# TOTAL PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITIES FROM THAI GLUTINOUS RICE LEAVE EXTRACTS

**JIRAPORN KRASAETEP**

**A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Chemistry**

**Mahasarakham University**

**October 2012**

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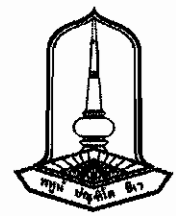

The examining committee has unanimously approved this thesis, submitted by Miss Jiraporn Krasaetep partial fulfillment of the requirements for the Master of Science degree in Chemistry, Mahasarakham University.

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Jiraporn Krasaetep

**ชื่อเรื่อง** สารประกอบฟีนอลิกรวมและฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากใบข้าวเหนียวไทย
**ผู้วิจัย** นางสาวจิราภรณ์ กระแสเทพ
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## บทคัดย่อ

งานวิจัยนี้ศึกษาฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากใบข้าวในแต่ละระยะการเจริญเติบโต ได้แก่ ระยะแตกกอ ตั้งท้อง และออกรวง จากข้าวเหนียวไทย 20 สายพันธุ์ รวมทั้งหาปริมาณฟีนอลิกรวม (TPC) ฟลาโวนอยด์รวม (TFC) และแอนโธไซยานินรวม (TAC) ด้วยวิธี โฟลินซิโอแคลธู (Folin-Ciocalteu) คอโลริมิทริกอะลูมิเนียมคลอไรด์ (Colorimetric Aluminum Chloride) และความแตกต่างของพีเอช (pH-differential) ตามลำดับ และทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี 2,2'-diphenyl-1-picrylhydrazyl (DPPH) และความสามารถในการรีดิวซ์เฟอร์ริกของสารต้านอนุมูลอิสระ (FRAP) ผลการทดลองพบว่า ปริมาณฟีนอลิกรวมอยู่ในช่วง 2.7236-5.9113 มิลลิกรัมสมมูลของกรดแกลลิกต่อน้ำหนักสด 1 กรัม ซึ่งในระยะออกรวงของใบข้าว No.11 มีปริมาณฟีนอลิกรวมสูงที่สุด ปริมาณฟลาโวนอยด์รวมอยู่ในช่วง 0.0051-0.0188 มิลลิกรัมสมมูลของคาร์เทชินต่อน้ำหนักสด 1 กรัม และใบข้าว No.1 ในระยะออกรวงมีปริมาณฟลาโวนอยด์รวมสูงที่สุด ค่า $IC_{50}$ ของสารสกัดอยู่ในช่วง 0.6497-5.0404 มิลลิกรัมต่อมิลลิลิตร และความสามารถในการต้านอนุมูลอิสระที่สูงที่สุดพบในสารสกัดจากใบข้าว No.14 ในระยะตั้งท้อง โดยที่สารสกัดทั้งหมดมีฤทธิ์ต้านอนุมูลอิสระน้อยกว่า BHA ($IC_{50}$ = 0.0044 มิลลิกรัมต่อมิลลิลิตร) ผลการทดสอบด้วยวิธี FRAP พบว่าสารสกัดจากใบข้าว No.14 ในทุกระยะการเจริญเติบโตมีความสามารถในการรีดิวซ์เฟอร์ริกสูงที่สุด และอยู่ในช่วง 61.2801-162.0832 มิลลิโมลาร์เฟอร์รัสต่อน้ำหนักสด 1 กรัม จากผลการทดลองแสดงให้เห็นว่าใบข้าวเหนียวพันธุ์ไทยเป็นแหล่งของสารพฤกษเคมีและสารต้านอนุมูลอิสระในธรรมชาติที่ดีที่อาจนำไปประยุกต์ใช้เป็นสารประกอบในอาหารเพื่อเสริมสุขภาพที่ดีได้

**คำสำคัญ** : ใบข้าวเหนียวไทย; ฤทธิ์ต้านอนุมูลอิสระ; ฟีนอลิก; ฟลาโวนอยด์; แอนโทไซยานิน

**TITLE** Total phenolic contents and antioxidant activities from Thai glutinous rice leave extracts

**CANDIDATE** Ms. Jiraporn Krasaetep

**DEGREE** Master of Science degree in Chemistry

**ADVISORS** Muntana Nakornriab, Ph.D.
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**UNIVERSITY** Mahasarakham University **YEAR** 2012

## ABSTRACT

This research was aimed to study antioxidant activities of some rice leave extracts from 20 cultivars Thai glutinous rice in different growth stages: tillering, booting and heading. The total phenolic contents (TPC), total flavonoid contents (TFC) and total monomeric anthocyanin contents (TAC) were investigated by using Folin-Ciocalteu assay, colorimetric aluminum chloride method and pH-differential method, respectively. Antioxidant activities of all extracts were also tested for radical scavenging capacity using a 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay and reductive capability by ferric reducing antioxidant power (FRAP) assay. The results indicated that the TPC was in the range of 2.7236 to 5.9113 mg GAE $g^{-1}$ of fresh weight (FW) which No.11 in heading stage found the highest of TPC value. The TFC in the extracts was in the range of 0.0051 to 0.0188 mg CE $g^{-1}$ of FW, and TFC of No.1 in heading stage showed the highest value. The $IC_{50}$ of methanolic extracts was 0.6497 to 5.0404 mg $mL^{-1}$ and the highest of free-radical scavenging activity found in No.14 of booting stage. All methanolic extracts have lower activities than that of BHA ($IC_{50}$ = 0.0044 mg $mL^{-1}$). With FRAP assay, the extract of No.14 in all stages showed the highest activity of 61.2801 to 162.0832 mM Fe(II) $g^{-1}$ of FW. The results indicated that leaves rice of Thai rice are the natural sources of phytochemicals and antioxidant compounds. It is promising that the extracts of Thai glutinous rice leaves might be applied as potential substances in functional foods for good health benefits.

**Keyword:** Thai glutinous rice leave; antioxidant activities; phenolic; flavonoid; anthocyanin

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# LIST OF ABREVIATIONS

| | |
|---|---|
| BHA | Butyliertes Hydroxyanisol |
| DPPH | 2,2'-diphenyl-1-picrylhydrazyl |
| CE | Catechin equivalent |
| cm | Centimeter |
| ˚C | Degree celcius |
| FRAP | Ferric reducing antioxidant power |
| Fe(II) | Ferrous |
| FW | Fresh weight |
| GAE | Gallic acid equivalent |
| g | Gram (s) |
| µL | Microliter (s) |
| mg | Milligram (s) |
| mL | Milliliter (s) |
| mM | Millimolar (s) |
| min | Minute (s) |
| M | Molar |
| MW | Molecular weight |
| nm | Nanometer |
| $g^{-1}$ | Per gram |
| $mL^{-1}$ | Per milliliter |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| TFC | Total flavonoid content (s) |
| TPC | Total phenolic content (s) |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Rice is one of the most consumed foods for one-third of the world population. It is belong to grass family, and a grain food for human (Zhai *et al.*, 2001; Zhu *et al.*, 2010). Approximately 95% of rice product is cropped in Asia (Chotimarkorn *et al.*, 2008; Sangkitikomol *et al.,* 2010). Black rice has high amounts of phenolic compounds, particularly natural anthocyanins compounds, such as cyaniding 3-glucoside, delephinidin 3-glucoside and peonidin 3-glucoside (Yawadio *et al.,* 2007; Tananuwong and Tewaruth, 2010), and also contains many beneficial components, including protein, several important amino acids, polyphenolic, flavonoids, vitamin E, phytic acid and γ-oryzanol (Ichikawa *et al.*, 2001; Zhang *et al.*, 2005). The functional properties of black rice include carcinogenic (Combet *et al.*, 2007), mutagenic (Macgregor and Jurd, 1978), free-radical scavenging and antioxidative effects (Frei and Becker, 2004; Zhang *et al.*, 2005) were reported.

Phenolic compounds are the secondary metabolites with a large range of structures and functions, but generally possess an aromatic ring bearing one or more hydroxyl substituent (Liu, 2007). The free phenolic compounds are proanthocyanidins or flavonoids, while the bound phenolic compounds are ester-linked to cell-wall polymers (Bonoli *et al.,* 2004a,b). Phenolic compounds commonly present in whole grains are phenolic acids and flavonoids (Al-Farsi and Lee, 2008). The common phenolic acids found in whole grains are ferulic acid, vanillic acid, caffeic acid, syringic acid and *p*-coumaric acid (Tian *et al.*, 2005), while flavonoids are flavonols, flavan-3-ols, flavones and flavanones (Lin and Tang, 2007).

This work was aimed to investigate the antioxidant activities of some rice leave extracts in different growth stages (tillering rice, booting rice and heading stage rice) of 20 Thai glutinous rice cultivars including the total phenolic contents (TPC) and total flavonoids content (TFC). Moreover the extracts were also tested for radical scavenging

capacity using a 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay and the chelating activity by ferric reducing antioxidant power (FRAP) assay.

## 1.2 Objectives of the research

To determine total phenolics, flavonoids anthocyanins content and antioxidant activities of some rice leave extracts in different growth stage (tillering rice, booting rice and heading stage rice) of 20 Thai glutinous rice cultivars.

## 1.3 Expected results obtained from the research

1.3.1 The total phenolics, flavonoids and anthocyanins content and antioxidant activity in leave of rice each growth stage of 20 Thai glutinous rice cultivars will be obtained from this work.

1.3.2 Data obtained from experiments can be used as basic knowledge for application of the rice extracts in medical and health care system.

## 1.4 Scope of the research

Methanolic extraction of rice leaves in different growth stage (tillering rice, booting rice and heading stage rice) of 20 Thai glutinous rice cultivars were investigated for their total phenolics and flavonoids content by using Folin-Ciocalteu assay and colorimetric aluminum chloride method, respectively. Moreover, their antioxidant activities were also measured using DPPH assay and FRAP assay.

## 1.5 Definition of terms

Antioxidant**:** Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged.

Free radical: Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules.

Phenolic compounds: Phenolic compounds possessing perfect antioxidants by virtue of the electron donating activity of the acidic phenolic hydroxyl group, which can stabilize unpaired electrons within its aromatic ring.

Flavonoids: Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms, and belong to the polyphenol family. The flavonoids consist of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids.

Thai glutinous rice: Thai glutinous rice or Thai sticky rice or sweet rice, a type of rice *Oryza sativa* Linn is turbid than the rice kernel such as black, red and white glutinous rice.

Black rice: Black rice or purple rice is contains anthocyanin pigments such as cyaniding and peonidin glucosides in the bran layer.

$IC_{50}$: $IC_{50}$ is amount of antioxidant needed to the initial $DPPH^{\cdot}$ concentration by fifty percentages.

# CHAPTER 2

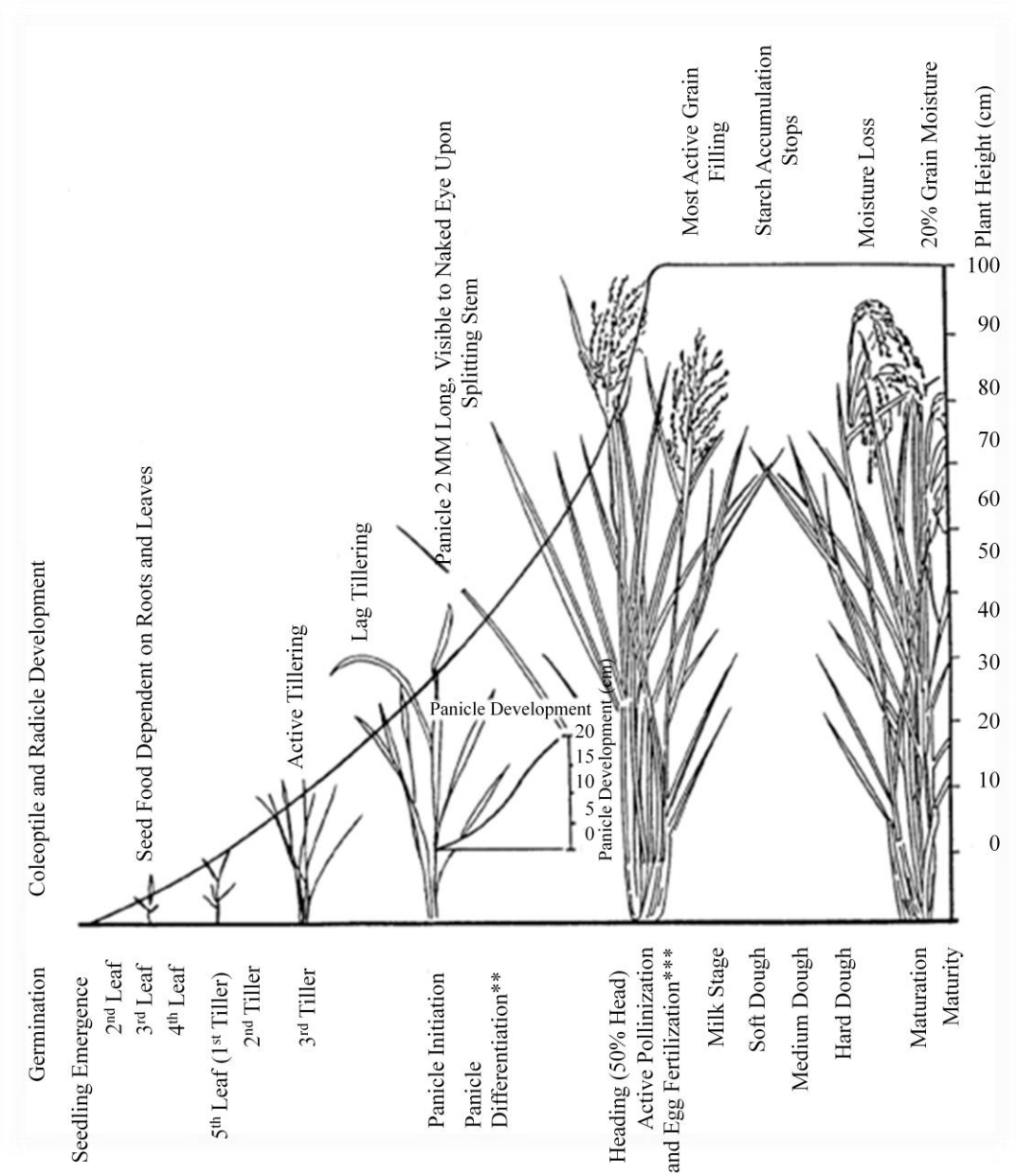
# LITERATURE REVIEW

## 2.1 Rice

Rice belongs to the Gramineae family and the genus *Oryza* comprised of about 23 different species, but only *Oryza sativa* Linn or Asian rice and *Oryza glaberrima* Steud or African rice are cultivated. Asia is a main source of *Oryza sativa*. It composed of *indica*, from tropical and temperate zone and *japonica* from temperate and subtropical Asia. *Oryza glaberrima* can only cultivate in South Africa. Rice is annual grass, leaves of monocotyledon and fibrous root system (Matsuo *et al.*, 1995).

2.1.1 Growth stage of rice

The stages of rice development can be divided in three large phases such as Vegetative, reproductive and grain filling and ripening stage showed in Figure 2.1 (Moldenhauer and Slaton, 2001).




†3 to 5 days.
*Under warm conditions use the lower number of days and for cool conditions use the larger number of days.
**The reproductive stage begins with panicle initiation.
***Stage III begins when 50% of the florets are pollinated.
****Variable time – 0 to 25 days (dependent upon variety).

**Figure 2.1** Development stages of the rice plant (Adapted from Moldenhauer and Slaton, 2001).

2.1.1.1 Vegetative stage

1) Seedling stage

Seedling stage is germinating development start until tillering development stage, general will to take time 25-30 days.

2) Tillering stage

The tillering stage will use time 30-50 days since seedling stage until maximum tillering stage.

2.1.1.2 Reproductive stage

This development stage can be divided in five phases such as panicle initiation, booting, heading, flowering and pollination stage. The reproductive stage usually lasts approximately 30 days in most varieties.

2.1.1.3 Grain filling stage

The grain filling and ripening or maturation stage follows ovary fertilization and is characterized by grain growth. This development stage can be the grouped in three phases such as milking, soft dough or heading and maturation stage. The grain filling reproductive stage usually lasts approximately 45-60 days in varieties.

## 2.2 Antioxidant activity

### 2.2.1 Free radicals

Free radicals are highly reactive compounds, spontaneous in the body during normal cellular metabolism by oxidation. Oxidant catalysts provide the stable electrons that are necessary for oxidation. The many common biological oxidant catalysts are iron and copper (Muramatsu *et al.*, 1995).

There are numerous types of free radicals that can be formed within the body. The most common is reactive oxygen species or ROS include; the superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), and hydrogen peroxide ($H_2O_2$). Superoxide anions are formed when oxygen ($O_2$) acquires an additional electron, leaving the molecule with only one unpaired electron. Hydroxyl radicals can be formed from $O_2^{\cdot -}$ and $H_2O_2$ via the Harber-Weiss reaction. The interaction of copper or iron and $H_2O_2$ also produce $OH^{\cdot}$. These reactions are found within the body and could easily interact (Campos-Martin *et al.*, 2006). Hydrogen peroxide is produced *in*

*vivo* by many reactions; it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted harmlessly as water.

### 2.2.2 Antioxidants

Antioxidants are classified into two broad divisions, soluble in water (hydrophilic) or lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Sies, 1997).

Phytochemicals are the bioactive; non-nutritive those have protective or disease preventive properties, naturally occurring plant compounds found in whole grains, fruits and vegetables (Liu, 2004). There are more than thousand known phytochemicals. It is well-known that plant produces these chemicals to protect itself. Some of the well-known phytochemicals are lycopene in tomatoes, isoflavones in soy and flavonoids in fruits. Phytochemicals are not essential nutrients and are not required by the human body for sustaining life. Some possible actions of phytochemicals are summarized in Table 2.1.

**Table 2.1** Some of the best known phytochemicals and their benefits and sources (Marcia, 2008).

| **Phytochemical** | **Potential Health Benefits** | **Food Source** |
|---|---|---|
| Anthocyanidins | Reduce risk of heart disease | Grapes, Raspberries, Blueberries, Cherries |
| Carotenoids | Encourage normal cell growth, Reduce risk of cancer | Yellow-orange vegetables and fruits, red fruits, green leafy vegetables |
| Catechins | Reduce risk of cancer | Green tea |
| Chalcones | Reduce risk of cancer | Licorice |
| Coumarins | Reduce risk of cancer | Carrots, Caraway, Celery, Parsley |

**Table 2.1** (Continued)

| **Phytochemical** | **Potential Health Benefits** | **Food Source** |
|---|---|---|
| Curcumins | Reduce risk of cancer, Reduce risk of heart disease, Antimicrobial | Turmeric, Ginger |
| Diallyl sulfide, Disulfides, Trisulfides | Reduce risk of cancer | Onions, Garlic, Chives, Leeks |
| Dithiolthiones | Reduce risk of cancer | Cruciferous vegetables |
| Ellagic acid | Reduce risk of cancer | Grapes, Strawberries, Raspberries, Nuts |
| Flavonoids | Reduce risk of heart disease, Reduce risk of cancer | Most fruits and vegetables |
| Isoflavones | Lower blood cholesterol, Reduce risk of cancer, Reduce risk of heart disease, Reduce risk of osteoporosis | Soy food (soybeans, tofu, soy milk, soy protein powder) |
| Alpha-linolenic acid | Lower blood cholesterol, Reduce hypertension, Reduce risk of heart disease, Reduce risk of cancer, Reduce inflammation, Immprove immune system | Vegetable oils (canola or soybean), Flax seed |
| Lignans | Lower blood cholesterol, Reduce risk of cancer | Soybeans, Flax seed, Sesame |
| Phenolic acids | Reduce risk of cancer | Berries, Grapes, Nuts, Whole grains |
| Phthalates, Polyacetylenes | Reduce risk of cancer | Caraway, Celery, Cumin, Dill, Fennel, Parsley |
| Phytates | Reduce risk of cancer | Grains, Legumes |

**Table 2.1** (Continued)

| **Phytochemical** | **Potential Health Benefits** | **Food Source** |
|---|---|---|
| Phytochemical | Potential Health Benefits | Food Source |
| Phytosterols | Reduce risk of cancer | Nuts, Seeds, Legumes |
| Saponins | Reduce risk of cancer | Beans, Herbs, Licorice root |
| Terpenoids | Reduce risk of cancer | Cherries, Citrus, Herbs (basil, oregano, thyme, sage) |

The following Table 2.2 gives the phytochemicals or phytochemical classes which provide the predominant source of coloring for the specified fruits or vegetables.

**Table 2.2** Dominant phytochemical pigments (Ben, 2006).

| **Color** | **Pigment** | **Fruits or Vegetables** |
|---|---|---|
| Red | Anthocyanins, Lycopene, Betacyanins | Strawberries, Raspberries, Cherries, Grapes, Cranberries, Pomegranates, Apples, Red Tomatoes, Pink Grapefruit, Watermelon Beets |
| Orange | Lycopene, Carotenoids | Carrots, Mangoes, Apricots, Cantaloupe, Pumpkin, Sweet Potatoes, Oranges, Tangerines |
| Blue/ Purple | Betacyanins | Blueberries, Plums, Eggplant, Concord Grapes |
| Yellow | Zeaxantin, Curcumin | Corn, Avocado, Turmeric (curry) |
| Green | Chlorophyll | Broccoli, Kale, Spinach, Cabbage, Asparagus, Green tea |
| Black | Thearubigins, Anthocyanins | Black tea Blackberries |

The antioxidants in plant to control the oxidative stress are caused by sunlight and oxygen, they are became a source of useful new compounds of antioxidant activity for human consumption. Plant derived antioxidants: tannins, quinines (Marwah

*et al.,* 2007), phenolic compounds (Que *et al.,* 2006; Okarter *et al.,* 2010; Vichapong *et al.,* 2010), flavonoids (Gorinstein *et al.,* 2007), catechins, oryzanol, tocopherols, tocotrienols (Okarter *et al.,* 2010; Butsat and Siriamornpun, 2010) and anthocyanins (Yawadio *et al.,* 2007; Daiponmak *et al.,* 2010) could delay of prevent to onset of degenerative diseases.

**2.2.2.1 Phenolic compounds**

Phenolic compounds or polyphenols constitute is one of the most numerous and widely-distributed groups of substances in the plant kingdom (Harborne, 1980). Polyphenols are products of the secondary metabolism of plant (Dai and Mumper, 2010; Mazid *et al.,* 2011). The phenolic compounds possess an aromatic ring bearing one or more hydroxyl substituent. Most of the major classes of plant polyphenols are listed in Table 2.3.

**Table 2.3** The major classes of phenolic compounds in plants (Harborne, 1980 and Balasundram *et al.,* 2006).

| **Number of carbon atoms** | **Basic skeleton** | **Class** | **Examples** |
|---|---|---|---|
| 6 | $C_6$ | Simple phenols, Benzoquinones | Catechol, Hydroquinone, 2,6-Dimethoxybenzoquinone |
| 7 | $C_6$-$C_1$ | Phenolic acids | Gallic acid, Salicylic acid |
| 8 | $C_6$-$C_2$ | Acetophenones, Tyrosine derivatives, Phenylacetic acids | 3-Acetyl-6-methoxybenzaldehyde, Tyrosol, p-Hydroxyphenylacetic acid |
| 9 | $C_6$-$C_3$ | Hydroxycinnamic acid, Phenylpropenes, Coumarins, Isocoumarins, Chromones | Caffeic acid, Ferulic acid, Myristicin, Eugenol, Umbelliferone, Aesculitin, Bergenon, Eugenin |

**Table 2.3** (Continued)

| Number of carbon atoms | Basic skeleton | Class | Examples |
|---|---|---|---|
| 10 | $C_6$-$C_4$ | Naphthoquinones | Juglone, Plumbagin |
| 13 | $C_6$-$C_1$-$C_6$ | Xanthones | Mangiferin |
| 14 | $C_6$-$C_2$-$C_6$ | Stilbenes, Anthraquinones | Resveratrol, Emodin |
| 15 | $C_6$-$C_3$-$C_6$ | Flavonoids, Isoflavonoids | Quercetin, Cyanidin, Genistein |
| 18 | $(C_6$-$C_3)_2$ | Lignans, Neolignans | Pinoresinol, Eusiderin |
| 30 | $(C_6$-$C_3$-$C_6)_2$ | Bifiavonoids | Amentoflavone |
| N | $(C_6$-$C_3)_n$ $(C_6)_n$ $(C_6$-$C_3$-$C_6)_n$ | Lignins, Catechol melanins, Flavolans (Condensed Tannins) | Lignins, Catechol melanins, Fiavolans (Condnsed Tannins) |

Phenolic compounds in plants have attracted great attention during the last decade due to their antioxidant contribution to human health (Shahidi and Ho, 2005). Phenolic compounds have been reported for health-related effects such as anti-bacterial (Ezoubeiri *et al.,* 2005), anti-mutagenic (Pedreschi and Cisneros-Zevallos, 2007), anti-carcinogenic (Kähkönen and Heinonen, 2003), anti-inflammatory (Elangovan *et al.,* 1994), cardiovascular diseases and certain cancers (Liu, 2004, 2007; Dykes and Rooney, 2007).The number, type and concentration of phenolics in plants exhibit extreme diversity as well as their structure. Hydroxybenzoic and hydroxycinnamic acid have a single-ring structure. However, flavonoids comprise three ring structures and can be further classified into anthocyanins, flavan 3-ols, flavones, flavanones and flavonols. Some flavonoids such as flavan 3-ols can be found in the form of dimers, trimers and polymers (Tsao and Deng, 2004). In plant, phenolics mainly occur as glycosylated forms through *O*-glycosidic bonds with a number of different sugars such as glucose, galactose, rhamnose, arabinose, xylose and rutinose (Justesen *et al.,* 1998). In addition, phenolic compounds are also present acylations with phenolic or

aliphatic acid, which complicates the identification task. Chemical structures of some phenolic acids showed in Figure 2.2 while flavonoids shown in Figure 2.3. Among these phenolic substances, flavonoids and in particular, anthocyanins are of interest because of their high occurrence in foods, especially in fruits, vegetables, and green leafy vegetables including green tea (Naczk and Shahidi, 2006).

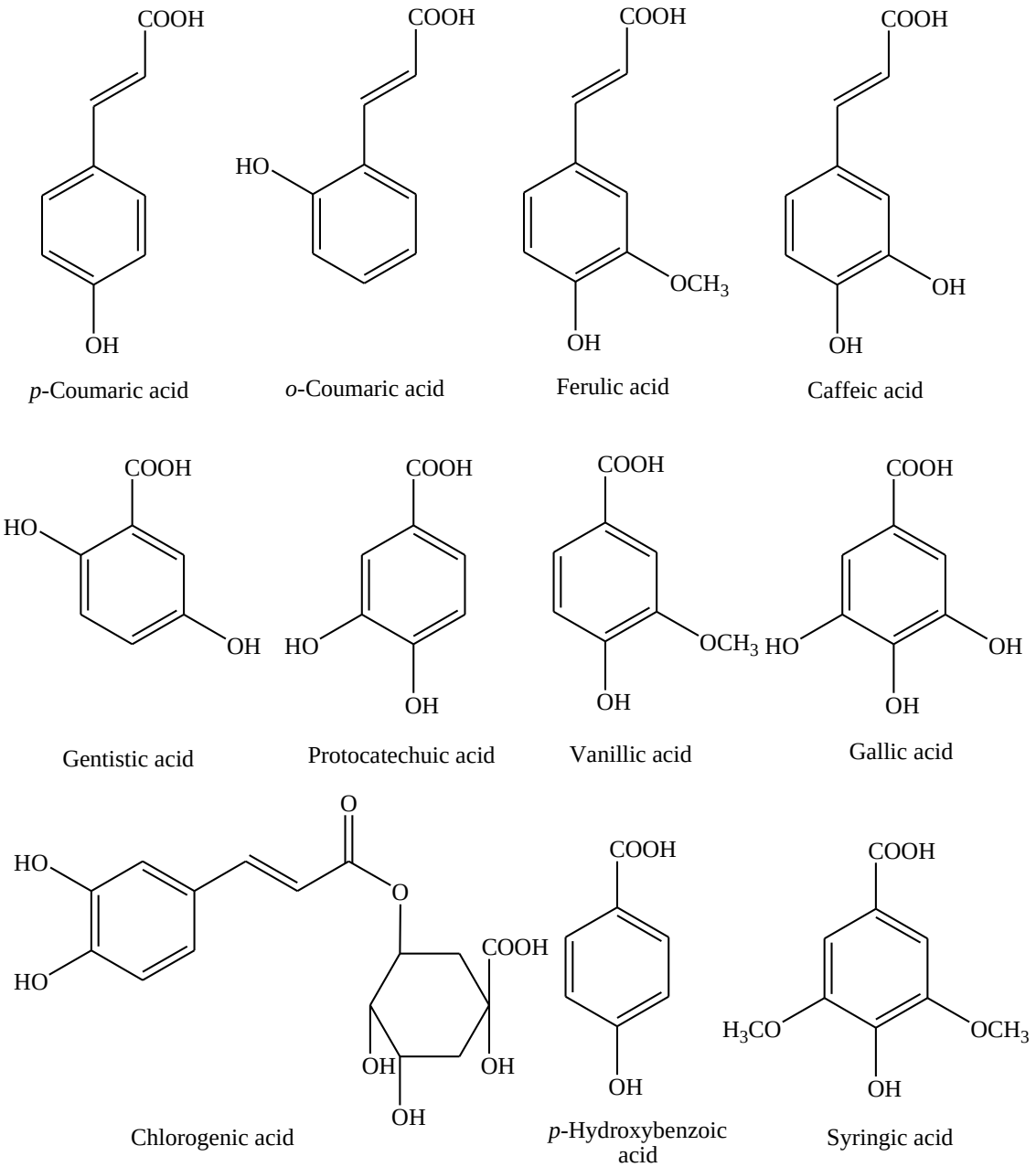



**Figure 2.2** Chemical structures of the phenolic acids (Adapted from Yu and Cheng, 2008).

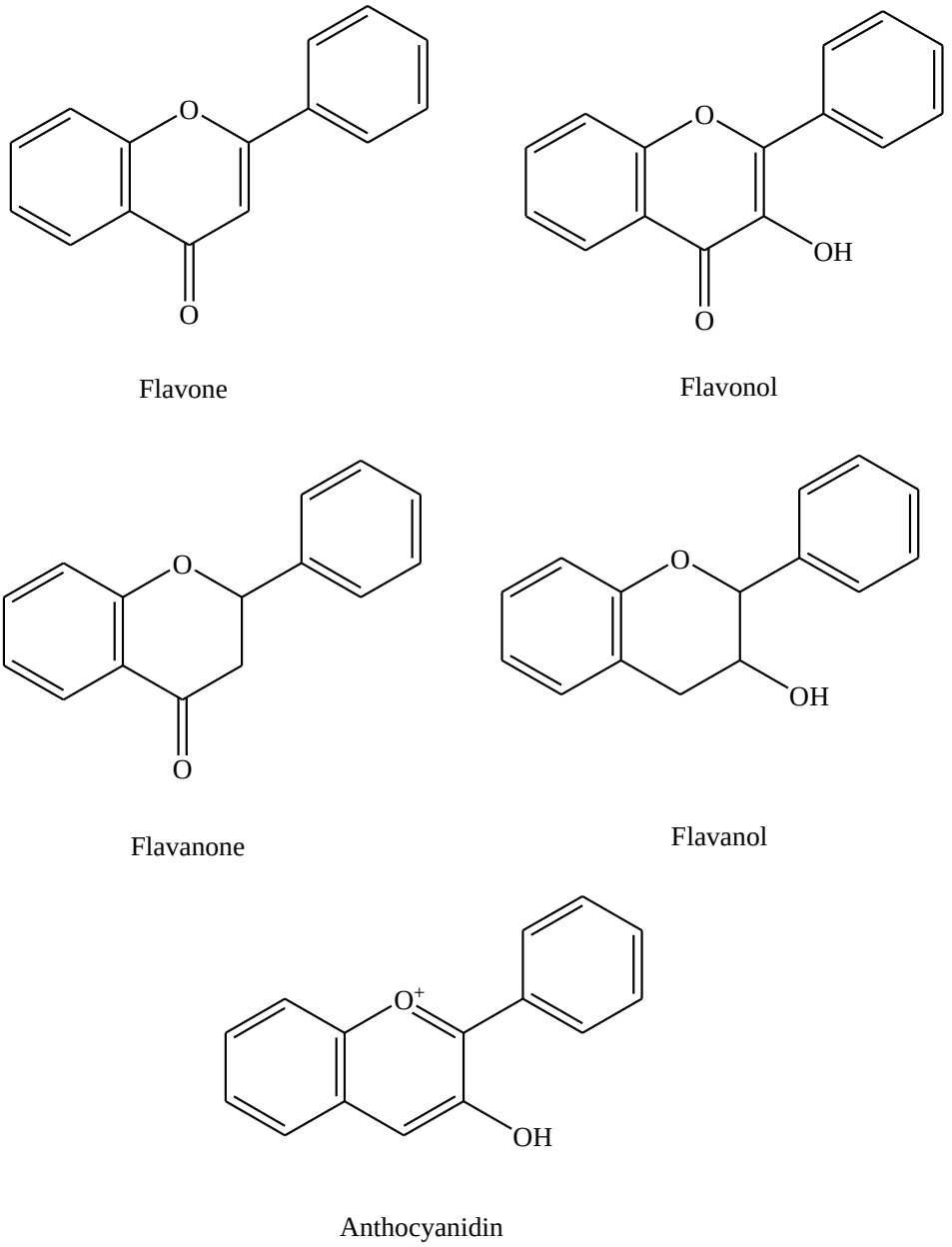



**Figure 2.3** Generic structures of major classes of flavonoids (Adapted from Balasundram *et al.*, 2006).

Phenolic compounds in rice

Many reports about phenolic compounds in rice were found recently. Most of results found that germinated brown rice and brown rice have more phenolic compounds than white rice (Tian *et al.*, 2004). Different from rice gain such as husk, bran, brown rice and milled rice were also investigated for their phenolic compounds

(Butsat and Siriamornpun, 2010; Vichapong *et al.,* 2010). The results also found that environmental and geographic land have affected on the phenolic content in rice (Daiponmak *et al.,* 2010). Moreover, the phenolic compounds in rice were varied depending on the color of rice gain (Muntana and Prasong, 2010).

**2.2.2.2 Flavonoids**

Flavonoids are a class of secondary phenolic metabolites, which are found in almost every plant. Many studies have suggested that flavonoids exhibit biological activities, including antioxidant, anti-carcinogenic and antimicrobial action (Parke, 1999; Pietra, 2000). Most of the beneficial health effects of flavonoids are attributed to their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species (Mellou *et al.,* 2005).

In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores (Harborne and Williams, 2000; Heim *et al.,* 2002). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Figure 2.4) and are classified according to substitutions (Figure 2.5). Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B- rings. Flavanones undergo a series of transformations affecting the heterocyclic C ring to give rise to other family members of flavonoids, including anthocyanins and catechin (Das, 1994). The immediate family members of flavonoids include flavones, isoflavones, and the 2,3-dihydroderivatives of flavone, namely flavanones, which are interconvertible with the isomeric chalcones (Sakakibara *et al.,* 2003; He *et al.,* 2008). Some flavonoids have been found to possess anti-lipoperoxidant (Terao, 1994), anti-tumoral (Deschner *et al.,* 1991; Elangovan *et al.,* 1994), anti-platelet (Tzeng *et al.,* 1991), anti-ischemic (Rump *et al.,* 1995), anti-allergic, and anti-inflammatory (Ferrándiz and Alcaraz, 1991; Middleton and Kandaswami, 1992) activities.

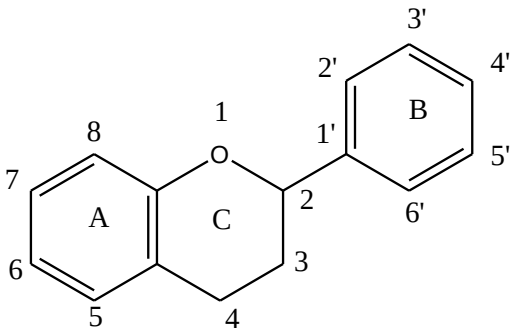



**Figure 2.4** Nuclear structures of flavonoids (Adapted from Cook and Samman, 1996).

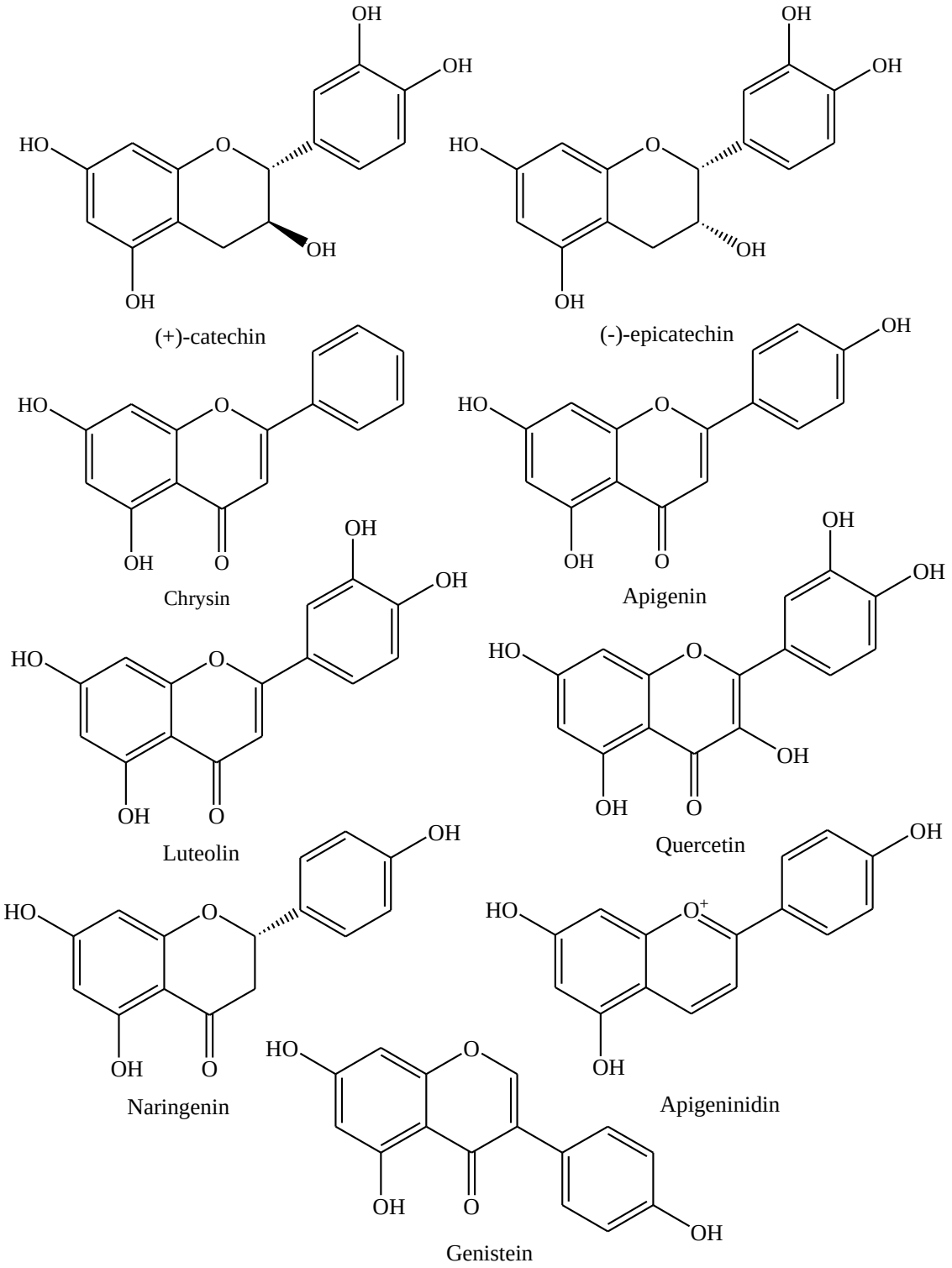



**Figure 2.5** Subclasses of flavonoids. Classification is based on variations in the heterocyclic C-ring. (Adapted from Hollman and Katan, 1999; Rice-Evans *et al.*, 1995,1996).

Flavonoid in rice extracts

The total flavonoid contents in rice were reported and have been focused in last decade. Many kinds of rice variety found to compose of flavonoid (Rao *et al.*, 2010). In addition, another composition of rice grain such as bran was also reported to contain flavonoid (Chotimarkorn *et al.,* 2008). The flavonoid contents were varied by the rice varieties, as well as the rice grain color (Shen *et al.,* 2009; Yafang *et al.,* 2011). The results found that flavonoid contents have the highest in black > red > white rice, respectively (Shao *et al.,* 2011; Kim *et al.,* 2010).

# CHAPTER 3

# RESEARCH METHODOLOGY

## 3.1 Equipments

All equipments used in this work are listed in Table 3.1.

**Table 3.1** List of equipments used in this work.

| Equipments | Model | Country |
|---|---|---|
| Visible spectrophotometer | Thermo Spectronic 4001/4 Spectrophotometer | USA |
| Rotary evaporator | Buchi Rotavapor R-210 | Switzerland |
| Adjustable air-displacement pipette | Gilson S.A.S. | France |
| Shaker | PSU-20, Platform Shaker | BIOSAN |
| pH Meter | 713 pH Meter Metrohm | Switzerland |

## 3.2 Chemicals

All chemical used in this work are listed in Table 3.2.

**Table 3.2** List of chemical used in this work.

| Name | Grade | Company | Country |
|---|---|---|---|
| Methanol ($CH_3OH$) | AR | Merck | Germany |
| Ethanol ($C_2H_5OH$) | | | |
| Hydrochloric acid fuming 37% (HCl) | | | |
| Sodium nitrite ($NaNO_2$) | | | |
| Aluminium chloride ($AlCl_3$) | | | |

**Table 3.2** (Continued)

| Name | Grade | Company | Country |
|---|---|---|---|
| Sodium hydroxide (NaOH) | AR | Merck | Germany |
| 2,2'-diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$) | AR | Sigma-Aldrich | Germany |
| Folin-Ciocalteu's reagent | AR | Carlo Erba Reagents | Spain |
| Sodium carbonate ($Na_2CO_3$) | | | |
| Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) | | | |
| Sodium acetate ($CH_3COONa \cdot 3H_2O$) | | | |
| Acetic acid glacial ($CH_3COOH$) | | | |
| *n*-Hexane ($CH_3(CH_2)_4CH_3$) | | | |
| 3,4,5-hydroxylbenzoic acid ($C_7H_6O_5$) | AR | Acros organics | USA |
| Butyliertes Hydroxyanisol ($C_{16}H_{16}O_2$) | | | |
| 2,4,6-Tri (2-pyridyl)-s-triazine ($C_{18}H_{12}N_6$) | | | |
| L-Ascorbic acid ($C_6H_8O_6$) | AR | Univar | Canada |
| Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) | AR | Univar | Australia |
| (±)-Catechin hydrate ($C_{15}H_{14}O_6$) | AR | Sigma-Aldrich | India |

### 3.3 Sample

The leaves of twenty Thai glutinous rice cultivars from different stages; tillering, booting and heading were harvested and collected as substrates for experiment. The order of relative water content of rice leaves was as follows: tillering stage (69.0384 ± 1.506 %), booting stage (61.9145 ± 1.859 %) and heading stage (60.4415 ± 1.997 %). All of rice cultivars are summarized in Table 3.3.

**Table 3.3** Colors of leaf and Thai glutinous rice used for experiments.

| **Local name** | **Varieties** | **Colors of leaf** | | |
|---|---|---|---|---|
| | | **Front** | **Margin** | **Under** |
| Niaw Dam (Ton Sung)-1 | Black glutinous rice No.1 | purple | purple | purple |
| Niaw Dam (Ton Sung)-2 | Black glutinous rice No.2 | purple | purple | purple |
| Khao' Kam No.7 | Black glutinous rice No.3 | green | purple | green |
| Niaw Dam (Entry 1) | Black glutinous rice No.4 | green | green | green |
| Niaw Dam (Entry 4) | Black glutinous rice No.5 | green | purple | green |
| Niaw Dam (Entry 6) | Black glutinous rice No.6 | green | purple | purple |
| Niaw Dam (Entry 8) | Black glutinous rice No.7 | green | purple | green |
| Niaw Dam (Entry 9) | Black glutinous rice No.8 | green | purple | green |
| Niaw Dam (Entry 10) | Black glutinous rice No.9 | green | purple | green |
| Niaw Dam (Entry 11) | Black glutinous rice No.10 | purple | green | green |
| Niaw Dam (Entry 12) | Black glutinous rice No.11 | green | purple | green |
| Niaw Dam (Entry 13) | Black glutinous rice No.12 | green | purple | green |
| Niaw Dam Noi (Entry 16) | Black glutinous rice No.13 | green | green | green |
| Niaw Dam Tap Mu (Entry 17) | Black glutinous rice No.14 | green | purple | purple |
| Niaw Dam (Entry 18) | Black glutinous rice No.15 | green | purple | green |
| Niaw Dam (Entry 22) | Black glutinous rice No.16 | green | purple | green |
| Niaw Dam (Entry 23) | Black glutinous rice No.17 | green | purple | green |
| Khao Klam (Entry 28) | Black glutinous rice No.18 | green | purple | green |
| Khao Klam (Entry 29) | Black glutinous rice No.19 | green | purple | green |
| Kor Khor 6 (ck6) | Thai glutinous rice No.20 | green | green | green |

## 3.4 Methods

### 3.4.1 Extraction of crude antioxidant

The leaves in different growth stages of rice samples were cut to 0.2-0.5 cm (5 g) and extracted overnight with 50 mL of 80% methanol. The extracts were done about 3 times and each for 60 min with intermittent shaking (155 rpm) at room temperature. The extracts were filtered through Whatman No. 1 filter paper. The extracts were then slowly concentrated until the final concentration is 1 mg $mL^{-1}$ under reduced pressure at 40˚C.

### 3.4.2 Total phenolic contents

The total phenolic contents (TPC) were measured by spectrophotometric method using the Folin-Ciocalteu reagent according to the modification method of Bonli *et al.,* (2004a,b). Briefly, a 50 µL of 125 mg $mL^{-1}$ methanolic extract was added to 3 mL of 10% Folin-Ciocalteu reagent (1:9 with deionized water). The mixture solution was incubated at room temperature for 15 min, and then 1.5 mL of 10% sodium carbonate solutions was added. The mixture was shaken and then incubated at room temperature for 15 min, before measuring at 750 nm using a spectrophotometer. The TPC was quantified against gallic acid standard calibration curve with triplicate and averages of values content. The results were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg GAE $g^{-1}$ of FW).

### 3.4.3 Total flavonoid contents

The total flavonoid contents (TFC) of extract were determined by a colorimetric aluminum chloride method described by Yang *et al.,* (2009). The 250 µL of 10 mg $mL^{-1}$ methanolic extracts was mixed with 1.25 mL of deionized water, 75 µL of 5% sodium nitrite solution. The mixture was then incubated for 5 min at room temperature. Then 150 µL of 10% aluminium chloride was added and standed for 6 min at room temperature. 500 µL of 1 M sodium hydroxide and 775 µL of deionized water were added to the mixture. The absorbance of the mixture was immediately measured at 510 nm using a spectrophotometer. TFC was calculated using the standard (±)-catechin curve, and expressed as milligrams of catechin equivalents per gram of fresh weight (mg CE $g^{-1}$ of FW).

### 3.4.4 Determination of antioxidant activity

#### 3.4.4.1 Free- radical scavenging activity

Free radical scavenging activity of extract was determined by using a stable 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH˙) following a modified method of Chan *et al.,* (2007). The 0.01, 0.05, 0.10, 0.50, 1.00 and 10.00 mg $mL^{-1}$ methanolic extracts were mixed with 3.0 mL of 0.1 mM DPPH radical, and incubated for 30 min at dark room temperature. Absorbance was measured at 517 nm, and calculated radical scavenging activity.

$$\text{Radical scavenging activity (\%)} = (1 - \frac{A_{sample}}{A_{control}}) \times 100$$

where $A_{sample}$ is the absorbance of sample and $A_{control}$ is the absorbance of solution without sample. BHA dissolved in methanol used as control solution. DPPH radical scavenging activity is expressed as 50% inhibition concentration $IC_{50}$ (mg $mL^{-1}$).

#### 3.4.4.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted with a modified method described by Benzie and Strain (1996). The 100 µL of 10 mg $mL^{-1}$ methanolic extracts were mixed with 3 mL of FRAP solution (300 mM acetate buffer (pH 3.6): 10 mM tripyridyltriazine solution (in 40 mM HCl): 20 mM ferric chloride solution of 10:1:1 by volume) and 300 µl of deionized water, then incubated at 37˚C for 4 min. The absorbance was then measured at 593 nm. The results were expressed as millimolar ferrous sulphate per grams of fresh weight (mM Fe(II) $g^{-1}$ of FW).

### 3.5 Data analysis

All experimental data were expressed as mean ± SD deviation of triplicate for each sample. The data of total phenolics, total flavonoids, total monomeric anthocyanin content and antioxidant activity of DPPH and FRAP assays, were analyzed for variance (ANOVA) procedures. Differences were considered significantly at $p < 0.05$. Statistical analysis was performed using SPSS software.

# CHAPTER 4

# RESULTS

## 4.1 Total phenolic content (TPC)

The total phenolic content (TPC) of rice leave extracts in different growth stage (tillering, booting and heading) of 20 Thai glutinous rice cultivars were investigated using Folin-Ciocalteu method. The TPC of the extracts from tillering stage were arranged from 2.7921-5.4495 mg GAE $g^{-1}$ FW (fresh weight). In this stage, black glutinous rice No.11 has highest value of TPC, while black glutinous rice No.15 was the lowest of TPC. In the booting stage, the highest of TPC was found in black glutinous rice No.14, whereas black glutinous rice No.13 showed the lowest value of TPC. For this stage, the TPC was arranged between 2.7236-4.9961 mg GAE $g^{-1}$ FW. At the heading stage, black glutinous rice No.11 showed the highest of TPC value, while black glutinous rice No.5 was the lowest of TPC. The distributions of TPC in this stage were arranged from 2.8110-5.9113 mg GAE $g^{-1}$ FW. The details of each TPC value showed in Table 4.1. The results indicated that those of TPC values were affected with rice cultivar as well as the growth stage.

**Table 4.1** Content of total phenolic of rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars. [a]

| Seed of Rice | TPC (mg GAE $g^{-1}$ of FW) | | |
|---|---|---|---|
| | Leaf of Tillering Rice | Leaf of Booting Rice | Leaf of Heading Stage Rice |
| Black glutinous rice No.1 | 4.2058 ± 0.048 | 4.2625 ± 0.042 | 4.3652 ± 0.107 |
| Black glutinous rice No.2 | 4.9440 ± 0.091 | 3.7523 ± 0.092 | 4.1810 ± 0.092 |

**Table 4.1** (Continued)

| Seed of Rice | TPC (mg GAE $g^{-1}$ of FW) | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.3 | 4.3546 ± 0.078 | 3.1239 ± 0.102 | 3.6991 ± 0.104 |
| Black glutinous rice No.4 | 3.7239 ± 0.078 | 2.7791 ± 0.059 | 3.6531 ± 0.079 |
| Black glutinous rice No.5 | 3.8456 ± 0.022 | 3.9625 ± 0.060 | 2.8110 ± 0.030** |
| Black glutinous rice No.6 | 3.9542 ± 0.059 | 3.6353 ± 0.044 | 3.9814 ± 0.122 |
| Black glutinous rice No.7 | 3.9389 ± 0.118 | 3.3389 ± 0.069 | 3.7322 ± 0.115 |
| Black glutinous rice No.8 | 3.2940 ± 0.114 | 3.7842 ± 0.072 | 3.7216 ± 0.047 |
| Black glutinous rice No.9 | 4.0523 ± 0.068 | 4.0771 ± 0.110 | 3.8255 ± 0.070 |
| Black glutinous rice No.10 | 3.1298 ± 0.083 | 3.5940 ± 0.072 | 3.2586 ± 0.106 |
| Black glutinous rice No.11 | 5.4495 ± 0.137* | 4.5058 ± 0.123 | 5.9113 ± 0.106* |
| Black glutinous rice No.12 | 4.1586 ± 0.032 | 3.0483 ± 0.039 | 3.7227 ± 0.078 |
| Black glutinous rice No.13 | 3.7145 ± 0.044 | 2.7236 ± 0.028** | 3.1924 ± 0.049 |
| Black glutinous rice No.14 | 4.4562 ± 0.134 | 4.9961 ± 0.117* | 5.1707 ± 0.013 |
| Black glutinous rice No.15 | 2.7921 ± 0.017** | 4.1326 ± 0.103 | 3.9117 ± 0.118 |

**Table 4.1** (Continued)

| Seed of Rice | TPC (mg GAE $g^{-1}$ of FW) | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.16 | 3.6412 ± 0.050 | 3.6731 ± 0.039 | 3.3306 ± 0.038 |
| Black glutinous rice No.17 | 3.8822 ± 0.068 | 3.9022 ± 0.108 | 4.1657 ± 0.096 |
| Black glutinous rice No.18 | 2.9208 ± 0.061 | 3.8539 ± 0.090 | 3.3554 ± 0.072 |
| Black glutinous rice No.19 | 2.8854 ± 0.031 | 3.0578 ± 0.063 | 3.8054 ± 0.109 |
| Thai glutinous rice No.20 | 3.0944 ± 0.016 | 3.7534 ± 0.100 | 2.9751 ± 0.037 |

[a] Each value is the mean of five replications ± standard deviation.

*, ** Means number the maximum and minimum in each column, respectively.

**4.2 Total flavonoids content (TFC)**

Table 4.2 showed the total flavonoid content (TFC) of rice leave extracts in different growth stage of 20 Thai glutinous rice cultivars. The rice leave extracts of tillering stage had the TFC values in range from 0.0051-0.0168 mg CE $g^{-1}$ of FW. In this stage, black glutinous rice No.11 was the highest value, while black glutinous rice No.19 was the lowest of TFC. The highest valve of TFC in booting stage was found in black glutinous rice No.18, while black glutinous rice No.3 was the lowest of TFC value. For this stage, the TFC was arranged between 0.0058-0.0146 mg CE $g^{-1}$ of FW. The heading stage showed the highest of TFC value in black glutinous rice No.1, whereas black glutinous rice No.8 and No.10 were the lowest values of TFC. The distributions of TFC in this stage were arranged from 0.0053-0.0188 mg CE $g^{-1}$ of FW. The rice leaves of black glutinous rice No.1 in heading stage contained the highest amount of flavonoid, whereas the lowest level was found in black glutinous rice No.8 and No.10 of heading stage and black glutinous rice No.19 of tillering stage.

**Table 4.2** Content of total flavonoid of rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars. [a]

| Seed of Rice | TFC (mg CE $g^{-1}$ of FW) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.1 | 0.0106 ± 0.0008 | 0.0090 ± 0.0003 | 0.0188 ± 0.0003* |
| Black glutinous rice No.2 | 0.0088 ± 0.0002 | 0.0088 ± 0.0004 | 0.0089 ± 0.0005 |
| Black glutinous rice No.3 | 0.0115 ± 0.0006 | 0.0058 ± 0.0002** | 0.0079 ± 0.0002 |
| Black glutinous rice No.4 | 0.0075 ± 0.0003 | 0.0067 ± 0.0003 | 0.0073 ± 0.0001 |
| Black glutinous rice No.5 | 0.0088 ± 0.0004 | 0.0086 ± 0.0019 | 0.0071 ± 0.0001 |

**Table 4.2** (Continued)

| Seed of Rice | TFC (mg CE $g^{-1}$ of FW) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.6 | 0.0123 ± 0.0005 | 0.0066 ± 0.0001 | 0.0096 ± 0.0002 |
| Black glutinous rice No.7 | 0.0097 ± 0.0006 | 0.0087 ± 0.0004 | 0.0104 ± 0.0004 |
| Black glutinous rice No.8 | 0.0068 ± 0.0002 | 0.0132 ± 0.0014 | 0.0054 ± 0.0001** |
| Black glutinous rice No.9 | 0.0084 ± 0.0001 | 0.0091 ± 0.0003 | 0.0095 ± 0.0002 |
| Black glutinous rice No.10 | 0.0083 ± 0.0001 | 0.0094 ± 0.0008 | 0.0053 ± 0.0001** |
| Black glutinous rice No.11 | 0.0168 ± 0.0004* | 0.0092 ± 0.0002 | 0.0118 ± 0.0002 |
| Black glutinous rice No.12 | 0.0160 ± 0.0006 | 0.0077 ± 0.0001 | 0.0103 ± 0.0008 |
| Black glutinous rice No.13 | 0.0063 ± 0.0003 | 0.0067 ± 0.0002 | 0.0091 ± 0.0005 |
| Black glutinous rice No.14 | 0.0123 ± 0.0012 | 0.0106 ± 0.0004 | 0.0095 ± 0.0005 |
| Black glutinous rice No.15 | 0.0079 ± 0.0000 | 0.0107 ± 0.0003 | 0.0085 ± 0.0002 |
| Black glutinous rice No.16 | 0.0072 ± 0.0004 | 0.0079 ± 0.0004 | 0.0088 ± 0.0009 |
| Black glutinous rice No.17 | 0.0063 ± 0.0004 | 0.0085 ± 0.0009 | 0.0065 ± 0.0002 |
| Black glutinous rice No.18 | 0.0075 ± 0.0003 | 0.0146 ± 0.0006* | 0.0058 ± 0.0003 |

**Table 4.2** (Continued)

| Seed of Rice | TFC (mg CE $g^{-1}$ of FW) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.19 | 0.0051 ± 0.0002** | 0.0084 ± 0.0001 | 0.0073 ± 0.0004 |
| Thai glutinous rice No.20 | 0.0075 ± 0.0003 | 0.0093 ± 0.0003 | 0.0078 ± 0.0001 |

[a] Each value is the mean of five replications ± standard deviation.

*, ** Means number the maximum and minimum in each column, respectively.

## 4.3 The antioxidant activities

### 4.3.1 DPPH radical scavenging activity

The free-radical scavenging activity of rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars were determined by the DPPH method. The scavenging activity of rice leave extracts and antioxidant standard as expressed by $IC_{50}$ values as shown in Table 4.4. The antioxidant standard, BHA, expressed the highest activity on DPPH radical scavenging with the $IC_{50}$ of 0.0044 mg $mL^{-1}$. The $IC_{50}$ of rice leaves extracts of tillering stage were in the range of 1.3163-3.6153 mg $mL^{-1}$ of FW. In this stage, black glutinous rice No.9 has the highest antioxidant activity, whereas black glutinous rice No.4 showed the lowest. In booting stage, black glutinous rice No.14 showed the highest of antioxidant activity, while black glutinous rice No. 4 was the lowest. For this stage, $IC_{50}$ was arranged between 0.6497-4.0261 mg $mL^{-1}$ of FW. At heading stage, black glutinous rice No.11 showed the highest of antioxidant activity, while black glutinous rice No.9 and No. 13 showed the lowest of antioxidant activity. The distributions of $IC_{50}$ in this stage were arranged from 1.2852-5.0404 mg $mL^{-1}$ of extract.

**Table 4.3** $IC_{50}$ of rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars. [a]

| Seed of Rice | $IC_{50}$ (mg $mL^{-1}$) ± SD | | |
|---|---|---|---|
| | Leaf of Tillering Rice | Leaf of Booting Rice | Leaf of Heading Stage Rice |
| Black glutinous rice No.1 | 1.8733 ± 0.019 | 1.4260 ± 0.019 | 2.3874 ± 0.020 |
| Black glutinous rice No.2 | 2.3825 ± 0.037 | 1.1612 ± 0.029 | 2.0569 ± 0.034 |
| Black glutinous rice No.3 | 3.2909 ± 0.166 | 2.5518 ± 0.053 | 2.2462 ± 0.027 |
| Black glutinous rice No.4 | 3.6153 ± 0.137** | 4.0261 ± 0.212** | 2.7887 ± 0.021 |

**Table 4.3** (Continued)

| Seed of Rice | $IC_{50}$ (mg $mL^{-1}$) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.5 | 2.8106 ± 0.050 | 2.2212 ± 0.033 | 3.1186 ± 0.082 |
| Black glutinous rice No.6 | 2.7614 ± 0.081 | 1.9023 ± 0.089 | 2.3566 ± 0.045 |
| Black glutinous rice No.7 | 2.2390 ± 0.092 | 2.1221 ± 0.039 | 1.8519 ± 0.043 |
| Black glutinous rice No.8 | 1.9800 ± 0.071 | 2.6963 ± 0.054 | 3.2527 ± 0.123 |
| Black glutinous rice No.9 | 1.3163 ± 0.040* | 2.0373 ± 0.019 | 5.0404 ± 0.187** |
| Black glutinous rice No.10 | 2.9849 ± 0.054 | 1.8813 ± 0.060 | 3.7498 ± 0.398 |
| Black glutinous rice No.11 | 1.5045 ± 0.013 | 1.1998 ± 0.020 | 1.2852 ± 0.026* |
| Black glutinous rice No.12 | 2.1953 ± 0.017 | 2.0250 ± 0.037 | 2.8590 ± 0.057 |
| Black glutinous rice No.13 | 2.2436 ± 0.031 | 3.7624 ± 0.162 | 4.9181 ± 0.312** |
| Black glutinous rice No.14 | 1.4265 ± 0.130 | 0.6497 ± 0.012* | 2.2099 ± 0.041 |
| Black glutinous rice No.15 | 3.0040 ± 0.1123 | 1.6157 ± 0.027 | 2.1285 ± 0.024 |
| Black glutinous rice No.16 | 2.3197 ± 0.054 | 1.5390 ± 0.025 | 3.1918 ± 0.062 |
| Black glutinous rice No.17 | 1.8230 ± 0.039 | 1.6720 ± 0.033 | 2.3390 ± 0.051 |

**Table 4.3** (Continued)

| Seed of Rice | $IC_{50}$ (mg $mL^{-1}$) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.18 | 2.4782 ± 0.034 | 1.4017 ± 0.016 | 3.3065 ± 0.050 |
| Black glutinous rice No.19 | 3.0330 ± 0.255 | 2.5093 ± 0.068 | 2.4523 ± 0.102 |
| Thai glutinous rice No.20 | 2.1113 ± 0.112 | 2.2438 ± 0.040 | 3.5088 ± 0.083 |
| BHA | 0.0044 ± 0.001 | | |

[a] Each value is the mean of five replications ± standard deviation.

*, ** Means number the maximum and minimum in each column, respectively.

### 4.3.2 Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars, expressed as millimoles of ferrous sulphate per gram of fresh weight as shown in Table 4.5. The reducing ability of the extracts from tillering stage of rice leaves was arranged from 64.4926-158.1893 mM Fe(II) $g^{-1}$ of FW. In this stage, black glutinous rice No.14 showed the highest ability, while black glutinous rice No.15 showed the lowest. The black glutinous rice No.14 and Thai sticky rice No.20 showed the highest of reducing ability, whereas black glutinous rice No.3, black glutinous rice No.13 and black glutinous rice No.4 were the lowest of booting stage. In this stage, FRAP values were arranged from 61.2801-125.9674 mM Fe(II) $g^{-1}$ of FW. The black glutinous rice No.11 showed the highest activity, while black glutinous rice No.5 was the lowest of heading stage. The distributions of FRAP in this stage were arranged from 61.2801-162.0832 mM Fe(II) $g^{-1}$ of FW.

**Table 4.4** FRAP of rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars. [a]

| Seed of Rice | FRAP (mM Fe(II) $g^{-1}$ of FW) ± SD | | |
|---|---|---|---|
| | Leaf of Tillering Rice | Leaf of Booting Rice | Leaf of Heading Stage Rice |
| Black glutinous rice No.1 | 75.2495 ± 2.691 | 92.1879 ± 2.541 | 97.6880 ± 3.213 |
| Black glutinous rice No.2 | 83.9620 ± 5.062 | 88.9267 ± 1.583 | 128.4497 ± 1.409 |
| Black glutinous rice No.3 | 87.2718 ± 4.992 | 62.1076 ± 3.701** | 92.0905 ± 2.636 |
| Black glutinous rice No.4 | 74.2760 ± 4.966 | 61.2801 ± 5.861** | 72.1343 ± 0.369 |
| Black glutinous rice No.5 | 76.1256 ± 3.901 | 110.4405 ± 4.006 | 61.2801 ± 2.122** |
| Black glutinous rice No.6 | 81.3336 ± 1.476 | 96.4712 ± 2.724 | 102.1660 ± 13.279 |

**Table 4.4** (Continued)

| Seed of Rice | FRAP (mM Fe (II) $g^{-1}$ of FW) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.7 | 91.0197 ± 2.298 | 90.3869 ± 3.148 | 110.4405 ± 0.172 |
| Black glutinous rice No.8 | 74.8114 ± 2.105 | 90.3870 ± 1.583 | 94.5242 ± 1.236 |
| Black glutinous rice No.9 | 86.0063 ± 2.234 | 96.3251 ± 2.801 | 92.2365 ± 0.697 |
| Black glutinous rice No.10 | 66.4395 ± 1.413 | 75.9796 ± 0.967 | 94.0862 ± 0.675 |
| Black glutinous rice No.11 | 101.8739 ± 2.772 | 109.9537 ± 1.686 | 162.0832 ± 1.880* |
| Black glutinous rice No.12 | 112.3874 ± 3.472 | 89.0728 ± 0.867 | 93.1614 ± 3.064 |
| Black glutinous rice No.13 | 78.5593 ± 1.695 | 61.7182 ± 0.555** | 82.1611 ± 1.750 |
| Black glutinous rice No.14 | 158.1893 ± 4.869* | 124.4098 ± 2.319* | 130.5914 ± 3.974 |
| Black glutinous rice No.15 | 64.4926 ± 4.121** | 102.8474 ± 3.418 | 83.7187 ± 2.082 |
| Black glutinous rice No.16 | 84.3027 ± 2.048 | 102.4093 ± 1.947 | 85.7630 ± 3.195 |
| Black glutinous rice No.17 | 85.3736 ± 1.747 | 102.5553 ± 1.200 | 113.0689 ± 1.326 |
| Black glutinous rice No.18 | 78.4132 ± 3.304 | 89.0728 ± 1.024 | 92.8206 ± 3.208 |

**Table 4.4** (Continued)

| Seed of Rice | FRAP (mM Fe(II) $g^{-1}$ of FW) ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.19 | 68.2405 ± 0.818 | 78.2185 ± 2.630 | 94.6702 ± 4.664 |
| Thai glutinous rice No.20 | 80.0195 ± 1.141 | 125.9674 ± 1.258* | 68.8732 ± 2.718 |

[a] Each value is the mean of five replications ± standard deviation.

*, ** Means number the maximum and minimum in each column, respectively.

# CHAPTER 5

# DISCUSSIONS AND CONCLUSIONS

## Discussions

Phytochemical, the secondary metabolites of plants play an important role in human health. There are many phytochemicals and each works differently. Some possible actions of phytochemicals are phenolic compounds, anthocyanins, carotenoids, flavonoids etc. Plant phenolic compounds are the most groups and diverse in structure but will consist of a hydroxyl group (-OH) directly bonded to an aromatic hydrocarbon group. These compounds have been reported for health-related effects such as anti-bacterial (Ezoubeiri *et al.,* 2005), anti-mutagenic (Pedreschi and Cisneros-Zevallos, 2007), anti-carcinogenic (Kähkönen and Heinonen, 2003), anti-inflammatory (Elangovan *et al.,* 1994), cardiovascular diseases and certain cancers (Liu, 2004, 2007; Dykes and Rooney, 2007). In this work, rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars indicated phenolic contents in the range from 2.7236-5.9113 mg GAE $g^{-1}$ FW. These values are higher than those phenolic found in pigmented rice grown in Southern Thailand (Yodmanee *et al.,* 2011), unpolished Thai rice strain of Leum Phua (Suwannalert and Rattanachitthawat, 2011), Thai white, red and black rice bran (Muntana and Prasong, 2010), unpolished Thai rice from the Organic Project Sukhothai Airport (Rattanachitthawat *et al.,* 2010), black glutinous rice Kam Doi Saked (Tananuwong and Tewaruth, 2010) or hempseed powder (Norajit *et al.,* 2011). This result suggested that the phenolic contents are varied depending on many factors such as rice cultivars, environments, other agricultural practices and also analyzed methods.

Flavonoids have been recognized as antioxidant and found to be impact on human nutrition and health (Pourmorad *et al.,* 2006). They are water soluble polyphenolic molecules containing 15 carbon atoms and secondary phenolic metabolites naturally present in plants. The basic structure of flavonoid allows a multitude of substitution patterns in the benzene rings: phenolic hydroxyls, *O*-sugars, methoxy groups, sulfates and glucuronides (Hollman and Katan, 1999). The total flavonoid

contents in the leaves of 20 Thai glutinous rice cultivars were arranged from 0.0051-0.0188 mg CE $g^{-1}$ FW. These values are higher than those of total flavonoid found in Korean pigmented rice cultivars (Kim *et al.*, 2010).

Antioxidants are vital substances which process the ability to protect the body from damage caused by free radical induced oxidative stress. These are an increasing interest in natural antioxidants, etc., polyphenols. In this study, the crude extracts obtained from solvent extraction were used for determination of relative antioxidant activities in 2 tests (DPPH radical scavenging assay and FRAP assay). 2,2-diphenyl-1-picrylhydrazyl or DPPH is a relatively stable free radical, has been widely used to examine the free radical-scavenging ability of tested samples (Bozin *et al.*, 2008). The concept of assay this in the color changed of DPPH solution from purple to yellow (Karagözler *et al.*, 2008; Jothy *et al.*, 2011). On the other hand, ferric reducing antioxidant power (FRAP) was also used for antioxidant analysis. The FRAP assay is simple, fast, and reproducible (Wong et al., 2006). The genetic diversity in antioxidant levels of rice in different growth stages was rarely reported. Changlian and co-workers (2006) reported the antioxidant activity Yunnan purple rice and Chijiaoruanzhan green rice indicated that purple rice had higher antioxidant activity than green rice. Daiponmak and co-workers (2010) reported the antioxidant activity of rice varieties under salinity stress by two classes of Thai rice lines: cyanic (Riceberry, Kham, and Khamdoisaket) and acyanic (KDML 105, Sinlek, and BC2F7#62-56) which $IC_{50}$ values was expressed as mM Trolox equivalents per gram of fresh weight. Comparison to these of antioxidant activity of other plant extracts, the leaves rice extracts showed higher their antioxidant activity than other plant. This might be concluded that the leaves rice should be used as high potential source of phytochemicals.

**Conclusions**

The study reported the investigation on the total phenolic, flavonoid, anthocyanin and antioxidant activity of extract from leave 20 cultivars Thai glutinous rice in different growth stage; tillering, booting and heading. The TPC of extract was analyzed by Folin-Ciocalteu method and TFC was analyzed by colorimetric aluminum chloride method. The antioxidant activities of leave rice extract were evaluated for

using the following test: 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing antioxidant power (FRAP) assay. The results indicated that TPC was 2.7236-5.9113 mg GAE $g^{-1}$ of FW, TFC was 0.0051-0.0188 mg CE $g^{-1}$ of FW, radical scavenging activity was 0.6497-5.0404 mg of FW and ferric reducing antioxidant power was 61.2801-162.0832 mM Fe(II) $g^{-1}$ of FW. All of the Thai glutinous leaves rice in different growth stage (tillering, booting and heading) showed high total phenolic, flavonoid, anthocyanin and antioxidant activity. It is promising that the extracts of Thai glutinous rice leave might be applied as potential substances in functional foods for good health benefits.

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# APPENDICES

# Appendix A
# Preparation of solutions

**1. Preparation of reagents for total phenolic content**

1.1 Preparation of 10% Folin-Ciocalteu reagent

A 10% Folin-Ciocalteu reagent was prepared by dilute 10 mL of Folin-Ciocalteu reagent in 900 mL of deionized water.

1.2 Preparation of 10% Sodium carbonate

A 10% sodium carbonate solution was prepared by dissolving 10.0502 g of 99.5% $Na_2CO_3$ in 100 mL of deionized water.

1.3 Preparation of Stock standard (2 mg $mL^{-1}$) gallic acid

Stock standard solution (2 mg $mL^{-1}$) of gallic acid was prepared by dissolving 0.0510 g of 98% gallic acid and made up to volume with deionized water in 25 mL volumetric flask.

**2. Preparation of reagents for total flavonoid content**

2.1 Preparation of 5% Sodium nitrite

A 5% sodium nitrite solution was prepared by dissolving 1.2886 g of 97% $NaNO_2$ in 25 mL of deionized water.

2.2 Preparation of 10% Aluminium chloride

A 10% aluminium chloride solution was prepared by dissolving 5.0505 g of 99% $AlCl_3$ in 50 mL of 50% methanol.

2.3 Preparation of Stock standard (2 mg $ml^{-1}$) (±)-catechin

Stock standard solution (2 mg $mL^{-1}$) of (±)-catechin was prepared by dissolving 0.050 g of (±)-catechin and made up to volume with methanol in 25 mL volumetric flask.

**3. Preparation of reagents for Free- radical scavenging activity (DPPH) assay**

3.1 Preparation of 0.1 mM DPPH (MW= 394.33)

A 0.1 mM DPPH was prepared by dissolving 0.0232 g of 85% DPPH in 500 mL and made up to volume with methanol in 500 mL volumetric flask.

3.2 Preparation of Stock standard 2 mg $ml^{-1}$ BHA

Stock standard solution (2 mg $mL^{-1}$) of BHA was prepared by dissolving 0.0521 g of 96% BHA and made up to volume with methanol in 25 mL volumetric flask.

**4. Preparation of reagents for Ferric reducing antioxidant power (FRAP) assay**

4.1 Preparation of 300 mM Sodium acetate buffer, pH 3.6

A 0.025 M sodium acetate buffer (pH 3.6) solution was prepared by dissolving 24.624 g of $CH_3COONa{\cdot}3H_2O$ in 500 mL of deionized water. The pH value of 0.3 M of the solution was adjusted using $CH_3COOH$ and made up to volume with deionized water in a 1000 mL volumetric flask.

4.2 Preparation of 10 mM TPTZ (MW= 312.32)

A 10 mM TPTZ solution was prepared by dissolving 0.0789 g of 99% TPTZ in 25 mL and made up to volume with 40 mM HCl in 25 mL volumetric flask.

4.3 Preparation of 20 mM Ferric chloride (MW= 162.21)

A 20 mM ferric chloride solution was prepared by dissolving 0.1655 g of 98% $FeCl_3$ in 50 mL and made up to volume with deionized water in 50 mL volumetric flask.

4.4 Preparation of 40 mM Hydrochloric acid (MW= 36.441; 37%; d= 1.19)

A 40 mM hydrochloric acid was prepared by dilute 3.30 mL of 37% HCl in 1000 mL and made up to volume with deionized water in 1000 mL volumetric flask.

4.5 Preparation of 10 mM Ferrous sulphate

Stock standard solution of 10 mM $FeSO_4$ was prepared by dissolving 0.0140 g of 99% $FeSO_4{\cdot}7H_2O$ in 5 mL and made up to volume with methanol in 5 mL volumetric flask.

# Appendix B

## Calibration curves of standard and example of methanolic extract in DPPH assay

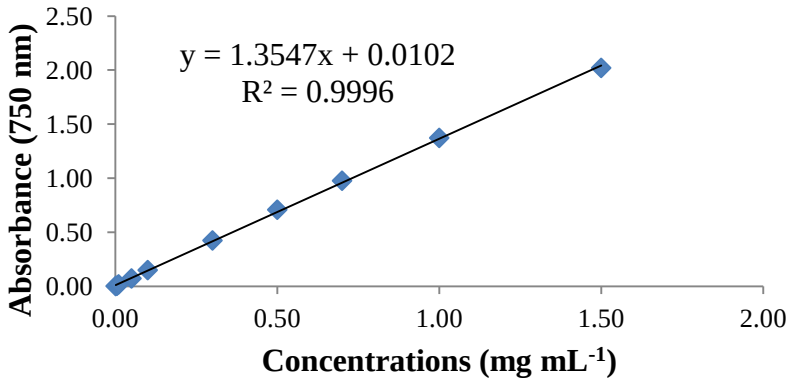


**Figure B1** Calibration curve of standard gallic acid.

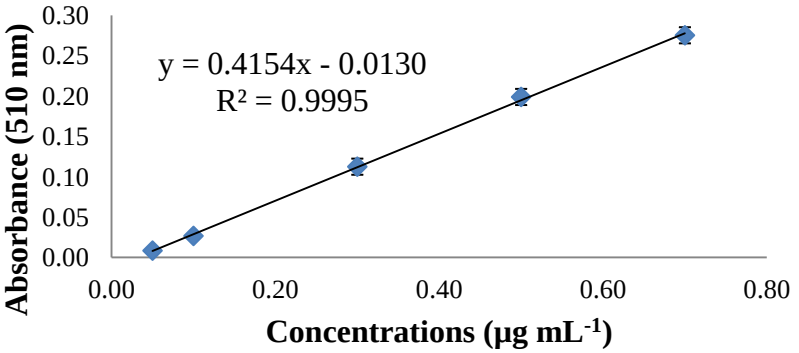


**Figure B2** Calibration curve of standard (±)-catechin.

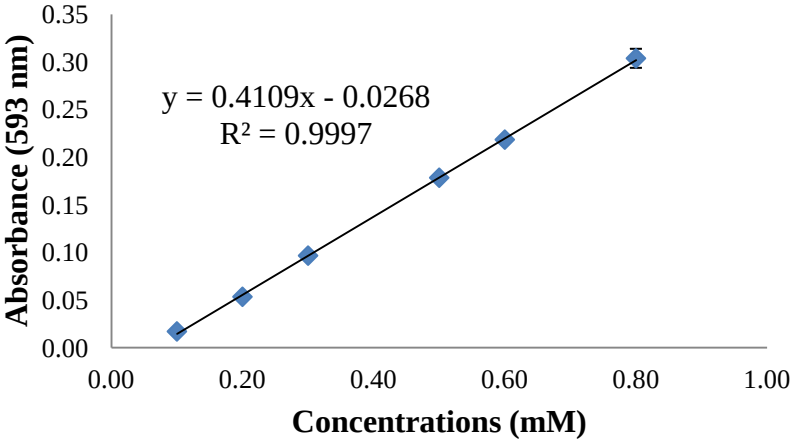


**Figure B3** Calibration curve of standard ferrous sulphate.

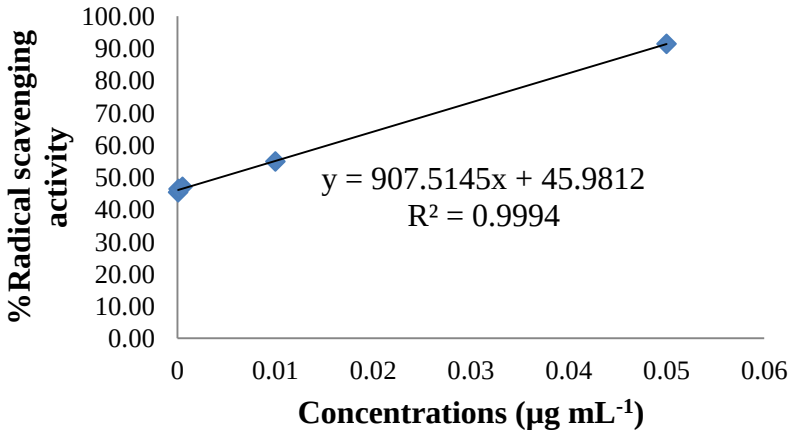


**Figure B4** Calibration curve of standard BHA.

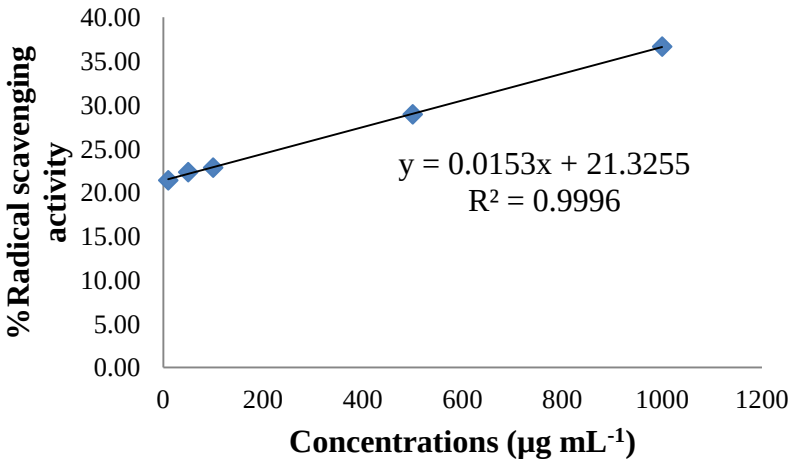


**Figure B5** Calibration curve of rice leaves methanolic extract of Thai black glutinous rice No.1 in tillering stage rice in DPPH assay.

# Appendix C

## The correlation coefficient of calibration curves in DPPH assay

**Table C** The correlation coefficient ($r^2$) of calibration curves in rice leave extracts in different growth stages of 20 Thai glutinous rice cultivars and BHA from DPPH assay. [a]

| Seed of Rice | $r^2$ ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.1 | 0.9991 ± 0.0005 | 0.9992 ± 0.0004 | 0.9745 ± 0.0040 |
| Black glutinous rice No.2 | 0.9935 ± 0.0023 | 0.9980 ± 0.0015 | 0.9992 ± 0.0004 |
| Black glutinous rice No.3 | 0.9946 ± 0.0024 | 0.9972 ± 0.0016 | 0.9994 ± 0.0004 |
| Black glutinous rice No.4 | 0.9891 ± 0.0113 | 0.9929 ± 0.0043 | 0.9986 ± 0.0004 |
| Black glutinous rice No.5 | 0.9950 ± 0.0056 | 0.9994 ± 0.0003 | 0.9959 ± 0.0030 |
| Black glutinous rice No.6 | 0.9969 ± 0.0019 | 0.9749 ± 0.0413 | 0.9985 ± 0.0005 |
| Black glutinous rice No.7 | 0.9981 ± 0.0007 | 0.9983 ± 0.0022 | 0.9933 ± 0.0029 |
| Black glutinous rice No.8 | 0.9850 ± 0.0171 | 0.9824 ± 0.0249 | 0.9731 ± 0.0229 |
| Black glutinous rice No.9 | 0.9971 ± 0.0019 | 0.9989 ± 0.0008 | 0.9622 ± 0.0129 |
| Black glutinous rice No.10 | 0.9937 ± 0.0021 | 0.9956 ± 0.0015 | 0.9145 ± 0.1329 |
| Black glutinous rice No.11 | 0.9989 ± 0.0010 | 0.9989 ± 0.0005 | 0.9990 ± 0.0004 |
| Black glutinous rice No.12 | 0.9971 ± 0.0016 | 0.9989 ± 0.0008 | 0.9981 ± 0.0011 |
| Black glutinous rice No.13 | 0.9988 ± 0.0009 | 0.9947 ± 0.0040 | 0.9954 ± 0.0050 |
| Black glutinous rice No.14 | 0.9933 ± 0.0061 | 0.9994 ± 0.0004 | 0.9965 ± 0.0013 |
| Black glutinous rice No.15 | 0.9945 ± 0.0039 | 0.9992 ± 0.0004 | 0.9981 ± 0.0013 |
| Black glutinous rice No.16 | 0.9981 ± 0.0011 | 0.9994 ± 0.0001 | 0.9929 ± 0.0014 |
| Black glutinous rice No.17 | 0.9976 ± 0.0010 | 0.9981 ± 0.0007 | 0.9990 ± 0.0008 |
| Black glutinous rice No.18 | 0.9915 ± 0.0050 | 0.9993 ± 0.0003 | 0.9985 ± 0.0010 |

**Table C** (Continued)

| Seed of Rice | $r^2$ ± SD | | |
|---|---|---|---|
| | **Leaf of Tillering Rice** | **Leaf of Booting Rice** | **Leaf of Heading Stage Rice** |
| Black glutinous rice No.19 | 0.8937 ± 0.0453 | 0.9973 ± 0.0021 | 0.9932 ± 0.0019 |
| Thai sticky rice No.20 (ck6) | 0.9880 ± 0.0123 | 0.9977 ± 0.0015 | 0.9891 ± 0.0028 |
| BHA | 0.9991 ± 0.0005 | | |

[a] Each value is the mean of five replications ± standard deviation.

# Appendix D

## Pictures of sample


No. 1 Niaw Dam (Ton Sung)-1


No. 2 Niaw Dam (Ton Sung)-2


No. 3 Khao' Kam No. 7


No. 4 Niaw Dam (Entry 1)


No. 5 Niaw Dam (Entry 4)


No. 6 Niaw Dam (Entry 6)


No. 7 Niaw Dam (Entry 8)


No. 8 Niaw Dam (Entry 9)


No. 9 Niaw Dam (Entry 10)


No. 10 Niaw Dam (Entry 11)


No. 11 Niaw Dam (Entry 12)


No. 12 Niaw Dam (Entry 12)


No. 13 Niaw Dam Noi (Entry 16)


No. 14 Niaw Dam Tap Mu (Entry 17)


No. 15 Niaw Dam (Entry 18)


No. 16 Niaw Dam (Entry 22)


No. 17 Niaw Dam (Entry 23)


No. 18 Khao Klam (Entry 28)


No.19 Khao Klam (Entry 29)


No. 20 Kor Khor 6 (ck6)

# Appendix E

# Research output

# PERCH-CIC

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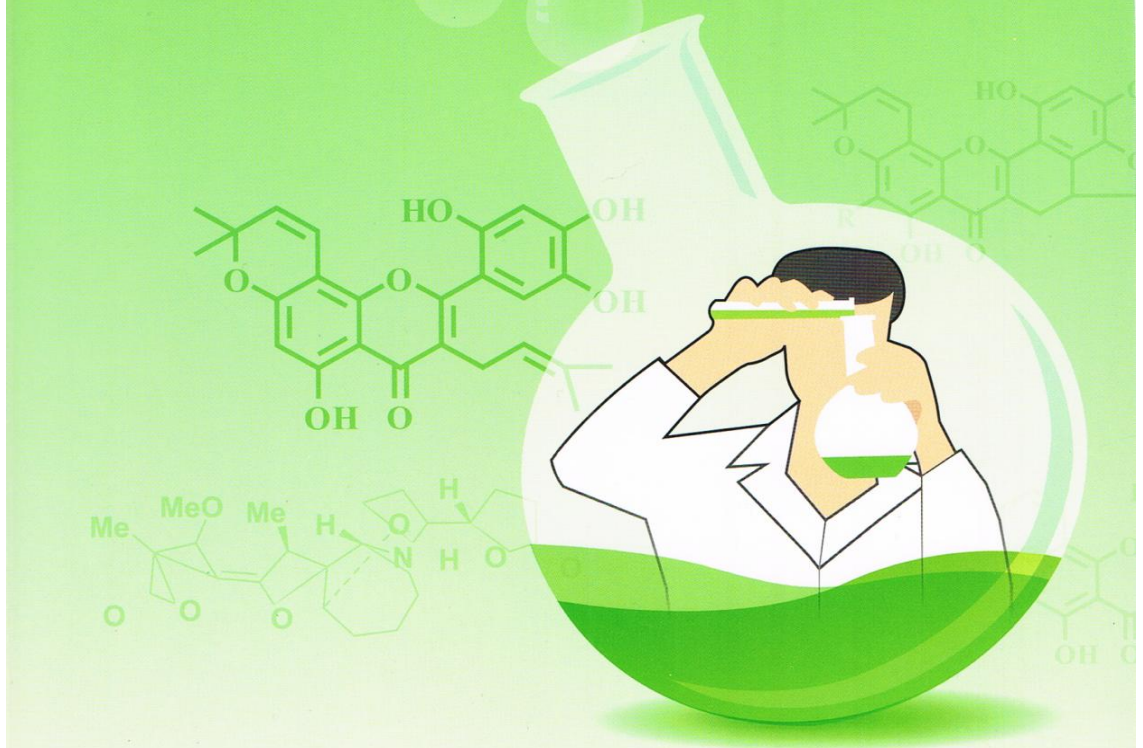

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# The total phenolic contents and their antioxidant activity from leaves in different growth stage of Thai glutinous rice cultivars

**Jiraporn Krasaetep[a,b], Muntana Nakornriab[a,b] and Darunee Puangpronpitag[a,c]**

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## Introduction and Objective

In Thailand, black rice is the send most common rice and grown in the Northeastern and Northern parts of country. The types of black rice include non-glutinous and glutinous rice. In contain high amounts of protein, phytofats, cellulose, minerals and pigmented. It is rich in anthocyanins, which are important to suppress oxidation in the body. The aims of this research work to investigate the total phenolic contents and antioxidand activity of leaves in different growth stage of Thai black glutinous rice cultivars.

## Methods

The total phenolic content (TPC) of the extracts was analyzed by Folin-Ciocalteau method (Bonli et al., 2004) and antioxidant activity was analyzed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity (Chan et al., 2007 and Lin & Yao, 2007).

## Results

The TPC was expressed as gallic acid equivalents and $EC_{50}$ as determined by DPPH assay from leaf rice extracts was shown in Table 1. The results indicated that the TPC was in the range of 3.67-4.89, 2.65-3.71 and 3.10-4.16 mg GAE/ g from the leaf of tillering, booting and heading stage rice extracts, respectively. The higher TPC levels have been detected in Black glutinous rice (No.2) from the leaf of rice extracts, and the lower in Black glutinous rice (No.13) from the leaf of rice extracts. For the DPPH radical concentration by 50%, the leaf of booting rice from Black glutinous rice (No.2) was the highest antioxidant activity ($EC_{50}$ = 1.77 mg/ml) while the leaf of heading stage from Black glutinous rice (No.13) was the lowest antioxidant activity ($EC_{50}$ = 13.44 mg/ml).

## Conclusions

From these results it can be concluded that, the leaves in different growth stage of Thai sticky rice cultivars such as tillering rice, booting rice, and heading stage rice, Black glutinous rice (No.2) from the leaf of rice extracts showed the high TPC and antioxidant activity.

**Keywords:** total phenolic content, antioxidant activity, Thai glutinous rice

## Selected References:

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## e-ABSTRACT

DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE, CHIANG MAI UNIVERSITY
& THE CHEMICAL SOCIETY OF THAILAND UNDER THE PATRONAGE OF
HER ROYAL HIGHNESS PRINCESS CHULABHORN MAHIDOL

**FAS-P-074**

# TOTAL PHENOLICS, FLAVONOIDS CONTENTS AND ANTIOXIDANT ACTIVITY FROM LEAVES IN DIFFERENT GROWTH STAGE OF THAI GLUTINOUS RICE CULTIVARS

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## ABSTRACT

To investigate the total phenolics and flavonoids contents in leaves of rice in different growth stages (tillering, booting and heading) from 7 Thai glutinous rice cultivars. The total phenolics and flavonoids contents of the extracts were analyzed by Folin-Ciocalteau method and colorimetric aluminum chloride method respectively. The antioxidant activities were measured by using DPPH radical and ferric reducing antioxidant power assay. Heading stage rice showed highest antioxidant activities in both assays. The highest total phenolics and flavonoids contents were found in tillering stage. In conclusion, Thai Black glutinous rice cultivars are strong antioxidants activities. These findings suggest that tillering stage of leaves in black rice should be useful in food industry or health products.

**Keywords** Antioxidant activity; Rice; Flavonoid; Phenolic

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# Antioxidant Activity and Total Phenolic Contents in Leaf of some Thai Rice Cultivars

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## Abstract

The methanolic extracts in each growth stage (tillering, panicle initiation, booting, milking, and maturation stage) from 14 kinds of Thai leaf rice cultivars (white, red, and black rice) are studied for their antioxidative activities and total phenolic contents. The total phenolic content of these extracts was analyzed by Folin-Ciocalteau method, and antioxidative activity was assessed by the 1,1-diphenyl-2-picrylhydrasyl (DPPH) free radical-scavenging activity, the inhibition of lipid peroxidation and metal chelating ability. The results revealed the highest total phenolic content in milking stage of methanolic extract from all white, red, and black rice (0.709–0.743, 0.948-0.991, and 1.061–1.132 mg GAE/mg of extract, respectively). Furthermore, milking stage of methanolic extracts also showed the greatest capability in antioxidant activity which black rice (*Entry 18*) indicated the highest potential in scavenging DPPH radicals and calculated in terms of $IC_{50}$ ($IC_{50}$ = 0.048 mg/mL) and lowest in white rice (*Jasmine 105*) ($IC_{50}$ = 0.556 mg/mL). In contrast, metal chelating ability was found in all stage of Thai rice cultivars (white, red, and black rice) in the range of 0.02-0.08 and 0.03-0.19 mg/ml, respectively. These findings suggest that milking stage of black rice should be developed further to be useful in food industry or health products.

**Keywords:** Thai leaf rice, DPPH, lipid peroxidation, metal chelating, phenolic contents

## Introduction

Rice (Oryza sativa L.) is one of the important foods of the world. There are different types of rice that contain color pigments, such as purple rice, black rice and red rice. Therefore the pigmented rice is some varieties of rice that have a color on the palea, lemma and another inside part such as pericarp tegmen and aleurone layer. It contains phytochemicals that are responsible for their colors. Generally, these colored compounds or pigments fall into a number of large groups such as chlorophylls, riboflavin, carotenoids, flavonoids and quinones. Most of these pigments are reported to from in plant for vital functions, which could benefit human health in two meaningful ways. Their important bioactivities include free-radical scavenging (Chiang *et al*., 2006), enhancement of the immune system (Choi *et al*., 2007), heart disease, cardiovascular disease, glycemic control, diabetes, and cancer prevention (Chen *et al*., 2006: Wang *et al*., 2006: Toyokuni *et al*., 2002). Pigmented rice is, thus, anticipated the greater functional dietary potential than that of the white rice (Nam *et al*., 2005). Many studies have been reported that black rice contains rich of anthocyanin and other polyphenolic compound much more abundantly than white rice (Ryu *et al*., 1998: Zhang *et al*., 2006). In addition, a significant positive correlation between in the black rice extract and their antioxidant activity was obtained (Hu *et al*., 2003: Ling *et al*., 2001).

Recently, Thai pigmented rice varieties have been growing popular and are demanding higher prices in the Asian rice market. Thus, this research aimed to study the antioxidant activity and total phenolic contents in each growth stage (tillering, panicle initiation, booting, milking, and maturation stage) from Thai leave rice cultivars extracts using a number of methods with different mechanisms. The total phenolic content was measured according to the Folin–Ciocalteu method using gallic acid as a standard. The total antioxidant activity was determined by means of the ferric thiocyanate method which is the measurement of the inhibition of linoleic acid peroxidation and metal ion chelating. To measured the radical and reactive oxygen scavenging capacity, a DPPH assay and the finally the chelating activity againt $Fe^{2+}$ was examined.

## Materials and methods

**Plant Materials.** Whole grains of 14 cultivars of Thai black rice, red rice and white rice were used. These were white rice (Jasmine 105, Goh-koh 6), red rice (SRN2007.No.2, SRN2007.NO.3, SRN2007.NO.4, SRN2007.NO.5), and black rice (Entry 1, Entry 2, Entry 8, Entry 11, Entry 20, Entry 30, SRN2007.NO.8, SRN2007.NO.20). All the rice was obtain from the Surin Rice Research Center, Surin province, Thailand, on 2008. The whole grains of 14 rice cultivars had been growth in Mahasarakham University, Mahasarakham province, Thailand, on 2009.

**Reagents and Chemicals.** All reagents include Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl, gallic acid, sodium carbonate, vitamin E, butylated hydroxyanisole, EDTA and all solvents (HPLC grade) were obtained from Fluka (Switzerland).

**Extraction of crude antioxidants.** The leaf of Thai rice cultivars (white, red, and black rice) in each growth stage (tillering, panicle initiation, booting, milking, and maturation stage) (25.0 g) were extracted with methanol (3×100 mL) for 30 min with intermittent shaking at room temperature. The extracts were combined and filtered through a 0.45 μm Nylon membrane filter. After which, the extracts were then slowly concentrated under reduced pressure, at temperature below 40 °C, on a rotary evaporator to yield the crude extracts. The crude samples were used for the determination of antioxidant activity.

**Total phenolic compounds.** The total phenolic contents of leaf in each growth stage from varieties of Thai rice were determined by spectrophotometric method using Folin–Ciocalteu's phenol reagent (Bonoli *et al*., 2004).The crude extracts in methanol (0.5 mL) were placed in a test tube and was diluted to 5.0 mL with a glass of distilled water. Folin–Ciocalteu's phenol reagent (5.0 mL) was added, and the contents of thetest tube were mixed thoroughly. After 3.0 min, 5 mL of 10% sodium carbonate solution was added, and the mixture was allowed to stand for 1 h with intermittent shaking. The absorbance of the blue color was measured in a Lamda 25 UV-VIS spectrophotometer (Thermo Spectronic 4001/4 Spectrophotometer, USA) at 750 nm. The concentration of total phenolic compounds was determined using the gallic acid equation (mg GAE/g of extract) obtained from the standard gallic acid calibration curve. This experiment was carried out three times, and the results were averaged for the different fractions in the leaf of Thai rice cultivars in each growth stage.

Antioxidative assay by the thiocyanate method. Antioxidative activity of leaf in each growth stage from varieties of Thai rice was carried out by using the linoleic acid system (Tsuda *et al*., 1993). In a well-stopped Erlenmeyer flask containing linoleic acid (0.13 mL) in a 0.2 M NaOH-phosphate buffer (10 mL, pH 7), the crude extracts in methanol (1 mg) from the different fractions of the leaf of Thai rice cultivars in each growth stage were added, and the volume increased to 25 mL with a glass of distilled water. The flasks were incubated at 40 °C for a two-week period, and the degree of oxidative was measured according to the thiocyanate method. The incubation mixture (0.2 mL) was reacted with $NH_4SCN$ (30%, 0.2 mL), 9.4 mL of 75% EtOH, and 0.2 mL of $FeCl_2$ ($2.53\times10^{-2}$ g/ 10 mL 3.5 % HCl) solution. The absorbance of the blue color (peroxide value) was measured in a Lamda 25 UV-VIS spectrophotometer (PerkinElmer, USA) at 500 nm. The control solution was prepared in a similar manner without the addition of any antioxidant, while α-tocopherol and butylated hydroxyanisole (BHA) at 200 μg per flask was used as a standard for comparison. This experiment was performed three times, and the results were averaged for the different fractions in the leaf of Thai rice cultivars in each growth stage. The percentage of inhibition of lipid peroxidation was calculated using the following equation:

$$\text{Inhibition (\%)} = [(A_c - A_s)/A_c] \times 100,$$

where $A_c$ is the absorbance of the control solution and $A_s$ is the absorbance in the presence of the leaf of Thai rice cultivars in each growth stage extracts

**DPPH free radical-scavenging activity.** The radical scavenging activity of leaf in each growth stage from varieties of Thai rice was measured using the method of Chan *et al*., 2007 and Liu *et al*., 2007. The crude extracts in methanol and α-tocopherol (5-40 mg/mL) were added to 1.5 mL of 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in ethanol. The mixture was shaken vigorously and was left to stand for 20 min at room temperature in the dark. The absorbance was measured in a Lamda 25 UV-VIS spectrophotometer (PerkinElmer, USA) at 517 nm. The control reaction contained all reagents except for the crude extracts.

The radical scavenging effect was calculated by the following equation:

$$\text{Scavenging effect (\%)} = [(A_c - A_s)/A_c] \times 100,$$

where $A_c$ is the absorbance of the control at 517 nm, and $A_s$ is the absorbance of the extract/standard at 517 nm. This experiment was repeated thrice, and the results were averaged for the different fractions in the leaf of Thai rice cultivars in each growth stage.

**Metal ion chelating activity.** The chelating of ferrous ion was measured using the method of Dinis *et al*., 1994. The crude extracts in methanol (5-25 mg/mL) were reacted with 0.05 mL of 2.0 mM $FeCl_2$. The mixture was then added with 0.2 mL of 5.0 mM ferrozine. After which, the reaction was shaken and incubated at room temperature for 10 min. The absorbance of the red color was measured in a Lamda 25 UV-VIS spectrophotometer (PerkinElmer, USA) at 562 nm. This experiment was carried out three times, and the results were averaged for the different fractions in the leaf in each growth stage from varieties of Thai rice. The percentage of metal chelating activity was calculated by the following equation:

$$\text{\% Metal chelating activity} = [(A_c - A_s)/A_c] \times 100,$$

where $A_c$ is the absorbance of the control at 562 nm, and $A_s$ is the absorbance of the extract/standard at 562 nm. This method was performed three times, and the results were averaged for the different fractions in leaf in each growth stage from varieties of Thai rice. EDTA was used as a positive control.

## Results

**Total phenolic contents.** The total phenolic content was measured by the Folin-Ciocalteu reagent method using gallic acid as the standard. A linear calibration curve of gallic acid resulted with a correlation coefficient of $R^2$ = 0.9990 over the concentration range 20-120 µg/mL. This linear equation was used to determine the total phenolic compounds in leaf in each growth stage from varieties of Thai rice. The total phenolic contents of leaf extracts from 14 varieties of Thai rice, was performed. Leaves from five growth stages of the 14 rice varieties were collected, which were in tillering, panicle initiation, booting, milking, and maturation stage. The average quantity of the total phenolic compounds found in crude extracts is shown in Table 1. The amount of total phenolics content of leaf in each growth stage from varieties of Thai rice extract were in the range of 0.27-2.24 mg GAE/mg of extract. The results

revealed the highest total phenolic content in milking stage of methanolic extract from all white, red, and black rice (0.71–0.75, 0.91-1.02, and 1.55–2.24 mg GAE/mg of extract, respectively). The order of total phenolic content of leaf in each growth stage from varieties of Thai rice extract is milking, booting, panicle initiation, tillering, and maturation stage, respectively.

**Table 1:** Total phenolic content in each growth stage from Thai rice leaves cultivars.

| Pigment of rice | Rice cultivars | Total phenol content ± SD (mg GAE/g of extract) | | | | |
|---|---|---|---|---|---|---|
| | | Tillering stage | Panicle initiation stage | Booting stage | Milking stage | Maturation stage |
| White | Jasmine 105 | 0.51±0.04 | 0.57 ± 0.09 | 0.69±0.08 | 0.71±0.09 | 0.35± 0.06 |
| | Goh-koh 6 | 0.65±0.05 | 0.52 ± 0.10 | 0.61±0.04 | 0.75±0.05 | 0.36± 0.04 |
| Red | SRN2007.No.2 | 0.74±0.12 | 0.89 ± 0.05 | 0.91±0.06 | 0.99±0.06 | 0.28±0.06 |
| | SRN2007.No.3 | 0.56±0.04 | 0.70± 0.05 | 0.78±0.10 | 0.91±0.06 | 0.27± 0.06 |
| | SRN2007.No.4 | 0.55±0.04 | 0.61 ± 0.06 | 0.77±0.07 | 0.95±0.08 | 0.34± 0.04 |
| | SRN2007.No.5 | 0.79±0.05 | 0.85 ± 0.07 | 0.91±0.07 | 1.02±0.05 | 0.27±0.05 |
| Black | Entry 1 | 1.24±0.04 | 1.44± 0.11 | 1.93±0.04 | 2.24±0.09 | 0.33 ±0.10 |
| | Entry 2 | 0.93±0.07 | 1.06 ± 0.09 | 1.25±0.10 | 1.55±0.07 | 0.34± 0.05 |
| | Entry 8 | 1.15±0.07 | 1.02 ± 0.04 | 1.32±0.10 | 1.66±0.10 | 0.36± 0.04 |
| | Entry 11 | 1.19±0.10 | 1.34± 0.05 | 1.57±0.04 | 1.91±0.03 | 0.41±0.06 |
| | Entry 20 | 1.08±0.09 | 1.17± 0.06 | 1.31±0.06 | 1.57±0.04 | 0.39± 0.06 |
| | Entry 30 | 0.98±0.03 | 1.24± 0.04 | 1.60±0.05 | 1.76±0.05 | 0.31± 0.04 |
| | SRN2007.No.8 | 1.32±0.09 | 1.58± 0.05 | 1.71±0.06 | 1.84±0.05 | 0.44± 0.07 |
| | SRN2007.No.20 | 1.59±0.08 | 1.61± 0.04 | 1.86±0.05 | 1.98±0.04 | 0.55± 0.04 |

**Antioxidative assay by the thiocyanate method.** Antioxidative assay of leaf in each growth stage from varieties of Thai rice extracts using the thiocyanate method. The results of antioxidative assays by the thiocyanate method carried out with the crude methanol extracts of the leaf rice compared with that of $\alpha$-tocopherol and butylated hydroxyanisole (BHA) are shown in Table 2. All of the leaf in each growth stage from pigmented Thai rice show strong antioxidant activities which are higher than $\alpha$-tocopherol but lower than BHA. The order of the inhibiting percentage is BHA (85%) > black > red > white in each growth stage from varieties of Thai rice. All the leaf in each growth stage from Jasmine 105 extracts exhibited the lowest inhibition of peroxidation, whereas all the leaf in each growth stage from Entry 1 extracts showed the highest inhibition of peroxidation. The order of antioxidative activities of leaf in each growth stage from varieties of Thai rice extract is milking, booting, panicle initiation, tillering, and maturation stage, respectively.

**Table 2:** Inhibition of lipid peroxidation (%) in each growth stage from Thai rice cultivars

| Pigment of rice | Rice cultivars | Tillering stage | Panicle initiation stage | Booting stage | Milking stage | Maturation stage |
|---|---|---|---|---|---|---|
| White | Jasmine 105 | 35 | 40 | 44 | 47 | 23 |
| | Goh-koh 6 | 35 | 41 | 46 | 50 | 23 |
| Red | SRN2007.No.2 | 38 | 43 | 51 | 60 | 19 |
| | SRN2007.No.3 | 37 | 41 | 50 | 61 | 20 |
| | SRN2007.No.4 | 36 | 42 | 54 | 61 | 20 |
| | SRN2007.No.5 | 37 | 44 | 54 | 62 | 18 |
| Black | Entry 1 | 45 | 53 | 67 | 75 | 12 |
| | Entry 2 | 42 | 55 | 65 | 72 | 17 |
| | Entry 8 | 46 | 53 | 65 | 70 | 19 |
| | Entry 11 | 43 | 51 | 62 | 69 | 17 |
| | Entry 20 | 44 | 52 | 65 | 70 | 15 |
| | Entry 30 | 45 | 50 | 65 | 71 | 17 |
| | SRN2007.No.8 | 44 | 52 | 64 | 69 | 16 |
| | SRN2007.No.20 | 43 | 58 | 67 | 73 | 14 |
| Standard | BHA | 55 | | | | |
| | α-tocopherol | 85 | | | | |

**DPPH scavenging activity.** In the DPPH radical-scavenging assay, Table 3 shows the concentrations of Butylated hydroxyanisole (BHA) and the leaf in each growth stage from varieties of Thai rice extract, at which the DPPH radicals were scavenged by 50 % ($IC_{50}$), the lower the $IC_{50}$, the higher the antioxidant activity. Furthermore, milking stage of methanolic extract also showed the greatest capability in antioxidant activity which black rice (*Entry 1*) indicated the highest potential in scavenging DPPH radicals ($IC_{50}$ = 0.09 mg/mL) and lowest in white rice (*Jasmine 105*) ($IC_{50}$ = 0.28 mg/mL) (Table 2).

**Table 3:** Antioxidant activity by DPPH assay in each growth stage from Thai rice cultivars

| Pigment of rice | Rice cultivars | $IC_{50}$ | | | | |
|---|---|---|---|---|---|---|
| | | Tillering stage | Panicle initiation stage | Booting stage | Milking stage | Maturation stage |
| White | Jasmine 105 | 0.55±0.14 | 0.39±0.14 | 0.30±0.15 | 0.28±0.16 | 3.18±0.15 |
| | Goh-koh 6 | 0.54±0.13 | 0.32±0.15 | 0.29±0.14 | 0.23±0.15 | 2.94±0.14 |
| Red | SRN2007.No.2 | 0.48±0.14 | 0.38±0.14 | 0.28±0.14 | 0.20±015 | 2.97±0.14 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | SRN2007.No.3 | 0.45±0.14 | 0.34±0.15 | 0.31±0.14 | 0.20±0.15 | 2.30±0.15 |
| | SRN2007.No.4 | 0.51±0.13 | 0.36±0.14 | 0.28±0.14 | 0.18±0.15 | 2.75±0.14 |
| | SRN2007.No.5 | 0.44±0.15 | 0.36±0.15 | 0.30±0.14 | 0.18±0.14 | 2.88±0.14 |
| Black | Entry 1 | 0.38±0.14 | 0.27±0.15 | 0.21±0.13 | 0.09±0.12 | 2.53±0.14 |
| | Entry 2 | 0.39±0.15 | 0.28±0.16 | 0.23±0.15 | 0.12±0.15 | 2.45±0.14 |
| | Entry 8 | 0.40±0.15 | 0.29±0.13 | 0.21±0.14 | 0.14±0.14 | 2.84±0.15 |
| | Entry 11 | 0.42±0.14 | 0.30±0.14 | 0.25±0.14 | 0.14±0.15 | 2.27±0.14 |
| | Entry 20 | 0.39±0.16 | 0.29±0.15 | 0.20±0.16 | 0.15±0.15 | 2.42±0.16 |
| | Entry 30 | 0.42±0.14 | 0.27±0.15 | 0.21±0.15 | 0.13±0.15 | 2.21±0.14 |
| | SRN2007.No.8 | 0.40±0.14 | 0.28±0.14 | 0.22±0.15 | 0.15±0.14 | 2.36±0.14 |
| | SRN2007.No.20 | 0.39±0.15 | 0.28±0.15 | 0.23±0.14 | 0.13±0.15 | 2.40±0.15 |
| Standard | BHA | 0.02 | | | | |

**Metal chelating activity.** The results from the metal chelating activity of the leaf in each growth stage from varieties of Thai rice extracts indicate that the chelating ability increased with the increased concentration of all leaf in each growth stage from varieties of Thai rice antioxidants in the range of 0-10 mg/mL and it was constant at concentration above 10.0 mg/mL as shown in Figure 3. The order of percentages of metal chelating was EDTA>black > red > white rice, again leaf of milking stage extract of the Entry 1 rice showed the highest percentage of metal chelating (89.96 %). EDTA was used as a reference. At a concentration of 15 mg/mL, which was, however, much lower than that of EDTA at the same concentration (94.98%).

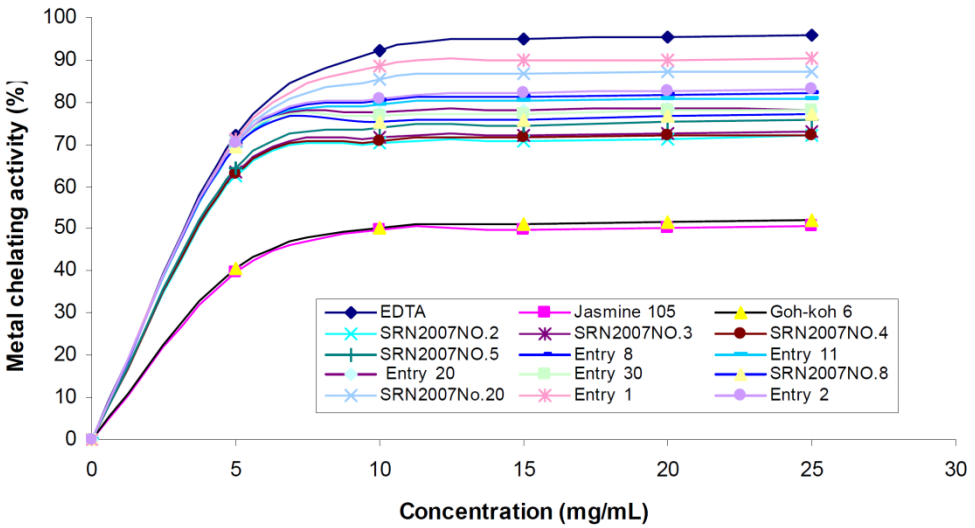


**Figure 1 A:** Booting stage) Antioxidative activity of the crude extracts of leaf in each growth stage from varieties of Thai rice as measured by the ferrozine assay

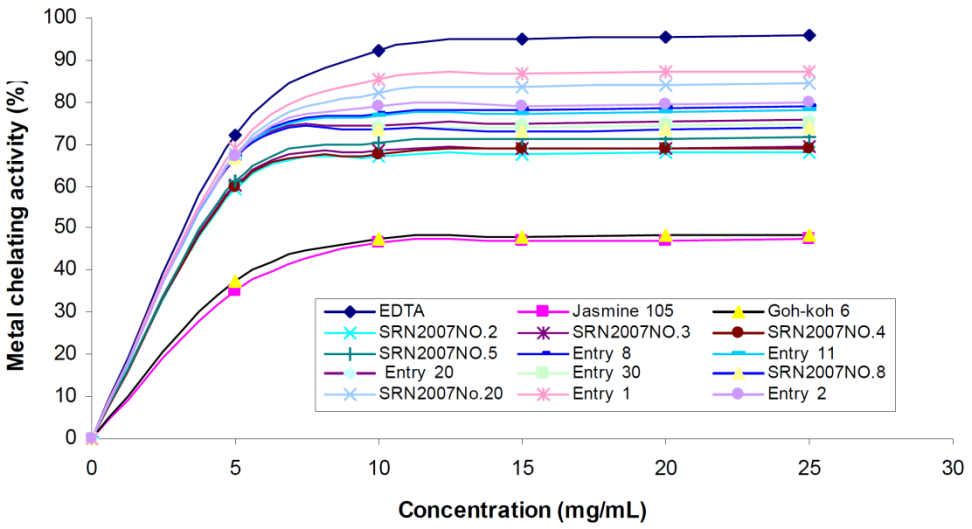


**Figure 1 B:** Milking stage) Antioxidative activity of the crude extracts of leaf in each growth stage from varieties of Thai rice as measured by the ferrozine assay

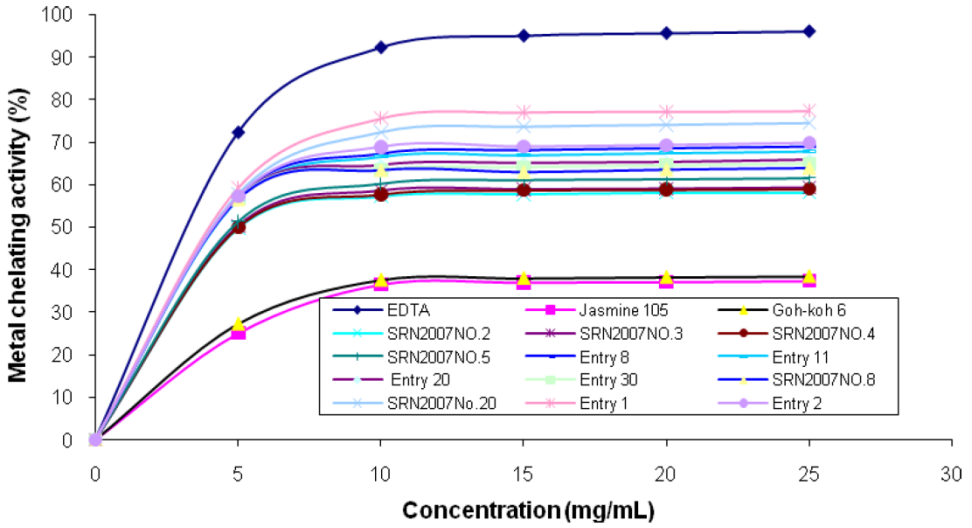


**Figure 1 C:** Panicle initiation stage) Antioxidative activity of the crude extracts of leaf in each growth stage from varieties of Thai rice as measured by the ferrozine assay

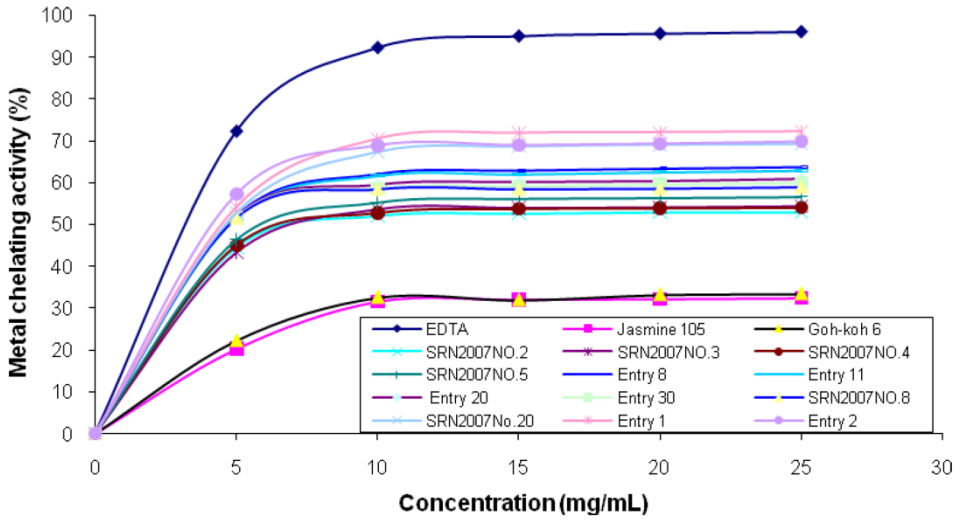


**Figure 1 D:** Maturation stage) Antioxidative activity of the crude extracts of leaf in each growth stage from varieties of Thai rice as measured by the ferrozine assay

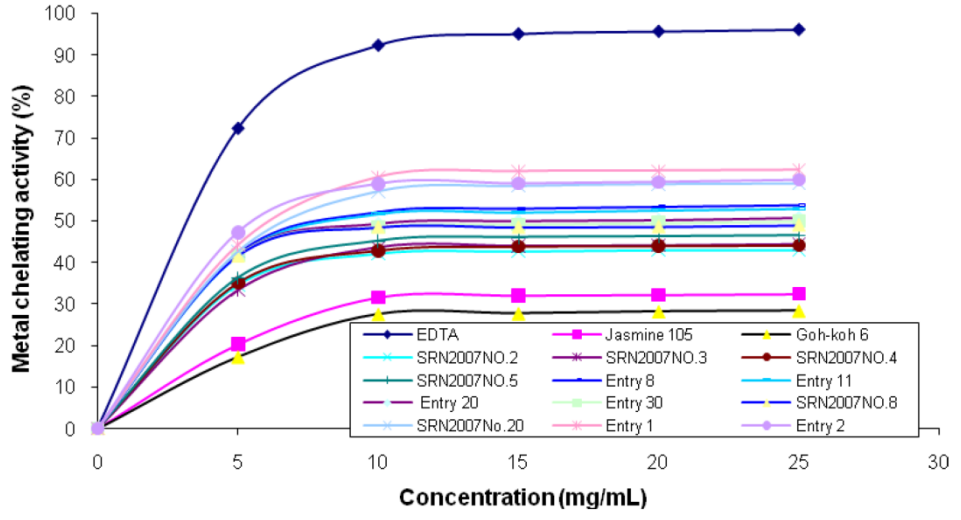


**Figure 1 E:** Tillering stage) Antioxidative activity of the crude extracts of leaf in each growth stage from varieties of Thai rice as measured by the ferrozine assay

## Discussion

The cultivars of pigmented rice, such as black or red rice have long history for people consumption, especially in Southeastern Asia (Hu *et al*., 2003). In addition,

pigmented rice, composed of high content of phenolic compounds (Oki *et al*., 2002; Clifford, 2000). The results from this study indicated the contents both total phenolic content and antioxidant activity were similar with many previously reported. The results from this study indicated the contents both total phenolic content and antioxidant activity were similar with many previously reported. Moreover, significantly different either total phenolic content or antioxidant activity compounds of black red rice were higher than that of white rice. In addition, leaves of Thai black rice cultivars contained the highest amount of phenolic compounds. A group of researchers reported (Itani *et al*., 2002) that the black rice is rich in anthocyanins such as cyanidin-3-*O*-*β*-D-glucoside, delephinidin-3-*O*-*β*-D-glucoside and pelagonidin-3-*O*-*β*-D-glucoside (Rabinkov *et al*., 1998), which are important to suppress oxidation in the body, and these benefits are not found in white rice. The order of the inhibiting percentage is black > red > white in each growth stage from varieties of Thai rice extracts using the thiocyanate method. It was suggested that the antioxidant activity of the rice came from free radicals that promote chain reactions during the linoleic acid peroxidation system (Chotimarkorn *et al*., 2008). The results in this experiment also showed that the black rice cultivar possessed the highest scavenging activity, followed by red rice, and white rice. Although the DPPH assay is not specific to any particular antioxidant components, the possible mechanism of hydrogen donating suggests that the radical-scavenging effects of black rice extracts might be due to the hydroxyl groups in the antioxidants of the extracts (Tananuwong *et al*., 2010: Sompong *et al*., 2011). The metal chelating activity of the leaf in each growth stage from varieties of Thai rice extracts was estimated by the ferrozine assay (Schlosnagle *et al*., *1982*). The leaf in each growth stage from black rice varieties showed the highest percentage of metal chelating, which can reduced the concentration of the catalyzing transition metal in lipid peroxidation, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential.

## Conclusion

The results presented in this study showed that leaf in each growth stage from varieties of Thai rice possess a relatively strong antioxidant activity of the leaf in each growth stage from varieties of Thai rice. Therefore, the leaf in each growth stage from varieties of Thai rice is a potential source of antioxidative phytochemicals and is a useful ingredient for nutraceutical or functional food products.

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**RESEARCH OUTPUT**

- **Krasaetep, J.**, Puangpronpitag, D., Nakornriab, M. (2011) The Total Phenolic Contents and Their Antioxidant Activity from Leaves in Different Growth Stage of Thai Glutinous Rice Cultivars. *PERCH-CIC CONGRESS VII Theme: Chemistry*, Environment and Society 2011, May 4-7, 2011, Jomtien Palm Beach Hotel & Resort Pattaya, Chonburi, Thailand. P. 221.

- **Krasaetep, J.**, Puangpronpitag, D., Nakornriab, M. (2012) Total phenolic, flavonoid contents and antioxidant activity from leaves in different growth stage of Thai glutinous rice cultivars. *The 6$^{th}$ PURE AND APPLIED CHEMISTRY INTERNATIONAL CONFERENCE 2012 "PACCON 2012 CHEMISTRY BEYOND BOUNDARIES*. January 11-13, 2012, The Empress Convention Center, Chiang Mai, Thailand. P. 69.

- **Krasaetep, J.**, Nakornriab, M., Puangpronpitag, D. (2012) Antioxidant Activity and Total Phenolic Contents in Leaf of some Thai Rice Cultivars. *International Journal of Applied Chemistry* 7, 285-296.