapid method for screening anthocyanin- pigmented cereals and quantifying total anthocyanins in these grains. They found that total anthocyanins content could be determined by measuring the absorbance directly at 535 nm. This method is suitable for pigmented cereals due to low sugar, amino acids and other small acids. The pH differential method and the single wavelength determination can be used to calculate total anthocyanins content based on the molecular weight (MW) and the molar extinction coefficient (ε) of either the main anthocyanin in the sample or cyanidin-3-glucoside, the most common anthocyanins in nature.

High performance liquid chromatography (HPLC) with UV-Vis or photodiode array (PDA) detector currently represents the most popular and reliable technique for analysis of anthocyanins. Reversed-phase (RP) columns have considerably enhanced HPLC separation of different types of anthocyanins and RP C18 columns are almost exclusively employed. It has been reported that column temperature affect the separation of individual anthocyanins, and constant column temperature is recommended for reproducibility (Stalikas, 2007). Acetonitrile and methanol are the most commonly used mobile phase. In many cases, mobile phase was acidified with acids such as acetic, formic, and phosphoric acids to minimize peak tailing. Gradient elution is widely used to separate individual anthocyanins.

The identification of anthocyanins has a critical role in taxonomic and adulteration studies besides in the quality evaluation of crude and processed food. Even though HPLC-PDA has been also used in the anthocyanin quantification, but the difficulty to obtain reference compounds and the spectral similarities of the anthocyanins are major challenges. Therefore, HPLC coupled mass spectrometry (MS), APCI-MS, ESI-MS or tandem mass spectrometry (MS-MS) and nuclear magnetic resonance (NMR) of ^{1}H and ^{13}C have become the preferred techniques for anthocyanins identification.

HPLC-ESI-MS and HPLC-MS/MS have been very powerful tools for the anthocyanins identification. In the past, numerous ionization methods have been developed for non-volatile or thermodynamically unstable samples such as anthocyanins. Fast atom bombardment (FAB) and electrospray ionization (ESI) are considered as smooth ionization sources because they cause very low fragmentation and allow exact molecular weight determinations. MS-MS is widely used for identification of anthocyanins. They produce structural information about a compound

by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. For instance, anthocyanins in wild blueberries and common foods in the United State were identified by HPLC-ESI-MS/MS according to molecular weight and MS fragmentation pattern, and by comparison with standards and published data for different anthocyanins with the same mass spectra (Nicoue, Savard, and Belkacemi, 2007; Wu and Prior, 2005).

2.2.4 Stability of anthocyanins

Anthocyanins pigments are instable and susceptible to degradation (Giusti and Wrolstad, 2003). Their stability is affected by several factors such as chemical structure, concentration of the pigment, pH, temperature, light intensity and quality, the presence of copigments, flavonoids, proteins, metal ions, enzymes, oxygen, ascorbic acid, sugars sulfur dioxide, and their degradation products (Brouillard, 1982; Mazza and Brouillard, 1990; Rein, 2005).

2.2.4.1 Structural effects

Regarding molecular structure, some anthocyanins are more stable than the others. The increased hydroxylation decreases stability, whereas increased methylation increases it, such as petunidin-3-glucoside, with two hydroxyl groups in the B-ring, was less stable than peonidin-3-glucoside which has one hydroxyl group in the same ring (Brouillard, 1982; Cabrita, Fossen, and Anderson, 2000). The hydroxyl and methoxyl group does not affect only the stability of anthocyanins but also the color display. The color of anthocyanins changes from pink to blue as the number of hydroxyl group increases. The type of glycosyl units or sugar moieties in the anthocyanins molecule also influences stability. It is found that anthocyanins from cranberry containing galactose were more stable than those containing arabinose during storage (von Elbe and Schwartz, 1996). In addition, the

sugar substitutions also affect the perceived color of the anthocyanin pigments. Anthocyanin 3-glucoside has higher intensity than the corresponding 3,5- and 5-glucoside, and an increasing number of glucose units gives rise to more yellow pigments (Mazza and Brouillard, 1987). Previous studies have shown that anthocyanins with acylating substitutes are more stable during processing and storage than other natural pigments (Giusti and Wrolstad, 2003; Rein, 2005). In addition, acylated anthocyanins are also more resistant to color fading with increased pH than unacylated analogs (Cevallos-Casals and Cisneros-Zevallos, 2004). Giusti and Wrolstad (2003) showed that acylated anthocyanins which are rich in raddishes, red potatoes, red cabbage, black carrots and purple sweet potatoes increase stability during processing and storage. These pigments may impart desirable color and stability for commercial food products. Their finding is in agreement with Cevallos-Casals and Cisneros-Zevallos (2004) who reported that the colorants from red sweet potato and purple carrot which are rich in acylated anthocyanins showed higher stability than colorants from purple corn and red grape are rich in non-acylated anthocyanins. The improved stabilization has been attributed to the stacking of the acyl groups with the pyrylium ring of the flavylium cation, thereby reducing susceptibility of nucleophile attack of water and subsequent formation of a pseudobase or a chalcone (Bąkowska-Barczak, 2005).

2.2.4.2 Concentration effects

It has been reported that increased anthocyanins promoted higher color stability (Giusti and Wrolstad, 2003). Skrede, Wrolstad, Lea, and Enerson (1992) investigated the stability of strawberry and blackcurrant syrups and they found that color stability was dependent upon total anthocyanin concentration rather than the different types of individual anthocyanins. Increased anthocyanin concentrations also

increase intensity by multifold. It was observed that increasing the concentration of anthocyanins resulted in their self-association which can improve the stability (Brouillard, 1982).

2.2.4.3 pH

Anthocyanins are more stable in acidic media than in alkaline solution and they can be found in different forms at varied pH (Figure 2.3). An increase in pH inflicts in a decrease of both the color intensity and the concentration of the flavylium cation, as it is hydrated by nucleophilic attack of water to the colorless carbinol form. The carbinol form does not absorb visible light because the molecule lost the conjugated double bond between the A- and B-ring (Brouillard, 1982; Rein, 2005). Changes in the color intensity of anthocyanin are more significant in the alkaline region (Cabrita et al., 2000).

2.2.4.4 Temperature

Heat treatment is one of the most widely used methods for preserving and extending shelf-life of foods. Therefore, temperature is one of the most important factors that affect stability of anthocyanins. The degradation rate of anthocyanins increases during processing and storage with the increasing heating temperature (Kirca, Özkan, and Cemeroğlu, 2007; Rein, 2005). Thermal degradation of anthocyanins follows first order kinetics (Rhim, 2002; Ahmed, Shivhare, and Raghavan, 2004). Abdel-Aal and Hucl (2003) reported that increasing the temperature from 65 to 95°C resulted in an increased degradation of blue wheat anthocyanins. Wang and Xu (2007) investigated the degradation of anthocyanins with increasing heating temperature and it is clear that the degradation of blackberry anthocyanins increased with increasing heating temperature and time.

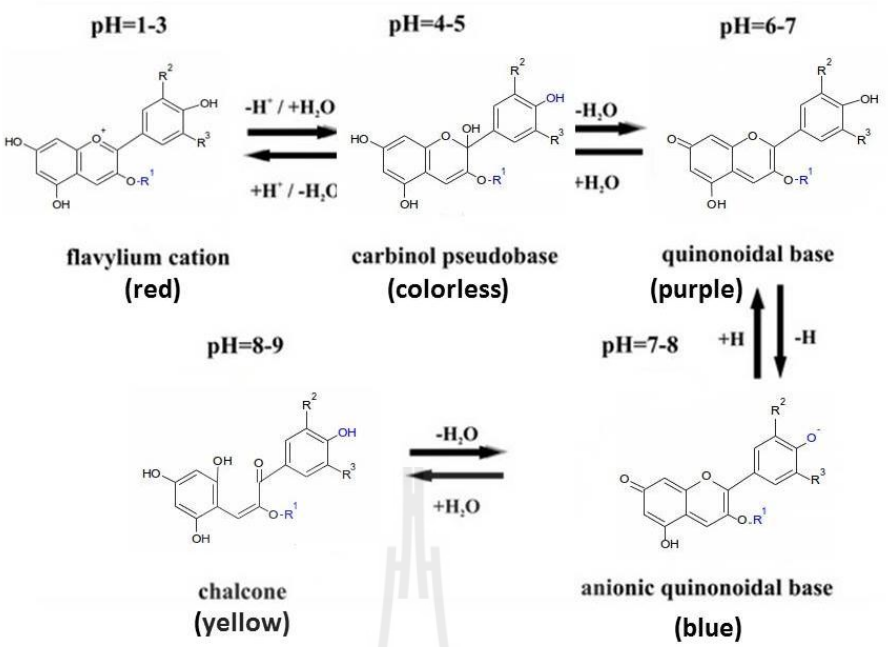


Figure 2.3 Changes of anthocyanin structure at various pHs.

Modified from: Ananga, Georgiev, Ochieng, Phills and Tsolova, 2013.

High temperature with high pH caused the degradation of cherry anthocyanins, resulting in the formation of degradation products such as benzoic acid derivatives and trihydrobezaldehyde (Seeram, Bourquin, and Nair, 2001). Increasing temperature at pH 2-4 induces the loss of the glycosyl moieties of the anthocyanins, by hydrolysis of the glycosidic bond. This leads to further loss of anthocyanin because aglycones are much less stable than their glycosidic forms. It is well known that anthocyanidins have lower stability than anthocyanins, especially in the neutral condition. They undergo rapid degradation by opening the C-ring, while the remaining B- and A-ring are then transformed to phenolic acids and aldehydes (Sadilova, Stintzing, and Carle, 2006). It has been reported that degradation products of cyanidin-3-glucoside and pelargonidin-3-glucoside were protocatechuic acid and phloroglucinaldehyde (Heimori et al., 2009; Sadilova, Carle, and Stintzing, 2007).

Moreover, vanillic acid was a degradation product from peonidin-3-glucoside (Stintzing and Carle, 2004). In general, the same structural factors which enhance pH stability of anthocyanins also increase their thermal stability (Von Elbe and Schwartz, 1996).

Anthocyanins are easily oxidized and susceptible to oxidative degradation during various steps of processing and storage (Patras, Brunton, O'Donnell, and Tiwari, 2010). Hiemori et al. (2009) reported that rice cooking using a rice cooker, pressure cooker, or on a gas range, caused a significant decrease of 74.2%, 79.8%, and 65.4% in cyanidin-3-glucoside, respectively. Various thermal processing treatments (blanching, boiling and steaming) of red cabbage resulted in a loss of 59%, 41%, and 29% anthocyanins, respectively (Volden, Borge, Bengtsson, Hansen, Thygesen, and Wicklund, 2008).

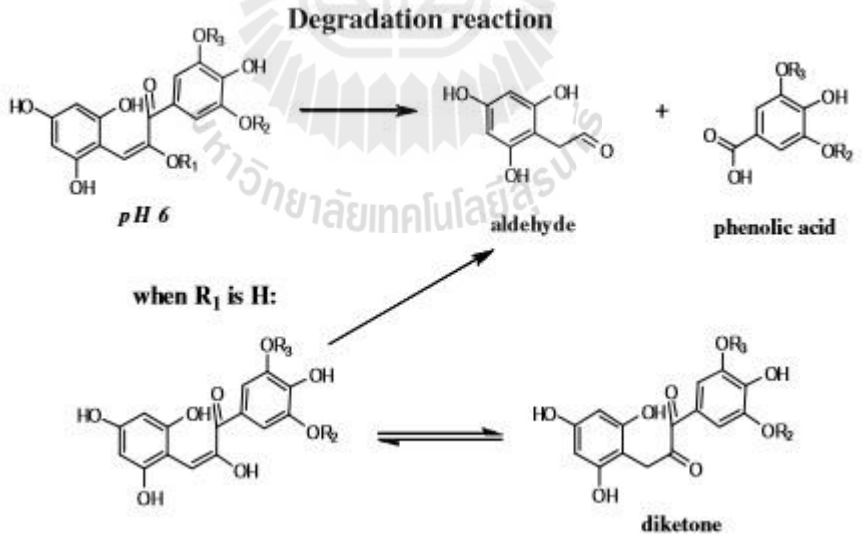


Figure 2.4 Degradation reactions for anthocyanins. Where R_1 = H or saccharide, R_2 and R_3 = H or methyl.

From: Castañeda-Ovando et al., 2009.

Besides adverse effect of temperature and storage, it was observed that it can also have a positive effect on anthocyanins. It has been shown that the anthocyanin content in strawberries and raspberries was increased in storage temperature over 0°C for eight days (Kalt, Forney, Martin, and Prior, 1999). Wang and Stretch (2001) reported that the highest anthocyanin content was found when stored different cranberry varieties at 15°C for 3 weeks.

2.2.5 Antioxidant activity

Anthocyanins showed ability to prevent lipid oxidation in different lipid environments, such as human low-density lipoprotein (LDL) *in vitro* and liposome and scavenging activity against various artificially generated free radical (Kähkönen and Heinonen, 2003). Therefore, it is important to physiological functions such as vision improvement, anticholesterolemia, and anticancer activities (Ichikawa et al., 2001).

Anthocyanidins and anthocyanins have shown to be possess antioxidant activity higher than vitamin C and E (Bagchi et al., 1998; Castañeda-Ovando et al., 2009). These compounds are able to capture free radicals by donation of phenolic hydrogen atoms (Chen, Chan, Ho, Fung, and Wang, 1996; Rice-Evans, Miller, and Paganga, 1996). The completely conjugated structure of anthocyanins that allows electron delocalization results in very stable radical products. The degree and position of hydroxylation and methoxylation in the B ring affect their stability and reactivity and thereby antioxidant actions.

Ichikawa et al. (2001) studied antioxidant properties of anthocyanins extracted from purple black rice (PBR). They analyzed hydroxyl radical scavenging, crocin bleaching activity and superoxide radical scavenging activity. They found that overall antioxidant potentials of anthocyanins extracted showed strong crocin bleaching activity similar to blueberry, at least 20 times stronger than Trolox

(reference antioxidant) at the same molar concentration. The highest activity was found in blueberry extract, but purple black rice (PBR) extract showed almost the same level of activity as blueberry. The purified cyanidin-3-glucoside of PBR also showed strong activity 75% of the activity in the original PBR extract indicating that anthocyanin cyanidin-3-glucoside contributes to the antioxidant activity of PBR through its strong superoxide radical scavenging activity. Hu et al. (2003) investigated the efficacy of an anthocyanins extract from black rice (*Oryza sativa* L. *indica*) to neutralize both reactive oxygen and nitrogen reactive species in chemical and cell culture models. They found that the extract of pigmented black rice containing known proportions of cyanidin-3-glucoside and peonidin-3-glucoside exhibited antioxidant activities and free radical scavenging capacities *in vitro* model systems. Black rice pigmented fraction (BRE) prevented supercoiled DNA strand scission which was induced by reactive oxygen species (specifically, peroxyl radical and hydroxyl radicals) and suppressed the oxidation of human low-density lipoprotein. In addition, BRE also suppressed the production of nitric oxide in the activated murine macrophage RAW264.7 cells, without introducing cytotoxicity. Therefore, black rice which contained anthocyanin pigments might have some health benefits associated with the relief of oxidative stress. Shen et al. (2009) evaluated total phenolics, flavonoids and antioxidant capacity of a large number of rice and to analyze their relationships with grain color, size and 100-grain weight. They found that total antioxidant capacity (using the ABTS assay) of methanolic extracts of red rice (0.291-2.963 mM TEAC) and black rice (2.527-5.533 mM TEAC) were higher than white rice (0.012-0.413 mM TEAC). Phenolic contents were positively correlated with the antioxidant capacity ($r = 0.962$) among all rice samples. Kong and Lee (2010) elucidated the distribution of the major antioxidant compounds in the milling fractions

of Korean black rice. They found that bran fraction of two cultivars, *Oryza sativa* cv. *Heugjinjubyeo* and *Oryza sativa* cv. *Heugkwangbyeo*, had high levels of free polyphenols (98.5 and 81.0 mg GAE per 1 g of dried sample) and flavonoids (19.8 and 15.1 mg (+)-catechin equivalents per 1 g of dried sample) as compared with endosperm fractions. This work concluded polyphenols and flavonoids group are considered as antioxidant. They did not determine individual polyphenols and flavonoids and their relationship on antioxidant activities. Tananuwong and Tewaruth (2010) found that the extraction of black glutinous rice at pH 6.8 using acetone-water mixture 70:30 (v/v) for 4 h resulted in the crude extract with highest antioxidant activities although its total phenolic contents and total monomeric anthocyanins (TMA) were not high. The studies revealed the pigmented rice cultivar exhibited various antioxidant capability, but the major individual phenolic compounds or anthocyanins contributed to high antioxidant activity has not been reported.

2.2.6 Nutraceutical properties

Epidemiological studies on the relationship between dietary habits and disease risk have shown that food has a direct impact on health. It is generally accepted that plant-derived foods, such as wine, fruits, nuts, vegetables, grains, legumes, spices, etc. exert some beneficial effects on human health, particularly on age-related diseases (Espín, García-Conesa, and Tomás-Barberán, 2007). As the human population lives longer, chronic age-related diseases, such as cardiovascular diseases, neurodegenerative diseases, type II diabetes, and several types of cancer (e.g. gastrointestinal cancer), known to be related to dietary habits, continue to expand. The capacity of some plant-derived foods to reduce the risk of chronic diseases has been associated, at least in part, to the occurrence of non-nutrient secondary metabolites (phytochemicals) that have been shown to exert a wide range of biological activities.

Their bioactivity has been associated with their antioxidant properties (capacity to scavenge free-radicals) which are involved in the onset development of many of the chronic degenerative diseases. Phytochemicals have low potency as bioactive compounds when compared to pharmaceutical drugs, but since they are ingested regularly and in significant amounts as part of the diet, they may have a noticeable long term physiological effect.

Numerous studies have shown that anthocyanins exhibited anticarcinogenic activity against multiple cancer cell types *in vitro* and tumor types *in vivo*. Potential cancer chemopreventive activities of anthocyanins revealed from *in vitro* studies including;

(1) Radical scavenging activity (antioxidant effects) (Elisa and Kitts, 2008).

(2) Stimulating of phase II detoxifying enzymes such as glutathione - related enzymes (Shih, Yeh, and Yen, 2007).

(3) Antiproliferation by blocking various stages of the cell cycle *via* effects on cell cycle regulator proteins e.g., p53, p21, p27, cyclin D1, cyclin A, etc. (Seeram et al., 2006).

(4) Anti-inflammatory effects of two inflammatory proteins, nuclear factor kappa B (NF-κB) and cyclooxygenase-2 (COX-2), is a common occurrence in many cancers (Bowen-Forbes, Zhang, and Nair, 2010).

(5) Anti-angiogenesis by inhibiting the process of forming new blood vessels from the existing vascular network (angiogenesis) which is an important factor in tumor growth and metastasis (Bagchi, Sen, Bagchi, and Atalay, 2004; Matsubara, Kaneyuki, Miyake, and Mori, 2005).

(6) Anti-invasiveness by reducing the expression of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA), both of which degrade extracellular matrix as part of the invasive process and, by stimulating the expression of a tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and inhibitor of plasminogen activator (PAI), both of which counteract the action of MMPs and u-PA (Chen et al., 2006).

(7) Induction of apoptosis (programmed cell death) by inducing apoptosis through both intrinsic (mitochondrial) and extrinsic (FAS) pathways (Katsube, Iwashita, Tsushida, Yamaki, and Kobori, 2003; Katsuzaki et al., 2003).

(8) Induction of differentiation by inducing cellular differentiation offers a cell-specific approach to cancer prevention and treatment, such as increasing adherence of cell to plastic, suggesting differentiation of leukemic cells into a monocyte/macrophage-like phenotype, resulting to reduce cell proliferation (Fimognari, Berti, Nüsse, Cantelli-Forti, and Hrelia, 2004; Wang and Stoner, 2008).

In vivo studies revealed that anthocyanins inhibited the development of cancer in carcinogen-treated animals. In most studies, the molecular mechanisms of tumor inhibition were not investigated in details. Animal models have been done on rats, mouse and mice to study anthocyanins inhibition on esophageal, colon and skin cancer. The mechanisms by which anthocyanins prevented these cancers are associated with cell proliferation, inflammation and angiogenesis (Afaq, Saleem, Kueger, Reed, and Mukhtar, 2005; Lala et al., 2006). Epidemiological studies in human have not provided obvious evidence of the anti-cancer effects of anthocyanins. For example, there was an examination the relationship between anthocyanidin intake and cancer risk in Italy by using a case-control study of 805 subjects with oral and pharyngeal cancer and 2,081 hospital controls without neoplasia (the pathological process that

results in the formation and growth of a tumor) was conducted. The results indicated no significant association between anthocyanidin intake and risk for oral or pharyngeal cancer. Anthocyanins and anthocyanidins from pigmented rice showed anticarcinogenic activity in multiple human cancer cells, such as human monocytic leukemia cells (Hyun and Chung, 2004), human hepatocellular carcinoma cell line, human tongue squamous cell carcinoma, and human cervical carcinoma (Chen et al., 2006).

Hyun and Chung (2004) tested cytotoxic activity on human monocytic leukemia cells. They isolated and identified two bioactive compounds, anthocyanidins: cyanidin and malvidin from pigmented rice, *Oryza sativa* cv. Heugjinjubyeo. Subsequently, they found that these compounds mediated the cytotoxicity through the arrest of the G_2/M phase of the cell cycle (anti-cell proliferation) and induction of apoptosis. They suggested that bioactive anthocyanidins isolated from *Oryza sativa* cv. *Heugjinjubyeo* could supply beneficial effects on health by inhibiting the growth of human monocytic leukemia cells. Chen et al. (2006) studied the effects of anthocyanins fraction (*Oryza sativa* L. anthocyanins; OAs), cyanidin 3-glucoside and peonidin 3-glucoside (extraction from black rice, *Oryza sativa* L. indica, in Taiwan) on cell invasion, motility, and adhesion, MMPs and u-PA expression, DNA binding activity and the nuclear translocation of AP-1 (activating protein-1) and NF-κB on human hepatocellular carcinoma cell lines (SKHep-1). They found that cells treated with OAs inhibited the cell invasion at the highest concentration of 200 μg/mL. Moreover, the inhibitory effect of OAs on cell motility was significant and also in a concentration-dependent manner. For cyanidin-3-glucoside and peonidin-3-glucoside, major anthocyanidins extracted from black rice, showed a marked inhibition on the invasion and motility of SKHep-1 cells. They explained that this effect was associated

with a reduced expression of matrix metalloproteinases-9 (MMP-9) and u-PA. In addition, these compounds also exerted an inhibitory effect on the DNA binding activity, the nuclear translocation of AP-1, and cell invasion on various cancer cells (SCC-4, Huh-7 and HeLa; human tongue squamous cell carcinoma, human hepatocellular carcinoma and human cervical carcinoma, respectively). From these results, it was evident that anthocyanins from OAs could inhibit the growth of human hepatocarcinoma cells (SKHep-1) *in vitro*, suggesting that the inhibition on invasion by peonidin 3-glucoside, or cyanidin-3-glucoside may be through a down-regulation of MMP-2, MMP-9, or u-PA expression of various cancer cells.

The lactate dehydrogenase (LDH) leakage assay, the neutral red, tuberculosis (TB) assay and the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay are the most common methods employed for the detection of antiproliferative activity or cell viability of toxic substances. *In vitro* MTT assay is one of the most used methods for preliminary screening. It determines the ability of viable cells to convert a yellow water soluble tetrazolium salt into insoluble purple formazan crystals by the mitochondrial dehydrogenase enzymes as shown in Figure 2.5. The MTT assay is a rapid, versatile, quantitative, and highly reproducible colorimetric method for mammalian cell viability (Liu and Zeng, 2009). Now all researchers used a rapid colorimetric assay, based on MTT salt by doing the experiments in a 96 well plate. After cell viability analysis by MTT assay, many researchers typically conduct further studies on the mechanisms of action on cancer cells. Flow cytometry has been used to determine anti-proliferation of cancer cells by cytotoxic components. Change or arrest of cell cycle in each phase (G1 S G2 and M phase) of cancer cells will be assessed. Subsequently, induction of apoptosis of cancer cells is performed by various methods, such as DNA fragmentation analysis. Apoptosis

can be induced in cells by the imposition of external stresses, such as bacteria toxins, heat shock, radiation, and oxidative stress, and failure of apoptosis is considered to contribute to the development of human cancer (McConkey and Orrenius, 1996; Que and Gores, 1996). The induction of apoptosis in tumor cells has become the means for cancer treatment because it has recently been suggested that cancer chemotherapeutics exert part of their pharmacological effects by triggering apoptotic cell death (Ahmad, Feyes, Nieminen, Agarwal, and Mukhtar, 1997).

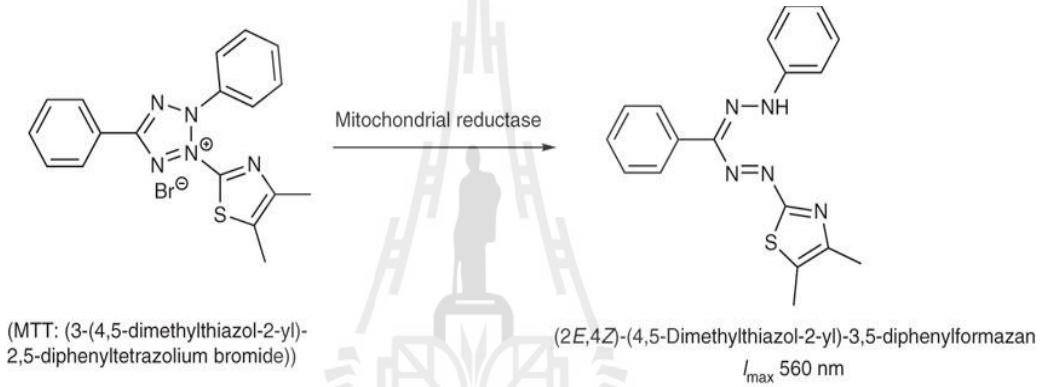


Figure 2.5 MTT reduction in live cells by mitochondrial reductase results in the formation of insoluble formazan, characterized by high absorptivity at 570 nm.

From: Ebada, Edrada, Lin, and Proksch, 2009.

2.3 Phenolic compounds

Phenolic compounds are considered as one of the main classes of secondary metabolites that are synthesized by plants during normal development and in response to stress conditions, such as infection, wounding, and UV radiation (Naczk and Shahidi, 2004). These compounds occur ubiquitously in plants and derived from phenylalanine and to a minor extent in some plants, also from tyrosine (Figure 2.6).

2.3.1 Chemistry of phenolic compounds

The term ‘phenolic’ or polyphenol’ can be defined as substances which possess an aromatic ring bearing one or more hydroxyl groups, including functional derivatives (esters, methylethers, glycosides etc.) (Ho, 1992). Phenolic compounds in plant foods belong to the families of phenolic acids, flavonoids, isoflavonoids, and tocopherols, among others (Shahidi and Naczk, 2004). Chemically, natural phenolic acids are distinguished by hydroxycinnamic and hydroxybenzoic acids structures. The most commonly encountered hydroxycinnamic acids are p-coumaric, caffeic, ferulic, and sinapic acids. Hydroxybenzoic acids mainly contain of p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids (Figure 2.7) (Qiu, Liu, and Beta, 2010).

Phenolic acids can be classified as free phenolic acids and bound phenolic acids (Zhou, Robards, Helliwell, and Blanchard, 2004). The level of free phenolic acids provided an index of grain resistance to *Sitophilus oryzae* attack in sorghum (Ramputh, Teshome, Bergvinson, Nozzolillo, and Arnason, 1999). Bound phenolic acids are typically involved in cell wall structure (Bunzel, Allerdings, Sinwell, Ralph, and Steinhart, 2002; Sun, Sun, and Zhang, 2001) where cross-linking of lignin components via phenolic acids appears to have a profound effect on the growth of the cell wall and its mechanical properties and biodegradability.

In fresh and processed plant foods, phenolic compounds are closely associated with sensory and nutritional quality. Generally, they contribute to astringency, undesirable color, flavor, odor and may also reduce the availability of minerals, such as zinc. Furthermore, phenolic compounds affect stability of products by undergoing oxidation during thermal processing and oxidized phenolics, such as quinines may combine with amino acids, resulting in loss of nutrient. However, many

phenolic compounds in plants are good sources of natural antioxidants. They are shown to have inhibitory effects on mutagenesis and carcinogenesis (Ho, 1992).

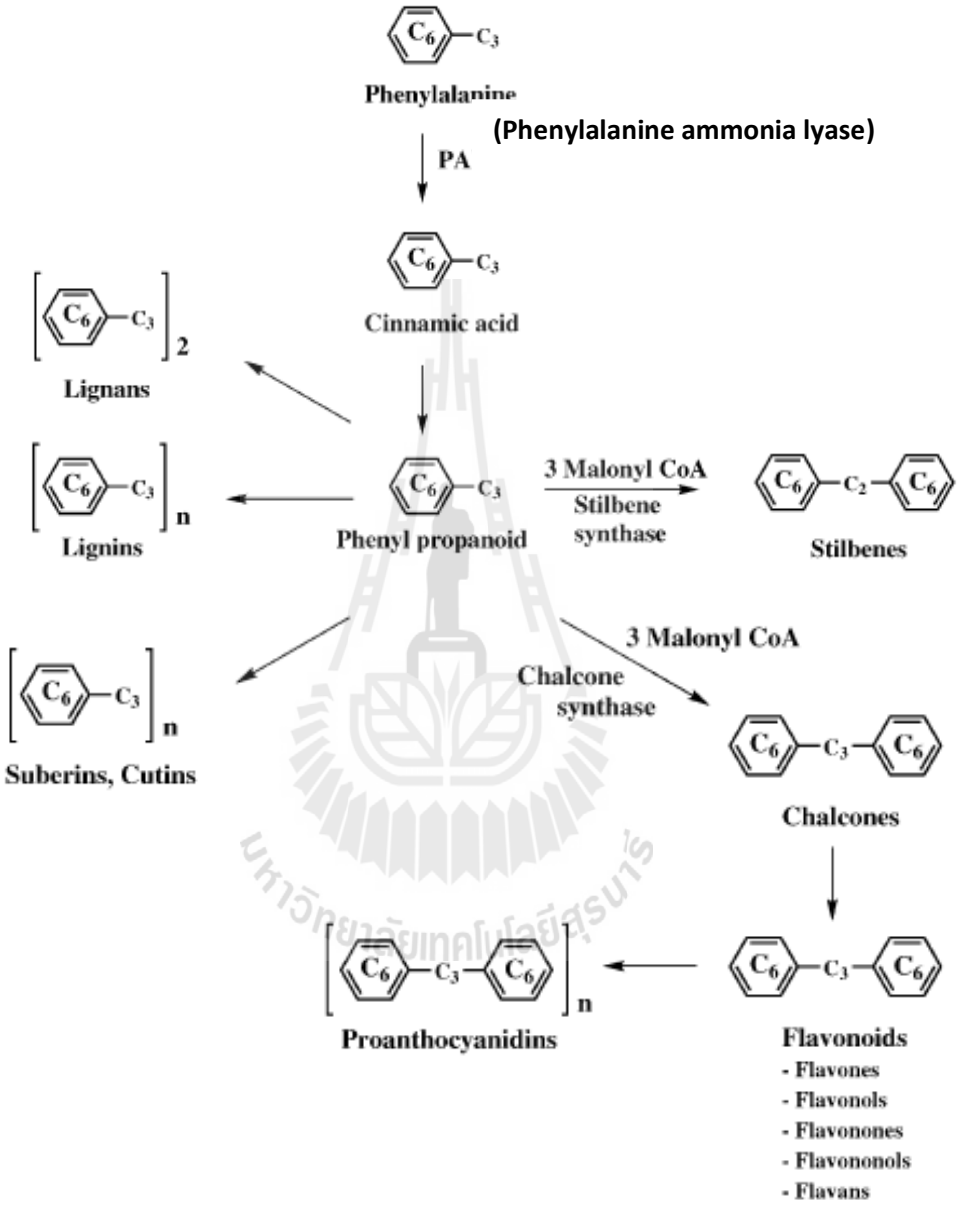
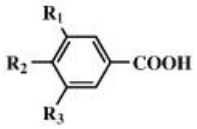


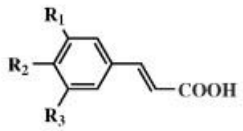
Figure 2.6 Production of phenylpropanoids, stilbenes, lignans, lignins, suberins, cutins, flavonoids, and tannins from phenylalanine.

From: Naczk and Shahidi, 2004.



Hydroxybenzoic acids

Acid		R_1	R_2	R_3
p-Hydrobenzoic	4-Hydroxybenzoic	H	OH	H
Protocatechuic	3,4-Dihydroxybenzoic	OH	OH	H
Vanillic	4-Hydroxy-3-methoxybenzoic	OCH_3	OH	H
Syringic	3, 5-Dimethoxybenzoic	OCH_3	OH	OCH_3
Gallic	3,4,5-Trihydroxybenzoic	OH	OH	OH



Hydroxycinnamic acids

Acid		R_1	R_2	R_3
p-Coumaric	4-Hydroxycinnamic	H	OH	H
Caffeic	3,4-Dihydroxycinnamic	OH	OH	H
Ferulic	4-Hydroxy-3-methoxycinnamic	OCH_3	OH	H
Sinapic	4-Hydroxy-3,5-dimethoxycinnamic	OCH_3	OH	OCH_3

Figure 2.7 Structures of phenolic compounds.

From: Fukumoto and Mazza, 2000.

Cereal grains contain a wide range of phenolic acids, ferulic and p-coumaric acids being the most significant quantities (Herrmann, 1989; Shahidi and Naczk, 2004; Zhou et al., 2004; Liyana-Pathirana and Shahidi, 2007; Qiu et al., 2010). The outer layers consists higher levels of phenolics than those located in the inner parts. Cell wall phenolics, such as lignins and hydroxycinnamic acids are linked to various cell components. These compounds contribute to the mechanical strength of cell walls, play a regulatory role in plant growth, morphogenesis, and in the cell response to stress and pathogens. Ferulic and p-coumaric acids, the major phenolic compounds, may be esterified to pectins and arabinoxylans or cross-linked to cell wall polysaccharides in the form of dimers, such as dehydroferulates and truxillic acid.

Rice endosperm cell wall contained 12 g/kg of esterified cinnamic acids comprising of 9 g/kg of ferulic acids, 2.5 g/kg of p-coumaric acids and 0.5 g/kg diferulic esters (Clifford, 1999; Shibuya, 1984). Most studies only focused on non-glutinous white rice. In pigmented rice, many researchers were interested to study phenolic compounds in:

(1) Determining total and individual phenolic compounds by colorimetric and instrument techniques (Tian, Nakamura, Cui, and Kayahara, 2005; Yawadio et al., 2007; Shen et al., 2009). They found that HPLC was suitable for the separation of individual phenolic acids in rice grains.

(2) Investigating antioxidant properties of phenolic compounds (Butsat and Siriamornpun, 2010; Qiu et al., 2010; Tananuwong and Tewaruth, 2010). They suggested that the antioxidant activities varied in each rice fractions, husk, bran, brown rice and milled. In addition, the antioxidant activity of soluble and insoluble in fractions is partially attributed to its phenolic acid profile.

(3) Elucidating effect of thermal and nonthermal process on phenolic compounds (Zhu et al., 2010), they suggested that suitable dose of irradiation might be carefully selected and use minimize the loss of antioxidant phenolic compounds in whole grain rice during storage.

(4) Evaluating nutraceutical properties such as the prevention diabetic complications by inhibiting the key enzyme, aldose reductase (Yawadio et al., 2007). They found that isolated compounds (anthocyanins, flavonoids, phenolic acids, and tocopherols) from black rice showed significant inhibitory activity against aldose reductase suggesting that these bioactive compounds might contribute significantly in combating diabetic complications as health-promoting food ingredients in food processing.

Zhou et al. (2004) determined the distribution of phenolic acids in three cultivars of fresh and aged rice (Koshihikari, Kyeema and Doongara varieties) grown in the Murrumbidgee Irrigation Area (MIA) of New South Wales, Australia during the 1999/2000. They found that ferulic acid and p-coumaric acid were predominant phenolic compounds and ferulic acids had higher levels (255-362 mg/kg grain) than p-coumaric acid (70-152 mg/kg grain). Brown rice contained higher amount of phenolic compounds than milled rice. Milled rice had low levels of ferulic acid about 61-84 mg/kg grain. Bound phenolic acids (insoluble form of phenolic acids by involving in cell wall structure where cross-linking of lignin components via phenolic acids appeared to have profound effect on the growth of the cell wall and its mechanical properties and biodegradability) consisted of 80-90% of total phenolic acids for brown rice and 53-74% for milled rice. When they considered the effect of temperature on phenolic acids, they found that storage led to a decrease in total and bound phenolic acid contents in both brown and milled rice, and the decline was greater at 37°C than at 4°C. Tian et al. (2005) determined phenolic compounds in brown rice and germinated brown rice (Koshihikari variety, in Nagano, Japan) by HPLC. They found that phenolic compounds; 6'-*O*-feruloylsucrose, 6'-*O*-sinapoylsucrose, ferulic, sinapinic, p-coumaric, chlorogenic (3-caffeoylquinic), caffeic, protocatechuic, p-hydroxybenzoic, vanillic, and syringic acids in rice could be separated by HPLC using C18 column by gradient elution. Yawadio et al. (2007) identified phenolic compounds isolated from Japanese pigmented rice (*Ozyza sativa* L. japonica) by HPLC and their aldose reductase inhibitory activities. They found that black rice showed two anthocyanidins, cyanidin-3-*O*-β-glucoside (85%) and peonidin-3-*O*-β-D-glucoside (15%), while pigmented brown rice showed phenolic compounds, ferulic acid (85.7%) and tocols (14.3%). For aldose reductase inhibitory activity of

isolated compounds, it was in the following decending order: cyanidin-3-glucoside > ferulic acid > peonidin-3-glucoside > tocopherol. These data suggested that black rice and pigmented brown rice possessed marked health benefits in the prevention of diabetic complications by inhibiting the key enzyme (aldose reductase) which involved catalyzing NADPH-dependent reductions of various sugar-derived carbonyl compounds to sugar alcohols (e.g. sorbitol) acting in the development of diabetic complications such as retinopathy and neuropathy. Zhu et al. (2010) studied the effect of γ-irradiation on phenolic compounds in the rice grains of three genotypes (black, red and white) and they found that the major phenolic compounds identified were p-coumaric acid, ferulic acid and sinapinic acid. Whereas two anthocyanins, cyanidin-3-glucoside and peonidin-3-glucoside, were identified in pigmented grain samples. From the results, γ-irradiation treatment at the highest doses decreased total contents of phenolic acids and anthocyanins, but a decrease of these compounds were not completely in a dose-dependent manner. Surprisingly, 6 and 8 kGy significantly increased total contents of anthocyanins and phenolic acids in black rice. They suggested that when storage rice grain by γ-irradiation treatment, suitable dose of irradiation should be carefully chosen as a feasible way to minimize the loss of phenolic acids and anthocyanins in whole rice grain.

2.3.2 Antioxidant activity

Natural antioxidants are primarily plant-polyphenol compounds which may occur in all parts of the plant. Plant phenolics are multifunctional and antioxidant activity is mainly due to their redox properties, which allow them to act as reducing agents (free radical terminators), hydrogen donators, and singlet oxygen quenchers. In addition, they act as a metal chelator (Rice-Evans, Miller, Bolwell, Bramley, and Pridham, 1995; Shahidi and Naczk, 2004). A relationship between the structures of

the phenolic compounds and their antioxidant activities has been established. The antioxidant activity of phenolic compounds and their esters depends on the structure and the number of hydroxyl groups in the molecule that would be strengthened by steric hindrance (Rice-Evans et al., 1996). It has been found that the derivatives of cinnamic acids showed higher antioxidant capacities than the derivatives of benzoic acid (Marinova and Yanishlieva, 2003). This is consistent with the electron withdrawing potential of the single carboxyl functional group attached to the aromatic rings, dampening the H-donation capacity of the hydroxyl group. Gallic acid, with 3-hydroxyl groups, was characterized by the highest antioxidant activity (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, and Bahorun, 2005). The antioxidant activities of the hydroxycinnamic acids were in the order: rosmarinic acid > chlorogenic acid > caffeic acid > ferulic acid > p-coumaric acid. Cuvelier, Richard, and Berset (1992) indicated that the presence of CH=CH−COOH group in cinnamic acids ensures greater efficiency than the COOH group in benzoic acid. They suggested that the double bond participated in stabilizing the phenoxyl radical by resonance.

Cereals contain a wide range of phenolic compounds by occuring in the grain primarily in the bound form as conjugates with sugars, fatty acids, or protein (White and Xing, 1997). For rice, phenolic antioxidants among varieties and milling fractions have been extensively studied. Most studies have been investigated on white rice cultivar producing in various geographical regions of the world and previous studies on phenolic compounds in rice can be described as follows:

Shen et al. (2009) evaluated total phenolics, flavonoids and antioxidant capacity of a large number of rice genotypes (481 accessions) and to analyze their relationships with grain color, size, and weight. Total phenolics, flavonoid contents and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS)

radical cation antioxidant capacity were different among various genotype. Their values increased in the order of white rice < red rice < black rice. Among all rice accessions, the grain color parameters had negative correlations with the phenolics, flavonoids and antioxidant capacity (p<0.001). They suggested that these data provided opportunities to increase the content of phenolics, flavonoids and antioxidant capacity by breeding, especially in white rice. Hiemori et al. (2009) investigated the influence of cooking methods on the stability of anthocyanins of black rice and they found that cooking black rice resulted in the thermal degradation of cyanidin-3-glucoside in concomitant with the production of protocatechuic acid.

2.4 Bioaccessibility

2.4.1 Definition of bioaccessibility

It is widely recognized that consumption of polyphenol-rich foods including fruits, vegetables and whole grains, has been related to reduce the risk of cardiovascular diseases and cancers (Bouay, Deußer, Hoffmann, and Bohn, 2012). The bioavailability of a dietary compound is dependent upon bioaccessibility, digestive stability and the efficiency of its transepithelial passage.

Bioaccessibility is defined as the amount of food constituent that is released from the solid food matrix and present in the gut (Chandrasekara and Shahidi, 2012). It is widely accepted that only polyphenols released from the food matrix by the actions of chemical environments, digestive enzymes and bacterial microflora (large intestine) are bioaccessible in the gut and, therefore, potentially bioavailable.

2.4.2 Bioaccessibiliy of anthocyanins

Anthocyanins are believed to be one of bioactive compounds preventing oxidation and cancer initiation (Kong et al., 2003). The beneficial effects of

anthocyanins rely on their bioaccessibility, bioavailability and metabolic fate. The bioavailability of a dietary compound is dependent upon its release of compounds from solid food matrices, referred as bioaccessibility, its digestive stability, cellular uptake, metabolism, and further transport in the circulatory system (Tagliazucchi, Verzelloni, Bertolini, and Conte, 2010). Digestion is a physical process that permits the extraction of macronutrients, micronutrients and phytochemicals from the food matrix, for subsequential absorption (Hinsberger and Sandhu, 2004). In human, the digestive process starts in the mouth under the effect of salivary α-amylase and lingual lipase, the initial degradation of polysaccharides and triglycerides during mastication occur (Bouayed et al., 2012; Hinsberger and Sandhu, 2004; Pederson, Bardow, Jensen, and Nauntofte, 2002). Subsequently, the food bolus is subjected to gastro-intestinal digestion, where digestive enzymes of the stomach and the small intestine, via secretions from liver/biliary system and pancreas, and later also colonic bacterial fermentation in the large intestine, together play a key role in the release of nutrients and non-nutrients (Biehler and Bohn, 2010). Importantly, it is widely accepted that not all constituents present in the food matrix may be completely bioaccessible (Saura-Calixto, Serrano, and Goñi, 2007). It has been reported that bioavailability differs greatly from one polyphenol to another, and for some compounds it depends on dietary source (Manach, Williamson, Morand, Scalbert, and Rémésy, 2005). *In vitro* bioaccessibility of grape was investigated and it was observed that *in vitro* gastric digestion resulted in an increase in bioaccessible anthocyanins 2.77 mg of cyaniding-3-glucoside/100 g of grapes (86.0% increase obtained by chemical extraction) (Tagliazucchi et al., 2010). The transition from the acidic gastric to the mild alkaline intestinal environment caused a decrease in the amount of bioaccessible anthocyanins,

and a decrease of bioaccessible anthocyanin after the incubation with pancreatic solution was observed. The *in vitro* bioaccessibility of anthocyanins of grapes was 7.6% when compared to total anthocyanins content obtained by chemical extraction. Bioaccessibility of anthocyanins from apple varieties was not different between by chemical extraction and following gastric digestion (Bouayed, Hoffmann, and Bohn, 2011). It was found that after the gastric phase, anthocyanins in apple were not detectable in the intestinal phase. A limit data of *in vitro* studies on bioaccessibility of anthocyanins from various foods have shown that the types of foods are important factor.

Several reports have highlighted poor bioavailability of several groups of anthocyanins, not exceeding the plasma concentrations of 1% of dose and correspondingly low levels of urinary excretion as intact or conjugated forms(Cooney, Jensen, and McGhie, 2004; Liang et al., 2012; Wu, Cao, and Prior, 2002). However, compounds that are present in plasma at low concentrations may be present in the gastro-intestinal lumen at much greater concentrations after direct consumption of a meal. In this case, the most important factors in determining the potential beneficial effects of polyphenols on the gut epithelial cells are their bioaccessibility and their stability under gastro-intestinal conditions. The information involving bioaccessibility of cooked pigmented rice which is rich in anthocyanins is not available at present. It is important data to know in order to evaluate the potential absorption and beneficial effects in human body. Therefore, the investigation of *in vitro* bioaccessibility of anthocyanins from cooked dark purple rice is one of the purposes for this work.

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CHAPTER III

COMPOSITION AND THERMAL STABILITY OF ANTHOCYANINS AND PHENOLIC COMPOUNDS OF THAI DARK PURPLE RICE

3.1 Abstract

Anthocyanins and phenolic compounds composition and their antioxidant activity were determined in various rice fractions, namely bran, whole rice and milled rice, of 2 cultivars of Thai dark purple rice, Malinil Surin No. 2 and No. 6. Anthocyanins and phenolic compounds were mainly located in bran fraction. Predominant anthocyanins were cyanidin-3-glucoside and peonidin-3-glucoside, while protocatechuic acid and vanillic acid were major phenolic compounds extracted by acidified methanol. Degradation of anthocyanins and a decrease of antioxidant activity occurred to a greater extent in the presence of water and hot air treatment counterpart at any studied temperatures of 60-90°C. Under hot water heating treatment, degradation of cyanidin-3-glucoside and peonidin-3-glucoside resulted in the formation of phenolic acids, protocatechuic and vanillic acids. Although thermal treatments resulted in the degradation of anthocyanins, protocatechuic and vanillic acids possessing antioxidant activity were formed.

3.2 Introduction

Rice, *Oryza sativa* L., is one of the most important cereals consumed by over half of the world's population (Hu, Zawistowski, Ling, and Kitts, 2003). There are many rice varieties worldwide. Pigmented rice is one of the cultivars widely consumed in many Asian countries. It has dark pericarp layer and is considered to be high in nutritional values and health-promoting compounds, particularly anthocyanins. Health-enhancing effects of anthocyanins are antioxidant activity (Kähkönen and Heinonen, 2003; Rice-Evans, Miller, and Paganga, 1996), anti-inflammatory (Tsuda, Horio, and Osawa, 2002), anti-atherosclerosis (Hiemori, Koh, and Mitchell, 2009; Xia et al., 2006), anticancer (Zhao, Giusti, Malik, Moyer, and Magnuson, 2004), hyperlipidemic (Guo, Ling, Wang, Liu, Hu, and Xia, 2006; Hiemori et al., 2009; Kwon et al., 2007), and hypoglycemia (Tsuda, Horio, Uchida, Aoki, and Osawa, 2003).

Cyanidin-3-glucoside (cy-3-glu), cyanidin-3-rhamnoside (cy-3-rham), cyanidin-3-rutinoside (cy-3-rut), cyanidin-3,5-diglucoside (cy-3,5-diglu), peonidin-3-glucoside (pn-3-glu), and malvidin-3-galactoside (mal-3-gal) were individual anthocyanins identified in Japanese, Korean, Canadian, and American pigmented rice varieties (Abdel-Aal, Young, and Rabalski, 2006; Cho, Paik, Yoon, and Hahn, 1996; Hiemori et al., 2009; Ryu, Park, and Ho, 1998; Yawadio, Tanimori, and Morita, 2007). Besides anthocyanins, phenolic acids typically found in the outer layer of rice grains are also nutritionally important. Generally, phenolic compounds found in white rice exist in free form, soluble conjugates as glycosides, and insoluble conjugates to polysaacharides. Predominant phenolic acids in rice are ferulic acid, p-coumaric acid and diferulate which are limited in fruits and vegetables (Adom and Liu, 2002). Composition and bioactivities of anthocyanins and phenolic acids in Thai pigmented

rice have not been fully realized. Moreover, the degree of removal of bioactive compounds of pigmented rice, namely anthocyanins and phenolic compounds, caused by milling process has not been well established.

Stability of anthocyanins and phenolic compounds is affected by several factors, such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins and metal ions (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodíguez, and Galán-Vidal, 2009; Rein, 2005). Hot air drying is a typical process for rice grain processing. Boiling is also commonly practiced in rice cooking. Heating in the presence or absence of water may have different effect on retention of anthocyanins and phenolic compounds as hot air and water transfer heat differently. In addition, these compounds likely undergo thermal degradation, generating new compounds. Abdel-Aal and Hucl (2003) reported that increasing the temperature from 65 to 95°C resulted in an increased degradation of blue wheat anthocyanins. In addition, a decrease of phenolic acids, especially hydroxycinnamic acids during kilning was reported (Maillard and Berset, 1995). The linkages between individual phenolic acids and/or lignin, arabinoxylan or other compounds could be broken by heat, resulting in the formation of off-flavors of p-vinylguaiacol from decarboxylation of ferulic acid (Naim, Striem, Kanner, and Peleg, 1988). Bioactivities of thermally-processed rice are likely to be different from those of raw rice. Bioactivities of cooked rice are more related to health-promoting effect as rice is mainly consumed after thermal treatment. Therefore, changes of the composition of anthocyanins and phenolic acids, and bioactivities of Thai pigmented rice after thermal processing should be studied. Objectives of this study were to characterize anthocyanins and phenolic compounds in various rice grain fractions of Thai dark purple rice and their antioxidant activities. In addition, the

influence of dry and hot water on the stability of anthocyanins and phenolic compounds in Thai dark purple rice was investigated.

3.3 Materials and methods

3.3.1 Samples

Two cultivars of dark purple non-glutinous rice (*Oryza sativa* var. *indica*), namely Mali Nil Surin rice No.2 (MNS2) and No.6 (MNS6) were obtained from Surin Rice Research Center (Surin, Thailand). These 2 cultivars have different production yield (369 and 224 kg/ 1600 m^2) and amylose content (13.50 and 13.22 %), respectively.

3.3.2 Chemicals

Folin-Ciocalteu reagent, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and gallic acid (GA) were purchased from Sigma (St. Louis, MO). Cyanidin-3-glucoside chloride, peonidin-3-glucoside chloride, ferulic acid (FA), p-coumaric acid (p-cou), vanillic acid (VA), p-hydroxybenzoic acid (p-hydroxy), syringic acid (Syr), protocatechuic acid (PCA), chlorogenic acid (Chl) were obtained from Extrasynthese (Genay Cedex, France). HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

3.3.3 Sample preparation

Paddy rice of two cultivars was dehusked and milled using a laboratory milling machine (Satake Co., Hiroshima, Japan), yielding bran, whole rice or husk-removed rice grains and milled rice. Whole rice and milled rice were lyophilized,

subsequently ground using an IKA laboratory milling machine (M20 Janke and Kunkel Co., Staufen, Germany), and sieved through a 60-mesh screen. The ground samples were kept at -20°C until use.

3.3.4 Heating conditions

Thermal stability of anthocyanins and phenolic acids of dark purple rice hot water was studied at 60, 70, 80, and 90°C. For hot air treatment, whole rice was heated at the studied temperatures for 60 min (Memmert Model BE-200, Schwabach, Germany). For hot water treatment, whole rice was added with distilled water preheated to the studied temperatures at a ratio 1:5 (w/w) and filled into a 250-mL erlenmeyer flask. Flasks were capped and placed in a thermostatic water bath (Grant Instruments, Cambridge, UK) set at a specified temperature for 60 min. After thermal treatments, samples (took everything, water plus rice for hot water treatment) were lyophilized, ground, sieved and stored at -20°C until use.

3.3.5 Extraction

Anthocyanins and phenolic compounds were extracted according to Abdel-Aal and Hucl (1999) with slight modifications. Three grams of lyophilized samples were extracted twice with 24 mL of methanol acidified with 1.0 N HCl (85:15, v/v) at a shaking speed of 1,200 rpm for 60 min at room temperature. The pH of the mixture was controlled at pH 1. The mixtures were centrifuged at 10,000×g for 20 min at 4°C, and supernatant was refrigerated at 4°C for 2 days to precipitate insoluble matters. The extract was recentrifuged at 10,000 ×g for 20 min at 4°C. Supernatants were brought to 50 mL with acidified methanol. Total anthocyanin content, extractable phenolic content and antioxidant activities were determined. Subsequently, 40 mL of the acidified methanol extracts were concentrated under a stream of nitrogen. The precipitates formed during N_2-flushing were separated by

centrifugation as described above. The concentrated extracts were adjusted to 4 mL with acidified methanol and vigorously mixed and filtered through a 0.45 µm Nylon Acrodisc syringe filter membrane for determination of individual anthocyanins and phenolic compounds using HPLC.

3.3.6 Determination of anthocyanins and phenolics

Total anthocyanin content (TAC) of each extract was determined using the spectrophotometric method according to Abdel-Aal and Hucl (1999). Absorbance of acidified methanol extract was measured at 535 nm. TAC (µg/g of sample) was calculated as cyanidin-3-glucoside as it was a major anthocyanin found in pigmented rice using the following equation:

$$TAC = A \times 288.21$$

Where, A is the absorbance reading.

Extractable phenolic contents (EPC) were determined as described by Waterhouse (2005). An aliquot (20 µL) of the appropriate diluted extract was mixed with 1,580 µL deionized water and 100 µL of Folin-Ciocalteu reagent was added and incubated at room temperature for 5 min. Three hundred µl of 20% (w/v) sodium carbonate solution was added to the mixture followed by incubation for 2 h at room temperature. Absorbance was measured at 765 nm. Total phenolic contents were expressed as mg gallic acid equivalents (GAE) per 100 g sample.

The concentrated extracts were analyzed for individual anthocyanins using liquid chromatography equipped with photo diode array detector (HPLC-PDA) (Agilent HP1100 system, Agilent Technologies, California, USA) according to Bordonaba and Terry (2008) with some modifications. Anthocyanins were separated on a Zorbax Eclipse XDB C_{18} column (5 µm, 150 × 4.6 mm i.d.; Agilent Technologies, Palo Alto, CA). Injection volume of the extract for analysis of individual anthocyanins

was 20 µL. Anthocyanins were eluted using a gradient mobile phase consisting of 2.5% acetonitrile and 5% formic acid (A) and acetonitrile (B) as follows: 0-10 min, 10% B; 10.00-10.01 min, 10-45% B; 10.01-15.00 min, 45% B; 15.00-15.01 min, 45-80% B; 15.01-18.00 min, 80% B; 18.00-18.01 min, 80-10% B; 18.01-22.00 min, 10% B at a flow rate of 1 mL/min. The separated anthocyanins were quantified at 520 nm. Peak identification and quantification were carried out using the external standards, cyanidin-3-glucoside (cy-3-glu) and peonidin-3-glucoside (pn-3-glu).

Individual phenolic compounds were determined according to the method described by Nave, Cabrita, and da Costa (2007). A gradient of 2.5% methanol and 0.5% formic acid (A) and methanol (B) was as follows: 0-11 min, 10-25% B; 11-12 min, 25-28% B; 12-15 min, 28-36% B; 15-17 min, 36-40% B; 17-19 min, 40-42% B; 19-24 min, 42-50% B; 24-31 min, 50-80% B; 31-35 min, 80% B; 35-40 min, 80-10% B; 40-45 min, 10% B at a flow rate of 1 mL/min. Phenolic acids were separated on a Zorbax Eclipse XDB C_{18} column. The injection volume was 20 µL and the wavelength was set at 280 nm. Quantification of phenolic acids was achieved using the external standards of GA, PCA, p-hydroxy, VA, Syr, p-cou and FA.

Individual anthocyanins and phenolic acids were also confirmed by liquid chromatography with mass spectrometry (LC-MS) using an Agilent 1100 LC/MSD SL quadrupole mass spectrometer (Palo Alto, CA, USA). The mass spectrometer was operated in the electrospray ionization (ESI) positive ion mode scanning mass range from 0-1000 m/z and 50-1,600 m/z for anthocyanins and phenolic acids, respectively. Operating condition of mass analyzer was set as follows: drying gas flow of 12.0 L/min, drying gas temperature of 275°C, drying gas pressure of 11.0 psi, nebulizer pressure of 45 psi and capillary voltage was 3 kV.

3.3.7 Antioxidant activity

3.3.7.1 ABTS radical scavenging assay

ABTS radical scavenging activity of each sample was determined according to Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) with slight modifications. Twenty µL of the extracts (with appropriate dilution if necessary) were mixed with 1.48 mL of the working solution, and a decrease of absorbance was measured at 734 nm after 6 min at 30°C using a spectrophotometer. This activity was expressed as mmol Trolox equivalents/ 100 g dry weight (Shen, Jin, Xiao, Lu, and Bao, 2009).

3.3.7.2 Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the modified method of Yang and Zhai (2009). Fifty µL of each extract (with appropriate dilution if necessary) was added to 0.95 mL of freshly prepared FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ in 40 mM HCl, and 1 part of 20.0 mM $FeCl_3 \cdot 6H_2O$ solution), and the reaction mixture was incubated at 37°C for 30 min. An increase in absorbance at 593 nm was measured. Results were expressed as mmol equivalents of Trolox/ 100 g dry weight.

3.3.8 Statistical analysis

Data with 3 replications were analyzed and performed using SPSS 17.0 software (SPSS Inc., Chicago, IL) and the significant differences at $p<0.05$ of means were compared by Duncan's multiple-range test (DMRT). All values were expressed as means ± S.D.

3.4 Results and discussion

3.4.1 Changes of anthocyanins and phenolics

MNS2 and MNS6 are dark purple rice and pure cultivars were selected from black non-glutinous rice (Mali Dam No.53). MNS2 had higher TAC than MNS6 in all fractions (Table 3.1). Bran fraction contained the highest TAC, while milled rice showed the lowest (Table 3.1). Pigmented rice is typically consumed in the form of whole rice whose TAC was about 2-8 times higher than that in milled rice. Anthocyanins were highly concentrated in the bran. Therefore, pigmented rice bran is a potential source for rice anthocyanin extraction. Anthocyanins are secondary metabolite of plants *via* the shikimic acid pathway. Two biochemical building blocks derived from photosynthesis, acetic acid and phenylalanine, are converted into anthocyanins by series of enzymes that are bound to cell membrane. Anthocyanins are further excreted on the other side of the membrane into vacuoles in the epidermal cell layer (Chen et al., 2006). Bran contains pericarp layer, aleurone layer and germ, whereas milled rice mainly contains endosperm and part of subaleurone layer (Saunder, 1985). Whole rice is a better source of anthocyanin than milled rice.

Previous studies have shown that anthocyanins pigment is located in the pericarp layer of rice grain (Chung and Shin, 2007; Hu et al., 2003; Xia et al., 2006). Abdel-Aal et al. (2006) reported that TAC of black USA sweet rice bran was about 25.35 mg/g of grain. In addition, TAC of purple rice bran was about 32.5 mg/g of bran (Jang and Xu, 2009).

Table 3.1 Total anthocyanin content and methanolic extractable phenolic content of 2 cultivars of Thai pigmented rice and their antioxidant activity.

Cultivar	Rice fraction	TAC (mg/100 g DW)	EPC (mg GAE/100 g DW)	$ABTS^{\cdot+}$ (mmol TE/100 g DW)	FRAP (mmol TE/100 g DW)
MNS2	Bran	728.21 ± 6.50^{f}	2671.1 ± 37.53^{f}	23.53 ± 0.12^{c}	21.71 ± 0.46^{d}
	Whole rice	203.69 ± 0.82^{d}	832.63 ± 9.64^{d}	4.76 ± 0.10^{b}	8.71 ± 0.14^{c}
	Milled rice	47.61 ± 0.40^{b}	265.67 ± 15.85^{b}	1.52 ± 0.03^{a}	2.20 ± 0.03^{a}
MNS6	Bran	433.28 ± 3.00^{e}	2279.5 ± 60.00^{e}	26.86 ± 0.69^{d}	27.37 ± 0.40^{e}
	Whole rice	109.62 ± 1.09^{c}	724.83 ± 11.65^{c}	5.56 ± 0.08^{b}	6.92 ± 0.00^{b}
	Milled rice	20.54 ± 0.17^{a}	186.5 ± 4.40^{a}	1.30 ± 0.03^{a}	1.75 ± 0.00^{a}

Different superscripts indicate significant difference in a column at $p<0.05$.

Phenolic content determined in this study reflects methanolic extractable phenolic compounds rather than total phenolic content in the sample. Phenolic compounds in rice exist as both free and bound forms (Adom and Liu, 2002). Bound phenolic acids are typically involved in cell wall structure. They are conjugated or cross-linked with lignin components and are mainly found in bran fraction (Zhou, Robards, Helliwell, and Blanch, 2004). Extractable phenolic acids in the present study seem to be as free phenolic acids which are located in the outer layer of the pericarp and can be extracted using organic solvents (Dykes and Rooney, 2007; Mattila, Pihlava, and Hellstrom, 2005). Among various rice fractions, bran showed the highest EPC, and milled rice was found to have the lowest value (Table 3.1). The results showed that anthocyanins and phenolic compounds are localized mainly in the external layers rather than in endosperm of the grains. Therefore, polishing or milling process significantly reduces phenolic content of pigmented rice. Phenolic compounds of light brown rice are different from those of pigmented rice. The formers contain mainly low molecular weight phenolics, including ferulic and p-coumaric acids, while the latters are predominant with higher molecular weight phenolics like anthocyanins (Deng, Xu, Zhang, Li, Gan, and Li, 2013; Tian, Nakamura, and Kayahara, 2004; Walter and Marchesan, 2011).

3.4.2 Identification of anthocyanins and phenolic acids

Two major peaks (I and II) of anthocyanin were detected in bran, whole rice and milled rice samples (Figure 1). Retention time of peak I and II corresponded to that of cy-3-glu and pn-3-glu, respectively. In addition, *m/z* of $[M + H]^+$ of peak I and II was found to be 449 and 463, coinciding with mass of cy-3-glu and pn-3-glu, respectively. Therefore, cy-3-glu and pn-3-glu were predominant anthocyanins in Thai

dark purple rice bran. In addition to cy-3-glu and pn-3-glu, cy-3-rham, cy-3,5-diglu, and mal-3-gal have been found in Japanese and Korean pigmented rice varieties (Ryu et al., 1998; Cho et al., 1996; Ha, Park, Lee, and Lee, 1999). Black rice produced in Canada contained cy-3-glu (88.15%), pn-3-glu (7.10%), cy-3-rut (0.87%) and two isomers of cyanidin-diglucosides (cy-diglu) (3.1% and 0.78%). These studies indicated that individual anthocyanins in pigmented rice varied with cultivars and geographical origin. Concentration of individual anthocyanins differed among fractions and cy-3-glu was the dominant anthocyanin in all fractions of Thai pigmented rice (Figure. 3.2). In addition, rice bran of 2 cultivars showed the concentration of cy-3-glu and pn-3-glu approximately 5-9 times higher than that of milled rice. In these 2 cultivars, the whole rice contained both anthocyanins approximately 2-4 times higher than milled rice. These results suggested that bran and whole rice are a good sources of anthocyanins in Thai pigmented rice.

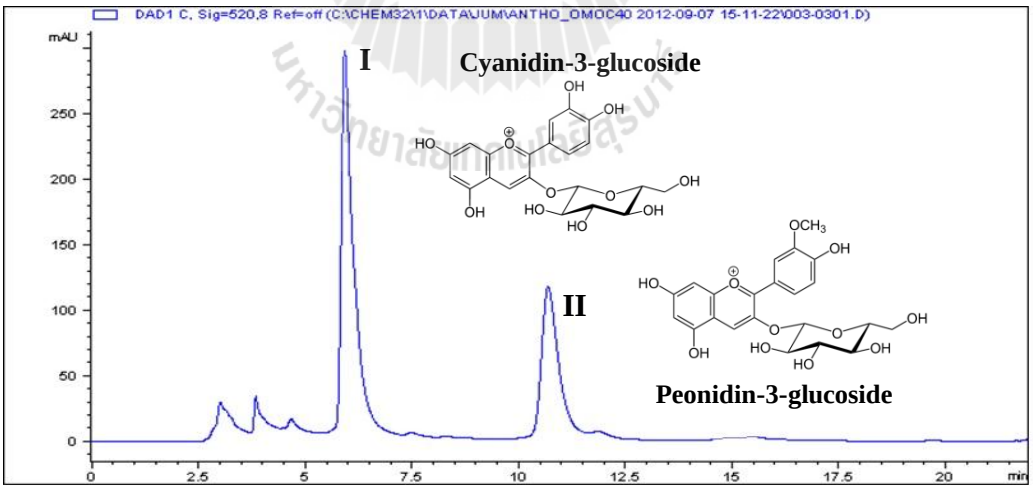

Figure 3.1 Typical HPLC chromatogram of major anthocyanins extracted from whole rice of Thai pigmented rice, MNS6, monitored at 520 nm.

For chromatogram of phenolic acids extracted from MNS6 milled rice, retention time and *m/z* of peak 3, 4, and 5 corresponding to 155, 169.1, and 195, revealed that they are PCA, VA and FA (Figure 3.3). Distribution of phenolic acids in various rice fractions of 2 cultivars is comparable (Table 3.2). PCA and VA were predominant phenolic acids extracted from bran and whole rice. Bran contained the highest amount of all phenolic acids detected, while milled rice showed the lowest contents. Therefore, milling process not only removed anthocyanins but also phenolic acids. FA, PCA and caffeic acid have been reported as free phenolic acids in pigmented rice (Hiemori et al., 2009; Morimitsu, Kubota, Tashiro, Hashizume, Kamiya, and Osawa, 2002). Furthermore, PCA or 3,4-dihydroxybenzoic acid was predominant phenolic acid in black japonica and heugjinjubyeo rice species (Chung and Shin, 2007; Hiemori et al., 2009). FA is a major phenolic acid in white rice and brown rice usually concentrated in the outer layers of the grains (Adom and Liu, 2002; Zhao and Moghadasian, 2008). Bound phenolic acids in cereal grains are typically involved in cell wall structure (Naczk and Shahidi, 1989). FA predominantly exists in insoluble bound form in rice grains (Mattila et al., 2005; Rao and Muralikrishna, 2001). FA may be esterified to arabinose residues in primary cell wall arabinoxylan or cross-linked to cell wall polysaccharides in the form of dimers, such as dehydroferulates and truxillic acid in the aleurone and pericarp layers (Clifford, 1999). Low concentration of FA was found in milled MNS rice, and FA was not detected in bran and whole rice. It is possible that small portion of FA exists in free form in endosperm while it covalently interacts with other components or self cross-linking to diferulic acid (DFA) in the pericarp layer. It has been reported that FA was found in milled rice of Thai white rice variety (Khao Dawk Mali 105) approximately < 5 mg/ kg using methanol as extractant (Butsat and Siriamornpun, 2010).

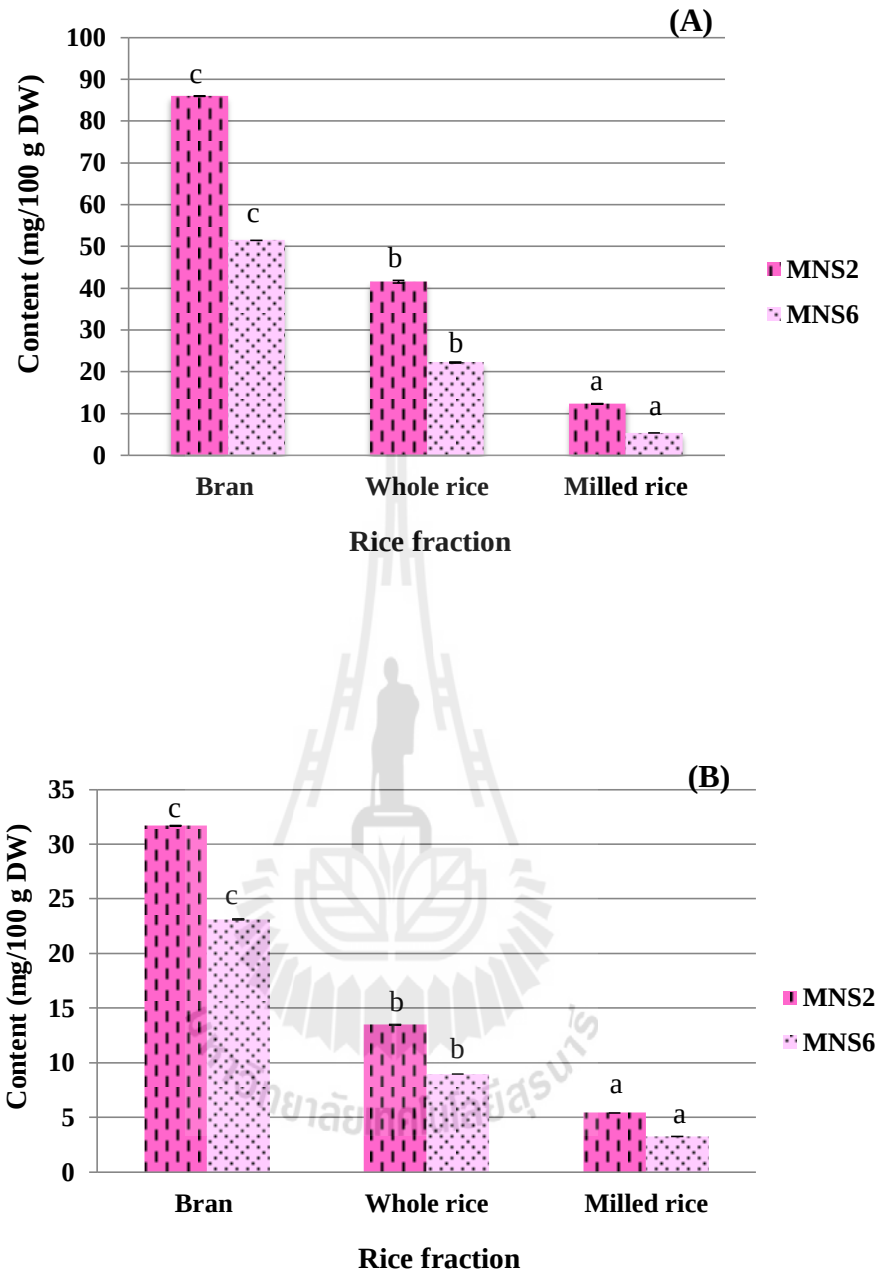

Figure 3.2 Distribution of cy-3-glu (A) and pn-3-glu (B) in each fraction of 2 cultivars of Thai pigmented rice (MNS2 and MNS6). Means values of different rice fractions (bran, whole rice and milled rice) in each cultivar marked with different letters are statistically different (p<0.05).

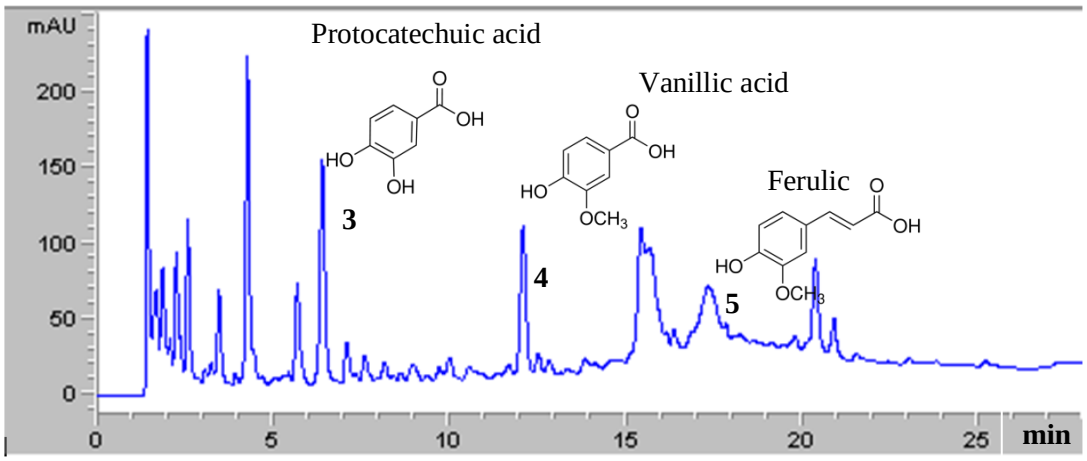

Figure 3.3 Typical HPLC chromatogram of methanolic soluble phenolic acids extracted from milled rice of Thai pigmented rice, MNS6, monitored at 280 nm.

Table 3.2 Distribution of phenolic acids in various rice fractions of Thai pigmented rice cultivars.

Cultivar	Fraction	Phenolic acid (mg/100 g dry weight)		
		Vanillic acid	Protocatechuic acid	Ferulic acid
MNS2	Bran	80.50 ± 0.29^{d}	113.85 ± 2.38^{c}	ND
	Whole rice	35.75 ± 1.39^{b}	49.79 ± 0.61^{b}	ND
	Milled rice	17.48 ± 0.12^{a}	18.48 ± 0.30^{a}	4.33 ± 0.55
MNS6	Bran	65.16 ± 5.56^{c}	103.56 ± 11.18^{c}	ND
	Whole rice	37.24 ± 1.29^{b}	50.94 ± 0.48^{b}	ND
	Milled rice	18.86 ± 0.93^{a}	19.19 ± 0.18^{a}	4.71 ± 0.55

ND = not detect. Different letters within a column are different ($p < 0.05$).

3.4.3 Antioxidant activity

Antioxidant activities of 2 cultivars were the highest in the bran fraction, corresponding to the highest content of anthocyanin and phenolic compounds. Nam, Choi, Kang, Kozukue, and Friedman (2005) reported that purple rice bran of two Korean pigmented rice varieties showed the scavenging effect by inhibiting superoxide anions, hydroxyl radicals and chelating of ferrous ions. Chung and Shin (2007) reported that the outer layer of purple rice, *Oryza sativa* cv. *Heugjinjubyeo*, have potent antioxidant activity due to the high concentration of anthocyanins, alkaloids and phenolic acids. Jang and Xu (2009) indicated that methanol or hydrophilic extracts of purple rice bran showed high total phenolic content and free radical scavenging activity.

It is well noted that the completely conjugated structure of anthocyanins allows electron delocalization, resulting in very stable radical products. The degree and position of hydroxylation and methoxylation in the B ring affect their stability and reactivity and thereby antioxidant actions (Kähkönen and Heinonen, 2003). Since it was estimated that about 85% of phenolic compounds in pigmented rice was anthocyanins (Hu et al., 2003), the main compounds contributing to such antioxidant activities are likely derived from anthocyanins.

3.4.4 Stability of anthocyanins and phenolic compounds

3.4.4.1 TAC, EPC and antioxidant activity

Degradation of TAC and EPC increased with heating temperatures, regardless of heating method. Hot air drying resulted in the degradation of anthocyanins and phenolic compounds in dark purple rice to a lesser extent than heating with water at any temperatures. Hot air drying leads to degradation of the anthocyanins due to high temperatures and high oxygen (Markakis, 1982). It is well

known that degradation of anthocyanins is primarily caused by oxidation and cleavage of covalent bonds, enhancing by thermal treatments. It has been reported that deglycosylation is the first thermal degradation step for anthocyanins. Sadilova, Stintzing, and Carle (2006) reported that cy-3-glu undergoes deglycosylation during heating, yielding cyanidin, which could further degrade to phloroglucinaldehyde and PCA.

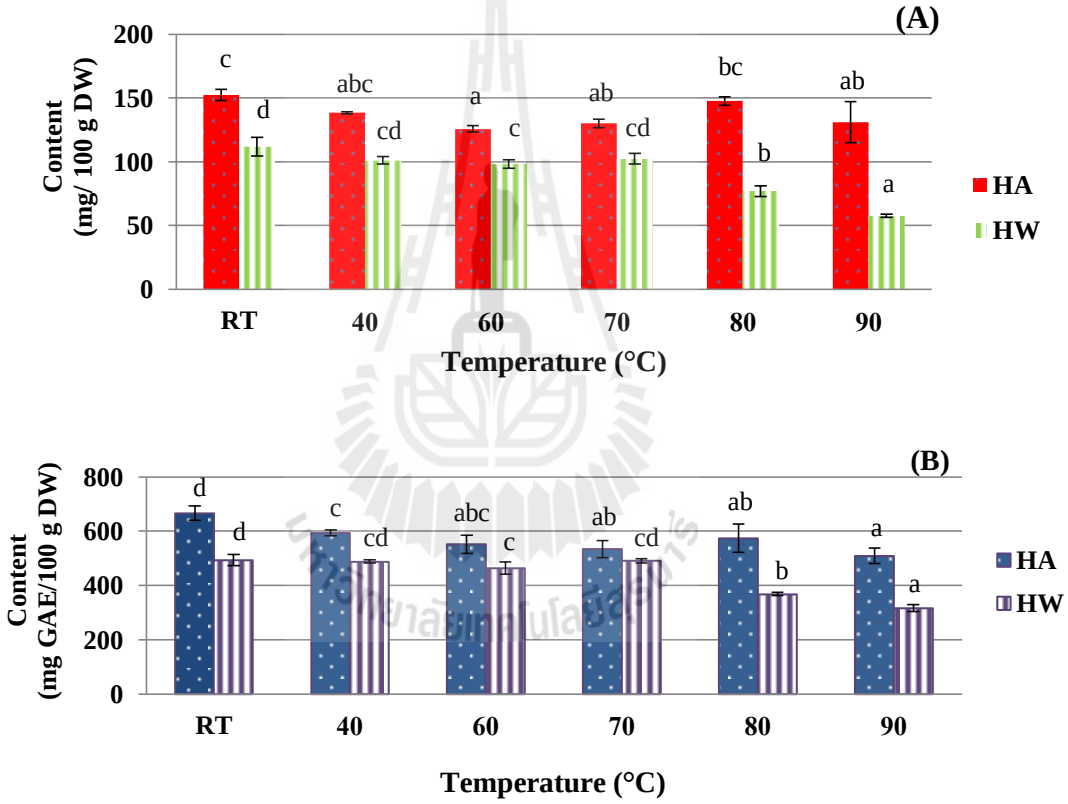

Figure 3.4 The content of anthocyanins (A), phenolic compounds (B) and ABTS radical scavenging activity (C) of MNS6, whole rice or husk-removed rice, under hot air (HA) and hot water (HW) at various temperatures for 60 min. Different letters indicate significant difference within heating method (p<0.05). RT= unheated sample.

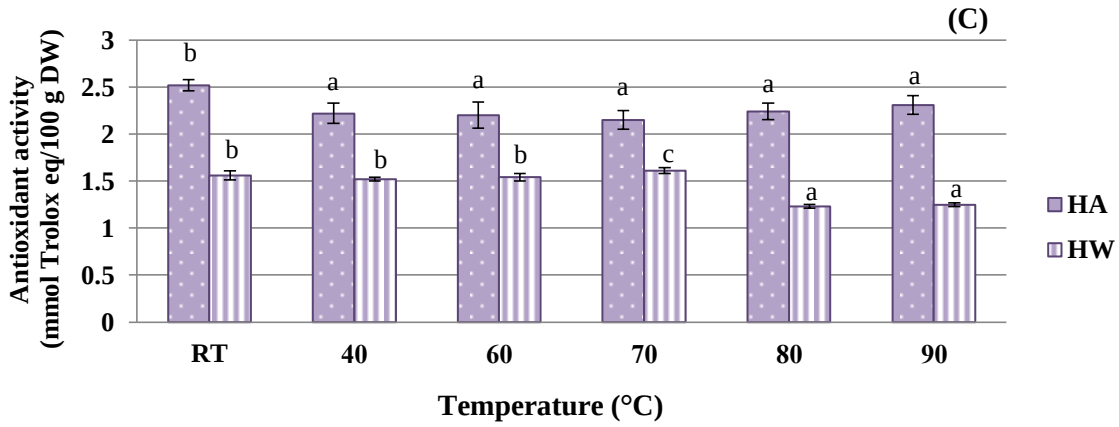

Figure 3.4 The content of anthocyanins (A), phenolic compounds (B) and ABTS radical scavenging activity (C) of MNS6, whole rice or husk-removed rice, under hot air (HA) and hot water (HW) at various temperatures for 60 min. Different letters indicate significant difference within heating method (p<0.05). RT= unheated sample (Continued).

In the presence of water, leaching efficiency of phenolics and anthocyanins in pigmented rice grains increased (Lamberts, Brijs, Mohamed, Verhelst, and Delcour, 2006). Higher temperature can increase membrane permeability in the rice cell wall, facilitating phenolic and anthocyanin extraction (Spanos et al., 1990). Moreover, bound phenolic acids may be released due to the breakdown of the cellular constitutes (Dewanto, Wu, Adom, and Liu, 2002). Arabinoxylans and lignin are the major matrix polysaccharides in the cell wall of rice grains and they form network structure between cellulose microfibrils (Zhou et al., 2004). Arabinoxylans possess FA, and lignins are conjugated with p-coumaric acid (p-Cou) (Maillard and Berset, 1995). Linkages between arabinoxylans and FA, and lignins and p-Cou were broken down by thermal treatments, resulting in the liberation of FA and p-Cou at high temperatures. These liberated phenolic compounds likely underwent further thermal degradation. FA

is one of hydroxycinnamic acids which are reconigized to undergo decarboxylation and oxidative reactions during thermal processing, It can be thermally degraded to generate aroma compounds such as 4-vinylguaiacol, guaiacol and vanillin (Jiang and Peterson, 2010).

At a low-moisture condition of solid foods and high temperature, production of furfural and 5-hydroxymethyl furfural increases, and this can degrade anthocyanins, pigment molecule (Stojanovic and Silva, 2007). Anthocyanins degradation under hot air might take place slower than heating in the presence of water. Rate of heat transfer and leaching out of anthocyanins and phenolics increase in the presence of hot water (Kronholm, Hartonen, and Riekkola, 2007; Petersson, Liu, Sjöberg, Danielsson, and Turner, 2010). Degradation of leached out anthocyanins and phenolics likely undergo thermal degradation to a greater extent than those in the rice grain matrix.

Antioxidant activity decreased at all samples studied heating when compared to that of unheated sample (RT). Antioxidant activity was the highest at 70°C under hot water heating. High concentration of anthocyanin and phenolic compounds was also found at this temperature, indicating that anthocyanin/phenolic compounds contributed to ABTS radical scavenging activity. Thermal degradation of anthocyanins results in the formation of polyphenolic degradation product. However, Maillard reaction products formed during heating may slightly contribute the antioxidant activity value (Elizalde, Bressa, and Rosa, 1992; Lamberts et al., 2006; Yen and Hsieh, 1995).

3.4.4.2 Changes of individual anthocyanins and phenolic acids

Individual anthocyanins decreased as heating temperature increased (Figure 3.5). During thermal treatment, deglycosylation of anthocyanins

normally takes place, forming sugar moieties and anthocyanidins (aglycons). The released reducing sugars may react with amino acids, resulting in the formation of Maillard browning reaction products (Chaovanalikit and Wrolstad, 2004). Maillard browning products and their intermediates possess antioxidant properties (Morales and Babbel, 2002). It is well known that anthocyanidins have lower stability than anthocyanins, especially in the neutral condition. Anthocyanidins undergo rapid degradation by opening the C-ring, while the remaining B- and A-ring are then transformed to phenolic acids and aldehydes (Sadilova et al., 2006). It has been reported that degradation products of cy-3-glu and pelargonidin-3-glucoside (pg-3-glu) were PCA and phloroglucinaldehyde (Heimori et al., 2009; Sadilova, Carle, and Stintzing, 2007). Moreover, VA was a degradation product from pn-3-glu (Stintzing and Carle, 2004). Phenolic acids in rice samples may further be transformed to aroma compounds upon thermal treatments. Jiang and Peterson (2010) reported that thermal degradation of FA in foods resulted in generation of aroma compounds such as 4-vinylguaiacol, guaiacol and vanillin.

The content of PCA and VA of MNS6 subjected to hot air drying at 80°C, was the highest ($p<0.05$, Figure 3.6A), while those at other temperatures were comparable ($p>0.05$). When rice was heated with water, PCA and VA increased with temperatures and reached the highest at 90°C hot water (Figure 3.6B). Thermal degradation of cy-3-glu leads to deglycosylation, resulting in the formation of cyanidin and free glucose. A- and B-rings of cyanidin which are unstable at neutral condition further transform to scission products, phloroglucinaldehyde and PCA, respectively (Figure 3.7) (Sadilova et al., 2006). Thermal degradation of pn-3-glu resulted in formation of VA (Figure 3.7) (Stintzing and Carle, 2004). The results indicate that the heating condition greatly influenced degradation of anthocyanins and

phenolic acids differently. It should be noted that a decrease of cy-3-glu and pn-3-glu occurred concomitantly with an increase in PCA and VA, suggesting that the latter phenolic compounds were likely formed via degradation of the formers.

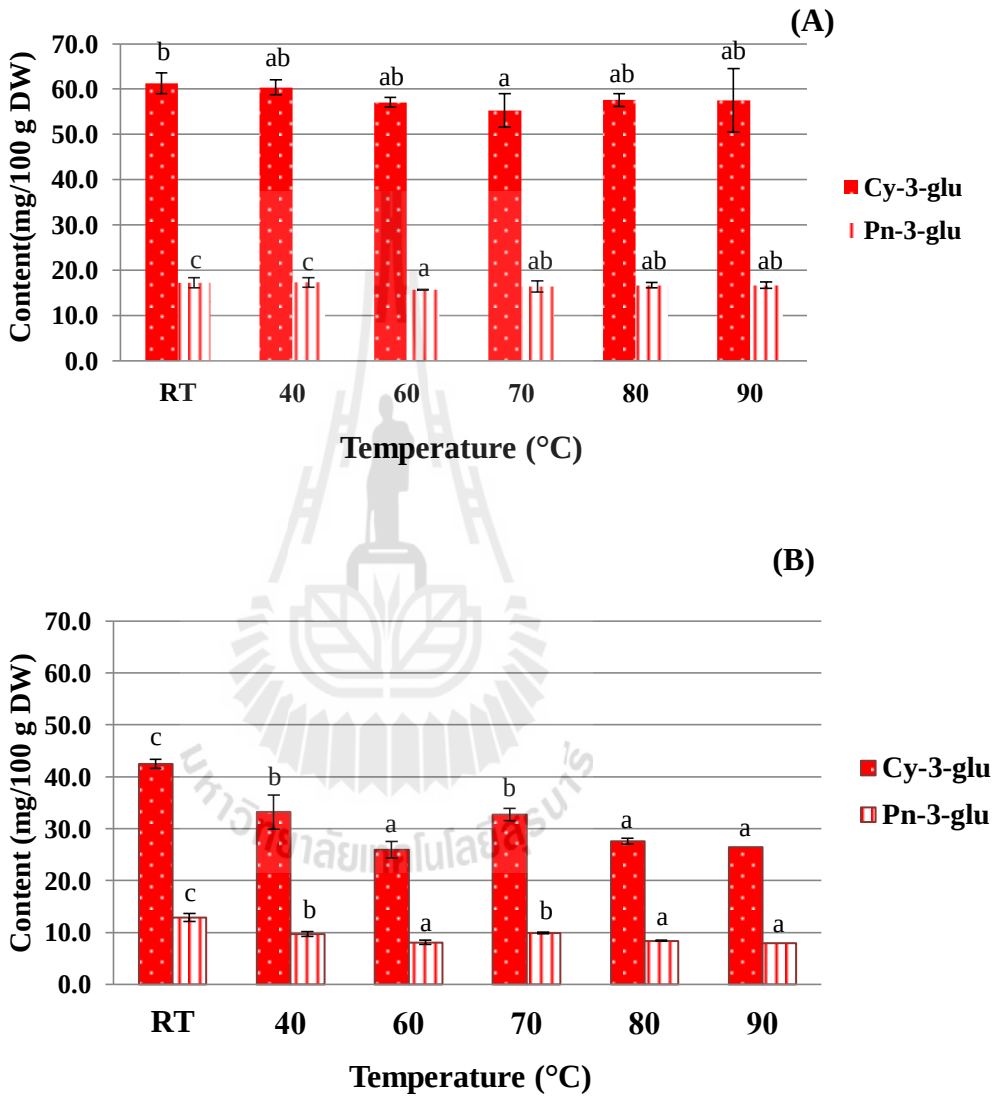

Figure 3.5 Changes of individual anthocyanins at various temperatures under hot air (A) and hot water (B) heating treatments. Different letters indicated differences among Cy-3-glu or Pn-3-glu (p<0.05). RT = untreated sample.

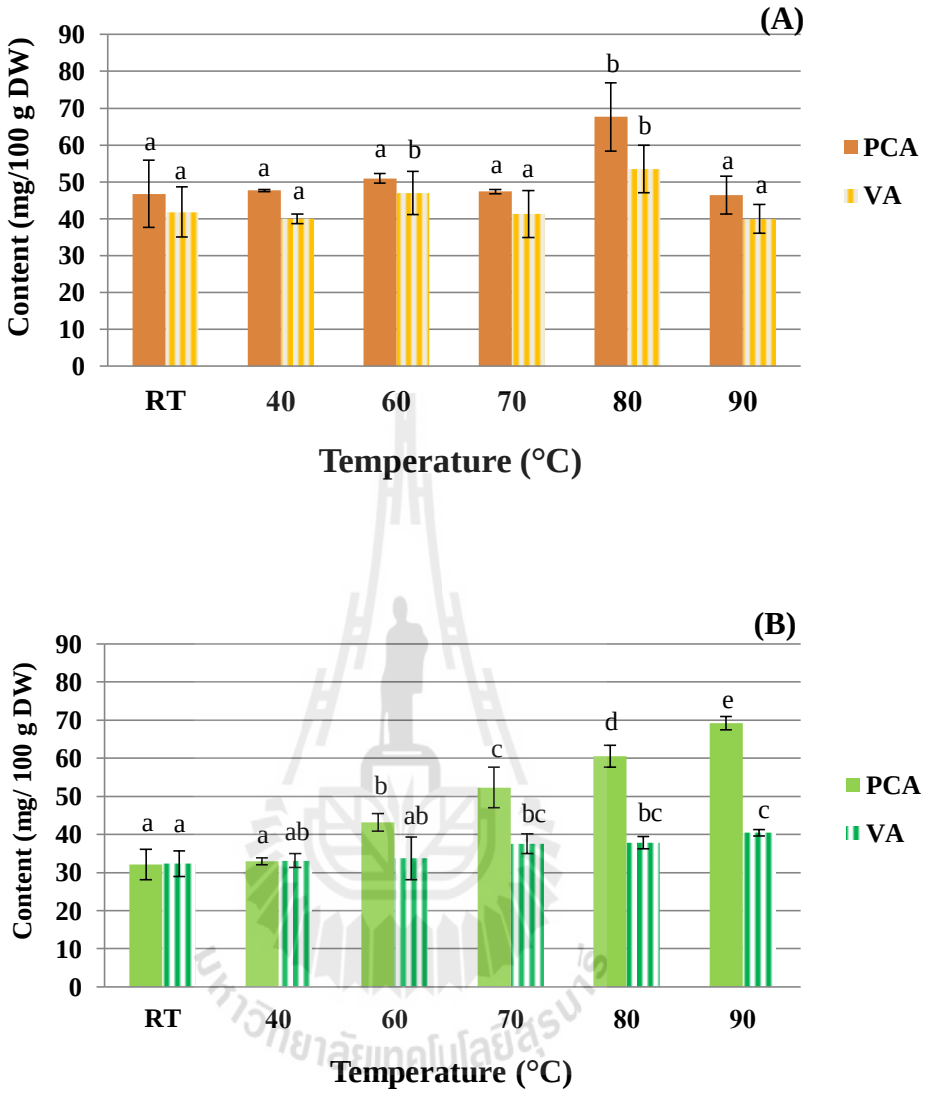

Figure 3.6 Changes of individual phenolic acids of MNS6 heated at various temperatures under hot air (A) and water (B) heating treatment. Different letters indicate significant difference (p<0.05). RT = untreated sample.

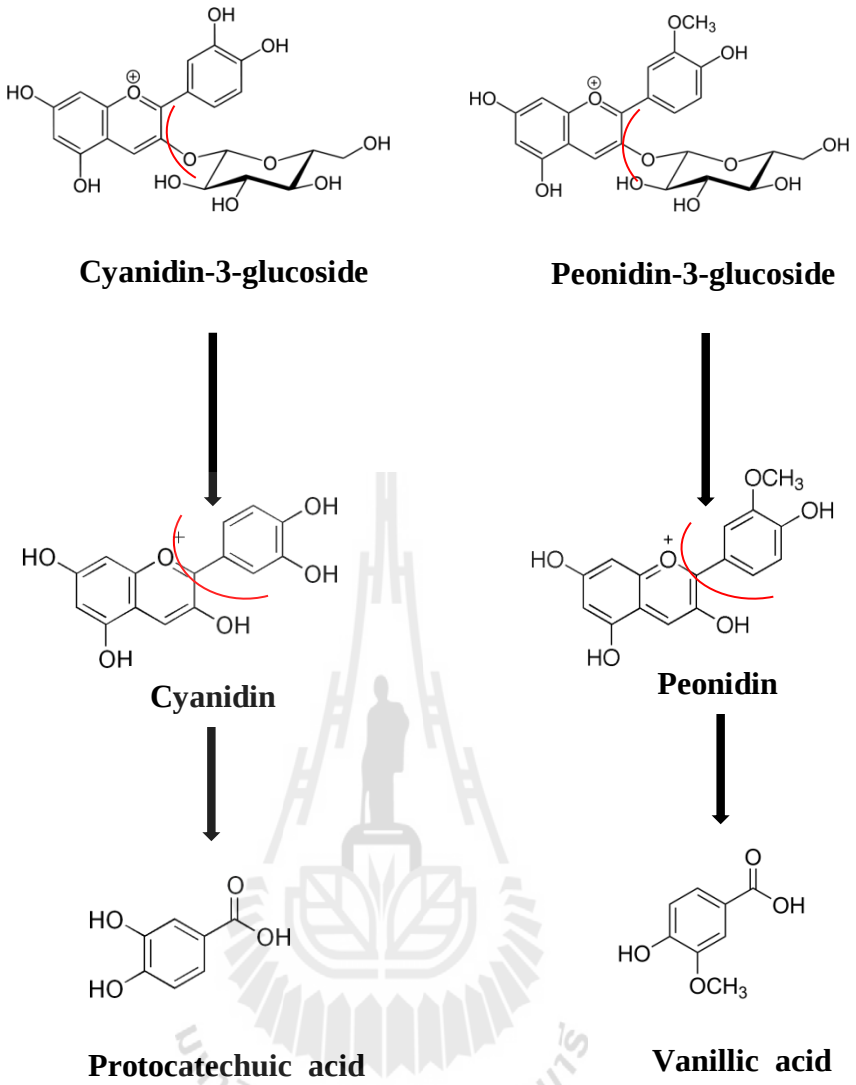

Figure 3.7 Schematic of the thermal degradation of cy-3-glu and pn-3-glu in Thai dark purple rice

Modified from: Hiemori et al., 2009; Sadilova et al., 2006; Stintzing and Carle, 2004.

3.5 Conclusions

Rice bran fraction of Thai pigmented rice, MNS2 and MNS6, contained the highest anthocyanins and phenolic compounds and exhibited the greatest antioxidant activities when compared to whole rice and endosperm. The main anthocyanins in bran

and endosperm of both cultivars were cy-3-glu, pn-3-glu, while PCA, and VA were the main phenolic compounds extracted by acidified methanol. Heating rice in hot water resulted in the greater loss of anthocyanins and antioxidant activity than hot air drying at the same temperature. PCA and VA, increased with heating temperature (60-90°C) in concomitant with a decrease of cy-3-glu and pn-3-glu, indicating the formation of phenolic compounds via thermal degradation of anthocyanins.

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CHAPTER IV

ANTIOXIDANT ACTIVITIES AND ANTIPROLIFERATIVE ACTIVITY ON HUMAN COLON CANCER CELLS OF THAI DARK PURPLE RICE COOKED BY VARIOUS METHODS

4.1 Abstract

Changes of anthocyanins, phenolic compounds, and antioxidant activities of Thai dark purple rice cooked by various methods, were investigated and evaluated for antiproliferative activity on human colon cancer cells. Cyanidin-3-glucoside (cy-3-glu) and peonidin-3-glucoside (pn-3-glu) are predominant anthocyanins, while protocatechuic acid (PCA), vanillic acid (VA), ferulic acid (FA), and p-coumaric acid (p-Cou) are major phenolic acids. Microwave cooking method resulted in a marked loss of phenolics, anthocyanins and antioxidant activities ($p<0.05$). A decrease of cy-3-glu was in concomitant with an increase of PCA. Methanolic extract of raw rice and rice cooked by autoclave showed the highest inhibition of Caco-2 cell proliferation with IC_{50} of 12.63 and 16.11 µg/mL. The results indicated that raw and autoclave cooked rice showed potent antiproliferation of colon cancer cells.

4.2 Introduction

Rice, *Oryza sativa* L., is the most important cereal crop and the staple food source being consumed by over half of the world's population (Hu, Zawistowski, Ling, and Kitts, 2003). Recently, purple and black rice has become popular due to their believe in health-promoting effect of anthocyanins and phenolic compounds (Heimori, Koh, and Mitchell, 2009). Cy-3-glu, cyanidin-3-rhamnoside, cyanidin-3-rutinoside, cyanidin-3,5-diglucoside, pn-3-glu, and malvidin-3-galactoside have been reported in Japanese, Korean, Canadian and American pigmented rice varieties (Cho, Paik, Yoon, and Hahn, 1996; Ryu, Park, and Ho, 1998; Abdel-Aal, Young, and Rabalski, 2006; Yawadio, Tanimori, and Morita, 2007; Hiemori et al., 2009). Phenolic acids are typically located at the outer layers of rice grains. Previous studies reported that FA and p-Cou are major phenolic acids in white rice (Herrmann, 1989; Shahidi and Naczk, 2004), while PCA was a major phenolic acid found in black and purple rice (Hiemori et al., 2009; Chung and Shin, 2007).

Phenolic compounds and anthocyanins are highly unstable (Giusti and Wrolstad, 2003). Thermal treatment is one of important factors affecting stability of these compounds. Rice is typically cooked by a rice cooker. Microwave heating can also be applied at the household level, while autoclave heating reflects the extreme case of canned products. Microwave heating relies on absorption of microwave energy by water, resulting in rapid heating rate. Autoclave cooking is the process where rice is subjected to superheated water. At a pressure of 15 psi above atmosphere pressure, water in a pressure vessel can reach a temperature of up to 121°C. The effect of these cooking methods on the stability of anthocyanins and phenolic compounds in Thai pigmented rice and their bioactivities has not been realized.

Anthocyanins and phenolic compounds have been recognized as health-enhancing substances due to their antioxidant activity, anti-inflammatory, antiartheriosclerosis, anticancer, hyperlipidemia, and hypoglycemic effects (Abdel et al., 2006; Hiemori et al., 2009, Guo, Ling, Wang, Liu, Hu, and Xia, 2007). Antioxidant activity of plant phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they can act as a metal chelator (Rice-Evans, Miller, Bolwell, Bramley, and Pridham, 1995; Shahidi and Naczk, 2004). Cytoprotective effect of pigmented rice on several human cancer cells have been investigated based merely on raw rice (Chen, Kuo, Chiang, Chiou, Hsieh, and Chu, 2006; Hui, Bin, Xiaoping, Chunye, Mantian, and Wenhua, 2010). Such effects of cooked pigmented rice have not been reported. Information obtained from cooked rice would be more pertinent to the health-promoting effect as it is a consumable form. Therefore, the objectives of this study were to investigate thermal stability of anthocyanins and phenolic acids, and antioxidant activity of Thai dark purple rice under electric rice cooking, autoclaving, and microwave heating. In addition, antiproliferative activity of raw and cooked rice on human colon cancer cell lines (Caco-2) was evaluated.

4.3 Materials and methods

4.3.1 Rice samples and chemicals

Non-glutinous dark purple rice cultivar (*Oryza sativa* L.) Mali Nil Surin No.6, was obtained from Surin Rice Research Center (Surin, Thailand). It was grown in August, 2010 and harvested at the end of the year. Folin-Ciocalteu reagent, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS),

potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and gallic acid (GA) were purchased from Sigma (St. Louis, MO). Cy-3-glu, pn-3-glu, FA, p-Cou, VA, p-hydroxybenzoic acid (p-hydroxy), syringic acid (Syr), PCA, chlorogenic acid (Chl) were obtained from Extrasynthese (GenayCedex, France). HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

4.3.2 Rice cooking

Paddy rice was dehulled using a laboratory milling machine (Satake Co., Hiroshima, Japan). Fifty grams of dehulled rice were cooked by each cooking apparatus; electric rice cooker, autoclave and microwave oven. Ratio of rice to water was varied with a cooking device in order to yield palatable cooked rice. Rice to water ratio of 1:5, 1:1.5, and 1:9 (w/v) was preliminarily determined to be optimum for electric rice, autoclave and microwave cooking, respectively. For microwave cooking, 50 g rice and 150 mL of potable water were added in a 400-mL microwavable glass container and heated in a microwave set at 800 W for 13 min. The same volume of water was added and heating was continued for another 13 min. Addition of water and heating was repeated the third time. For autoclaving, rice and the set volume of water was added into a 450-mL glass screw cap bottle and subjected to 121°C, 15 psi, for 15 min. Total cooking time of electric rice cooker, autoclave and microwave oven was 50, 60, and 39 min, respectively. Three replications were performed for each cooking device. About 25 g of cooked rice was immediately lyophilized upon cooking. Lyophilized samples were ground using an IKA laboratory milling machine (M20 Jankeand Kunkel Co., Staufen, Germany), and sieved through a 60-mesh screen. The ground samples were kept at -20°C until use.

4.3.3 Extraction of anthocyanins and phenolic compounds

Anthocyanins and free phenolic compounds were extracted according to Abdel-Aal and Hucl (1999) with slight modifications. Three grams of lyophilized samples were extracted twice by 24 mL of methanol acidified with 1.0 N HCl (85:15, v/v) at a stirring speed of 1,000 rpm for 60 min at room temperature. The pH of the mixture was control at pH 1 during extraction. The mixtures were centrifuged at 10,000×g for 20 min at 4°C and supernatant was refrigerated at 4°C for 2 days to precipitate insoluble matters. The extract was re-centrifuged at 10,000×g for 20 min at 4°C. Supernatants were brought up to 50 mL with acidified methanol. Anthocyanin content, free phenolic content and antioxidant activities were determined. A part of supernatant (40 mL) was concentrated under a stream of nitrogen. The precipitates formed during N_2-flushing were separated by centrifugation as described above. The concentrated extracts were adjusted to 4 mL with acidified methanol and vigorously mixed and filtered through a 0.45 µm Nylon Acrodisc syringe filter membrane for determination of individual anthocyanins and free phenolic acids by HPLC.

4.3.4 Extraction of bound phenolic compounds

Bound phenolic acids were extracted according to Mattila, Pihlava, and Hellström (2005) with slight modifications. The residue remaining after anthocyanin extraction was added with 42 mL of the mixture of acidified methanol and 10% acetic acid (85:15 v/v). The mixture was homogenized (Polytron® PT3100, Lucerne, Switzerland) at 10,000 rpm for 5 min and sonicated for 30 min. Volume was brought to 132 mL with deionized water. Subsequently, 30 mL of 10 N NaOH were added and the homogenate was stirred for 24 h at 25°C, and was adjusted to pH 2. Bound phenolic compounds were extracted by adding a mixture of cold diethyl ether and ethyl acetate (1:1) at a ratio of the extract to solvent of 1:2. Extraction was carried out for

3 times and organic layers were combined, evaporated to dryness under N_2 gas and dissolved in methanol before HPLC analyses.

4.3.5 Spectrophotometric determination of anthocyanins and phenolic compounds

Total anthocyanin content (TAC) was determined according to Abdel-Aal and Hucl (1999). Absorbance of acidified methanol extract (A) was measured at 535 nm. TAC (µg/g of sample) was calculated as cy-3-glu as it was a major form found in pigmented rice using the following equation;

$$TAC = A \times 288.21$$

Extractable phenolic content (EPC) was determined using Folin-Ciocalteu as described by Waterhouse (2005). Absorbance was measured at 765 nm. EPC was expressed as mg gallic acid equivalents (GAE) per 100 g lyophilized sample.

4.3.6 Determination of antioxidant activity

4.3.6.1 ABTS radical scavenging assay

ABTS radical scavenging activity was determined according to Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) with slight modifications. The radical cation $ABTS^{\cdot+}$ was generated by persulfate oxidation of ABTS as stock solution (7 mM ABTS and 2.45 mM potassium persulfate which was allowed to stand in the dark at room temperature for 12-16 h before use). Working solution was diluted with 80% ethanol to reach absorbance of 0.7 ± 0.02 at 734 nm. Twenty µL of the extracts (with appropriate dilution, if necessary) were mixed with 1.48 mL of the working solution, and a decrease of absorbance was measured immediately at 734 nm after 6 min at 30°C in the dark. The control was prepared using 80% ethanol without $ABTS^{\cdot+}$ solution. Results were expressed as Trolox equivalent antioxidant capacity (mmol Trolox equivalents per 100 g lyophilized sample).

4.3.6.2 Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the modified method of Yang and Zhai (2009). Fifty µL of sample (with appropriate dilution, if necessary) were added to 0.95 mL of freshly prepared FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ in 40 mM HCl, and 1 part of 20.0 mM $FeCl_3 \cdot 6H_2O$), and the reaction mixture was incubated at 37°C for 30 min. An increase in absorbance at 593 nm was measured. Results were also expressed as mmol of Trolox equivalents per 100 g of lyophilized sample.

4.3.7 HPLC and LC-MS/MS analysis

Individual anthocyanins, free and bound phenolic acids, were analyzed using HPLC equipped with DAD (Agilent HP1100 system, Agilent Technologies, Palo Alto, California, USA) using a Symmetry ® C_{18} column (3.5 µm, 4.6 × 75 mm, Waters, Ireland) and a Zorbax Eclipse XDB C_{18} column (5 µm, 150 × 4.6 mm i.d.; Agilent Technologies, Palo Alto, CA), respectively, with injection volume of 20 µL.

Anthocyanins were eluted using a gradient mobile phase consisting of 5% (v/v) formic acid in water (A) and 5% (v/v) formic acid in acetonitrile (B) as follows: 0-10 min, 20% B; 10.00-2.00 min, 20-30% B; 12.00-14.00 min, 30-50% B; 14.00-16.00 min, 50-95% B; 16.00-19.00 min, 95-0% B; 19.00-19.50 min, 0% B at a flow rate of 1 mL/min and column temperature was set at 40°C. The separated anthocyanins were monitored at 520 nm. Peak identification of each anthocyanin was based on retention time of anthocyanin standards, including cy-3-glu and pn-3-glu. Individual free phenolic acids were eluted using a gradient of 2.5% (v/v) methanol and 0.5% (v/v) formic acid (A) and methanol (B) as follows: 0-11 min, 10-25% B; 11-12 min, 25-28% B; 12-15 min, 28-36% B; 15-17 min, 36-40% B; 17-19 min, 40-42% B; 19-24 min, 42-50% B; 24-31 min, 50-80% B; 31-35 min, 80% B; 35-40 min, 80-10% B;

40-45 min, 10% B at a flow rate of 1 mL/min (Nave, Cabrita, and Da Costa, 2007). Individual bound phenolic acids were separated using the condition described by Mattila et al. (2005). Gradient elution was performed with a mobile phase 50 mM H_3PO_4 at pH 2.5(A) and acetonitrile (B) as follows: 0-5 min, 5% B; 5-17 min, 5-15% B; 17-40 min, 15-20% B; 40-60 min, 20-50% B; 60-65 min, 50% B; 65-67 min, 50-5% B and post time for 6 min before the next injection. The temperature of the column was set at 35°C. The flow rate of the mobile phase was 0.7 mL/min. Phenolic acids were monitored at 280 nm. GA, PCA, p-hydroxy, VA, Syr, p-Cou and FA were used as standards.

Identity of individual anthocyanins and phenolic acids was confirmed by high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Waters Alliance 2695 Waters Corporation, Milford, MA, USA) equipped with Q-Tof Premier (Micromass, city, UK). The column and the conditions for the separation of individual anthocyanins and phenolic acids were the same as analysis by HPLC. The mass spectrometer was operated in the electrospray ionization (ESI) positive ion mode for anthocyanins and negative ion mode for phenolic acids and scanning mass range from 50-1500 m/z. MS operating conditions were as follows: fragment ions were generated from precursor ion by collision-induced dissociation (CID). Argon and nitrogen were used as the collision gas and nebulizing gas, respectively. A desolvation temperature was 500°C at a flow rate of 100 L/h and cone gas N_2 flow rate was 100 L/h.

4.3.8 Cell antiproliferation capacity

Methanolic rice extracts were prepared using solid phase extraction (C18 Sep-Pak solid cartridge, 5 g, Waters Corporation, Milford, MA, USA). The C18 cartridge was activated with 2 column volumes of methanol followed by 3 column

volumes of acidified deionized water (0.01% HCl in deionized water, v/v). The C18 cartridge was washed by 40 mL of 0.01% (v/v) HCl solution to remove sugars and other polar compounds. Anthocyanins and phenolics were then eluted using 40 mL acidified methanol (0.01% HCl in methanol, v/v). Methanol was removed by a rotary evaporator at 40°C. Concentrated anthocyanin and phenolic extracts were lyophilized and stored at -20°C until use.

Human colon adenocarcinoma cancer cell lines (Caco-2, Cat. No. HTB-37) was purchased from the American Type Culture (ATCC, Manassas, VA, USA). Cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non essential amino acids, 0.1 unit/mL bovine insulin, 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were cultured in the medium and incubated at 37°C in a fully humidified, 5% CO_2 incubator. Fresh medium was changed 2-3 times per week. Each lyophilized sample was dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 10 mg/mL. Samples were then serially diluted using the culture medium.

Caco-2 cancer cells were plated at a density of 3.0×10^3 cells/well in a 96-well plate and incubated for 48 h. Anthocyanin-phenolic rice extract (APE) at various concentrations (0.78-100µg/mL) were tested on the cells and incubated for 24 h. The control was prepared by incubating cells in 0.5% DMSO. After 24 h, APE or DMSO was removed from cells, and fresh medium was added. Cells were further incubated for 24 h and cell viability was determined. Briefly, 50 µL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) in PBS at 5 mg/mL was added to each well and incubated for 4 h. Medium and MTT were then aspirated from the wells, and formazan was solubilized with 200 µL of DMSO and 25 µL of Sorensen's glycine buffer, pH 10.5. The optical density of blue color of formazan was

read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm. Data were analyzed with the SoftMax Program (Molecular Devices, Sunnyvale, CA, USA) to determine the IC_{50} for each sample. The IC_{50} value is expressed as the concentration of sample required to kill 50% of the cells as compared to respective controls.

4.3.9 Statistical analyses

Experimental data were analyzed by a one way analysis of variance (ANOVA). Statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL) and the significant difference between means was compared by Duncan's multiple-range test (DMRT).

4.4 Results and discussion

4.4.1 Changes of anthocyanin and phenolic content

TAC and EPC decreased upon all cooking methods ($p<0.05$, Figure 4.1). The highest loss of TAC and EPC was observed in rice cooked by microwave and autoclave heating ($p<0.05$). Exposure to high temperature for a long time in microwave heating and sterilization condition in autoclave treatment resulted in significant loss of anthocyanins. Zhao, Li, Xu, Wu, Liao, and Chen (2013) reported that degradation of malvidin-3-glucoside and malvidin-3,5-diglucoside, major anthocyanins in grape berries under microwave treatment were 257 and 19 times faster than those heated in a 98 ± 2°C water bath. Heat generation under microwave treatment is caused by the friction of rotating water molecules. In addition, polar molecules having large dissipation factor (tan δ) can absorb microwave energy efficiently (Mudgett, 1986). Phenolic acids and anthocyanins are polar compounds, which are likely to absorb microwave energy, leading to marked degradation. Among

3 cooking devices tested, an electric rice cooker appeared to retain the highest anthocyanins and free phenolic compounds of 35% and 53.2%, respectively.

4.4.2 Antioxidant activity

Antioxidant activities of rice cooked by various methods dramatically decreased from the raw sample (p<0.05, Figure 4.2). A decrease of ABTS radical scavenging activity occurred to a greater extent than FRAP value. A decrease of antioxidant activity of rice cooked by an electric rice cooker occurred to the lowest extent, while microwave heating resulted in the highest loss (p<0.05). This corresponded to a decrease of extractable phenolic compounds and total anthocyanins (Figure 4.1). These results indicate that extractable phenolic compounds and anthocyanins contributed to antioxidant activity of Thai pigmented rice (Walter and Marchesan, 2011).

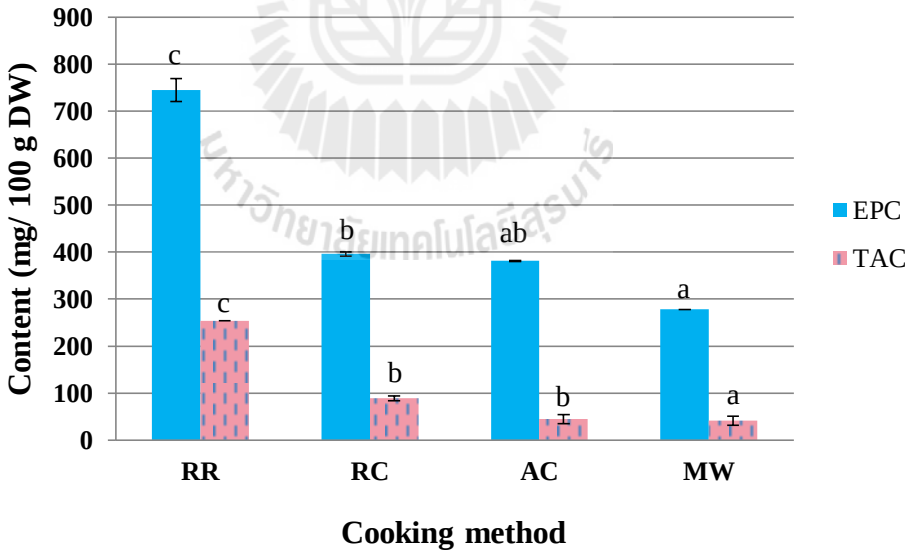

Figure 4.1 Extractable phenolics and anthocyanins in raw rice (RR) and rice cooked by various methods. RC = electric rice cooking, AC = autoclave cooking, MW = microwave heating. Different letters indicate differences among EPC or TAC (p<0.05).

4.4.3 Changes of individual anthocyanins and phenolic acids

Cy-3-glu and pn-3-glu were predominant anthocyanins (Figure 4.3) and positively identified by LC-MS/MS with parent ion $[M+H]^+$ at m/z 449 and 463, and main fragment ions at m/z 287 and 301, respectively. Cy-3-glu and pn-3-glu appeared

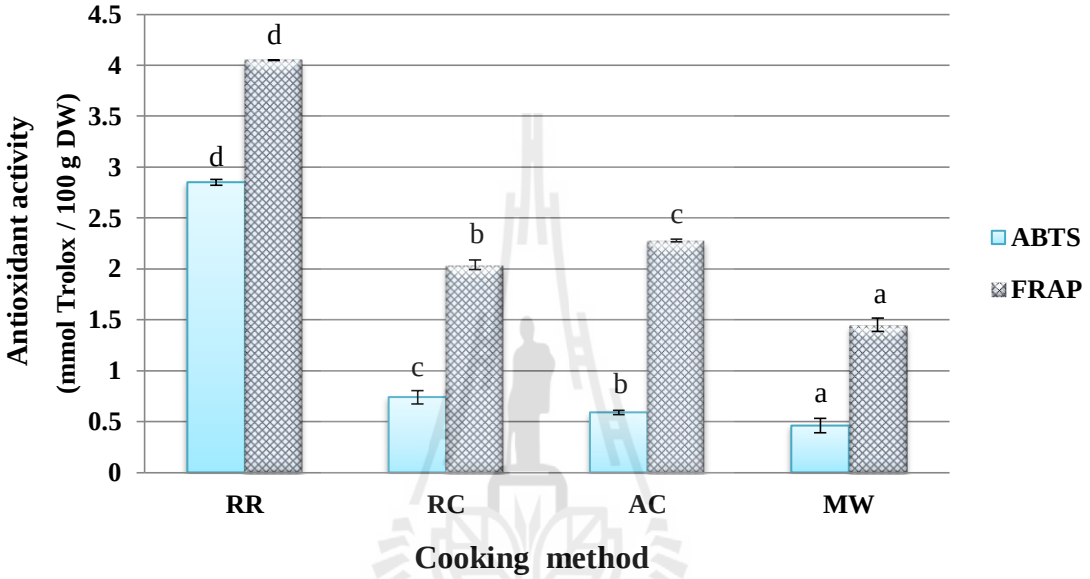

Figure 4.2 ABTS radical scavenging activity and FRAP value of raw rice and rice cooked by different cooking methods. Means of ABTS and FRAP values with different letters indicate significant difference (p<0.05). Abbreviations are the same as described in Figure 4.1.

with peak 1 and peak 2 (Figure 4.3). Cy-3-glu and pn-3-glu drastically decreased upon cooking (p<0.05, Figure 4.4). Rice cooked by microwave and autoclave heating showed the highest reduction in cy-3-glu and pn-3-glu of about 93% and 88%, respectively. Reduction of cy-3-glu and pn-3-glu in rice cooked by an electric rice cooker was lower with the loss of 74.2% and 41.7%, respectively. Heimori et al. (2009) also found the similar extent of cy-3-glu reduction of black rice cooked by an

electric rice cooker. These results indicate that cy-3-glu was less stable than pn-3-glu when subjected to thermal treatments. The structural effect is an important factor affecting the stability of anthocyanidins and anthocyanins. It has been reported that the increased hydroxylation of the aglycone stabilizes the anthocyanidin such as delphinidin is more stable than cyanidin in acidic methanol (Dao, Takeoka, Edwards, Berrios, and De, 1998). However, delphinidin-3-glucoside was reported to be less stable than cy-3-glu. In addition, an increasing methylation of the OH groups weakens the stability of the anthocyanins. In a buffered solution cy-3-glu was more stable than petunidin-3-glucoside (pt-3-glu), and pn-3-glu was more stable than malvidin-3-glucoside (mal-3-glu). Therefore, we could not describe the thermal stability between cy-3-glu and pn-3-glu by structural effects, we need for further study.

Major extractable phenolic acids in Thai dark purple rice were PCA, VA, while VA, p-Cou and FA were predominant bound phenolic acids (Figure 4.3). These phenolic acids were confirmed by negative ion mode of LC-MS/MS and showed molecular ion $[M-H]^-$ at *m/z* 153.1, 167, 163 and 193.1, and their main fragment ion were 109, 123, 119, and 149.1, respectively. Several studies have been reported that FA and p-Cou are major phenolic compounds in cereal grains, particularly brown rice (Herrmann, 1989; Shahidi and Naczk, 2004). In addition, it is well known that FA in rice is a major bound phenolic involved in cell wall structure. FA may be esterified to pectins and arabinoxylans or cross-linked to cell wall polysaccharides in the form of dimers, such as dehydroferulates and truxillic acid in the aleurone and pericarp layers (Clifford, 1999; Shibuya, 1984).

Microwave cooking resulted in the greatest loss of FA, VA and p-Cou. On the other hand, the concentration of PCA significantly increased about 1.21-3 times

after cooking (p<0.05, Table 4.1). The highest content of PCA was found in autoclave-cooked rice, which was in concomitant with the highest antioxidant activity based on

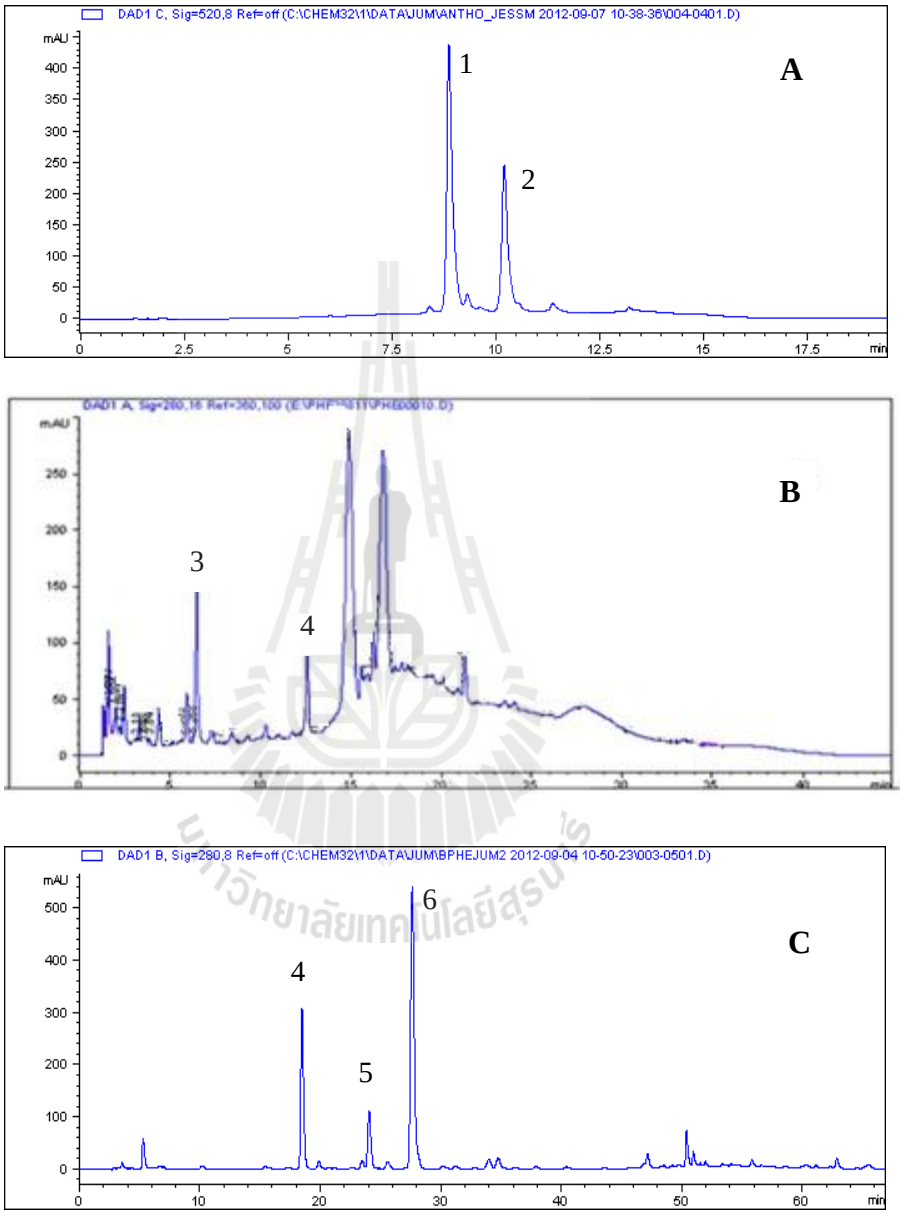

Figure 4.3 Representative HPLC chromatograms of anthocyanins (A), extractable phenolic acids (B) and bound phenolic acids (C) in raw rice. Peak 1, cy-3-glu; peak 2, pn-3-glu; peak 3, PCA; peak 4, VA; peak 5, p-Cou; peak 6, FA.

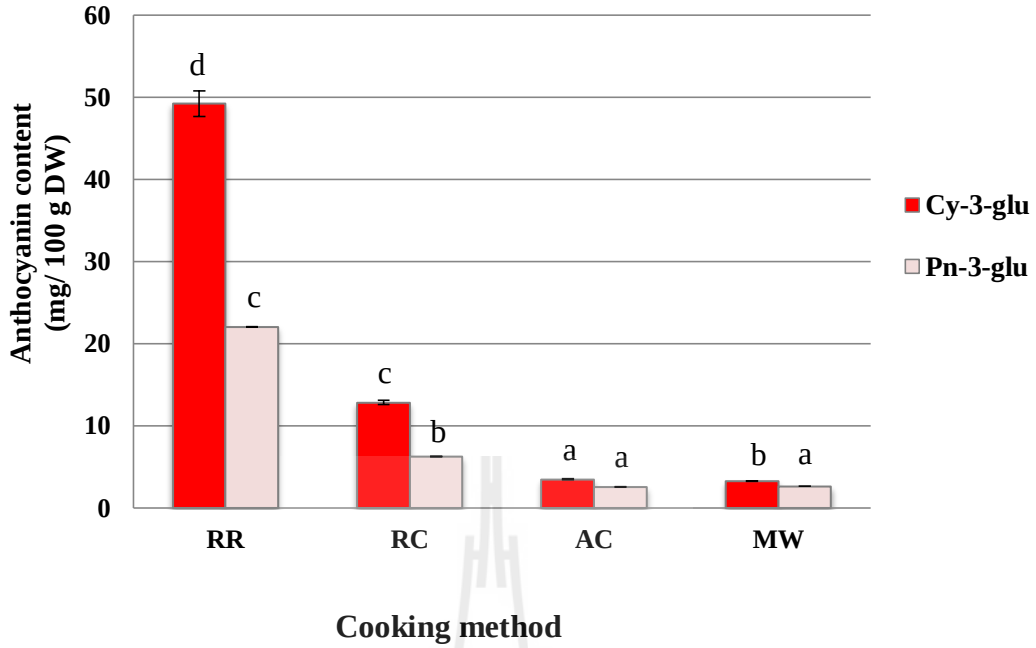

Figure 4.4 Degradation of cy-3-glu and pn-3-glu in raw rice and rice cooked by different cooking methods. Different letters indicate significant difference (p<0.05). Abbreviations are the same as described in Figure 4.1.

FRAP assay. Heating under saturated steam (15 psi, 121°C) induced degradation of anthocyanins to PCA (Heimori et al., 2009). Thermal degradation of cy-3-glu leads to deglycosylation, resulting in the formation of cyanidin and free glucose. A- and B-rings of cyanidin which are likely unstable at neutral condition further transform to scission products, phloroglucinaldehyde and PCA, respectively (Sadilova, Stintzing, and Carle, 2006). Moreover, thermal degradation of pn-3-glu resulted in formation of VA (Stintzing and Carle, 2004). However, VA content decreased upon cooking method. This could probably be due to further degradation of VA to volatile compounds.

Bound VA underwent thermal degradation at various cooking methods (Table 4.1). A decrease of hydroxycinnamic acids (p-Cou and FA), major bound phenolic acids in rice samples, at high temperatures could probably be due to the breakage between p-Cou and lignin and between FA and arabinoxylans (Maillard and Berset, 1995). Naim, Striem, Kanner, and Peleg (1988) reported that FA was sensitive to thermal degradation in a model solution of orange juice heated at 70°C. In addition, it has been reported that volatile compounds such as, 4-methyl-, 4-ethyl-, 4-vinylguaiacols, guaiacol and vanillin were products of thermal degradation of ferulic acid (Fiddler, Paker, Wasserman, and Doerr, 1967; Jiang and Peterson, 2010).

4.4.4 Antiproliferation of colon cancer cells

Extracts prepared for human colon cancer cell (Caco-2) treatment were acidified methanol extract after passing through C18 cartridge. Amino acids, sugars and small polar compounds were mainly removed from the extract. The concentrations of phenolics and anthocyanins of lyophilized powder for Caco-2 cell treatment are shown in Table 4.2. The content of anthocyanin extracted from various cooked rice samples was lower than that of raw rice, while anthocyanin and phenolic content were the lowest in microwave-cooked rice.

Rice extract obtained from various cooking methods reduced Caco-2 cell viability in a concentration-dependent manner (Figure 4.5). Raw rice showed the highest antiproliferation activity with IC_{50} ~12.63 µg of lyophilized powder/mL. Rice cooked by autoclave heating also showed inhibition comparable to that of raw rice. The lowest inhibitory effect was observed in sample cooked by microwave heating. Thai dark purple rice cooked under sterilization condition appeared to have the highest potent antiproliferation of colon cancer cells. This could be attributed to the presence of anthocyanins and phenolic compounds. The autoclave-cooked rice showed phenolic

content approximately 6 times higher than that of raw rice (Table 4.2). The microwave method resulted in the lowest phenolic compounds and anthocyanins. Therefore, microwave heating may markedly degraded anthocyanins and phenolic compounds (Table 4.2), could result in the lowest anticancer activity.

Synergistic interaction between anthocyanins and phenolics of anthocyanin-rich extracts of cranberry and chokeberry explained their antiproliferative effect on HT-29, human colon cancer cells (Seeram, Adams, Hardy, and Heber, 2004; Jing, Bomser, Schwartz, He, Magnuson, and Giusti, 2008). However, anthocyanins are still the primary chemoprevention components in anthocyanin-rich foods. The lowest IC_{50} of raw rice in concomitant with the highest anthocyanin content indicated that anthocyanins could be the significant bioactive compound contributing to antiproliferative activity. The autoclave-cooked rice also showed the lowest IC_{50} and the highest phenolic content. This implied that phenolic compounds were likely bioactive compounds inhibiting proliferation of colon cancer cells in the autoclave-cooked rice. Major phenolic compound in the methanolic extract of autoclave-cooked rice was PCA, which was a degradation product of anthocyanins (Table 4.1). It could be postulated that PCA could be one of important phenolic compounds inhibiting colon cancer proliferation.

Anthocyanins and their aglycons have been reported to exhibit antiproliferative activity towards multiple cancer cells *in vitro* and *in vivo* (Hui et al., 2010; Seeram et al., 2006; Hyun and Chung, 2004; Kong, Chia, Goh, Chia, and Brouillard, 2003). Degradation of anthocyanins led to formation of PCA and VA which acted as antioxidants and could, in turn, contribute to antiproliferative activity of Caco-2 cells. Pure PCA showed significant antioxidant activity in a concentration-dependent manner (Chung and Shin, 2007; Natella, Nardini, Di Felice, and Scaccini,

1999). In addition, it acted as an apoptosis inducer in human gastric carcinoma cells by showing a dose- and time-dependent inhibitory effect on the proliferation (Lin, Chen, Huang, and Wang, 2007). Antiproliferative effect of VA on human cancer cells has not been clearly described. Regarding the chemical structure of VA, phenol ring consists of one hydroxyl (OH) and methoxy (-OMe) group which is different from PCA containing 2 OH groups. It was found that the presence and number of OH ring substituents in polyphenolic derivatives could determine their corresponding biological activity (Fiuza et al., 2004). Methylation of these OH groups, which are mainly responsible for the antioxidant characteristics, abolished cytotoxicity (Nam, You, Kim, Hong, Kim, and Ahn, 2001). VA may also exhibit potent antioxidant due to its similarity of OH structure to other potent phenolic compounds. The inhibitory effect of VA on Caco-2 proliferation may lower than that of PCA due to the substitution of OH group by a methyl group in VA structure resulting in lower cytotoxicity. However, although high temperature treatment induces degradation of anthocyanin in Thai dark purple rice, it generates phenolic compounds that showed antiproliferation of colon cancer cells.

Table 4.1 Changes of free and bound phenolic contents of dark purple rice cooked by different methods.

Cooking method	Free phenolic acid (mg/100 g DW)		Bound phenolic acid (mg/100 g DW)			Total VA (mg/100 g DW)
	PCA	VA	VA	p-Cou	FA	
RR	5.00±0.02[a]	4.38±0.03[b]	59.07±0.04[d]	8.96±0.01[d]	74.13±0.40[d]	63.45±0.01[d]
RC	10.06±0.03[c]	5.84±0.03[c]	33.09±0.04[c]	4.63±0.02[c]	44.30±0.20[c]	38.93±0.06[b]
AC	15.45±0.10[d]	8.55±0.01[d]	31.06±0.01[b]	4.44±0.03[b]	41.72±0.30[b]	39.61±0.01[c]
MW	6.08±0.10[b]	3.46±0.06[a]	27.42±0.27[a]	4.25±0.02[a]	40.63±0.35[a]	30.88±0.50[a]

Different letters in the same column indicate significant difference (p<0.05).

Abbreviations are the same as described in Figure 4.1.

Table 4.2 Phenolic and anthocyanin content of various rice extracts used for Caco-2 treatments.

Sample	Phenolic content (mg GAE/ 100 g DW)	Anthocyanin content (mg/ 100 g DW)
RR	279.4 ± 10.72[a]	64.46 ± 0.17[d]
RC	460.0 ± 16.67[b]	40.93 ± 0.29[c]
AC	1875 ± 48.22[c]	20.31 ± 0.14[b]
MW	267.7 ± 12.5[a]	10.96 ± 0.11[a]

Different superscript letters in the same column indicate significant difference (p<0.05).

Abbreviations are the same as described in Figure 4.1.

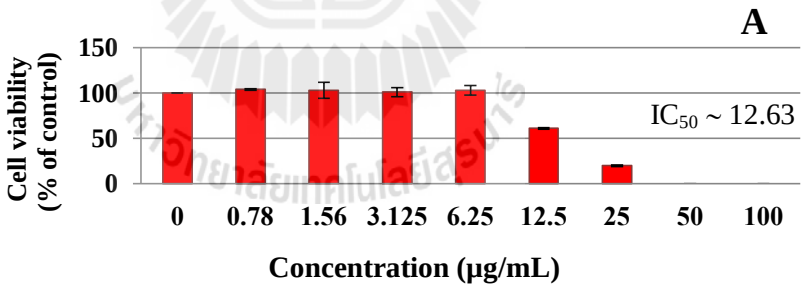

Figure 4.5 The effect of raw and cooked Thai dark purple rice on cell viability of colon human colon cancer cells (Caco-2). Cell viability of raw rice (A); rice cooked by an electric rice cooker (B); autoclave (C) and; microwave oven (D). Data represented the mean ± S.D. with 3 replications.

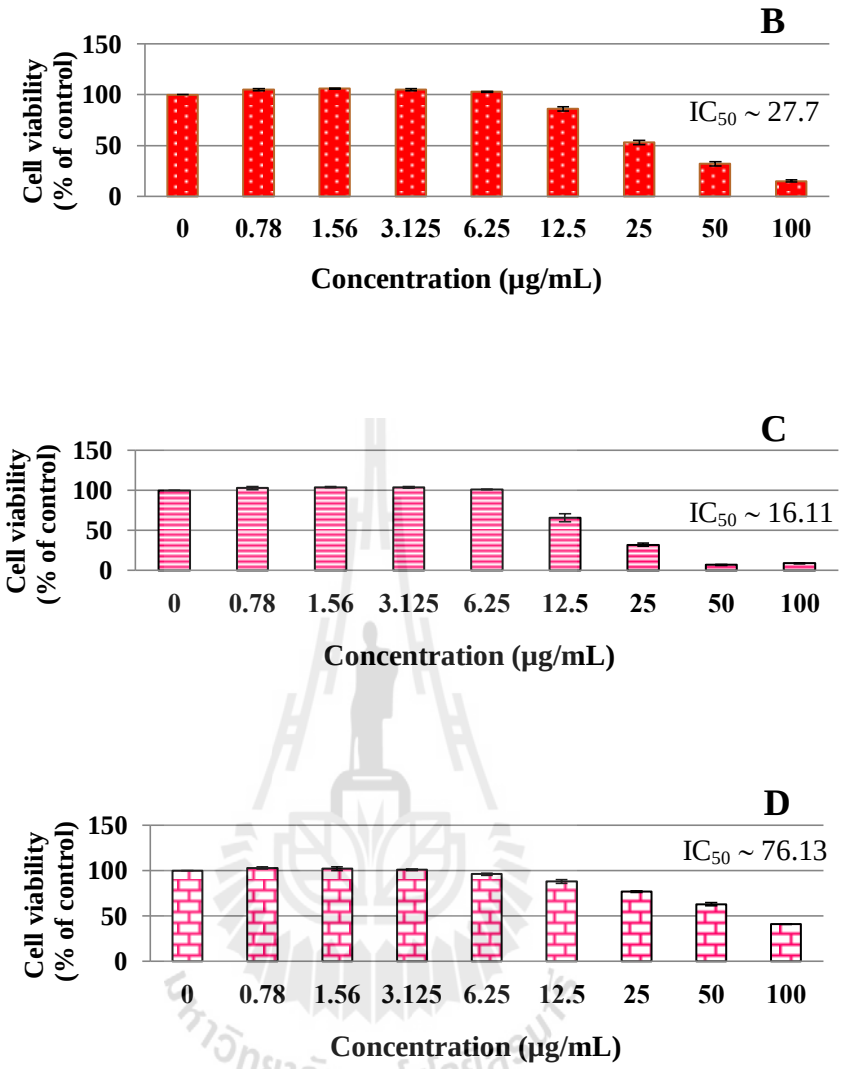

Figure 4.5 The effect of raw and cooked Thai dark purple rice on cell viability of colon human colon cancer cells (Caco-2). Cell viability of raw rice (A); rice cooked by an electric rice cooker (B); autoclave (C) and; microwave oven (D). Data represented the mean ± S.D. with 3 replications (Continued).

4.5 Conclusions

All studied cooking methods resulted in a decrease of total anthocyanins and extractable phenolic compounds. However, microwave cooking drastically decreased anthocyanins and phenolic content as well as antioxidant activities. Degradation of major anthocyanins, cy-3-glu and pn-3-glu, of cooked dark purple rice resulted in an increase of free phenolic acids, PCA and VA. Rice cooked by the autoclave heating showed the highest extractable PCA. Raw and autoclave-cooked rice showed the highest antiproliferation of colon cancer cells. The main bioactive compound of raw rice is anthocyanins, while autoclave-cooked rice is phenolic acids. The cooked Thai dark purple rice could be a potential source of bioactive compounds which plays an important role for chemoprevention of human cancer.

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CHAPTER V

IN VITRO BIOACCESSIBILITY OF ANTHOCYANINS OF COOKED PIGMENTED RICE AND ITS ANTIPROLIFERATIVE EFFECT AGAINST COLON CANCER CELLS

5.1 Abstract

The bioaccessibility of cooked dark purple rice based on *in vitro* oral and gastro-intestinal condition was investigated. Phenolics and anthocyanins were released from cooked dark purple rice after oral, gastric and pancreatic digestion. Phenolics were released from the matrices of cooked dark purple rice by both digestive environments and enzymes, whereas anthocyanins were released mainly by chemical environment of digestion. At the end of digestion, the bioaccessible phenolics and anthocyanins were 62.50% and 10.67%, respectively. ABTS radical scavenging activity markedly decreased after pancreatic digestion. Anthocyanin extract significantly inhibited proliferation of HCT116 and HT-29 colon cancer cell lines *in vitro*. The concentration of anthocyanin extract required to kill 50% (IC_{50}) of the HCT116 and HT-29 colon cancer cells compared to the control after 72 h of incubation were 37.20 and 37.19 µg/mL, respectively. These results suggested that anthocyanins in cooked dark purple rice may play a role in the chemoprotective action.

5.2 Introduction

It is widely recognized that consumption of polyphenol-rich foods including fruits, vegetables and whole grains, has been related to several health benefits, such as reducing the risk of cardiovascular diseases and cancers (Bouay et al., 2012). Thai dark purple rice (*Oryza sativa* L. indica) is rich in anthocyanins in the pericarp and aleurone layers and has been regarded as a healthy food in Asia. Anthocyanins are believed to be one of bioactive compounds preventing oxidation and cancer initiation (Kong, Chia, Goh, Chia, and Brouillard, 2003). The health benefit of anthocyanins relies on their bioaccessibility, bioavailability and metabolic fate. The bioavailability of a dietary compound is dependent upon its release of compounds from solid food matrices referred to as bioaccessibility, its digestive stability, cellular uptake, metabolism, and further transport in the circulatory system (Tagliazucchi et al., 2010). Digestion is a physical process that permits the extraction of macronutrients, micronutrients and phytochemicals from the food matrix for absorption (Hinsberger and Sandhu, 2004). In human, the digestive process starts in the mouth under the effect of salivary α-amylase and lingual lipase (Bouayed et al., 2012; Hinsberger and Sandhu, 2004; Pederson et al., 2002). Subsequently, the food bolus is subjected to gastro-intestinal digestion, where digestive enzymes of the stomach and the small intestine, via secretions from liver/biliary system and pancreas, and later also colonic bacterial fermentation in the large intestine, together play a key role in the release of nutrients and non-nutrients (Biehler and Bohn, 2010). It is widely accepted that not all constituents present in the food matrix may be completely bioaccessible (Saura-Calixto., 2007). Several reports have highlighted poor bioavailability of several groups of anthocyanins (<1% of dose in the serum) and correspondingly low levels of urinary excretion as intact or conjugated forms (Cooney, Jensen, and McGhie, 2004; Liang

et al., 2012; Wu, Cao, and Prior, 2002). Therefore, the most important factors in determining the potential health benefit of polyphenols on the gut epithelial cells are their bioaccessibility and their stability under gastro-intestinal conditions.

Anthocyanins and anthocyanidins are suggested to be involved in the inhibitory of colon cancer cell lines *in vitro* and *in vivo*, respectively (Jing et al., 2008; Kang, Seeram, Nair, and Bourquin, 2003). Anthocyanin-rich food sources have been shown to possess antiproliferative activity of colon cancer cells. Cyanidin and malvidin isolated from Korean dark purple rice (*Oryza* sativa cv. *Heuginjubyeo* (Gramineae) also inhibited proliferation of U937, human monocytic leukemia cells by arresting of the G2/M phase of cell cycle and induction of apoptosis (Hyun and Chung, 2004). Recently, it has been reported that anthocyanin-rich extract from Chinese raw black rice reduced the viability of breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-453 (Hui et al., 2010). They suggested that the anticancer effects of anthocyanins-rich extract *in vitro* and *in vivo* were based on apoptosis induction and angiogenesis suppression. Thai pigmented rice, Mali NilSurin, has been reported to be a rich source of anthocyanins, such as cyanidin-3-glucoside and peonidin-3-glucoside. After raw rice was cooked by an electric rice cooker, the loss of anthocyanins was observed and antioxidant activities decreased. However, some phenolic acids increased after cooking. Degradation of cyanidin-3-glucoside and peonidin-3-glucoside resulted in the formation of protocatechuic acid and vanillic acid which possess antioxidant activity. Therefore, bioactivities of cooked rice could be different from those of raw rice which have been extensively studied.

The objective of this study was to investigate the bioaccessibility of anthocyanins from cooked purple rice using an *in vitro* model. Changes of the antioxidant activity during the digestion were also elucidated. In addition, the

antiproliferative activity of anthocyanin extract of cooked Thai purple rice on human colon cancer cell lines *in vitro* was assessed.

5.3 Material and methods

5.3.1 Rice samples and chemicals

Thai dark purple non-glutinous rice (*Oryza sativa* L., Mali Nil Surin No.6) was obtained from Surin Rice Research Center (Surin, Thailand). It was grown in 2011 and harvested at the end of the year. Pepsin (250 units/mg solid), pancreatin from porcine pancrease (4× USP specifications), α-amylase (~30 units/mg solid), bile salt mixture, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, 2,4,6-tri(pyridyl)-1,3,5-triazine (TPTZ), Folin-Ciocalteu phenol reagent, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO). All solvents and reagents of analytical and superior were obtained from Fisher Scientific (Fair Lawn, NJ). The artificial saliva consisted of $NaHCO_3$ (5.208 g), $K_2HPO_4.3H_2O$ (1.369 g), NaCl (0.877 g), KCl (0.447 g), $CaCl_2.2H_2O$ (0.441 g), mucin (2.160 g), and 200,000 U of α-amylase in 1 L of distilled water, and was adjusted to pH 7 (van Ruth, Roozen, and Cozijnsen, 1994).

5.3.2 Human cancer cell lines

The HCT116 and HT-29 cell lines derived from a colorectal carcinoma and colorectal adenocarcinoma were obtained from American Type Culture Collection (Manassas, VA). They were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) and 1.4% penicillin/streptomycin, at 37°C and 5% CO_2 atmosphere.

5.3.3 Rice cooking

Paddy rice was dehulled using a laboratory milling machine (Satake Co., Hiroshima, Japan). Fifty grams of dehulled rice were cooked by AROMA®rice cooker Model No. ARC-687D-1NG (Aroma Housewares Co., San Diego, CA) in the mode of cooking brown rice. The rice to water ratio of 1:3 was found to be optimum. Three replications of cooking were performed. Cooked rice samples were kept at -20°C until use.

5.3.4 Chemical extraction

Cooked rice (5 g) was ground by motar and pestle and filled in a 125 mL Erlenmeyer flask. Fifteen mL of distilled water was added and adjusted to pH 2 and free phenolics were extracted by stirring at 1,000 rpm, at room temperature for 30 min. After centrifugation at 3000 × g, 20°C for 20 min, supernatant was recovered and the insoluble fraction was further extracted by 15 mL of acidified methanol (85% methanol and 15% of 1 N HCl). Supernatant was recovered and insoluble fraction was further extracted by 15 mL of acetone. Each fractions were determined for total phenolic (TPC), total anthocyanin content (TAC) and antioxidant activity.

5.3.5 *In vitro* digestion

In vitro digestion of cooked rice samples were carried out according to Tagliazucchi et al. (2010). Briefly, ground cooked rice (10 g) were added 10 mL of artificial saliva containing α-amylase, incubated at 37°C in a shaking water bath for 1 h. Samples were centrifuged at 13,500 × g for 20 min. Supernatant was kept in a refrigerator for the analysis of TPC, TAC and antioxidant activity. Simulated gastric fluid containing 2 mg/ml of NaCl and 300 U/mL of pepsin was added in the volume of 15 ml. The pH was adjusted to 2.0 with concentrated HCl and the solution was

incubated at 37°C in a shaking water bath for 2 h. The samples in digestion flask were cooled in ice and then centrifuged at 13,500 × g for 20 min, and the supernatants were kept in a refrigerator for the analyses mentioned above. The pH of samples was then brought to 7.5 with 0.5 M $NaHCO_3$ at the end of gastric digestion before adding 18 ml of 0.8 mg/mL pancreatin and 25 mg/mL bile salts in 0.1 M $NaHCO_3$. The mixture was subsequently incubated at 37°C in a shaking water bath for 2 h. Samples were adjusted to pH 2 immediately at the end of pancreatic digestion and centrifuged at 13,500 × g for 20 min and supernatants were collected for the analyses. The control before digestion (CBD) was prepared by mixing ground cooked rice with water before determining TPC, TAC, and antioxidant activity.

5.3.6 Determination of total phenolic and total anthocyanin content

TPC was determined as described by Waterhouse (2005). An aliquot (20 µL) of the appropriately diluted digested sample and chemically extracted sample were mixed with 1,580 µL deionized water and 100 µL of Folin-Ciocalteu (FC) reagent was added and incubated at room temperature for 5 min. Three hundred µL of 20% (w/v) sodium carbonate solution was added to the mixture followed by incubation for 2 h at room temperature. Absorbance was measured at 765 nm. Total phenolic contents were expressed as mg gallic acid equivalents (GAE) per 100 g sample.

TAC was determined using the spectrophotometric method according to Abdel-Aal and Hucl (1999). Absorbance of appropriately diluted digested sample and chemically extracted sample were measured at 535 nm. TAC (µg/g of sample) was calculated as cyanidin-3-glucoside as it was a major anthocyanin found in pigmented rice using the following equation:

$$TAC = (A/\varepsilon) \times (vol/1{,}000) \times MW \times (1/sample\ wt) \times 10^6$$

Where, A is the absorbance reading.

5.3.7 Antioxidant activity determination

5.3.7.1 ABTS radical scavenging assay

ABTS radical scavenging activity of each sample was determined according to Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) with slight modifications. Twenty µl of the appropriately diluted samples were mixed with 1.48 mL of the working solution, and a decrease of absorbance was measured at 734 nm after 6 min at 30°C using a spectrophotometer. The activity was expressed as mmol Trolox equivalents/ 100 g cooked rice (Shen, Jin, Xiao, Lu, and Bao, 2009).

5.3.7.2 Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the modified method of Yang and Zhai (2009). Fifty µl of appropriately diluted samples were added to 0.95 mL of freshly prepared FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ in 40 mM HCl, and 1 part of 20.0 mM $FeCl_3 \cdot 6H_2O$ solution), and the reaction mixture was incubated at 37°C for 30 min. An increase in absorbance at 593 nm was measured. Results were expressed as mmol Trolox equivalents / 100 g sample.

5.3.8 Anthocyanin extract

Anthocyanin extract of dark purple rice was extracted according to the method described by Abdel-Aal and Hucl (1999) with slight modifications. Eighteen g of lyophilized sample of cooked purple rice were extracted twice by 144 mL of methanol acidified with 1.0 N HCl (85:15, v/v) at a stirring speed of 1,000 rpm for 60 min at room temperature. The pH of the mixture was control at pH 1 during

extraction. The mixtures were centrifuged at 10,000×g for 20 min at 4°C. Supernatants were brought up to 300 mL with acidified methanol.

An anthocyanin extract was prepared using solid phase extraction (C_{18} Sep-Pak solid cartridge, 5 g, Waters Corporation, MA, USA). The C18 cartridge was activated with two column volumes of methanol followed by three column volumes of acidified deionized water (0.01% HCl in deionized water, v/v). The C18 cartridge was washed by 40 mL of acidified deionized water to remove sugars and other polar compounds. Phenolic acids were removed using with ethyl acetate. Subsequently, anthocyanins were then eluted by 40 mL acidified methanol (0.01% HCl in methanol (v/v)). Methanol was removed by a rotary evaporator at 40°C. Anthocyanin extract were lyophilized, stored at -20°C, and use for cell culture study.

5.3.9 Cell antiproliferation assay

Lyophilized samples were dissolved in the mixture of dimethylsulfoxide (DMSO) and ethanol (50:50% v/v) to attain concentration of 15 mg/mL. Samples were then serially diluted using the mixed solvent of DMSO and ethanol. HCT116 and HT-29 cancer cells were plated at a density of 8.0×10^3 cells/well in a 96-well plate and incubated for 24 h. Anthocyanins extract at various concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µg/ml) were tested at incubation times of 24, 48, and 72 h. As the set incubation time was attained, the extracts and medium were removed from cell cultures. Subsequently, the cell viability was determined using Tetrazolium-dye based colorimetric microtitration (MTT) assay. Briefly, 10 µL of MTT in PBS at 5 mg/mL was added to the culture medium in each well and the cells were incubated for 2 h. Medium and MTT were then aspirated from the wells, and formazan were solubilized with 100 µL of DMSO. The optical density of blue color of formazan was read by a microplate reader at a wavelength of 550 nm. Data were analyzed with the

Microsoft Excel to determine the IC_{50} of each sample at each incubation time. IC_{50} is expressed as the concentration of sample required to kill 50% of the cells as compared to respective controls (cancer cells in medium without tested compounds).

5.3.10 Statistical analyses

The data were presented as mean ± SD and experiments were carried out in triplicate. The statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL). Differences of $p<0.05$ were considered significant and the significant difference between means was compared by Duncan's multiple-range test.

5.4 Results and discussion

5.4.1 *In vitro* bioaccessibility of cooked dark purple rice

TPC and TAC as well as antioxidant activity of cooked purple rice by chemical extraction are shown in Table 4.1. TPC and TAC greatly varied with solvent used. Acidified water is likely to extract free phenolics. The highest TPC was obtained when acidified methanol was used. Anthocyanins are believed to be one of major phenolic compounds in the acidified methanol extract. Acidified methanol system destroys the cell membranes, simultaneously dissolves anthocyanins, and stabilizes them. To assure that anthocyanins in rice sample were extracted completely. Rice sample were extracted by acetone in the last step. A acetone has been used to extract proanthocyanidins in the cereal grains and anthocyanins in vegetables and fruits to avoid the problem from pectins (Garcia-Viguera, Zafrilla, and Tomás-Barberán, 1998).

The first physical transformation of food matrices during eating occurs in the mouth, and mastication is considered the initial step in the digestion of foods. In the present study, after simulated mastication, phenolics and anthocyanins were released about 47.1 mg GAE/100 g of cooked rice and 0.89 mg/100 g of cooked rice,

respectively. These amounts corresponded to 8.4% and 5.5% of the phenolics and anthocyanins, respectively, obtained from chemical extraction (Table 5.1). It has been reported that most phytohemicals in the whole grain of pigmented rice are mainly present in the bran layers of seed coat, pericarp, aleurone, nucellus, and the germ. A significant amount of insoluble phenolic compounds are bound covalently to cell wall components such as cellulose and arabinoxylan (Miller, Rigelhof, Marquart, Prakash, and Kanter, 2000). Anthocyanins located within the vacuole of the plant cells along with other phenolic compounds are enclosed by tonoplast and cytoplasmic membranes which are, in turn, encapsulated by the plant cell wall (Rodriquez, Jaramillo, Heredia, Guillen, Jemenez, and Fernandez-Balanos, 2004). This complex arrangement of phenolics and anthocyanins may result in small release after mastification.

Table 5.1 The content of total phenolic, anthocyanin, and antioxidant activity of cooked dark purple rice after chemical extraction.

Extractant	Total phenolic mg GAE/100 g of cooked rice	Anthocyanins mg/100 g of cooked rice	ABTS µmol Trolox equivalent/ 100 g of cooked rice	FRAP µmol Trolox equivalent/ 100 g of cooked rice
Acidified water	19.60 ± 0.51	1.88 ± 0.04	79.09 ± 2.09	86.06 ± 1.71
Acidified methanol	195.22 ± 9.62	29.06 ± 0.74	421.98 ± 25.76	175.44 ± 22.33
Acetone	4.90 ± 0.20	0.45 ± 0.03	24.11 ± 0.59	26.68 ± 0.80
Total	**219.72 ± 10.33**	**31.39 ± 0.81**	**525.18 ± 28.44**	**288.18 ± 24.84**

Data are mean ± standard deviation (n = 3).

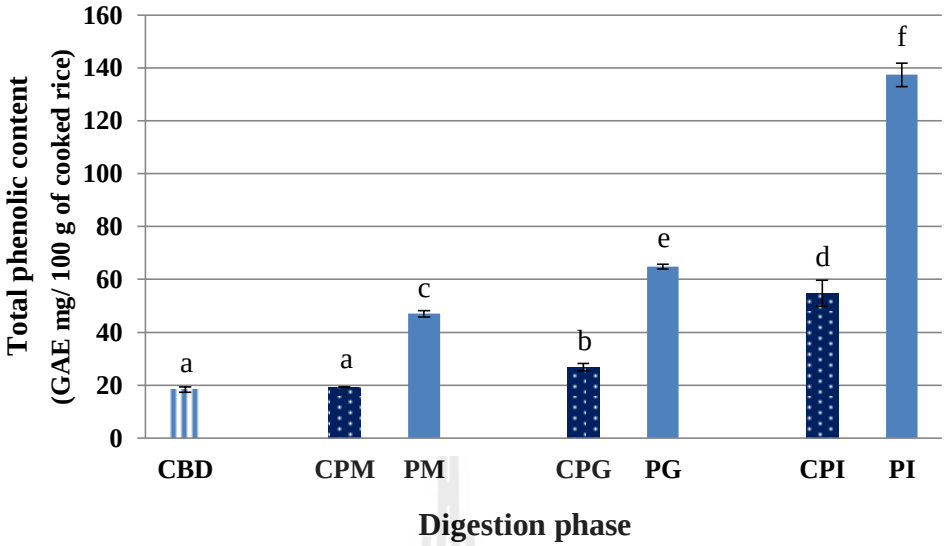

Figure 5.1 Changes of total phenolic content (TPC) of MNS6 during *in vitro* digestion compared to the control. CBD: control before digestion, PM: mouth condition, PG: gastric condition, PI: intestinal condition. CPM, CPG, and CPI: control of mouth, stomach and small intestinal digestion in the absence of digestive enzymes. Different letter indicate significant difference among samples ($p<0.05$).

A decrease of anthocyanins was observed when compared to the control before digestion (Figure 5.2). It is possible that there were an interaction between anthocyanins and artificial saliva constituents, including α-amylase. It is well known that proteins can interact with different classes of flavonoids, such as catechin, epicatechin, anthocyanins, and procyanidins (condense tannin), generate astringency sensation affecting the content of these flavonoids released (Dinnella, Recchia, Fia, Bertuccioli, and Monteleone, 2009; Laurent, Besançon, and Caporiccio, 2007). Salivary proline-rich proteins (PRPs) and histamine-rich proteins (HRPs) can bind with phenolic compounds (Charlton et al., 2002). Wiese, Gärtner, Rawel, Winterhalter, and

Kulling (2009) reported that cyanidin-3-glucoside bound in the immediate vicinity of tryptophan residues in the substrate binding site of amylase.

The amounts of bioaccessible phenolics after oral, gastric and pancreatic digestion were 21.4%, 29.5% and 62.5% when compared to chemical extraction (100%) (Figure 5.1). While only 2.8%, 9.5%, and 10.7% of extractable anthocyanins were obtained from the respective phase of digestion (Figure 5.2). The results indicated partial release of phenolics and anthocyanins from cooked dark purple rice. However, after each phase of oral, gastric and pancreatic digestion resulted in an increase of the bioaccessibility of phenolics and anthocyanins when compared to the respective control without enzyme digestion. Human digestive system begins in the mouth with chewing and chemical digestion with saliva containing amylase and lipase, and ends in the small intestine. Phenolics bound to the cell wall structure of rice can be broken by mechanical and enzymatic action. Amylase in saliva may play a role to release phenolics in the oral digestion. Amylase treatment helps to break down carbohydrates, allowing the release of bound phenolics (Saura-Calixto, Serrano, and Goni, 2007). In the gastric phase, chemical environment at acidic pH appeared to increase the release of phenolics. In addition, some of phenolics bound with proteins may be released at this point by the pepsin action which enhanced the release of phenolics from the food matrix. Protein-phenolic interactions in food derived plants have been reported (Kroll, Rawel, and Rohn, 2003; Xu and Diosady, 2000; Cheynier, 2005). Phenolics can bind to proteins through different mechanisms such as hydrogen bonding, covalent bonding, hydrophobic interactions and ionic bonding in aqueous media (Loomis and Batta, 1966; Mason, 1955, Hagerman and Butler, 1978; Rubino, Arntfield, Nadon, and Bernatsky, 1996; Xu and Diosady, 2000). Thus, an increase of bioaccessible phenolics in the gastric phase was found in this study. At the end of digestion, alkaline condition

alone of intestinal environment also increased the release of phenolics. Pancreatin containing pancreatic enzymes, trypsin, amylase and lipase, may be the main step for breaking down phenolics that are linked with carbohydrates, dextrins, proteins and lipids, resulting in an increase of released phenolics in the small intestine. Low molecular weight phenolics such as phenolic acids may also be released from solid matrix of cooked dark purple rice into the digesta of pancreatic digestion. Tagliazucchi et al. (2010) demonstrated that flavonoids are stable at the intestinal alkaline pH value, which is different from anthocyanins.

In the present study, the transition from the acidic to the mild alkaline environment did not cause a decrease in the amount of bioaccessible phenolics and anthocyanins. In addition, at the end of gastric and pancreatic digestion, the bioaccessible anthocyanins were not comparable (Figure 5.2). It is likely to conclude that the form of anthocyanins may be stable at the alkaline environment in the small intestine. The results showed that bioaccessibility of anthocyanins after pancreatic digestion was quite low, which is in agreement with others. Tagliazucchi et al. (2010) reported that the bioaccessible anthocyanins of grapes after pancreatic digestion were 7.6% of that obtained by chemical extraction. In the present study, water was used in each phase of digestion. Water is not the best solvent for anthocyanin extraction in cereal grains resulting in low bioaccessibility (Table 1).

It should be noted that an increase in bioaccessible phenolics and anthocyanins was also observed in the absence of digestive enzymes for each digestion phase (oral, gastric, and pancreatic). It was speculated that these bioactive compounds are released as a sequence of the chemical environment during digestion. Based on this study, both chemical environments and digestive enzymes contributed to the release of

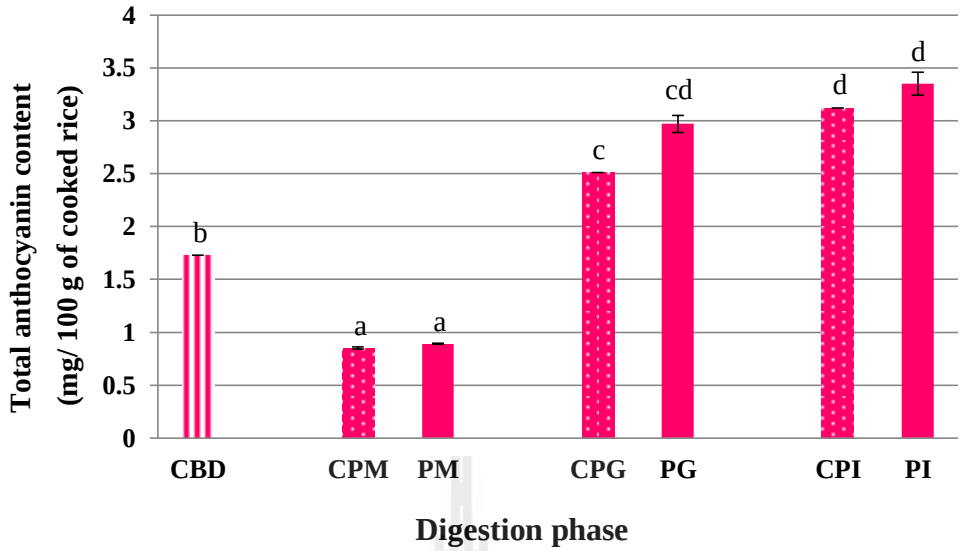

Figure 5.2 Changes of total anthocyanins content (TAC) of purple cooked rice cultivars during *in vitro* digestion compared to the control. CBD: control before digestion, PM: mouth condition, PG: gastric condition, PI: intestinal condition. CPM, CPG and CPI: control of mouth, stomach and small intestinal digestion in the absence of digestive enzymes. Different letters indicate significant difference among samples.

phenolics from the matrices of cooked dark purple rice, resulting in bioaccessibility of phenolics in the oral and gastro-intestinal digestion. On the other hand, the control digestion samples showed comparable bioaccessibility of anthocyanins to samples with enzymes (p>0.05). This indicated that the release of anthocyanins during the *in vitro* oral, gastric, and pancreatic digestion was mostly caused by chemical environments, rather than digestive enzymes. This was in agreement with Tagliazucchi et al. (2010) who reported that the chemical environment was the only dominant factor affecting the extraction of anthocyanins during the in vitro gastric and pancreatic digestions of grape.

Many studies showed that the transition from gastric to pancreatic phase caused a decrease in the amount of bioaccessible phenolics and anthocyanins (Bermúdez-Soto, Toma´s-Barberán, García-Conesa, 2007; Bouayed, Deußer, Hoffmann, and Bohn, 2012; Tagliazucchi et al., 2010; Tagliazucchi, Verzelloni, and Conte, 2012) which were in disagreement with this study. Tagliazucchi et al. (2010) reported that 15% of polyphenols was lost during the transition from gastric to intestinal environment, and pure phenolics such as gallic acid and caffeic acid were degraded, while flavonoids (catechin and quercetin) were not significantly degraded under pancreatic conditions. Anthocyanins at neutral or slightly basic pH can be attributable to the formation of the colorless chalcone pseudobase (McDougall et al., 2005). Chalcone formation is also favored by elevated temperatures and prolonged exposure may enhance degradation between the B and C rings, resulting in the destruction of anthocyanins chromophore (Clifford, 2000; Strack and Wray, 1993).

The degradation of anthocyanins after pancreatic digestion was not observed in this study, and the content in this phase was comparable to that after gastric digestion. It can be postulated that no degradation of anthocyanins took place in the pancreatic phase. Some investigations suggest that the co-pigmentation of anthocyanins with other compounds (co-pigments) is the main mechanism of stabilization of color in plants at neutral pH (Davies and Mazza, 1993; Mazza and Brouillard, 1990). The co-pigments are rich systems in π-electrons which are able to associate with flavylium ions (are rather poor in electrons), resulting in protection for the water nucleophilic attack in the 2 position of the flavylium ion (Matsufuji, Otsuki, Takeda, Chino, and Takeda, 2003). Co-pigments are generally colorless and can be flavonoids, alkaloids, amino acids, organic aids, nucleotides, polysaccharides, metals or another anthocyanin. In this study found that phenolics were released in pancreatic

phase higher than that gastric phase, and phenolic acids may be dominant extractable compounds released from food matrices. Therefore, it is possible that anthocyanins can form co-pigmentation with phenolic acids such as protocatechuic, vanillic, p-coumaric and ferulic acids to be acylated anthocyanins. It has been reported that acylated anthocyanins were more stable than non-acylated form after pancreatic digestion (McDougall et al., 2005). In the presence of acyl groups hinders the hydrolysis of flavylium cation form to the colorless carbinol (Bridle and Timberlake, 1997). The stability of anthocyanins containing in foods may play an important role to the bio accessibility and bioavailability to exert beneficial effects on the gastro-intestinal tract. This study indicated that only 10.67% of anthocyanins from cooked dark purple rice were bioaccessible at the end of pancreatic digestion. Anthocyanins may be physically or chemically bound to other constituents, of cooked pigmented rice, rendering difficulty in extraction during the oral and gastro-intestinal digestion.

5.4.2 Changes in antioxidant activity during simulated digestion

Antioxidant activity determined by ABTS assay increased during oral and gastric digestion, while decreased at the intestinal environment (Figure 5.3). The results indicated that extractable phenolics and anthocyanins acted either as hydrogen or electron donors. Antioxidant activity appeared to correlate well with the content of phenolic and anthocyanin during oral and gastric digestion. At pH 7-8 of pancreatic phase, the flavylium cation, aglycone of anthocyanin, can alternatively be transformed to quinoidal bases through proton transfer reaction and can further be converted to the quinonoid anions and chalcone (Heredia, Franchia-Aricha, Rivas-Gonzalo, Vicario, and Santos-Buelga, 1998). Therefore, various forms of anthocyanins such as quinoidal and chalcone may play an important role in the antioxidant action of anthocyanins (Heredia et al., 1998; Lapidot, Harel, Akiri, Granit, and Kanner, 1999). The completely

conjugated structure of anthocyanins that allow electron delocalization results in very stable radical products, shows good antioxidant activity (Kähkönen and Heinonen, 2003).

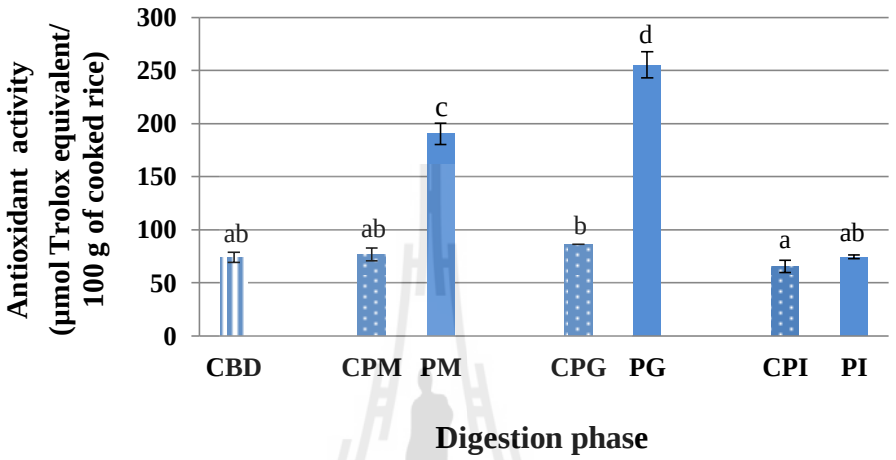

Figure 5.3 Antioxidant activity determined with ABTS assay of purple cooked rice during *in vitro* digestion compared to the control. CBD: control before digestion, PM: mouth condition, PG: gastric condition, PI: intestinal condition. CPM, CPG and CPI: control of mouth, stomach and small intestinal digestion in the absence of digestive enzymes. Different letter indicate significant difference among samples.

Antioxidant activity measured with the FRAP assay increased after oral and gastric digestion. Whereas a slight decrease of FRAP value was observed after the pancreatic digestion. The results showed that this antioxidant activity was related to anthocyanins and phenolic content in the oral and gastric, not pancreatic digestion. This study indicated that phenolics and anthocyanins released during simulated oral and gastro-intestinal digestion showed electron donating ability. Alkaline condition in pancreatic digestion at small intestine might induce conjugation of phenolic compounds, leading to lower electron donating ability.

The results obtained from this study indicated that 62.5% and 10.7% of phenolics and anthocyanins in cooked purple dark rice are readily bioaccessible in the gut and could be potentially bioavailable. *In vitro* bioaccessibility of anthocyanins from cooked dark purple rice in the present study was comparable that obtained from grapes (Tagliazucchi et al., 2010).

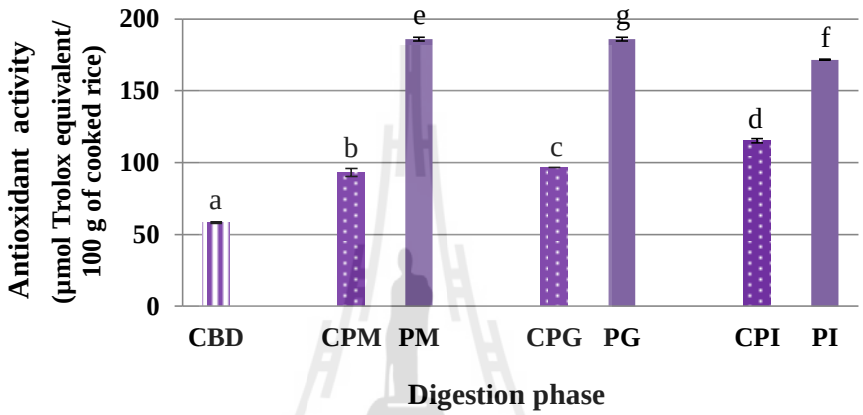

Figure 5.4 Antioxidant activity determined by FRAP assay of purple cooked rice during *in vitro* digestion compared to the control. CBD: control before digestion, PM: mouth condition, PG: gastric condition, PI: intestinal condition. CPM, CPG and CPI: control of mouth, stomach and small intestinal digestion in the absence of digestive enzymes. Different letter indicate significant difference among samples.

5.4.3 Effect of anthocyanin extract of cooked purple rice on cell proliferation

TAC of lyophilized extract after passing through C18 was 5.19 g/kg lyophilized sample. The anthocyanin extracts decreased proliferation of HCT116 and HT-29 colon cancer cells in a dose-dependent manner (Figures 5.5 and 5.7). Significant reductions in proliferation of HCT116 and HT-29 by the anthocyanin

extract of cooked purple rice were observed in the concentration ranging from 25 to 100 µg/mL (Figures 5.5 and 5.7). The antiproliferative activity also depended on time of exposure. (Figures 5.6 and 5.8) The longer the exposure time the lower the IC_{50} values of both cell lines.

Bioaccessibility and the serum bioavailability of many anthocyanins are quite low (Mcdougall, Ross, Ikeji, and Stewart, 2008). Therefore, this study was designed to evaluate the effective inhibition of human colon cancer cells of anthocyanin extract from cooked dark purple rice at relatively low concentrations. Major portion of ingested anthocyanins or other polyphenols survive in the upper gastrointestinal tract and reach to the colon where they could be subject to fermentation by colonic microflora (Aura et al., 2005; Karle et al., 2006). Anthocyanins from many food sources were evaluated for antiproliferative activity on human cancer cells such as breast, liver and leukemia cells by many researchers. Anticancer activity of raw dark and purple rice on various human cancer cells might result from arresting of cell cycle, inducing apoptosis, suppressing angiogenesis and inhibiting cell invasion (Chen et al., 2006; Hui et al., 2010; Hyun and Chung, 2004). This study demonstrated antiproliferation of colon cancer cells in vitro by anthocyanin extract from cooked pigmented rice.

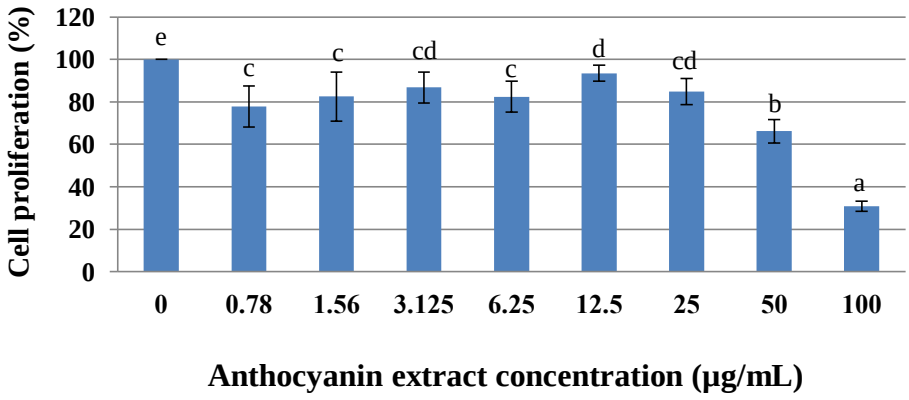

Figure 5.5 The effect of cell proliferation after anthocyanin extract treatment on HCT116 colon cancer cells. The cells were treated with or without different concentrations of anthocyanin extract from cooked purple rice for 24 h in a dose dependent manner.

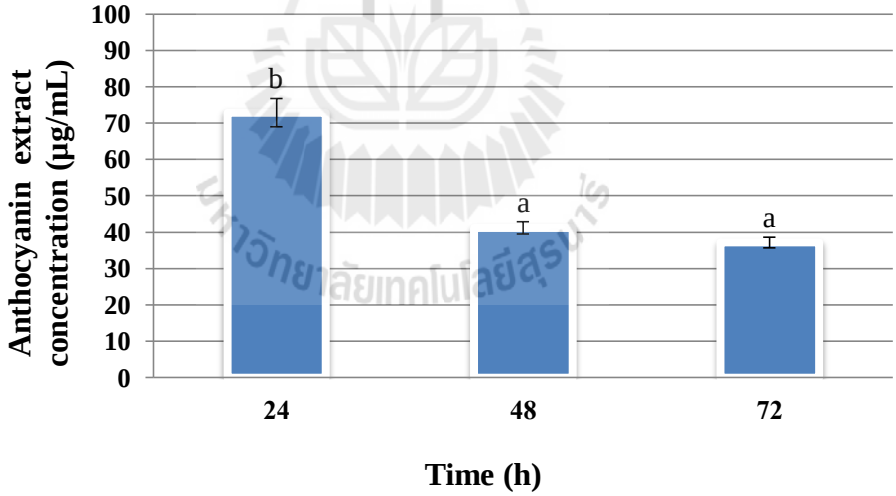

Figure 5.6 IC_{50}, treatment with different anthocyanin extract concentrations of dark purple rice on HCT116 colon cancer cells in a time-dependent manner. Values are expressed as means ± standard error. Control and test compounds were assayed in triplicate for each concentration and replicated three times for 24, 48, and 72 h.

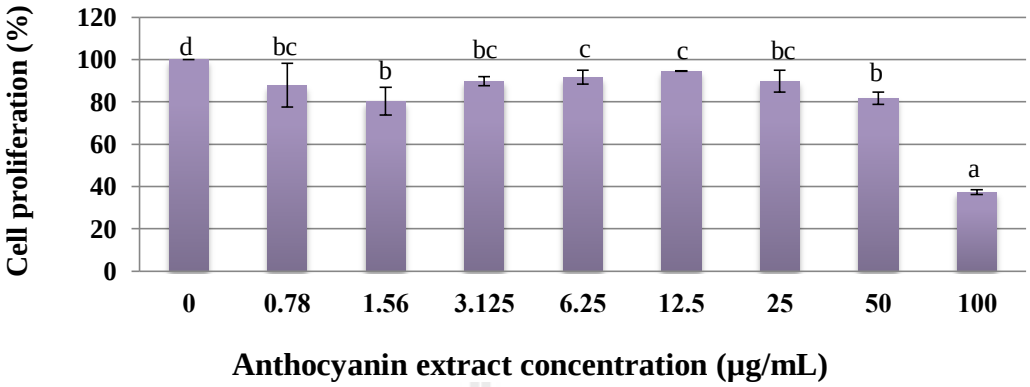

Figure 5.7 The effect of cell proliferation after anthocyanin extract treatment on HT-29 colon cancer cells. The cells were treated with or without different concentrations of anthocyanin extract from cooked purple rice for 24 h in a dose dependent manner.

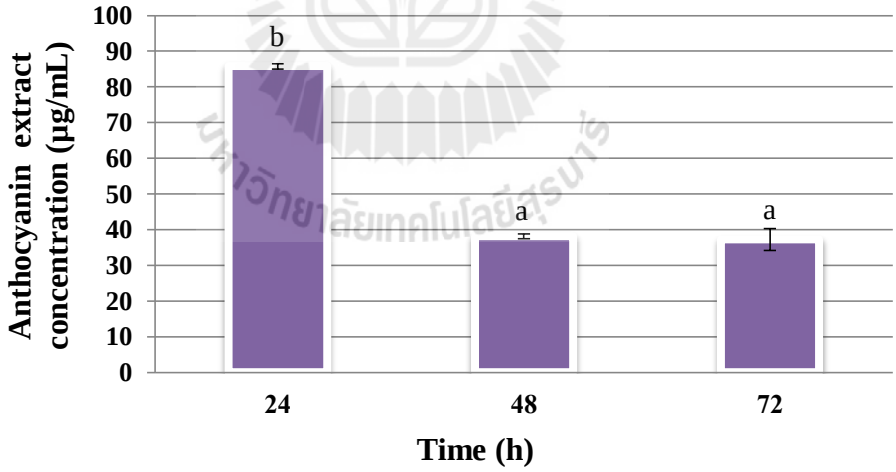

Figure 5.8 IC_{50}, treatment with different anthocyanin extract concentrations of dark purple rice on HT-29 colon cancer cells in a time-dependent manner. Values are expressed as means ± standard error. Control and test compounds were assayed in triplicate for each concentration and replicated three times for 24, 48, and 72 h.

5.5 Conclusions

In vitro oral and gastro-intestinal digestion resulted in an increase in the release of phenolics, anthocyanins, and antioxidant activities. The amount of bioaccessible phenolics and anthocyanins was 62.50, 10.67, % of that obtained from chemical extraction. Phenolics of cooked rice were released by environments of simulated oral and gastro-intestinal digestion as well as digestive enzymes, whereas the release of anthocyanins was mainly derived from chemical environments at alkaline pH. The released phenolics and anthocyanins reduced free radicals by either hydrogen donation or electron donation. Anthocyanin extracted from cooked rice inhibited proliferation of HCT116 and HT-29 colon cancer cell lines *in vitro* in dose- and exposure time-dependent manner. Anthocyanins in cooked dark purple rice may play an important role in the chemoprotective action.

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CHAPTER VI

SUMMARY

Brans of 2 cultivars of Thai dark purple rice, namely Mali Nil Surin No. 2 and Mali Nil Surin No.6, are rich in anthocyanins, phenolics and exhibited antioxidant activities. Predominant individual anthocyanins analyzed by HPLC-DAD and confirmed by LC-MS were cyanidin-3-glucoside (cy-3-glu) and peonidin-3-glucoside (pn-3-glu), while protocatechuic acid (PCA) and vanillic acid (VA) were major phenolic acids extracted by acidified methanol. Anthocyanins and antioxidant activity decreased to a greater extent when heating husk-removed rice in the presence of water as compared to hot air treatment at any studied temperatures of 60-90°C. Under hot water treatment, PCA and VA increased with heating temperatures in concomitant with a decrease of cy-3-glu and pn-3-glu, respectively, indicating that the degradation of these anthocyanins resulted in the formation of such phenolic acids.

All studied cooking methods, namely electric rice cooker, autoclave and microwave heating, resulted in a decrease of anthocyanin, phenolic content and antioxidant activities. Microwave cooking showed the greatest loss of both anthocyanins and phenolics (p<0.05). PCA and VA are major free phenolic acids, while ferulic acid and p-coumaric acid are major bound phenolic acids of raw and cooked dark purple rice in MNS6. Methanolic extracts of raw rice and rice cooked by autoclave heating showed the highest inhibition of Caco-2 cell proliferation. The

results indicated that raw and autoclave cooked rice showed potent chemoprevention in inhibition the proliferation of human colon cancer cells.

The bioaccessibility of phenolics and anthocyanins of cooked dark purple rice increased during *in vitro* mouth and gastro-intestinal digestion. Phenolics were released from the matrices of cooked dark purple rice by the action of digestive enzymes and environments, whereas anthocyanins were mostly released by the digestive environments. The released phenolics and anthocyanins during simulated in oral and gastro-intestinal digestion exhibited antioxidant activities assessed by ABTS and FRAP assay. At the end of entire phase of digestion, the bioaccessibility of phenolics and anthocyanins were 62.50% and 10.67% respectively. Anthocyanin extracts of cooked dark purple rice showed antiproliferative effects on human colon cancer cells, HCT116 and HT-29. The IC_{50} value of the HCT116 and HT-29 colon cancer cells was 37.2 and 37.19 µg/mL after 72 h of incubation, respectively. Anthocyanin-rich Thai dark purple rice could be a potential candidate as a functional food.

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