Selenium Biofortified Ricegrass (*Oryza sativa* L.) Juice Powder with an Enhancement of Its Selenium Content, Bioactive Compounds Content and Biological Properties

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ชื่อวิทยานิพนธ์ การผลิตผงน้ำคั้นจากต้นอ่อนข้าวเสริมแร่ธาตุซีลีเนียมขณะปลูกซึ่งมีการเพิ่มขึ้นของปริมาณแร่ธาตุซีลีเนียม ปริมาณสารออกฤทธิ์ทางชีวภาพและคุณสมบัติทางชีวภาพ

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บทคัดย่อ

ในปัจจุบันต้นอ่อนพืชได้รับความสนใจในการนำมาบริโภคทั้งในรูปของทานสดและน้ำคั้น เนื่องจากมีหลักฐานทางวิทยาศาสตร์ยืนยันว่าพืชในระยะนี้เป็นระยะที่มีการผลิตสารออกฤทธิ์ทางชีวภาพสูงสุด น้ำคั้นจากต้นอ่อนข้าวสาลีเป็นน้ำคั้นจากพืชในระยะต้นอ่อนที่ได้รับความนิยมมากที่สุด มีการศึกษาคุณสมบัติทางชีวภาพและระบุประโยชน์มากมาย แต่ข้าวสาลีเป็นพืชที่มีการปลูกมากในแถบอากาศเย็น ข้าวเจ้าเป็นพืชในตระกูลเดียวกันกับข้าวสาลี ในแถบเอเชียข้าวเจ้าเป็นแหล่งคาร์โบไฮเดรตหลักและเป็นธัญพืชที่มีการทำเกษตรกรรมมากที่สุด หากแต่การนำน้ำคั้นต้นข้าวในระยะต้นอ่อนมาบริโภคดังเช่นต้นอ่อนข้าวสาลียังไม่เป็นที่นิยม ยังไม่มีการผลิตในเชิงการค้าและยังไม่ทราบถึงประโยชน์ของต้นอ่อนข้าวมากนัก การศึกษาในตอนที่ 1 จึงมีวัตถุประสงค์เพื่อศึกษาปริมาณสารสำคัญและสมบัติการต้านอนุมูลอิสระของน้ำคั้นจากต้นอ่อนข้าวเปรียบเทียบกับน้ำคั้นจากต้นอ่อนข้าวสาลี ผลการทดลองพบว่าน้ำคั้นจากต้นอ่อนข้าวมีปริมาณคลอโรฟิลด์และวิตามินซีต่ำกว่าหากแต่พบสารประกอบฟีนอลิคในปริมาณที่สูงกว่าน้ำคั้นจากต้นอ่อนข้าวสาลีเมื่อวัดด้วยเทคนิคโครมาโตกราฟีและมีฤทธิ์ในการต้านอนุมูลอิสระที่ใกล้เคียงกัน เมื่อเปรียบเทียบจากต้นทุนการผลิตที่ต่ำกว่าและคุณค่าที่ได้รับ น้ำคั้นจากต้นอ่อนข้าวเจ้าจึงมีความเป็นไปได้ในการผลิตเพื่อเป็นอาหารเพื่อสุขภาพชนิดใหม่ ในการนำพืชมาใช้ประโยชน์เป็นอาหารเพื่อสุขภาพนั้น ปริมาณสารสำคัญในพืชและคุณสมบัติทางชีวภาพของพืชเป็นสิ่งที่สำคัญเป็นอย่างมาก ปัจจุบันมีเทคโนโลยีต่างๆมากมายในการเพิ่มฤทธิ์ทางชีวภาพของพืช จากการทบทวนวรรณกรรมพบว่าการเสริมแร่ธาตุในพืชต่างๆขณะปลูกสามารถเพิ่มพูนปริมาณแร่ธาตุในรูปของสารอินทรีย์ กระตุ้นการผลิตสารสำคัญในพืชบางชนิดและส่งผลต่อการเพิ่มขึ้นของคุณสมบัติทางด้านชีวภาพโดยสามารถทำได้ง่ายและใช้ต้นทุนต่ำ

แร่ธาตุซีลีเนียมเป็นแร่ธาตุที่มีความสำคัญในระบบการต่อต้านอนุมูลอิสระในร่างกายของมนุษย์ ซีลีเนียมเป็นที่นิยมในการบริโภคเป็นอาหารเสริมเพื่อประโยชน์ในการต้านอนุมูลอิสระและต้านการเกิดมะเร็งอย่างมีประสิทธิภาพ การเสริมแร่ธาตุซีลีเนียมในพืชในปริมาณที่เหมาะสมนั้นได้มีการรายงานว่า

สามารถเพิ่มปริมาณสารประกอบฟีนอลิค คลอโรฟิลล์ วิตามินซี กลูโคซิโนเลทและเพิ่มคุณสมบัติทางชีวภาพอีกด้วย ดังนั้นการศึกษาในตอนถัดไปจึงต้องการศึกษาการเสริมแร่ธาตุซีลีเนียมที่ระดับความเข้มข้น 0, 10, 20, 30 และ 40 มิลลิกรัมต่อลิตร ในต้นอ่อนข้าวขณะปลูกเพื่อปรับปรุงคุณค่าทางโภชนาการของน้ำคั้นจากต้นอ่อนข้าวสายพันธุ์ชัยนาท 1 ซึ่งมีราคาถูกและไม่นิยมบริโภคในรูปของเมล็ดข้าว ผลการทดลองพบว่าการเติมแร่ธาตุซีลีเนียมมีขีดจำกัดที่ 40 มิลลิกรัมต่อลิตรและการเติมที่ระดับนี้สามารถเพิ่มปริมาณซีลีเนียมโดยเฉพาะแร่ธาตุซีลีเนียมอินทรีย์ได้สูงสุด การเติมแร่ธาตุซีลีเนียมที่ระดับ 10 มิลลิกรัมต่อลิตร สามารถเพิ่มปริมาณสารประกอบฟีนอลิคและกิจกรรมการต้านอนุมูลอิสระได้สูงสุดเมื่อเทียบกับชุดการทดลองอื่นๆ หากแต่ไม่แตกต่างมากนัก ดังนั้นชุดการทดลองควบคุม (RG0) ชุดที่มีปริมาณฟีนอลิคสูงสุด (RG10) และชุดที่มีปริมาณซีลีเนียมสูงสุด (RG40) จึงถูกนำไปทดสอบคุณสมบัติทางชีวภาพต่อไปในระบบเซลล์ของสิ่งมีชีวิตและจุลินทรีย์ สารสกัดจากน้ำคั้นต้นอ่อนข้าวถูกนำมาวิเคราะห์เพื่อระบุชนิดของสารประกอบฟีนอลิคหลักที่พบด้วยเทคนิคแมสเสปกโตรสโกปี ผลการทดลองพบว่าสารหลักในน้ำคั้นจากต้นอ่อนข้าวเป็นสารที่อยู่ในกลุ่มฟลาโวนไกลโคไซด์ ผลการทดสอบคุณสมบัติทางชีวภาพของสารสกัดจากน้ำคั้นต้นอ่อนข้าว พบว่าสารสกัดจากทั้งสามชุดการทดลองมีคุณสมบัติในการเพิ่มปริมาณของจุลินทรีย์โปรไบโอติค แลคโตบาซิลลัส แพลนทารัมที่ระยะเซลล์แบ่งตัวทวีคูณและสามารถยืดอายุของเชื้อจุลินทรีย์ก่อนเข้าระยะเซลล์ตาย สามารถยับยั้งการเจริญของเซลล์มะเร็งลำไส้ เมื่อนำมาทดสอบในเซลล์เม็ดเลือดขาวพบว่าสารสกัดไม่เป็นอันตรายต่อเซลล์และสามารถลดการเกิดปฏิกริยาออกซิเดชั่นของไขมันที่ผนังเซลล์ โดยสามารถเพิ่มปริมาณเอนไซม์กลูต้าไทโอนเปอออกซิเดสและคะตะเลสและยังสามารถลดการเกิดสารไนตริกออกไซด์ซึ่งเป็นสารที่เกิดจากกระบวนการอักเสบในเซลล์เม็ดเลือดขาวได้ จากการทดสอบคุณสมบัติในการต่อต้านพิษของแคดเมียมในเซลล์ไตตามช่วงเวลาต่างๆ ได้แก่การเติมสารสกัด ก่อน ร่วมและหลังจากการเติมแคดเมียมพบว่า สารสกัดสามารถช่วยลดการตายของเซลล์ที่เกิดจากการโดนพิษแคดเมียมได้เมื่อเติมสารสกัดเข้าไปก่อนและร่วมกับแคดเมียมโดยไปลดการเกิดปฏิกริยาออกซิเดชั่นของไขมันที่ผนังเซลล์และลดการแตกหักของสารพันธุกรรมเมื่อทำการทดสอบด้วยวิธีโคเมทเอสเสโดยสามารถลดความยาวของหางโคเมทและปริมาณสารพันธุกรรมที่แตกหักได้ สารสกัดในชุดการทดลองที่ RG40 พบว่ามีประสิทธิภาพดีที่สุด เนื่องจากมีสารประกอบฟีนอลิคที่มีฤทธิ์ในการยับยั้งการเกิดอนุมูลอิสระและยังมีการเสริมคุณสมบัติต่างๆ จากการมีปริมาณแร่ธาตุซีลีเนียมในปริมาณที่สูงกว่าชุดอื่นๆ ซึ่งซีลีเนียมเป็นองค์ประกอบสำคัญของเอนไซม์กลูต้าไทโอนเปอออกซิเดส เอนไซม์ในร่างกายที่ทำหน้าที่ต้านอนุมูลอิสระในระดับเซลล์ ดังนั้นผลการทดลองในครั้งนี้ชี้ให้เห็นว่า น้ำคั้นต้นอ่อนข้าวที่ผลิตจากข้าวพันธุ์ชัยนาท 1 ที่มีการเสริมแร่ธาตุซีลีเนียมที่ระดับ 40 มิลลิกรัมต่อลิตร (RG40) สามารถนำไปใช้ผลิตเป็นอาหารเพื่อสุขภาพชนิดใหม่ที่มีประโยชน์และเป็นการช่วยเพิ่มมูลค่าให้กับข้าวในอนาคตได้

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Major program Functional Food and Nutrition

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ABSTRACT

In the present day, sprouts have been paid interested to consume in the form of fresh vegetables or as juice since sprouting stage is claimed to be the stage that plants contained the highest bioactive compounds. Wheatgrass juice is the most consuming sprout juice which providing lots of pharmaceutical benefits. However, it can mostly grow in temperate areas. Rice is the plant in similar family to wheat (Poaceae). Among Asian countries, rice is being the most consumable carbohydrates source and the major agricultural crops. However, the consumption of ricegrass juice alike wheatgrass juice are not widespread. None of commercial products are available in the market and less information on its beneficial effects are reported. Therefore, the study was designed to compare the bioactive compounds content and the antioxidant activities of ricegrass juice compared with wheatgrass juice. Results indicated that ricegrass juice contained lower level of chlorophyll and ascorbic acid while contained higher level of phenolics detected by HPLC compared with wheatgrass juice and the comparable level of antioxidant activities. When dedicate the cost of ingredients and the probable benefits achievement, ricegrass juice could be a potential candidate to produce as new functional food.

To produce the functional plant foods, bioactive compounds content and biological properties of plants are mostly concerned. Recently, new technologies to improve biological properties has been stated. According to the review of the literature, the bio-fortification is a well-accepted method to improve organic mineral content, trigger the production of bioactive content and enhance the bioactivity using low production cost and can be easily applied. Selenium (Se) is an important trace element in the human antioxidant

system. It is well known for antioxidant boosting and effective cancer treatment. The bio-fortification of Se into plants indicated the improvement in phenolics, ascorbic acid, and glucosinolate content as well as antioxidant and anti-cancer properties. Therefore, the experiment was further conducted on the effect of Se bio-fortification at 0, 10, 20, 30 and 40 mg/L into ricegrass and investigated the bioactive compounds content and biological properties. Results showed that the fortification of Se into ricegrass had a limitation at 40 mg/L. The addition of 40 mg/L Se can increase the highest content of Se compounds, especially in the organic form. The addition of 10 ppm Se could increase the highest level of total phenolic content (TPC) and antioxidant activities but not to the large extent. Consequently, the treatment of control (RG0), the treatment which contained the highest in TPC (RG10) and the highest in Se content (RG40) were chosen to the study on biological properties in cell lines and microorganisms model. Ricegrass juice extract was firstly identified on its predominantly polyphenols using mass spectroscopy (LCMS) and found that flavone glycosides were the foremost group. Results on biological properties revealed that ricegrass juice provided the effect of enhancing probiotic bacteria number namely, *Lactobacillus plantarum*, on its late-log phase and can prolong their life-cycle. It could also inhibit HT-29 colon cancer cells, and propose role in reducing lipid peroxidation and nitric oxide production in RAW264.7 murine macrophage cells. Moreover, while examined the effect of ricegrass juice against cadmium toxicity in HEK293 kidney cells, the extract could reduce the number of cell death while pre-incubation and co-incubation with cadmium by reducing the lipid peroxidation rate as well as reduce the damaging to DNA determined by comet assay. The extract RG40 exhibited the greatest ability on all activities because it contained an extra amount of Se which is the cofactor of GPx, the antioxidant enzymes in the human body in combination with polyphenols compounds in ricegrass. Therefore, it strengthened the antioxidant protection to cells. The outcomes of this study indicated that ricegrass juice bio-fortified with Se at 40 mg/L produced from Chainat1 had potential to produce as new functional food which provides tons of benefits and offered the added value to rice in the future.

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LIST OF ABBREVIATIONS

µg = microgram

µg/g = microgram per gram

µg/ml = microgram per milliliter

µl = microliter

µmol = micromole

AAPH = 2,2'-azobis (2-amidinopropane) dihydrochloride

ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

ACN = acetonitrile

Ag = silver

ALP = alkaline phosphate

ANOVA = analysis of varience

AT = active tillering

b = booting

BSA = bovine serum albumin

C_a = Chlorophyll a

C_b = Chlorophyll b

Ca = calcium

$CaCO_2$ = calcium carbonate

CAT = catalase

CCl_4 = carbon tetrachloride

CC_{50} = half maximal cytotoxicity concentration

CFU/g = colony forming unit per milliliter

CML (K562) cells = chronic myeloid leukemia cell line

Cd = cadmium

$CdCl_2$ = cadmium chloride

CRD = completely randomized design

d = day

LIST OF ABBREVIATIONS (Cont.)

DPPH = 1,1-diphenyl-2-picrylhydrazyl

DTNB = 5,5'-dithiobis-2-nitrobenzoic acid

DRI = dietary reference intakes

DMEM = Dulbecco′s modified eagle′s medium

DMSe = dimethyl-selenide

DNA = deoxyribonucleic acid

DW = dry weight

EDTA = ethylenediaminetetraacetic acid

EDTA-Na_2 = ethylene diamine tetraacetic acid disodium

ESI = eletron spray ionized

E. = *Escherichia*

F = flowering

FBS = fetal bovine serum

Fe = iron

g = gram

G = germination

GluE = glucose equivalent

GABA = gamma amino butyric acid

GCMS = Gas Chromatography Mass Spectrometry

GPx = glutathione peroxidase

GSH = glutathione

h = hour

H_2O_2 = hydrogen peroxide

HCl = hydrochloric acid

Hg = mercury

HPLC = High Performance Liquid Chromatography

I = iodine

LIST OF ABBREVIATIONS (Cont.)

IBD = inflammatory bowel diseases

IC_{50} = half maximal inhibition concentration

ICP-OES = Inductively Coupled Plasma Optical Emission Spectroscopy

IDI = iodothyronine deiodinase

IT = initiation of tillering

IP = initiation of panicle primordia

IU = International unit

K = potassium

kcal/mol = kilocalories per mole

kg = kilogram

L = Liter

LAI = leaf area index

LNA = L-nitro arginine

LMA = low melting point agarose

LPS = lipopolysaccharide

M = molar

MDA = malondialdehyde

MeHg = methylmercury

mg = milligram

Mg = magnesium

mg/g = milligram per gram

mg/L = milligram per liter

min = minute

ml = milliliter

mm = millimeter

mM = millimolar

mmol/L = millimole per litter

LIST OF ABBREVIATIONS (Cont.)

MS = mass spectroscopy

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Mn = manganese

mo = month

MRS broth = de Man, Rogosa and Sharpe broth

N = nitrogen

NBT = nitro blue tetrazolium

NCI = National Cancer Institute

ND = not detected

nm = nanometer

NO = nitric oxide

NOS = nitric oxide synthase

NPC = Nutritional Prevention of Cancer

$OH^{\bullet}$ = hydroxyl radicals

ºC = degree Celsius

P = phosphorus

PAL = phenylalanine ammonia lyase

PBS = phosphate buffer saline

PM = physiological maturity

p- = *para*

PYE = pyrogallol equivalent

RPMI = Roswell Park Memorial Institute

RT = retention time

RG = Ricegrass

ROS = Reactive Oxygen Species

s = second

S = sulfur

LIST OF ABBREVIATIONS (Cont.)

w

Se = selenium

Se-Met = selenomethione

SGOT = Serum Glutamic Oxaloacetic Transaminase or AST (Aspartate transaminase)

SGLT = Sodium-Glucose Linked Transporter

SOD = superoxide dismutase

T cells = T lymphocyte

TA = total titratable acidity

TBA = thiobarbituric acid

TBARS = thiobarbituric acid reactive substances

TCA = trichloroacetic acid

TE = Trolox equivalent

TPC = total extractable phenolic content

TPTZ = 2,4,6-tris(2-pyridyl)-s-triazine

TVC = total viable count

US = United states

UN = unknown compound

UV = ultraviolet

v/v = volume per volume

v/v/v = volume per volume per volume

w/v = weight per volume

WHO = world health organization

WG = wheatgrass

Zn = zinc

γ = gamma

λ = wavelength

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Chronic diseases are the greatest world health problems which cause premature death and mortality worldwide for more than 60% of total death (WHO, 2005). Risk factors for chronic diseases in daily life are currently hard to avoid, besides non-healthy lifestyles such as smoking, the consuming poor diet even low in physical activity were the crucial factors. In the past, food consumption aimed to provide energy as the body's fuel. However, a new direction in the consumption of human seems to need the daily nutrition which can provide a variety of nutrients and the bioactive compounds, demonstrate the benefits to health, promote well-being and reduce the risk of various chronic diseases. Towards the consumers' demand for a healthy diet, functional food has been introduced as a food which contains components responsible for positive biological function in the body (Hasler, 1998). Among groups of diet, fruits and vegetables have been much interested since they have been proved to provide many phytochemicals substances that can protect our body against diseases. Sprouts or young plant of cereals, grains or legumes are currently interested as new kind of vegetables since the young stage of these plants is a noteworthy period to consume as a powerful source of helpful nutrients according to the fact that pre-jointing stage of plant growth was educated as the stage that contains the highest nutritional values per gram of plant. After this stage, plants regularly use those nutrients in the cellulose synthesis (Padalia *et al.*, 2010). Wheatgrass or young wheat shoots (*Triticum aestivum*) is the most familiar plant consuming at this stage. It has been introduced from western countries and widespread nowadays for the benefits from consumption of its fresh juice. It is a rich source of chlorophyll, amino acid, minerals, vitamins, enzymes and many phytochemical compounds which claim for various clinical utilities such as blood builder and therapy for anemia, possible use as anti-cancer and anti-

inflammatory substance as well as claims to use as a detoxifying agent. However, wheat is mostly grown in Europe and United States where the appropriate temperate zones are.

For Asian population, rice (*Oryza sativa* L.) is the principal energy source. Similarly, rice was in the grass family, same as wheat. Few studies on rice were stated that young rice plant or in another word "ricegrass" also contains chlorophyll and phenolic constituents as well as compounds that are responsible for the antioxidant activities (Benjawan *et al.*, 2011, Rattanapon *et al.*, 2016b). Rice is the major goods of Thailand's agriculture for a century and farmer is praised as the backbone for Thai nationality. Consequently, Thailand has a lot of supply for rice which is suggested to be a lower cost ingredient rather than wheat. Therefore, an introducing of ricegrass as a functional food product from Thai rice is interesting. Moreover, it can offer the added value to rice especially for some cultivars which are less consuming as grains. Chainat1 rice is a highly productive rice as it is non-sensitive to photoperiod and resistant to insects (Vetayasuporn, 2012). However, it contained high amylose content which affects the texture of cooked rice to be hard and crumbly, thus not preferring for daily consumption as cooked rice. The Phatthalung rice research center stated that Chainat 1 production was extremely reduced compared with other varieties, thus the center strongly recommended for the research and development of this rice cultivar to support the alternative utilization.

As the bioactive compounds of a plant-based diet have been receiving substantial interest currently for the health improvement, the strategies on how to improve those phytochemicals production in plants to promote plant nutritional values are more significant. Bio-fortification of plants with micronutrients is another scheme to produce functional food product which generates minerals or vitamins-rich crops using an inexpensive technique and can provide the supplementation majority in the organic form which is believed to be safer than inorganic medicinal supplement as well as more bioavailable in human body. Besides, the bio-fortification may also possibly improve the nutritive values and biological properties of plants from the combination effects of phytochemicals compounds and an increased content of minerals.

Selenium (Se) is an essential trace mineral which is known to be the most efficacy antioxidant minerals (McDowell *et al.*, 2007). It is incorporated to protein in human body to form seleno-proteins which are a part of a primary antioxidant enzyme called glutathione peroxidase (GPx). Se, therefore, plays the main function in the body regulation, prevents body against oxidative stress and further benefits as anti-cancer substance (Ip and Ganther, 1994), anti-heart diseases substance (Flores-Mateo *et al.*, 2006). Moreover, Se also strengthens the immune system (McKenzie *et al.*, 1998) and promotes thyroid function (Gärtner *et al.*, 2002). This element is now widely produced as supplementation in medicinal form. It can be the choice for people who want to improve their health status. However, the cost of the supplement is rather high for common population and consumers tend to aware the risk from consuming varieties of supplement. Thus, there is a limitation usage in realistic.

The bio-fortification of Se has been applied in various plants such as potatoes (Lei *et al.*, 2014), broccoli (Ávila *et al.*, 2013), green tea (Molan *et al.*, 2009, Xu *et al.*, 2003) even in spinach (Saffaryadi *et al.*, 2012) and showed the potential in increasing Se content, some bioactive substances which results in improving total nutraceutical profits to those plants. Different plant species relate to unlike Se accumulating ability in their tissues. Cereal grains are reported as one of a good source for bioavailable of Se which included wheat, barley, rye, oat and rice. Because of they are the main staple food for the entire world population, thus based on previous literatures, bio-fortification of Se in this plant species focused mainly on an increasing of Se content in the final grains which supposed to primarily relief the deficiency problem. Yet, the other stages of cereal plants development are less regard. The bio-fortification of Se into rice grain and grow them until it reaches the stage of ricegrass is the noticeable idea that expected to produce new kind of functional food rich in phytochemicals and Se compounds which can be improved in the total biological properties against some of the chronic difficulties.

1.2 Review of literature

1.2.1 Chronic health problems

Chronic diseases have long been known for the largest world health problems. Chronic diseases are long-lasting symptoms that can only be controlled but cannot be cured by any medication (Kuh and Shlomo, 2004). According to WHO (2005), approximately 60 % of the world population or over 35 million people were dying due to at least one chronic health conditions with 80% occurred in low and middle-income countries. In Thailand, the accumulated number of patients who have suffered from top 5 chronic diseases includes diabetes mellitus, hypertension, ischemic heart disease, stroke and chronic lower respiratory disease in 2012, in accordance with the report from Thailand ministry of public health, was accounted for almost 6 million (Thonghong *et al.*, 2012). The death rate from chronic diseases worldwide is currently expanding and expected to increase substantially over the next 2 decades (Yach *et al.*, 2004). The foremost chronic diseases across the global include cardiovascular diseases, cancer, diabetes and respiratory diseases (Halpin *et al.*, 2010). In the present day, chronic diseases may occur more rapidly to young ages of people due to the environmental changes such as pollution and UV rays which are unavoidable factors and the increasing of people's health-damaging behaviors included particularly tobacco use, extensively alcohol consumption, physical inactivity, mental stress and especially, poor eating habits (Willcox *et al.*, 2004).

1.2.2 Functional Foods

Eating healthy diet is still believed to be the best way to promote healthfulness of people in all generations. The honorable quote by Hippocrates, the physician who has been praised as a father of western medicine also supports this fact.

"Let food be thy a medicine and medicine be thy a food"

In the past food were primarily be the recognized for their essential nutrients for normal body regulation and function. However, during the past 2 decades, consumer attitudes on food has changed from an emphasis on satisfying hunger to emphasize on the promoting use of foods to encourage well-being and ability to reduce the risk of diseases

(Niva, 2007). The alteration of this diet trend may perceptibly cause by human's health awareness effects, an increasing of aging population and higher health care cost. People are more likely to choose healthy choices to stay healthy and prevent themselves away from diseases. Those factors lead to an introducing of a new category of food products which is called "Functional food". It means food that contain components or substances which affected one or a limited number of specific function on the body in a targeted way (Roberfroid, 1999).

1.2.3 Sprouts or cereal grasses

Cereal grains are the main carbohydrates dishes for world population for a century. Nowadays, young stages of cereal species or known as grass has been much attention since the laboratory results claimed that it is a stage that plants contain the highest concentration of nutrients content per grams of the sample as seen in Figure. 1 (Seibold, 2008). This stage can be called as a pre-jointing stage; the theoretical hypothesis behind this fact was supposed that plants on their young stage used pool of nutrients in the seeds to synthesis the protecting substances to survive from harm or any dangerous disturbances as well as to prepare themselves for the jointing of new leaf while after this stage nutrients in the seeds were primarily use in the cellulose synthesis targeting for the extension (Padalia *et al.*, 2010).

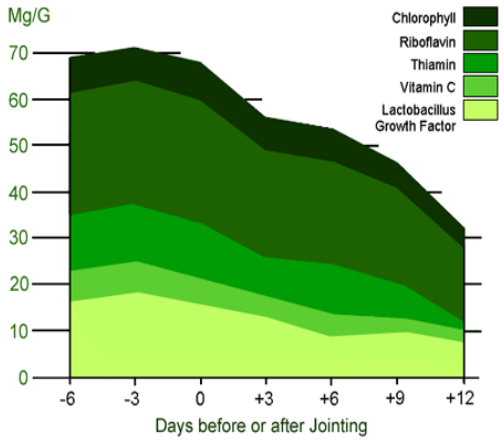

Figure 1. Nutrients content in cereal grass versus day of jointing stage

Source: Seibold (2008)

1.2.4 Wheatgrass

Wheatgrass is the most familiar plant consuming at the pre-jointing stage. Wheatgrass is the word denote a young plant of wheat (Figure 2). The scientific name is *Triticum aestivum* Linn., a subspecies of family Poaceae (Padalia *et al.*, 2010). Wheatgrass has been firstly introduced in the western country since the 1930s. The consumption of wheatgrass juice aims to supply health benefits and be a part of raw food diet (Murphy, 2006). It is scientifically proved to contain a high concentration of chlorophyll, vitamins, minerals and some enzymes. Therefore, it has been used as herbal medicine to treat some diseases as well as consume to fulfill the portion of vegetable recommendation in each day.

Figure 2. Wheatgrass and wheatgrass juice

Wheatgrass is commonly found in the temperate areas of Europe and United States, though it is also cultivated in India and the north part of China. In the last few years, wheatgrass consumption has been widespread throughout the world and becoming in demand for people as the most famed functional drink with powerful therapeutic properties. Moreover, the quantity of paperwork for the investigation of phytochemical substances and useful biological properties of this plant was enlarged more than twice from 2008 to 2014. Wheatgrass can be stated as the powerful food because the consumption of only single serve (4g) which provides only 15 kcal can responsible for macronutrients include essential amino acids, almost all vitamins and minerals included very high content of vitamin A, B_1, B_2, B_3, B_5, B_6 and E. Additionally, it also rich in Fe, Zn, Mn as well as Cu contents.

1.2.4.1 Chemical constituents

a. Chlorophyll

Chlorophyll is the most abundant natural pigments which respond to the color of green plants and plays main role in the oxygenic photosynthesis (Scheer, 1991). Chlorophyll is specified as the main bioactive compounds in wheatgrass as it is accounted for approximately 70% of total chemical compounds in wheatgrass (Padalia *et al.*, 2010) Chlorophyll has resembled molecular structure to heme, the small pigment of human blood molecules which responsible for oxygen transportation. Heme and chlorophyll contained similar tetra pyrrole ring structure (Figure 3). The resemblance of these two molecules may profit to therapeutic properties relating to the deficiency of hemoglobin. In addition, chlorophyll has long history used as a traditional medicine for many purposes as detoxifying agent (Wigmore, 1985), wound healing substances (Smith and Livingston, 1943), antioxidant booster (Endo *et al.*, 1985), anti-inflammatory agents (Park *et al.*, 2007) as well as useful for cancer therapy (Ferruzzi and Blakeslee, 2007).

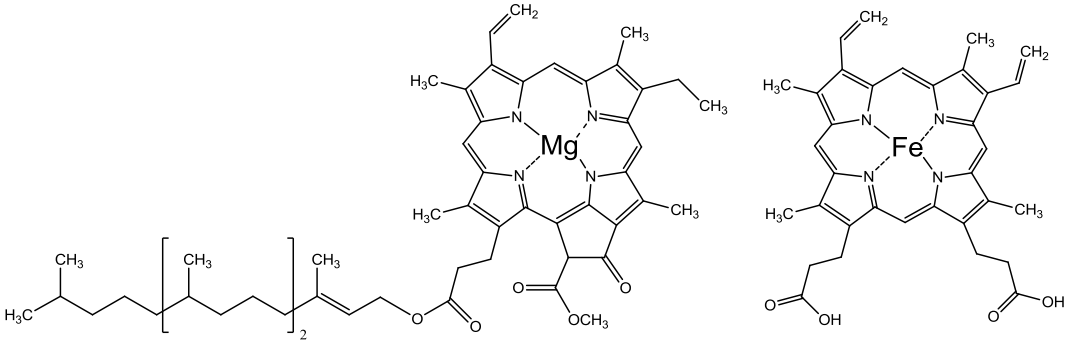

Figure 3. Structure of chlorophyll molecule (a) in plant compares to heme (b) in blood
Source: Padalia *et al.* (2010)

b. Other bioactive compounds

Wheatgrass is observed to possibly contain the group of alkaloids, flavonoids, glycosides, amino acids, saponins, tannins, phytosterols, triterpenoid, coumarin as well as gum and mucilage (Ashok, 2011, Durairaj *et al.*, 2014, Mohan *et al.*, 2013). The

water-soluble extract of wheatgrass is found to be greater than alcoholic (methanol) soluble extractive value at about 71.11% compared to just 9.2% (Ashok, 2011). Later works in the recent years have been studied extensively and indicated the structure of some compounds in many groups of phytochemicals using the Gas chromatography mass spectroscopy (GC-MS) analysis. Known phytochemical compounds of wheatgrass, as well as their biological activities which may exert benefits, showed in Table 1.

Table 1. Known phytochemical constituents of wheatgrass and their biological properties

Type	Constituent	Biological property	References
Terpene	α-amyrin/β- amyrin	Anti-inflammatory Antioxidant Gastroprotective Hepatoprotective Anti-hyperglycemic	Mohan *et al.* (2013), Durairaj *et al.* (2014), Jain and Jain (2014)
	Caryophyllene/ Caryophyllene oxide	Anti-cancer Anti-bacterial and fungi Anti-inflammatory Immuno-modulator Anti-platelet aggregation	Mohan *et al.* (2013), Durairaj *et al.* (2014)
Sterol	Gamma sitosterol	Influence in the synthesis of cholesterol Cytotoxic sensitizing	Mohan *et al.* (2013), Durairaj *et al.* (2014), Jain and Jain (2014)
Flavonoid	Apigenin/ Quercetin/ Luteolin	Blood pressure reduction Antioxidant Anti-inflammatory Improve endothelial function Anti-platelet aggregation	Padalia *et al.* (2010)., Mohan *et al.* (2013), Zendehbad *et al.* (2014)

Type	Constituent	Biological property	References
Indole compounds	Choline	Control phospholipid concentration Synthesis of acetylcholine	Padalia *et al.* (2010)
	Laetrile	Benefit for cancer	Padalia *et al.* (2010)

1.2.4.2 Health benefits of wheatgrass

a. Blood builder in thalassemia

Thalassemia syndromes are genetically autosomal recessive blood disorder that categorized by an impaired gene to produce hemoglobin, the molecule that carries and transport the oxygen in red blood cells. Therefore, fewer and unusual red blood cells which are short-lived and easily to be destroyed circulate in the body (Tangvarasittichai, 2011). People with this condition are thus receiving inadequate oxygen. Wheatgrass juice has been stated to potential increase hemoglobin level due to a high level of chlorophyll, which is the resemble molecule of heme in hemoglobin. Moreover, it has been appealed to reduce blood transfusion and increase the interval of blood transfusion in patients excessively (Singh *et al.*, 2010). Another clinical study by Marawaha *et al.* (2004) also claimed that the use of 100 ml wheatgrass juice on daily basis can reduce by 25% in transfusion requirement and 29.5% increase in interval time between transfusions. Besides, the use of wheatgrass as a source of antioxidant substances included antioxidant enzymes, vitamin C, and E, as well as bioflavonoids, may be another reason for thalassemia clinical improvements because those substances can consolidate the membrane of red blood cells, improve the integrity and give rise to prolong their survival time in the body.

b. Detoxifying properties and hepatoprotective effects

The liver is responsible for the main function in detoxifying hazardous stuff such as toxins and heavy metals. Dietary chlorophyll is the substance which can enhance the production of new hemoglobin. Therefore, it can reintroduce the blood, then cleanse and detoxify dirt in the liver and bloodstream. Particularly chlorophyllin, one of dietary

chlorophyll has been studied broadly and claimed for the ability to detoxify the carcinogen aflatoxin from the liver which further benefit to protect against cancer (Egner *et al.*, 2001). Moreover, various types of amino acids and bioflavonoids found in wheatgrass can neutralize toxic substances such as cadmium, mercury, strontium, nicotine as well as polyvinyl chloride by managing them into the form that can simply eliminate out from the body (Wigmore, 1985). The hepatoprotective effects of wheatgrass have also been reported by the administration of 100 mg/kg body weight dose per day in a CCl_4 treated rat. The outcome in terms of SGOT, SGLT, ALP, and Bilirubin in serum indicated a recovery of liver from the damage of CCl_4 significantly (Jain *et al.*, 2007).

c. Antioxidant properties

Wheatgrass contains many antioxidant substances that provide benefit in preventing free radicals attack. The outstanding substances on antioxidant activities in wheatgrass have been mentioned on polyphenol compounds, carotenoids and vitamin C (Urbonavičiūtė *et al.*, 2009). The ethanolic extract of wheatgrass at dose 100 mg/kg body weight has been treated to diabetic Wistar albino rat and indicated a significant decrease in the level of lipid peroxides measured with thiobarbituric acid reactive substances (TBARS) reagent (Mohan *et al.*, 2013). The potent of wheatgrass as an antioxidant substance may be the reason for explaining some others wheatgrass health claims.

d. Anti-inflammatory properties

Inflammatory bowel diseases (IBD) are the inflammation of digestive tract included small intestine and colon. It can cause the accumulation of micro-particles and form to antigenic particles which trigger the inflammatory system to release cytokines (Baumgart and Carding, 2007). Wheatgrass juice has been considered for the treatment of IBD, according to bioflavonoids compounds namely, apigenin and luteolin which can act as anti-inflammatory agents (Funakoshi-Tago *et al.*, 2011). Ulcerative colitis is one kind of IBD which affects primarily the colon and rectum. 100 ml of wheatgrass juice consumption for 1 mo has been studied in a randomized, placebo-controlled trial in the management of this symptom. Results showed 78% improvement in wheatgrass juice treatment group compared to 30% of placebo group by assessing for the sigmoidoscopic

evaluation, number of bowel movement and rectal bleeding. However, only small groups of patients were analyzed (n=19) (Ben-Arye *et al.*, 2002).

e. Anti-cancer properties

Many dietary complexes have been recognized as natural cancer healing substances. Wheatgrass is also one of a food which claimed for anti-cancer properties due to many reasons included a high level of antioxidant substances, increasing the oxygen supply in the body and the creation of alkalinity atmosphere. The antioxidant substances which strengthen the body defense system in wheatgrass may include chlorophyll, vitamin C, vitamin E, and phenolics. Chlorophyll in wheatgrass may enhance the synthesis of hemoglobin, thus result in an enhancing of oxygen supply to all the body including cancer cells which are highly vulnerable to high oxygen concentration and toxic to cancer cells (Matés and Sánchez-Jiménez, 2000). Wheatgrass has also been appealed for an induction of apoptosis against Caco2, mice colon cancer cell line (Lakshmi *et al.*, 2014) as well as CML (K562), human chronic myeloid leukemia cell line (Aydos *et al.*, 2011). Moreover, the administration of wheatgrass into induced colon cancer mice for 30 d showed a satisfied result by decrease level of lipid peroxidation, increase level of glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) which are important in an enhancing of antioxidant defense system (Lakshmi *et al.*, 2014).

1.2.4.3 Limitation of wheatgrass

According to voluminous scientific data support the dominant profit of wheatgrass, demand in the consumption of them has been increased substantially. Therefore, there are many farm operators worldwide which are adverted in wheatgrass production. However, cultivation of wheatgrass in some areas that climate is not proper, thus not worthwhile. The places where wheatgrass is mostly grown were specifically in Europe and United States where the appropriate temperate zones are. Wheatgrass naturally does not contain any gluten like wheat, however, mistake from harvesting and processing method might be a reason of contaminating with gluten-containing wheat kernels. As well as wheatgrass powder may possibly contain gluten from the addition of wheat containing stabilizer in the final product. Thus, wheatgrass consuming has now been considered and

watched out from people with coeliac diseases. People with the severity of a wheat-related allergy is needed to carefully be consulted with a healthcare professional before use. Moreover, the strong flavor and taste of wheatgrass juice have been reported that hard to be acceptable for some group of people (Ben-Arye *et al.*, 2002).

1.2.5 Rice

Rice (*Oryza sativa* L.) is one of the most consumed cereal crops for almost half of world population, especially for Asian population, rice is the principal energy source. Rice production in Asia is accounted for approximately 90% of world production (Milovanovic and Smutka, 2017). China and India are the leading of rice producers while Thailand is also a world recognized rice producer which produce approximately more than 30 million tons of rice every year as well as an exporter to the world marketplace, especially the Jasmine white rice 105 (Petchseechoung, 2017). Rice is the major goods of Thailand's agriculture for a century and farmer is praised as the backbone for nationality. Consequently, Thailand has a lot of supply for countless varieties of rice.

1.2.5.1 Type of rice

Rice is the plant in grass family Poaceae. Two main species of rice included *Oryza sativa L.* which is the common type that cultivated throughout the world and another one is *Oryza glaberrima* which mainly grown in African. The *Oryza sativa L*. is originated in India and it can be further divided into three main types comprise of Indica, Japonica, and Javonica. Japonica rice is mostly grown in the central and northern of China, Japan, Korea and Thailand. It is a short-grain and high in amylopectin hence becoming sticky after cooking, while Indica rice is widely grown in tropical regions. Both species can be grown in subtropical regions. Additionally, Javonica is broad-grained grown in tropical climates (Smith and Dilday, 2003). Within each variety, there are many cultivars, each favored for purposes or regions

1.2.5.2 Rice ecology (*Oryza sativa L.*)

Rice is a monocotyledon, an annual plant which generated from the seeds with long slender leaves, hollow and terminates panicle or flower cluster (Figure 4). The

rice plant can grow to 1 to 1.8 m tall, depending on the variety of rice and soil fertility. The edible seed of them called grain were about 5–12 mm long and 2–3 mm thick.

Figure 4. Rice *(Oryza sativa L.*)

Rice is the only cultivated cereal plant which can adapt to growing in both flooded and non-flooded soils. Moreover, it can be grown under a wide range of climate and geographical conditions on all five continents. Thus, rice can be separated by growing environmental condition mostly on water availability into 4 groups.

1. Upland rice – rice which is prone to drought and can tolerate low temperature
2. Lowland rice, rain-fed – rice which is grown only in season rice field, using rain as a source of water supply which is dependable
3. Lowland rice, irrigated – rice which can grow for all year by controlling water supply using irrigation
4. Floating rice – rice grown in deep water for more than 50 but less than 100 cm

In Thailand, lowland rice with rain-fed type is accounted for 70% of total rice cultivation while 24% is grown in lowland rice with irrigated. Moreover, type of rice can be separated on the response on photoperiod into two types including sensitive to photoperiod rice which can grow only in season period and can be harvested the crop once per year while another type is non-sensitive to photoperiod which can flower for grain achievement depends only on time of cultivation.

1.2.5.3 Growth and development of rice

The growth cycle of the rice plant is divided into three stages included vegetative, reproductive, and ripening growth stage. Vegetative growth is the phase from seeds germination extends to first panicle initiation. Main processes during this phase are increasing in plant height, tillering, root growth, leaf weight and expanding leaf area index (LAI) which mainly affected by the protein synthesizing. Carbohydrate supply is required for the growth of tiller buds. After the third leaf has completely emerged, it can produce its own photosynthesis. The morphology of lowland rice seedling at tillering stage showed in Figure 5.

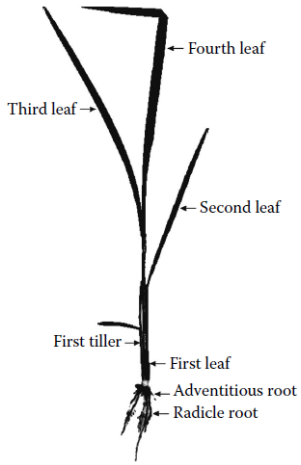

Figure 5. Morphology of lowland rice seedling at tiller initiation growth stage
Source: Fageria *et al.* (2007)

In the second stage, the reproductive growth stage included the panicle initiation, booting, panicle emergence, and flowering. It is also characterized by elongation, a decrease in tiller number, and the emergence of the last leaf on the tiller. This stage generally lasts about 30 d in rice cultivars having 130–140 d of the growth cycle. Adverse environmental conditions such as N deficiency, drought, low light and very low or high temperatures can reduce panicle size and hence grain yield reduction (Counce *et al.*, 2000). The ripening stage in rice extends from flowering to physiological maturity. The dry weight

of the caryopsis increases rapidly up to 15–20 d after flowering in the tropics and 25–30 d after flowering under temperate conditions. During the ripening growth stage, the morphogenesis of the rice plant is already completed, and photosynthesis is accumulated in the panicles in the form of starch. Mobile carbohydrates, proteins, and mineral nutrients, which are stored in leaves, stems, and roots of the plant, also move into the panicles. More than 85% of the rice grain is a carbohydrate, primarily starch (Hayashi, 1995). The starch accumulated in rice grains originates from carbohydrates assimilated by the leaves after flowering or during grain filling, as well as from the carbohydrates stored in the shoot prior to flowering. All stages of rice growth and development showed in Figure 6.

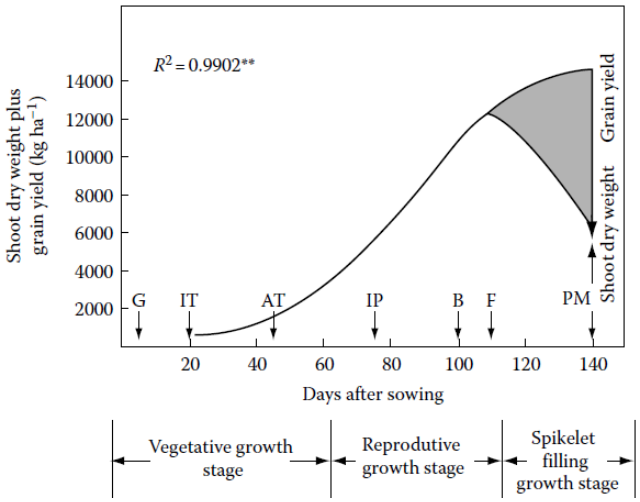

Figure 6. Stage of rice growth and development for 140 days old cultivation (G = germination, IT = initiation of tillering, AT = active tillering, IP = initiation of panicle primordia, B = booting, F = flowering, and PM = physiological maturity)
Source: Fageria *et al.* (2007)

Unhusked grain is known as paddy rice while husked or hulled rice, usually termed brown rice. Brown rice is milled to remove the outer layers, including the aleuronic layer and the germ, after that it is polished to produce white rice. Paddy, on milling, gives approximately 20% husk, 50% whole rice, 16% broken rice, and 14% bran and meal. The endosperm is highly digestible and nutritious and on average contains about 8% protein.

1.2.5.4 *Oryza sativa* L., cv. Chainat 1

Chainat 1 cultivar was firstly introduced by Chainat Rice Research Center, Thailand from the combination of 3 different cultivars included IR13146-158-1, IR15314-43-2-3-3, and BKN6995-16-1-1-2 since 1982. Chainat 1 was produced with the aim of increasing yield from the regular cultivars. It responses well to N fertilizer and can be gained high production, tolerates well to ragged stunt disease and rice blast as well as bear to brown planthopper which results in fewer pesticides usage. It is classified as non-sensitive photoperiod, lowland, and off-season rice; thus, it can grow all year using irrigation.

Price of paddy rice according to the latest announcement from Thailand Rice Department in 2014, rice which is non-sensitive to the photoperiod, including Chainat 1 were the cheapest one ranging from 7,500 – 8,000 baht/ton while Jasmine rice cost about 10,000 – 12,000 baht/ton. Moreover, pigmented rice such as Sangyod Phattalung rice cost about 20,000 – 25,000 baht/ton (Rice Department, 2014). Additionally, the quantity of Chainat 1 left over in Thailand rice research centers' warehouse was the lowest compared with others in the same group such as Pathumthani 80, Phitsanulok 2, Kor Khor 41, Kor Khor 49 (Rice Department, 2015) which possibly means that Chainat 1, at the present is less produced and not favored by farmers. This fact may cause by its hard texture, crumbling and less flavor when cooked which may not attractive to consumers who mostly prefer soft and fragrance rice (Kongseree *et al.*, 1999). Thus, less demand from consumers also influencing in less production of this rice cultivars. Therefore, an introducing of Chainat 1 rice cultivar to produce some other products rather than eating as grains is interesting to retain this Thai traditional rice cultivar for the next generations. Moreover, the fact that Chainat 1 is modified to gain higher yield may be a predictable point to contain some extra bioactive compounds in plants and further benefit to the products produced from their shoots.

Chainat 1 rice was reported to rich in bioactive compounds including phenolic compounds such as ferulic acid, *p*-cresol, syringic acid, caffeic acid, gallic acid,

p-hydroxybenzoic acid and vanillic acid while ferulic acid and syringic acid were the major phenolic compounds found (Vichapong *et al.*, 2010). Phenolic compounds content of Chainat 1 rice determined by HPLC is shown in Table 2. In addition, it has been reported that Chainat 1 rice contained the total tocopherol content as 0.02 μg/g rice seed which higher than RD6 and RD31 variety. Moreover, high level of total γ-oryzanol content approximately 17 μg/g rice seeds which were higher than some rice varieties including RD31, Chainat 2 and Sangyod Phatthalung were also claimed on its high nutritive values (Yodpitak *et al.*, 2013).

Table 2. Phenolic compound profiles determined by HPLC technique in Chainat 1 rice

Phenolic compounds Content	**(mg/100 g dry weight)**
Gallic acid	0.20
Protocatechuic acid	0.05
p-Hydroxybenzoic acid	0.14
Vanillic acid	0.08
Caffeic acid	0.22
Syringic acid	0.49
p-Coumaric acid	0.28
Ferulic acid	0.89
Guaiacol	0.14
p-Cresol	0.80
o-Cresol	0.19
3,5-Xylenol	0.18

Source: Vichapong *et al*. (2010)

1.2.6 Ricegrass

Similarly, rice is in the grass family as wheat. With a lot of rice supply in Thailand and rice seeds are also being distributed in the lower cost than wheat seed for

approximately 6 times. Therefore, it can be suggested being a suitable ingredient to produce as ricegrass correspondingly to wheatgrass. Moreover, there was a study on Thai rice research stated that rice seedling or in another word young rice plant, rice shoot, rice leaves or can be called as "ricegrass" (Figure 7) also contains chlorophyll and phenolic constituents as well as some compounds responsible for the antioxidant activities.

Figure 7. Ricegrass

A study by Khumkha (2009) in the nutritional contents of ricegrass and wheatgrass has been stated that ricegrass contains vitamin B1, vitamin C, phenolic compounds, chlorophyll, carotenoids in a lesser extent than wheatgrass. However, the level of gamma-aminobutyric acid (GABA) in ricegrass is higher (3.07 mg/100 g) compare with wheatgrass (0. 76 mg/ 100g) . Also, the experiment in comparison of nutritional values among the extracted juice from the shoot of wheat, Hommali 105, Supanburi 1, Hangtabtim, black glutinous rice as well as white glutinous rice, wheatgrass showed higher chlorophyll content as well Ca content than others. However, Mg and Fe content of wheatgrass were lower than Thai ricegrass. In addition, Hangtubtim showed the highest antioxidant activities (Benjawan *et al.*, 2011). There are only small numbers of study interested in the value of rice to produce as ricegrass while countless evidences attention on wheatgrass investigation for their phytochemical profiling as well as their possible use in the treatment of diseases. Owing to some evidence suggest on resembling of ricegrass and wheatgrass nutritional contents, ricegrass may be a good choice to introduce as well as further investigated on their phytochemical contents intensively, their biological activities and therapeutically used in the future.

1.2.7 Food fortification

Fortified food is one of the well-defined functional food products. The fortification of food stated by World Health Organization (WHO) is "The addition of one or more essential nutrients to a food for preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups" (Liyanage and Hettiarachchi, 2011). Fortified foods have more advantages over the supplementation of inorganic tablets or capsules from the simplicity of providing specific nutrients to foods that people normally consumed in daily life or the foods that already widespread in the market, therefore this may not extremely affect the cost of overall products that much and consumers do not need to change their regular behavior or eating habit to consume this product. According to consumer's sight, the fortified products are positively considered as more worthwhile and useful things than conventional products as well as harmless than those of supplements. If consumed regularly, fortified foods can maintain body stores of nutrients more efficiently and more effectively than supplements.

1.2.8 Bio-fortification

There are several types of food fortification separated by different fortify procedures. Among these fortification methods, bio-fortification is the most sustainable method by providing superior micronutrients to foods start from the staple crops, the first step of mostly food ingredients production. Bio-fortification may apply using the traditional breeding technology such as the use of micronutrients fertilizers in soil or solution or enhancement of micronutrients bioavailability by manipulating the level of pro-nutrients component or biotechnology which is the technique of modifying crop initially from gene levels (Kiremidjian-Schumacher *et al.*, 1996). Among these marvelous methods, the basic one, soil and foliar application are the most popular techniques because they can easily be used in practices by farmers and achieving satisfies outcomes. There are numerous advantages of this approach over other techniques. Firstly, staple foods predominate in the diets of the most population. Bio-fortification of micronutrients into crops can reach to the large population by utilizing their regular diet. Bio-fortification is

the techniques by using the plant as intermediates to convert inorganic nutrients and other additives into complex organic molecules within the plant issues. This process increased the efficiency of absorption by human digestive tracts through digestion of plant-based macromolecules by forming a food complex which human body can easily recognize. Therefore, the bio-fortification crop is better than the substitution of nutrients alone during food processing in term of bioavailability. Another incredible profit from fortified plants is nutritional improvement varieties among various plants which further improve the precise and valuable of plants. Studies on minerals bio-fortified to plants which can improve some of the bioactive compounds and their biological properties have been widely investigated. Moreover, bio-fortification may increase farm productivity by increasing yield and the rate of growing rapidly in some plant products and possibly help in resisting some diseases and environmental stressors (Hoffmann and Berry, 2008). Therefore, bio-fortification of micronutrients into the plant is a fascinating approach to promoting well-being health to the population. Based on previous works on bio-fortifying several minerals into plants, iron (Fe), iodine (I) and zinc (Zn) are the target one because of the most population around the world are suffered mainly from those minerals deficiency. Other elements such as calcium (Ca), copper (Cu) and selenium (Se) are lacked from food sources only in some areas; therefore, less consideration has been concerned on bio-fortifying these minerals. Among all minerals, Se has been known as a greatest antioxidant element by being involved in many endogenously antioxidant enzymes (McDowell *et al.*, 2007). Antioxidants are the initial step of protecting the cells against damage, thus Se could promote many biological activities and benefits to human's health. For that reason, it is one of a good candidate to apply with bio-fortification technique.

1.2.9 Selenium

1.2.9.1 Selenium chemistry

Selenium (Se) is an essential trace element which classified as a metalloid, with properties of both metals and non-metals. The chemical and physical properties of Se are comparable to sulfur (S) since they both have similar atomic size, bond-energies, ionization potentials, electron affinities as well as outer valence shell electron

configurations (Javasit, 2010). Se can exist as elemental Se (Se^0), selenide (Se^{2-}), selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}). Commercial available forms of Se are H_2Se, H_2SeO_3, H_2SeO_4, Na_2SeO_3, Na_2SeO_4, $SeCl_2$, and SeF_4 (Rayman, 2000).

1.2.9.2 Role of selenium

Se is an essential trace mineral required by a human that is reported to have the main role in the antioxidant system, it is thought to have various benefits on human's health. In human's body, Se is incorporated with proteins mostly cysteine and methionine to form "seleno-proteins" (Figure 8) which play a vital role in preventing health risk, they cooperate the function in the reduction of DNA damage and oxidative stress (Weeks *et al.*, 2012). The chief seleno- enzyme which works on antioxidant mechanisms is called glutathione peroxidase (GPx). Glutathione peroxidase is a primary antioxidant enzyme which has the main function in converting hydrogen peroxide (H_2O_2) which is one of the reactive oxygen species (ROS) molecules into nontoxic H_2O before it can produce damaging free radicals like hydroxyl radicals (OH·), therefore it can stop the occurrence of chain reaction which destroys the cell membrane. The step of antioxidant mechanism showed in Figure 9. In accordance to the GPx, Se is also a part of other seleno- proteins which have various functional roles in the human body. The lists of known seleno-proteins and their functions showed in Table 3.

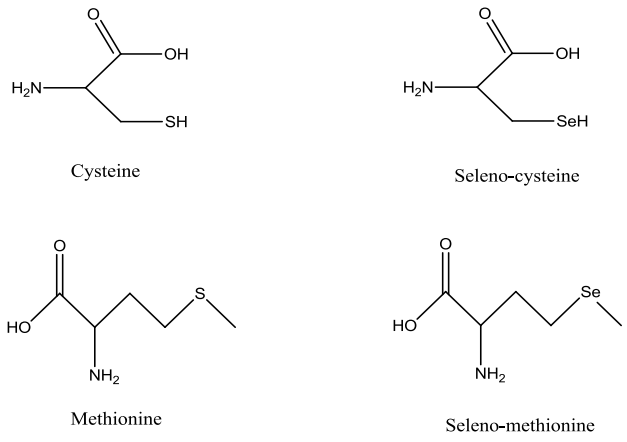

Figure 8. The structure of cysteine, methionine, selenocysteine, and seleno-methionine

Source: Rayman (2005)

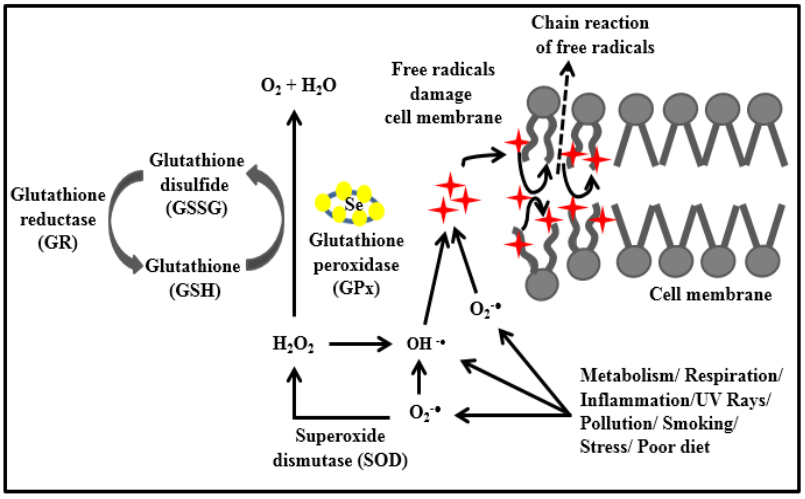

Figure 9. The role of glutathione peroxidase (GPx) in the antioxidant system

Source: Christensen (2006)

Table 3. Functional properties of known seleno-proteins

Seleno-protein	Function
Glutathione peroxidases (GPx) GPx1 (cytosolic) GPx2 (gastrointestinal) GPx3 (plasma) GPx4 (phospholipid)	Antioxidant enzymes: convert hydrogen peroxide, lipid and phospholipid peroxides into the water which stop the propagation of further oxidative damage to biomolecules such as lipids, lipoproteins, and DNA
Thioredoxin reductases (TrxR)	Required for DNA synthesis; maintenance the intracellular redox state and regulate gene expression
Iodothyronine deiodinases (IDI)	Required for the production and regulation level of active thyroid hormone (T_3, reverse T_3 and T_4)
Selenoprotein P (SEPP1)	A major contributor to plasma Se, transports Se from the liver via the plasma
Selenoprotein S (SEPS1)	Important for anti- inflammatory properties, protect the cell from stress- induced apoptosis, linked to glucose metabolism and insulin sensitivity

Seleno-protein	Function
Selenoprotein W	Needed for muscle function
Selenophosphate synthetase	Required for biosynthesis of selenoprotein
15kDa selenoprotein (Prostate Epithelial)	Protected secretory cells against the development of carcinoma
18 kDa selenoprotein	Important selenoprotein, found in kidney and a large number of other tissues.
34 kDa selenoprotein (DNA-bound spermatid)	Glutathione peroxidase- like activity which protects the developing sperm

Source: Rayman (2000)

1.2.9.3 Selenium intakes and deficiency

Se intake recommendation varies enormously worldwide depends on Se content in each geographic area (Thomson, 2004). If rely on the data from the US, Se intake recommended for healthy men and women is approximately 55 µg daily (Food and Nutrition Board, 2000) as well as in Europe (Scientific Committee for Food, 1993) but higher in Australia at about 70-85 µg per day due to incidence of deficiency in some areas (Truswell *et al.*, 1990). However, the possible amount which states to prevent some of the chronic diseases is required not only optimal but extra. Evidence from the US Nutritional Prevention of Cancer (NPC) suggested for the supplement of Se above recommended level (200 µg) possess as an anti-cancer effect (Duffield-Lillico *et al.*, 2003). Low consumption of Se leads to Se deficiency which causes an illness called Keshan disease, the myocardial necrosis that finally results in the weakening of the heart. Keshan disease is found mostly in China and Russia (Rayman, 2008). Se deficiency also causes the symptoms hypothyroidism including fatigue, mental slowing, and miscarriages (van Rij *et al.*, 1979). However, Se intakes exceed to 400 µg daily can be toxic to human and leads to the

symptom called selenosis which the condition includes garlic odor breathing, gastrointestinal disorders, hair loss and neurological injury (Hamilton, 2004). Thus, appropriate Se nutrition is important in maintaining superior health. The Dietary Reference Intakes (DRI) of Se at each age of life showed in Table 4.

Table 4. Dietary Reference Intake (DRI) of selenium in each group of ages

Group	DRI
Children age 1-3	20 µg/day
Children age 4-8	30 µg/day
Children age 9-13	40 µg/day
Children age 14- adults	55 µg/day
Pregnant women	60 µg/day
Breastfeeding women	70 µg/day

Source: Food and Nutrition Board (2000)

1.2.9.4 Health benefits of selenium

Se is concerned to participate roles in human health as claims by the evidence of large prospective study in the US (Bleys *et al.*, 2008), the beneficial effect of high Se status is stated over the mortality rate. Serum Se levels in total 13,887 representative US adult participants in the Third National Health and Nutrition Examination Survey were measured and followed up for mortality up to 12 years. Results showed the association between serum Se levels and all-cause mortality. An increase of serum Se levels up to 135 µg/L were associated with decreased mortality. However, higher serum Se levels may be associated with increased mortality.

a. Anti-cancer properties

Along with plenty of clinical studies related to health benefits of Se, most of the researchers emphasized on the powerful effect of Se as anti-cancer substance. Interventions on Se have shown benefit in reducing the risk of cancer incidence and

mortality in all-cause of cancers (Rayman, 2005). For example, Se supplementation at dose 200 µg showed 63% reduction in the incidence of prostate cancer in at-risk men over 10 years' period (Clark *et al.*, 1998). This may possibly due to the mechanism of Se in diverse function included the reduction of DNA damage (Waters *et al.*, 2005), reduction of oxidative stress, suppress the inflammation (Rayman, 2000), enhancement the response of immune system by increasing the numbers of cytotoxic lymphocytes and natural killer cells (Kiremidjian-Schumacher *et al.*, 1996). Besides, nutrigenetic researchers found that seleno-methionine, organic Se can activate tumor suppressor protein p53 gene which can inhibit the proliferation and induce the early apoptosis of cancer cells (Smith *et al.*, 2003).

b. Boosts immunity

Se has been pronounced for the immunity stimulant effects according to its highly potential as an antioxidant which can regulate the enemies in the body like reactive oxygen species (ROS) (Hoffmann and Berry, 2008). The belief that Se can boost the immune system has also been supported by studies involving aging immunity (Ventura *et al.*, 1993). In addition, Se may enhance the number of T lymphocytes (T-cells) which play role in cell-mediated immunity according to the experiment that 400 µg Se supplements as Se-yeast has been administrated to elderly participants in Arizona and can significantly increase 27% of total T-cells compared with the placebo (Wood *et al.*, 2000). In addition, results from 200 µg sodium selenite used during surgery led to significant enhance cell-mediated immune responsiveness while the placebo showed reverse results (Kiremidjian-Schumacher *et al.*, 2000).

c. Anti-cardiovascular diseases

The research whether Se participates in the role of cardiovascular disease protection remains inconclusive. Although a meta-analysis of 25 observational studies by Flores-Mateo *et al.* (2006) showed a significant inverse effect association between Se status and risk of coronary heart diseases, some of the studies have not observed the similar results (Salvini *et al.*, 1995). Therefore, the upcoming interventions which are large and reliable studies for this aspect still need to run further. However, the possible reasons to support

the effects of the anti-vascular disease of Se in some intervention might due to the prevention of lipid peroxidation which can further inhibit the platelet aggregation and reduce overall inflammation of blood vessel.

d. Thyroid regulation and anti-autoimmune thyroid diseases

Se has various roles in thyroid glands. One of the seleno-enzymes called iodothyronine deiodinase (IDI) is the main enzyme producing the active thyroid hormones T_3 and T_4 which is needed to regulate the normal function of this gland. Moreover, Se in form of GPx3 also acts as the protector of thyroid cells from hydrogen peroxide. Se supplementation also showed the helpful effect against Hashimoto's thyroiditis which is the most common form of autoimmune thyroid diseases (Toulis *et al.*, 2010) as well as Graves' disease, the autoimmune hyperthyroidism (Marcocci *et al.*, 2011).

e. Improve brain function

Se has a responsibility in the brain function. Selenoprotein P (SEPP1) is important for the neuroprotective task by enhancing neuronal survival and preventing apoptotic cell death (Burk and Hill, 2009). Lower serum of Se was observed in the children with epileptic seizures and febrile seizures (Ashrafi *et al.*, 2007). For adults, low Se status is reported to be involved with an increased risk of cognitive decline in elderly 60-70 years from the EVA study (Marcocci *et al.*, 2011). Besides, data from the cross-sectional study showed that low nail Se concentration of 2,000 Chinese elderly was associated with a low cognitive score in the survey test (Gao *et al.*, 2007).

f. Anti-dote properties

Se has been stated as a good antidote agent to a range of heavy metal toxicities including mercury (Hg), cadmium (Cd) and silver (Ag) (Mukherjee *et al.*, 1988). The protective effect of sodium selenite against methylmercury (MeHg) neurotoxicity in the brain of rat confirmed this fact. In the group of rats treated with MeHg+Se, the nerve fiber damage was significantly reduced compared with the group treated with MeHg alone and no other neuronal change was reported (Chang, 1983). For the antidote of Cd by Se,

there is a study in pregnant hamster investigate the protective effect of Se against cadmium toxicity by comparing the treatment of Cd alone and Cd+Se. The result showed that in the group of Cd+Se treatment can reduce Cd toxicity symptoms includes lower embryo size and foetotoxic indices (Włodarczyk *et al.*, 2000). Moreover, Se cooperates with Zn has been confirmed for the defense against Cd in a liver cell of rat (Imed *et al.*, 2008). Because of Se is the main ingredient of glutathione peroxidase (GPx) which protect the organisms against a certain type of damage. Role of GPx thus can possibly explain the effect of Se in the detoxifying of these heavy metals (Bjørklund, 2015).

1.2.9.5 Selenium in plants

The issue whether Se is a necessary micronutrient required by plants or not remains ambiguous (Kamboj, 2000). However, the role of Se as a beneficial element in plants that can accumulate some other compounds in plants has been considered. Se concentration in different species of plant is high variation. Uptake and accumulation of Se depend on factors included affinity of species to absorb and metabolize Se as well as form and concentration of Se used (Rayman, 2012).

Three different groups of plant separated by the ability to uptake Se in plant tissues are non-accumulator, secondary accumulator and primary accumulator (Bleys *et al.*, 2008). Most of the crops such as fruits and vegetables are classified as non-accumulator plant species which accumulate < 25 mg Se/kg dry weight. The second group is facultative or secondary absorbers such as plants belong to species *Aster*, *Grindelia* and *Mentzelia* can accumulate Se from 25 to 100 mg Se/kg dry weight and the last group, Se accumulator plant species can accumulate from 100 to 10,000 mg Se/kg dry weight. This group includes plants in *Astralagus* species which is a member of Fabaceae genus as well as *Stanleya*, a member of Brassicaceae. Therefore, Se accumulator plant type is considered causing selenosis, or Se toxicity to animals that are herbivore.

Se is primarily taken up from the soil by plants as an inorganic form which is selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}) or as organic compounds such as the seleno-amino acid (Ellis and Salt, 2003). Se incorporates into plant proteins, in the form of seleno-cysteine or seleno-methionine at usual levels which are the organic form of Se. Selenate is

first reduced to selenite, which further reduced to selenide. Selenide is then transformed to seleno-cysteine in a similar way to that of sulfur via sulfate transporter in the root plasma membrane. According to close chemical and physical properties of Se and S, their uptake route is similar. Uptake of SeO_4^{2-} and SO_4^{2-} is controlled by the same carrier with similar affinity for both ions. Therefore, both atoms are a competitive candidate for the same binding sites and showed the antagonistic relationship (Clark *et al.*, 1998). Thereafter, seleno-cysteine is metabolized to seleno-methionine then further metabolized to seleno-methyl selenocysteine.

In addition, plants also have the biochemical processes which can get rid of excess Se to volatile Se compounds in the form of dimethyl-selenide (DMSe) which is less toxic than inorganic Se species (Ganther *et al.*, 1966). This process is called "Volatilization" or recognized as a detoxification process (McChesney *et al.*, 2007). A schematic of Se metabolism in plants is shown in Figure 10. Plants are therefore beneficial to use as the intermediate to process inorganic forms of Se into organic forms which are more bio-available in the human body and can be supplementary use as a safe source of Se food supplement (Hartikainen, 2005).

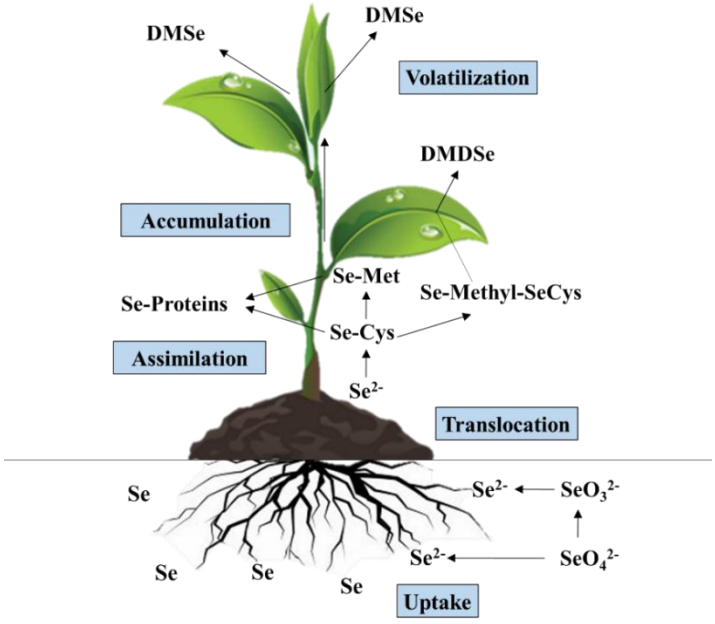

Figure 10. Schematic of Se metabolism in plants

Source: Dumont *et al.* (2006)

1.2.10 Selenium biofortification into plants

The bio-fortification of micronutrients to plant primarily aims to relief nutrients deficiency problem. While Se deficiency occurred in some parts of the world, the biofortification of this mineral also prior to supplying enough Se to the population. However, the by-products from supplying this mineral into the plant have also been observed widely and becoming more interesting nowadays. Universal studies were visibly claimed for the benefits of Se biofortification in plants for an improvement in the content of phytochemical substances, biological properties, as well as effects in preventing some of the diseases. The uptake and accumulation of Se in plants are firstly related to the type of plant which can accept Se amount differently. Moreover, the variable also depends on the concentration of Se in the soil where plants have been grown as well as the chemical form of Se (Rayman, 2008). Thus, the consistent assessment of Se fortified in each plant sources is essential.

1.2.10.1 A form of selenium use

Sodium salts are the usual form which is used for the bio-fortifying purpose. Sodium selenate and sodium selenite are both inorganic chemical forms of Se which are dominantly found in soil. Therefore, plants were mainly taken up Se from these two forms (Ellis and Salt, 2003). Selenate is more stable, highly absorbable by plants for approximately 50-80%. It is highly mobile in xylem transport into shoot but little was integrated to organic form (Li *et al.*, 2008). Moreover, it is less soluble in water and the cost is rather high. While selenite is white, completely water-soluble compounds which the absorption is about 50%. Even selenite is less absorbable by plants, there is evidence suggested that selenite fortification can be further transformed rapidly to a larger amount of organic Se than fortified with selenate (Lavu *et al.*, 2013). Furthermore, the price is reasonable to use as the source of biofortification to plants. Plants can also uptake the organic Se compounds in the form of selenomethionine, which is less available in the soil. Though it is well absorbed by plants, the price is high and too much of Se absorption by the plant may cause toxicity to consumers.

1.2.10.2 Effect on plant phytochemicals and bioactivity

The biofortification of Se in various plants has been done mostly in Se accumulating plants such as garlic, onion, broccoli, and chives (Pyrzynska, 2009). The study from Ip *et al.* (2000) reported the effects of Se fortified in garlic on cancer chemoprevention in a rat model. Some studies also investigated Se fortified in broccoli to reduce the risk of cancer. According to the study which investigates the effect of Se enriched broccoli feed in rats (n=90) by Burk and Hill (2009), the result confirmed higher on prevention of precancerous lesions in the colon of rats. In the same way, Se bio-fortified to broccoli in another study has also been reported to increase the Se-methyl selenocysteine and glucosinolates which are both anti-cancer compounds (Ávila *et al.*, 2013). Moreover, there were also some studies in other kinds of plant which obtain satisfy outcomes such as sodium selenite fortified to spinach at 1 mg Se/L showed an enhancing in the chlorophyll a and b contents by 87 and 165 % while 10 mg/L showed the highest values of total phenolic compounds in spinach (Saffaryadi *et al.*, 2012). However, ha igher level of Se may exert toxic effects. The fortification of Se hydroponically in lettuce has also been investigated. Results explained that low concentration (1.264 mg/L) of Se favored the biomass growth and antioxidant activities as measured by rthe ate of SOD and CAT activities (Ramos *et al.*, 2010). In China, several studies conducted on the Se fortification in tea leaves. Se fortified green tea one showed an enhancing of the antioxidant activity rather than the regular one (Xu *et al.*, 2003). Moreover, Se fortified green tea has been stated by Molan *et al.* (2009) for the first time to have prebiotic effects by promoting gthe rowth of *Lactobacillus rhamnosus* and *Bifidobacterium breve* when added in MRS broth compared with the normal incubation. *Lycium chinense* tea leaves were also being stated that chlorophyll, carotenoid and chlorogenic acid content was increased as affected of sodium selenite biofortification (Dong *et al.*, 2013). Additionally, a total phenolic component of purple potatoes was also enhanced significantly (p=0.002) by Se during plantation (Lei *et al.*, 2014). Besides, ethe ffect of Se in increasing total phenolic contents can also be confirmed in mushroom, namely *Lentinula edodes* study (Turło *et al.*, 2010).

Likewise, Se has been fortified roughly to the plant family Poaceae or the grass family. Agronomic grasses which are grown for edible seeds are called cereals or grains such as wheat, barley, oat, rye, and rice. Though these plants were classified as Se medium accumulating species, they were recognized as the foremost dishes to supply energy and carbohydrate for entire world population as well as rich in protein, vitamins, and minerals more than any other kind of crops. Thus, a large portion of them can supply plenty of Se for population, especially for plant-based diet people. Rice is the most widely consumed grain in the world, especially in tropical areas such as Asia and Africa while wheat is the primary cereal for temperate zones. Therefore, based on review literature, bio-fortification on cereals always focus mainly on an increasing of Se content in the final grains to be a large source of Se which expect to primarily relief deficiency problem (Chen *et al.*, 2002b, Gupta *et al.*, 1993, Lyons *et al.*, 2005). Only a little work has mentioned on a development of their biological effects and less regard on some other stages of cereal plants development.

Various studies interested in an enrichment of Se to cereals and analyze for Se speciation after fortifying to investigate the Se species in final grains. A recent study by Wang *et al.* (2013) stated that 10.5 g of Se/hectare fortification can enhance rice grains Se content by up to 51 times with no toxic effects and specified that the major form of Se in rice grains was absorbable organic Se included seleno-methionine, methylseleno-cysteine, and selenocysteine. The same study patterns were also observed in wheat, agronomic Se fortification 100 g of Se/hectare showed benefits on Se grain concentration as well as highlighted on the transformation of inorganic compounds into seleno- methionine (Galinha *et al.*, 2014) which reflected the value of the plant as a good mediator. The effect of foliar and soil biofortification was also comparable in some studies for the effectiveness in providing Se into grains. The outcome showed an improvement in grain yield but non-significant different between these 2 methods, however soil biofortification into rice with sodium selenate demonstrated higher shoot dry matter and higher Se accumulation in grain (Boldrin *et al.*, 2013). Few studies which stated on biological effects of these grasses plant family while fortified with Se related to antioxidant properties. An increasing of *in vitro*

antioxidant capabilities of rice grain fortified with sodium selenite measured by DPPH, ABTS and AAPH method were observed (Wang *et al.*, 2013). Moreover, the study in ryegrass fortified with at 1 mg/kg Se as selenic acid displayed an inhibiting of lipid peroxidation by enhancing glutathione peroxidase level (GPx) activity but not with superoxide dismutase (SOD). However, the oxidative stress was found when fortified 10 mg/kg of Se and over which results in yield losses in ryegrass (Hartikainen *et al.*, 2000). Since the bio-fortification of Se into plants could result in an improvement of plant bioactivity which Se might play some role too. Thus, this study aims to investigate if the Se fortification can improve the total quality of ricegrass juice which could further benefits to the human consumption as a functional healthy drink.

1.3 Objectives

1. To compare the bioactive compounds contents and antioxidant activities of ricegrass juice and wheatgrass juice
2. To investigate the effect of different concentration of Se bio-fortification into ricegrass on growth characteristics, bioactive compounds content, Se content and antioxidant activities
3. To identify the main polyphenols components in ricegrass juice
4. To evaluate the effect of Se bio-fortified ricegrass juice on probiotic growth enhancer properties in *Lactobacillus plantarum* and anti-cancer properties in HT-29 colon cancer cells
5. To evaluate the effect of Se bio-fortified ricegrass juice on antioxidant activities and nitric oxide inhibition in RAW264.7 macrophage cells
6. To evaluate the effect of Se bio-fortified ricegrass juice on protective effect against Cd toxicity and DNA damage in HEK embryonic kidney cells

CHAPTER 2

INVESTIGATION OF PHYTOCHEMICAL CONSTITUENTS, PHENOLIC PROFILES AND ANTIOXIDANT ACTIVITIES OF RICEGRASS JUICE COMPARED WITH WHEATGRASS JUICE [1]

2.1 Abstract

Cereal grass has been brought attention as a new functional food. Wheatgrass juice was known as a superfood which provides lots of advantages for human health. However, juice from young ricegrass has not been widely known for consumption, though it is in the similar family to wheat (Poaceae). Accordingly, it should be introduced as an economical functional drink in Asian countries. However, there are only a few apparent evidence which stated on the nutritive values of ricegrass juice. The objective of this study was to investigate the chemical compositions, major phytochemical constituents, bioactive compounds content, specific phenolic profiles, and *in vitro* antioxidant activities of ricegrass juice compared with wheatgrass juice. Data revealed that both grass juice extracts exhibited the group of phenol, tannin, and saponin. Wheatgrass juice can be detected for a higher level of ascorbic acid and chlorophyll while ricegrass juice contained larger amounts of phenolic compounds content detected by HPLC. However, wheatgrass juice exposed slight higher antioxidant activities for all methods. This may propose the synergistic effects of ascorbic acid, chlorophyll, and phenolic compounds in wheatgrass juice, while the main composites exhibited antioxidant activities in ricegrass were phenolic compounds. Even if ricegrass juice gave less statistically ability on antioxidant activities, they still had comparable levels of antioxidant activities. With the reduction in the cost of raw materials and contribution of high nutritional values, ricegrass juice could be introduced as an antioxidant boosting drink in competition to wheatgrass juice.

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[1] The content of this chapter has been published in *Functional Food in Health and Diseases*

2.2 Introduction

Plants are important sources of natural remedies which have been used by human for ages since they contain many biologically active compounds, for example, polyphenols, vitamins, terpenes, organic acids, etc. that work vigorously against several kinds of diseases such as cancer, atherosclerosis, diabetes, and many more (Ahmad *et al.*, 2006). In the recent years, young cereal sprouts have been getting increasing attention as a new potent plant for consumption as vegetables because the sprouting stage is believed to be the stage that plants contain the highest content of readily available compounds such as amino acids, vitamins, and enzymes since after this stage plant starts to use these compounds to produce cellulose as a structure of growing stems (Seibold, 2008). Additionally, plants at young stage produce a high level of phytochemicals substances to protect themselves from danger and these compounds exert various biological benefits to human health. Therefore, the sprout is a fresh, simple, and full of nutrition vegetable, can be suggested as a good choice of healthy eating diet. Furthermore, the consumption of sprout juice is prominent, as it is a simple manner to supplement the powerful nutritive and advantageous compounds in small quantities.

Wheatgrass juice (*Triticum aestivum* L.) is the most popular consumed sprout juice in the world. It has been widely investigated for lots of pharmaceutical effects on human health, including blood builder agents for thalassemia, detoxifying agents, antioxidant substances, and possible use as anti-inflammatory and anti-cancer substances (Padalia *et al.*, 2010). However, wheat is mostly grown in the low-temperature zones include some parts of United States, Canada, and Europe. For Asian countries, rice (*Oryza sativa* L.) is the principal energy food source. Thus, there is the plentiful resource of rice which is a lower cost ingredient than wheat in this region. In the same manner, rice is one of the cereal plants in the grass family (Poaceae) similar to that of wheat, rye, and barley. Some literacies also reported on discovering phenolic compounds and some biological properties such as antioxidant properties and DNA protective properties in ricegrass juice (Khanthapoka *et al.*, 2015, Rattanapon *et al.*, 2016). Consequently, the introduction of the young ricegrass juice as an innovative functional drink is fascinating. Moreover, it can

offer the added value for some rice cultivars which their grains are low in price because of low palatability, hard texture, being less fragile, and lack of identity.

However, rice sprouts or ricegrass at a young stage still have not been widely consumed as food. There is no report to prove whether the number of bioactive molecules and biological properties of young ricegrass juice is in comparison to that of wheatgrass juice. Moreover, little is known about the type of phytochemical constituents as well as specific phenolic compounds found in both young ricegrass and wheatgrass. Therefore, this study aimed to investigate the chemical composition of fresh ricegrass and wheatgrass, as well as determine the type of phytochemical constituents, bioactive compound contents, the presence of specific phenolic compounds using HPLC and *in vitro* antioxidant activities of ricegrass juice compared with wheatgrass juice.

2.3 Materials and methods

2.3.1 Chemicals

Chemicals used for determination of chemical compositions, bioactive compounds, and antioxidant activity were purchased from Sigma (Germany)

2.3.2 Plant materials

Rice seeds (*Oryza sativa* L. cv. Chainat 1) obtained from Patthalung Rice Research Center, Thailand and wheat seeds (*Triticum aestivum* L. cv. Fang 60) were soaked in water for 24 h to stimulate the germination. After rinsing the water, seeds were placed in the plantation tray with moist and allowed to germinate for 48 h. Germinated seeds were grown hydroponically without any supporting materials and fertilization under natural light with a day/night average temperature of 32/25 ºC, relative humidity 65±8.5% in the partially closed system. Plants were watered twice a day and harvested at day 8.

2.3.3 Chemical compositions

The chemical composition of fresh ricegrass and wheatgrass included moisture, protein, fat, carbohydrate, ash, and fiber contents of samples were analyzed followed the method of AOAC (2000). Total sugar content was determined using phenol-sulfuric method (Dubois *et al.*, 1956). Total reducing sugar content was measured using

Nelson's method (Nelson, 1944). Glucose was used as the standard for both total sugar and total reducing sugar determination at the range 20-100 μg/ml.

2.3.4 Extraction procedure

Ricegrass and wheatgrass were extracted with water at a ratio of 1:2 (w/v) using juicer machine (Hurom DA-900) at room temperature. The sample was filtered with Whatman No. 4 filter paper to get rid of residues and followed by centrifugation at 10,000 x g for 20 min. Fresh juices were tested for some chemical analysis. The others were subjected to freeze-drying then stored at 4 ºC.

2.3.5 Phytochemical screening

Freeze dried powder of ricegrass and wheatgrass juice (10 mg/ml) were tested for the presence of numerous phytochemical constituents using the following precipitation and coloration methods (Harborne, 1973, Sofowora, 1993).

2.3.5.1 Alkaloid test

1 ml extract was added with 5 ml of 1% hydrochloric acid and heated on a steam bath at 60 ºC for 15 min. After filtration, 1 ml filtrate was separated into 3 tubes. 1 ml of Dragendorff's reagent, Mayer's reagent, and Wagner's reagent were added to each tube. The orange, yellow and turbid brown color precipitate indicated the presence of alkaloid.

2.3.5.2 Phenol and Tannin test

1 ml of the extract was added with 1 ml of 3% $FeCl_3$ was added. Formation of greenish black indicated the presence of hydrolyzed tannin including phenol or blue color indicated the presence of condensed tannin.

2.3.5.3 Terpenoid test

5 ml of the extract was mixed with 2 ml chloroform and carefully added with 3 ml concentrated H_2SO_4. A reddish-brown coloration between upper and lower layer revealed the presence of terpenoid.

2.3.5.4 Sterol test

2 ml of extract was added to 2 ml of concentrated H_2SO_4. Red precipitation indicated the presence of the steroidal ring.

2.3.5.5 Saponin test

2 ml of the extract was mixed with 5 ml of distilled water and shaken vigorously for 5 min. Persistence of foams for 30 min indicated the presence of saponin.

2.3.5.6 Coumarin test

The filter paper was soaked in 10% NaOH and hang upon the tube containing 5 ml of the extract and evaporated for 30 min in water bath. The soaked filter paper was examined after placing under UV light for 10 min. Fluorescence spot indicated the presence of coumarin.

2.3.6 Ascorbic acid content

The ascorbic acid content of grasses juice was analyzed using indo-phenol colorimetric method (AOAC, 2000). The juice was diluted with 2% meta-phosphoric acid to the appropriate concentration. 5 ml of diluted samples were added with 10 ml of 1.7 mM 2, 6 - dichloroindophenol solution, mixed well and measured for the absorbance using spectrophotometer at 518 nm. Ascorbic acid (0-400 μg/ml) was used as a standard.

2.3.7 Chlorophyll and carotenoid content

The total chlorophyll, chlorophyll a (C_a) and chlorophyll b (C_b) were analyzed using the defined method of Arnon (1949). Carotenoid was calculated using Lichtenthaler and Wellburn (1983) equation. Briefly, 0.5 to 1 ml of grasses juice were made up of 80% acetone to the volume of 10 ml. The mixtures were then centrifuged at 5000xg for 2 min. The absorbance of supernatants was measured with a spectrophotometer at 470, 645, 663 nm. The concentration of pigments was calculated from the following equations when A is absorbance (nm):

Total chlorophyll = $20.2\ (A_{645}) + 8.02\ (A_{663})$

Chlorophyll a (C_a) = $12.7\ (A_{663}) + 2.69\ (A_{645})$

Chlorophyll b (C_b) = $22.9\ (A_{645}) – 4.68\ (A_{663})$

Carotenoid = $(1000(A_{470}) - 3.27C_a - 104C_b)/229$

2.3.8 Total extractable phenolic content

The total extractable phenolic content of the extracts was measured using a Folin-Ciocalteu method from Singleton and Rossi (1965). Concisely, 20 µl of the extract was added to 96-well plate. Next, 100 µl of Folin-Ciocalteu reagent (10% v/v) and 80 µl of Na_2CO_3 (7.5% w/v) were added and mixed thoroughly. After incubation for 30 min in the dark at ambient temperature, the absorbance was measured at 765 nm using the microplate reader. The total phenolic content was expressed as mg of pyrogallol equivalent (PYE)/g extract (20-80 µg/ml).

2.3.9 HPLC analysis

Both aqueous ricegrass and wheatgrass extract were acidic hydrolyzed using 6M HCl at 70ºC for 3 hours before the analysis. HPLC-DAD investigation was performed on a 1200 Agilent technologies HPLC system (Germany). Reversed-phase chromatographic analyses were carried out under gradient conditions using a C-18 column (250 mm × 4.6 mm) packed with 5 µm diameter particles. The phenolic profiles were carried out using HPLC water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B). The elution profile had the following proportion of acetonitrile (v/v): 0.00–10.00 min, 0%–10.0%; 10.00–15.00 min, 10.0%; 15.00–20.00 min, 10.0%–15.0%; 20.00–30.00 min, 15.0%–25.0%; 30.00–35.00 min, 25.0%; 35.00–50.00 min; 25.0%–0%. The flow rate was 0.8 ml/min at the temperature 40 ºC with the injection volume of 20 µl. Identification of phenolics was performed by comparing retention times with those of standards. Stock solutions of pyrogallol, protocatechuic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, coumaric acid, and ferulic acid were prepared and run for a linear equation. Quantification was performed by peak integration.

2.3.10 Antioxidant activities

2.3.10.1 DPPH radical scavenging activity

2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity was determined using the modified method of Brand-Williams *et al.* (1995). Briefly, 150 µl of the extract was added with 150 µl of 0.2 mM DPPH in 95% ethanol. The mixture was mixed and stand for 30 min in the dark. The absorbance was determined at 517 nm using the microplate reader. Standard curves Trolox with concentration 1-10 µg/ml were prepared, respectively. The activity was reported as mg Trolox equivalent (TE)/g extract.

2.3.10.2 ABTS radical scavenging activity

2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay was determined to follow the method of Arnao *et al.* (2001). The stock solutions of 7.4 mM ABTS solution and 2.5 mM $K_2S_2O_8$ solution were prepared. The ABTS radical solution was generated by mixing both stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The radical solution was diluted to obtain an absorbance of 1.1±0.02 at 734 nm before performing the assay. 15 µl of extract was mixed with 285 µl of ABTS radical solution and left at room temperature for 2 h in the dark. The absorbance was measured at 734 nm using the microplate reader. Standard curves of Trolox with concentration 10-100 µg/ml were prepared, respectively. The activity was reported as mg Trolox equivalent (TE)/g extract.

2.3.10.3 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was performed according to a method of Benzie and Strain (1996). FRAP solution was freshly prepared from the mixture of 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution at the ratio 10:1:1 (v/v/v) and then warmed at 37ºC for 30 min before use. 15 µl extract was added with 285 µl of FRAP solution and stand for 30 min in the dark. The absorbance was read at 593 nm using microplate reader. Standard curves Trolox with concentration 10-100 µg/ml were prepared, respectively. The activity was reported as mg Trolox equivalent (TE)/g extract.

2.3.10.4 Ferrous ion chelating activity

Ferrous ion chelating activity was determined using the method of Ebrahimzadeh *et al.* (2009). 277.5 µl of extract was mixed with 7.5 µl of 2 mM $FeCl_2$ and 15 µl of 5 mM ferrozine for 10 minutes at room temperature. The absorbance was read at 562 nm using microplate reader. A standard curve of EDTA at concentration 20-40 µg/ml was prepared. The activity was expressed as mg EDTA equivalent (EDTAE)/g extract.

2.3.11 Sensory acceptability

Ricegrass juice and wheatgrass juice were subjected to sensory evaluation employing the attributes of appearance, color, odor, taste and overall with a 9-point hedonic scale from 1 = disliked much to 9 = liked much. The samples were served in random order for 50 panelists consisting of scientists and students in Faculty of Agro-Industry, Prince of Songkla University who familiar with drinking vegetable juice.

2.3.12 Statistical analysis

All data were subjected to the statistical analysis using paired sample t-test. Statistical analyses were carried out using the SPSS statistical software (SPSS, Inc., Chicago. IL).

2.4 Results and discussion

2.4.1 Chemical compositions

The chemical compositions of ricegrass and wheatgrass are presented in Table 5. Significant differences ($p< 0.05$) in crude protein, fat, carbohydrate, ash and sugar contents were observed among these two plants. The difference may attribute to the genetic variation in each plant (Kantety *et al.*, 2002). Wheatgrass contained a higher level of protein, fat, total sugar and reducing sugar content than ricegrass while ricegrass had a higher level of carbohydrates. While the percentage of crude fiber which was accounted for a part of carbohydrate did not significantly different ($p<0.05$). Therefore, it was predicted that ricegrass mainly composed of carbohydrate in the form of starch. In addition, the observation of both plant physical characteristics revealed that ricegrass leaves were more rigid and tough rather than wheatgrass leaves may due to the accumulation of higher

insoluble fiber such as cellulose, hemicellulose, and lignin. Ash percentage of ricegrass appeared slightly higher than wheatgrass which may build up from a large number of minerals in rice pericarp.

Table 5. Proximate composition of fresh ricegrass and wheatgrass

Composition	Unit	Ricegrass	Wheatgrass
Moisture content	% FW	77.65 ± 0.16 [b]	85.28 ± 0.30 [a]
Crude protein	% DW	23.27 ± 0.52 [b]	30.73 ± 0.62 [a]
Crude fat	% DW	9.91 ± 0.60 [b]	14.53 ± 0.74 [a]
Ash	% DW	5.58 ± 0.72 [a]	4.49 ± 0.12 [b]
Total carbohydrate	% DW	61.24 ± 0.65 [a]	50.25 ± 0.57 [b]
Crude fiber	% DW	22.91 ± 0.59 [a]	22.42 ± 0.66 [a]
Total sugar	mg GluE/ g FW	8.48 ± 0.09 [b]	15.34 ± 0.07 [a]
Reducing sugar	mg GluE/ g FW	5.30 ± 0.05 [b]	9.67 ± 0.04 [a]

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same row indicated significant differences ($p< 0.05$). DW: dry weight. FW: fresh weight. GluE: glucose equivalent.

2.4.2 Phytochemical screening

The qualitative phytochemical screening of ricegrass and wheatgrass juice was performed on 7 groups of compounds (Table 6). It was found that both ricegrass and wheatgrass juice extract contained the group of phenol, tannin, and saponin but not alkaloid, terpenoid, sterol, and coumarin. Results indicated the presence of similar phytochemical groups in both kinds of grass. As the juices were extracted using water which is high polarity solvent, most of the compounds dissolved in the juice were hydrophilic molecules. It has been reported earlier that tannin, soluble phenolic compounds and saponin were presented in the solution using water as solvent (Tiwari *et al.*, 2011). Though some of less polar compounds may be partially extracted from the mechanical forces while juicing, only small number of compounds cannot be detected from those

coloration and precipitation test. Since these procedures require a large number of compounds to exhibit positive results. Results in this study were not consistent to the study of Ashok (2011) who reported that 8 days grown wheatgrass juice contained alkaloid as well as another study from Durairaj *et al.* (2014) who stated that alkaloid, flavonoid, and terpenoid were found in the juice produced from dried powder wheatgrass. This may due to several reasons such as raw material preparation, extraction method, age, and variety of plant, plantation condition, fertilizer, and irrigation.

Table 6. Phytochemical screening of aqueous extracted ricegrass and wheatgrass

Constituent	Ricegrass juice	Wheatgrass juice
Alkaloid	-	-
Phenol & Tannin	+	+
Terpenoid	-	-
Sterol	-	-
Saponin	+	+
Coumarins	-	-

Remark: (+): Positive, (-): Negative

2.4.3 Bioactive compounds content

The content of bioactive compounds found in ricegrass compared with wheatgrass is shown in Table 7. Wheatgrass contained almost double level of total chlorophyll, chlorophyll a and chlorophyll b to ricegrass. This was supported by a large number of amino acids present in wheat seeds. As known that amino acids play a major role in chlorophyll biosynthesis (Yaronskaya *et al.*, 2006). High level of chlorophyll which exhibited dark green color to wheatgrass was claimed to possess outstanding benefits as a blood builder agent due to the structure of chlorophyll was closely related to heme molecules in the blood (Padalia *et al.*, 2010). While ricegrass proposed their color as light green- yellow which may due to the enclosing in a lower amount of chlorophyll and containing a higher number of carotenoids. In addition, accumulation of higher chlorophyll found in wheatgrass may due to it is a plant normally grown in the dry and temperate zone

with lower light intensity, thus it possibly need to produce the condensed amount of pigments to absorb light to supply sufficient energy for the survival while ricegrass was faced with higher light intensity.Wheatgrass juice comprised twice amount of ascorbic acid (vitamin C) to ricegrass juice may relate to higher accumulation of sugar which was a substrate to produce ascorbic acid. The total extractable phenolic content (TPC) of both kinds of grass were studied using Folin-Ciocalteu method. Wheatgrass exhibited a marginally higher level of TPC expressed as mg pyrogallol equivalent/g extract. However, it was found that this method was the nonspecifically reaction to phenolic molecules. There were some scientific data pointed out that reducing sugars, some compounds such as ascorbic acid, amino acids, organic acids and Fe (II) molecules can interfere the Folin-Ciocalteu analysis and drawback to the overestimation of phenolic compounds (Berker *et al.*, 2013). Due to a great amount of reducing sugar and ascorbic acid in wheatgrass, the level of TPC may not indicate the actual number of phenolic molecules in the samples. Thus, the study was subjected to further analyze for the specific compounds using HPLC.

Table 7. Bioactive compounds content of ricegrass and wheatgrass

	Unit	**Ricegrass**	**Wheatgrass**
Total chlorophyll	mg/g DW	0.911 ± 0.011^{b}	1.804 ± 0.105^{a}
Chlorophyll a	mg/g DW	0.674 ± 0.007^{b}	1.363 ± 0.080^{a}
Chlorophyll b	mg/g DW	0.379 ± 0.021^{b}	0.715 ± 0.042^{a}
Carotenoids	mg/g DW	0.073 ± 0.014^{a}	0.050 ± 0.014^{b}
Ascorbic acid	mg/ g DW	0.189 ± 0.001^{b}	0.400 ± 0.017^{a}
TPC	mg PYE/ g extract	31.44 ± 0.44^{b}	33.74 ± 0.34^{a}

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same row indicated significant differences ($p < 0.05$). DW: dry weight. PYE: pyrogallol equivalent. TPC: Total extractable phenolic content.

2.4.4 HPLC qualitative and quantitative of phenolic compound profile

The chromatogram of phenolic compounds detected in both ricegrass juice and wheatgrass juice extracts compared with nine external standards are shown in Figure 11. According to the HPLC qualitative results, four similar phenolics compounds noticed in both ricegrass and wheatgrass juices were pyrogallol, vanillic acid, syringic acid, and ferulic acid. In addition, the pattern of other unknown compounds (UN) in both kinds of grass was mostly alike. However, the type of foremost phenolic compounds found was still unknown (UN1-UN4). To sum up, the majority phenolic compounds found in both grass juices were phenolic acids while flavonoid compounds were authorized not to notice using this specifical method (data not shown). The similar in the types of compounds of both plants may attribute to the similar family which plants belong to, accordingly containing assemble substrates in the seeds and generate closely related molecules.

The quantitative of specific phenolic compounds of the juice from ricegrass and wheatgrass detected by HPLC-DAD are shown in Table 8. Identified ricegrass juice extract was significantly ($p< 0.05$) higher level of pyrogallol, vanillic acid and ferulic acid while wheatgrass juice extract contained a greater amount of syringic acid. Moreover, the prominent unknown compounds (UN1-UN4) found in ricegrass juice were significantly observed for the greater peak area rather than wheatgrass juice which indicated the huge amount of existing phenolics. Thus, it can be concluded that ricegrass juice enclosed with the outstanding content of phenolic acids exceeding to wheatgrass. When related to TPC data, it can be confirmed that TPC data alone may lead to untruthful interpretation on phenolic compounds content which significantly related to antioxidant activity.

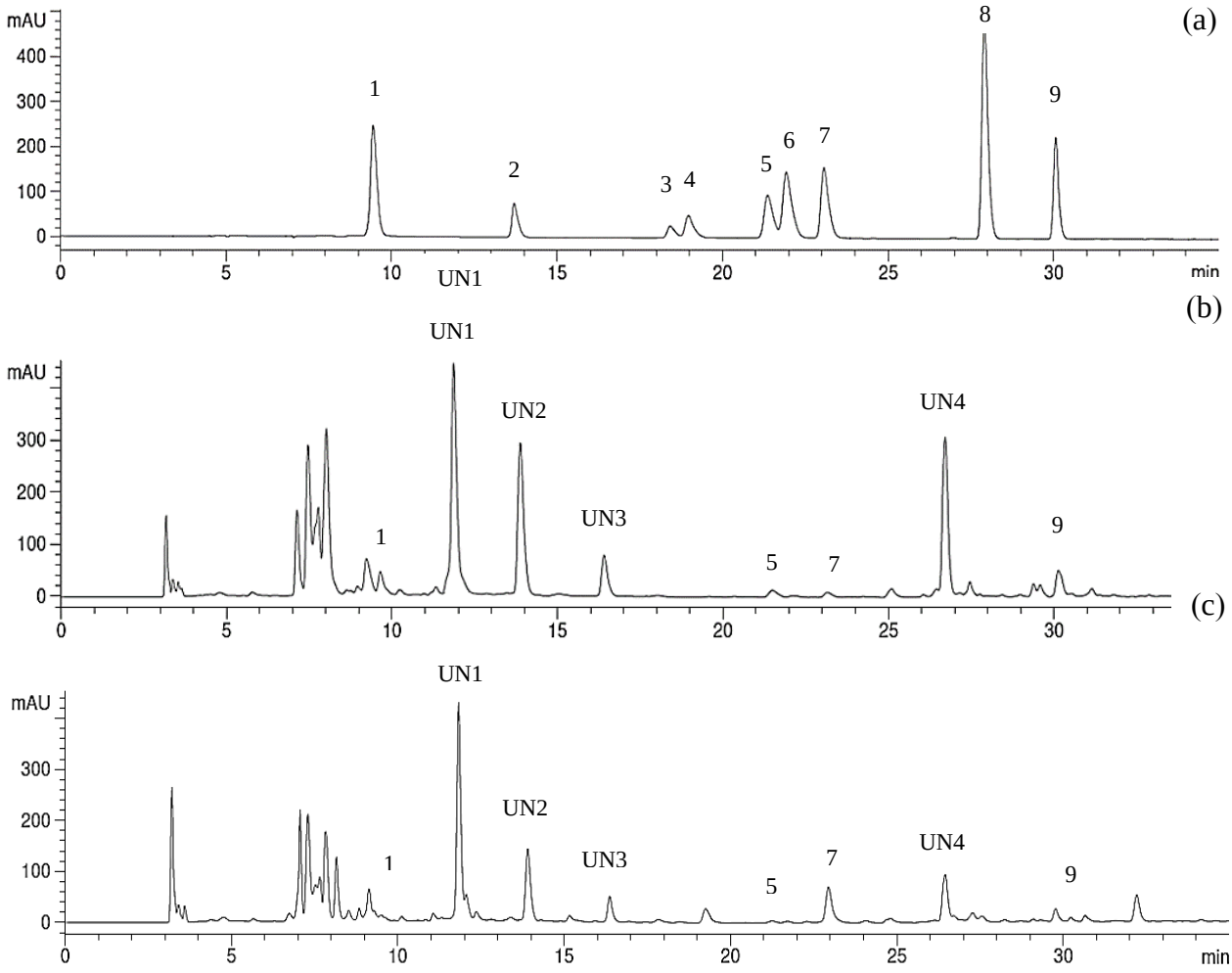

Figure 11: HPLC Chromatogram of (a) Standard phenolic compounds. Peaks: 1-pyrogallol; 2-protocatechuic acid; 3-catechin; 4-chlorogenic acid; 5-vanillic acid; 6-caffeic acid; 7-syringic acid; 8- *p*-courmaric acid; 9-ferulic acid. (b) Ricegrass aqueous extract (c) Wheatgrass aqueous extract detected at 280 nm. UN: unknown compounds

Table 8. Retention time and concentration of specific phenolic compounds of acidic hydrolyzed of aqueous extracted ricegrass and wheatgrass as detected by HPLC-DAD

Phenolic compounds	λ_{max} (nm)	Ricegrass juice extract		Wheatgrass juice extract	
		RT (min)	Conc. (mg/g)	RT (min)	Conc. (mg/g)
Pyrogallol	267	9.452 ± 0.009^{A}	10.382 ± 0.03^{a}	9.494 ± 0.001^{A}	4.084 ± 0.092^{b}
Protocatechuic acid	280	ND	ND	ND	ND
Catechin	280	ND	ND	ND	ND
Chlorogenic acid	280	ND	ND	ND	ND
Vanillic acid	280	21.096 ± 0.108^{A}	0.641 ± 0.015^{a}	21.192 ± 0.082^{A}	0.394 ± 0.008^{b}
Caffeic acid	280	ND	ND	ND	ND
Syringic acid	280	22.811 ± 0.087^{A}	0.604 ± 0.009^{b}	22.863 ± 0.028^{A}	1.416 ± 0.016^{a}
p-coumaric acid	280	ND	ND	ND	ND
Ferulic acid	320	29.905 ± 0.005^{A}	0.062 ± 0.001^{a}	29.990 ± 0.007^{A}	0.044 ± 0.001^{b}

Each value was expressed as the mean ± standard deviation. Different capital letters indicated significant differences in retention time (p<0.05). Different small letters indicated significant differences in concentration (p< 0.05). RT: retention time. ND: not detected.

2.4.5 Antioxidant activities

The antioxidant activities of ricegrass and wheatgrass juice extract were analyzed using various methods to represent the different ability to prohibit the oxidation process and expressed as Trolox equivalent per mg sample, a universal standard for *in vitro* antioxidant system (Table 9).

DPPH and ABTS assay were widely methods to determine the ability to scavenge DPPH radical which generated in ethanol and ABTS radical which well-generated in water by giving hydrogen atom and electron, respectively. It was found that wheatgrass juice extract exhibited lightly higher ability to scavenge radical than ricegrass juice extract in both systems (p< 0.05). FRAP assay is the one which determines the ability of antioxidant as a reductant. The results revealed that the reducing power of ricegrass juice was slightly lower than wheatgrass juice (p< 0.05).

Phenolic compounds contain hydroxyl groups on the aromatic ring. Due to the resonance property of them, they can donate protons and/or electrons to other unstable molecules (Vinson *et al.*, 2001). Consequently, phenolic compounds in ricegrass and wheatgrass might play important roles in the antioxidant activities. According to the experiment, the high level of phenolic compounds found in ricegrass displayed high antioxidant activities. However, it has been pointed out that not only phenolic molecules but other compounds such as chlorophyll, ascorbic acid, and organic acids also revealed anti- oxidation ability. In addition, wheatgrass juice showed greater ability to chelate ferrous ion, almost doubled to that of ricegrass. This may be contributed to the large amount of chlorophyll which is one of the most efficient natural chelating molecules (Kephart, 1955). Das *et al.* (2012) reported that the DPPH scavenging activity of wheatgrass juice is higher when freeze-dried where its ascorbic acid is more retained compared with when oven dried. As a result, high level of ascorbic acid and chlorophyll synergizing with phenolic compounds may propose higher antioxidant activities of the wheatgrass juice. While the main function of being antioxidant molecules in ricegrass juice was supposed to relate to phenolic compounds.

Table 9. Antioxidant activities of ricegrass and wheatgrass juice extract

Method	Unit	Ricegrass juice extract	Wheatgrass juice extract
DPPH	mg TE/g extract	4.65 ± 0.12^{b}	5.51 ± 0.04^{a}
ABTS	mg TE/g extract	38.06 ± 0.38^{b}	39.77 ± 0.27^{a}
FRAP	mg TE/g extract	35.62 ± 0.03^{b}	37.45 ± 0.98^{a}
FCA	µmol EDTAE/g extract	10.47 ± 0.14^{b}	25.59 ± 0.35^{a}

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same row indicated significant differences ($p< 0.05$). EDTAE: Ethylenediaminetetraacetic acid equivalent. TE: Trolox equivalent.

Nevertheless, antioxidant activities may not only be related to the bioactive molecules but also variable among different cultivars of rice or wheat, growing environment, fertilization, etc. Additionally, the juice from some pigmented rice such as Kum Doisaket, Rice berry and Kum Noi have been previously reported to display higher percentage of DPPH radical scavenging activity and FRAP compared with wheatgrass juice due to high anthocyanin content, though some had no significant difference or lesser ability than wheatgrass juice (Khanthapoka *et al.*, 2015). However, the price of those pigmented rice grains was quite high. Though ricegrass juice in this study was produced from rice cultivar Chainat1 which was statistically qualified to have lower antioxidant power than wheatgrass juice, there was not noticeably different. Furthermore, Chainat1 which is the cheapest rice (0.5$/kg) showed the lower cost of production per beneficial in antioxidant activity compared with wheatgrass which the seeds roughly cost 3.6$/kg in Thailand. Therefore, Chainat1 rice is a very promising candidate. Additionally, there are various methods available for the improvement of bioactive compounds which can possibly offer superior antioxidant activities, such as agronomic bio-fortification, seed priming or using natural and chemical elicitation while applying to plant food which can be further studied.

2.4.6 Consumer acceptability

The sensory acceptability of ricegrass juice compared with wheatgrass expressed as the score for appearance, color, odor, taste and overall acceptability were shown in Figure 12. The results showed that ricegrass juice got the higher score on all attributes higher than wheatgrass juice. While the overall acceptability did not significantly different ($p<0.05$). It was observed that wheatgrass juice had an extremely low score in odor characteristics. This may due to a strong greeny smell of wheatgrass juice. Moreover, some panelists also remarked that wheatgrass juice had a strange odor which is not acceptable to consume at all. Panelists rather like more the color of ricegrass juice which is slightly yellow-green compared with the very dark green of wheatgrass juice. However, no significant difference score on overall liking may refer to a high variation of the score for wheatgrass juice for some who extremely like and dislike.

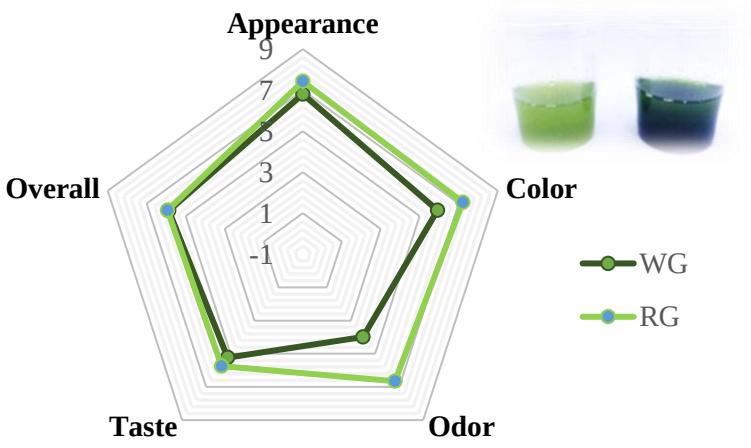

Figure 12. Sensory acceptability of ricegrass juice compared with wheatgrass juice

2.5 Conclusion

Ricegrass juice though had a slightly lower ability on anti-oxidation activity compared with wheatgrass juice, it contained a higher level of phenolic acid molecules included pyrogallol, vanillic acid, and ferulic acid. Moreover, when comparing with wheatgrass juice, ricegrass juice had lower production cost (rice seeds 16 baht/kg compared with wheat seeds 120 baht/kg) and a higher score on consumer acceptability. Then, ricegrass juice can be developed competitively to wheatgrass juice as an antioxidant boosting drink.

CHAPTER 3

INFLUENCE OF SELENIUM BIO-FORTIFICATION ON NUTRITIONAL COMPOSITIONS, BIOACTIVE COMPOUNDS CONTENT AND ANTI-OXIDATIVE PROPERTIES OF RICEGRASS [1]

3.1 Abstract

Young ricegrass (*Oryza sativa* L.) can be introduced as a functional food product, as sprouts have gained significant interest in recent times due to their high nutritional values. Bio-fortification of selenium is one strategy to enhance plant bioactivity. However, the level of selenium used varies among species of plants. Thus, the proper level needs to be explored. To investigate the influence of selenium bio-fortification on nutritional compositions, bioactive compounds content, and anti-oxidative properties of young ricegrass. Sodium selenite ranging 0, 10, 20, 30, and 40 mg Se/L has been hydroponically bio-fortified into ricegrass, grown for 8 days, and then investigated for changes of growth characteristics, selenium content, accumulation of bioactive compounds, and anti-oxidative properties. Results revealed that selenium bio-fortified exogenously increased the accumulation of selenium in ricegrass by 529% at 40 mg Se/L treatment without negative changes in the leaves' biomass at the day they were harvested. However, the root part weight slightly decreased when the selenium level increased. Selenium at a concentration of 10 and 20 mg Se/L can stimulate the production of phenolic compounds and antioxidant activities in young ricegrass as measured by DPPH, ABTS, FRAP, and chelating assay. Conversely, the higher level of selenium fortification reducing the accumulation of phenolics in ricegrass may due to pro-oxidant expression. Selenium bio-fortification can be used as a useful technique to improve quality of ricegrass plantation. 10 mg Se/L treatment was ideal to trigger the synthesis of phenolics which exhibited high antioxidant activities. However, 40 mg Se/L treatment was the most effective to produce Se plant foods.

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[1] The content of this chapter has been published in *Functional Food in Health and Diseases*

3.2 Introduction

Sprouts or young plant of cereals, grains, or legumes are currently relevant and interesting functional foods, as these plants at the beginning of the growing stage are associated with large amount of quality bioactive compounds such as amino acids, trace elements, vitamins, and various phytochemicals (Pająk *et al.*, 2014). Consequently, the consumption of sprouts in usual dietary may deliver countless health benefits. Ricegrass is a brand-new sprout which was shortly introduced as a substitution to wheatgrass, especially in high rice producing areas because it is being an economy ingredient as well as the purpose of utilizing low-cost variety rice seeds. Additionally, young ricegrass has been described to possess beneficial biological properties included antioxidant (Rattanapon *et al.*, 2016a) and DNA protective properties (Khanthapoka *et al.*, 2015) which may probably due to numerous secondary metabolite compounds like phenolic compounds. Now, the study of phytochemicals has become the foremost research issues in the field of functional foods. The development of effective strategies to improve the level of useful metabolites in edible plants without gene modification is increasingly relevant and significant. Elicitation is one of the most effective approaches to induce the synthesis of bioactive secondary metabolites in plants, as plants automatically respond to all kind of unknown compounds to ensure their survival via the production of these protective substances (Namdeo, 2007). The type of elicitor is varied from biotic to abiotic one. As known that minerals are the basic elements required for all living cells. The bio-fortification is the procedure which normally use to increase the micronutrients content in plants during plantation by adding the mineral into soil or solution. Additionally, this technique can also be applied, as plant elicitation techniques using those minerals to induce the physiological changes and trigger the synthesis of phytochemical compounds which can possibly improve the biological properties of plants from the synergistic effects of phytochemical compounds and an increased content of minerals.

Selenium (Se) is an essential trace mineral required by a human. It has a main function in the antioxidant system and claims for the outstanding efficiency of antioxidant minerals involving many endogenously antioxidant enzymes (McDowell *et al.*,

2007). Likewise, in plants, Se is also known to offer a protective role against oxidative damage. Therefore, various plants worldwide have been bio-fortified to perform despite unusual condition such as drought stress, UV-B stress, even heavy metals stress (Kumar *et al.*, 2014, Soleimanzadeh, 2012, Yao *et al.*, 2010). Moreover, the bio-fortification of Se at the appropriate level also showed the superior beneficial effects to some common plants, including the stimulation of total phenolic compounds accumulation in purple potatoes (Lei *et al.*, 2014), an improvement of antioxidant and anticancer properties in green tea (Molan *et al.*, 2009, Xu *et al.*, 2003), the increasing of glucosinolate and Se-methylselenocysteine content, which are the anticancer substances in broccoli sprouts and florets (Ávila *et al.*, 2013). However, the ability of each plant to accumulate Se varies among species. Thus, the favourable level of fortified Se still needs to be studied. As rice grain is the reference source of Se in diet, the accumulation of Se in their sprouts has yet to be performed. The bio-fortification of Se into ricegrass is a significant idea that is expected to provide a source of organic Se supplementation and stimulate the bioactive compounds content in plants, in addition to providing greater biological properties. Hereafter, the goal of this present study was to investigate the influence of the different concentration of Se bio-fortification into rice grain on growth characteristics, Se content, nutritional components, bioactive compound contents included phenolic compounds, chlorophyll, carotenoid and ascorbic acid content as well as the anti-oxidative properties. Moreover, the activity of phenylalanine ammonia lyase (PAL), which was the initial enzymes responsible for phenolic compounds biosynthesis, was determined. The present study was meant to deliver advantageous evidence on the level of Se fortified to rice grain to further produce Se bio-fortified ricegrass in the form of juice or juice powder as a functional food product.

3.3 Materials and methods

3.3.1 Chemicals

Chemicals used for determination of nutritional values, bioactive compounds, and antioxidant activity were purchased from Sigma (Germany).

3.3.2 Plantation

Paddy rice (*Oryza sativa* L. cv. Chainat 1) obtained from the Phatthalung Rice Research Center, Thailand was soaked in water for 24 h. After they were being drained and washed with distilled water, a total 100 g soaked rice seeds were spread on the plantation tray and left in darkness for 48 h. Sodium selenite solutions varying from 0, 10, 20, 30, and 40 mg Se/L equivalent were applied at ratio 1:1 v/w into germinated rice seeds. No apparent toxicity was observed based on the preliminary experiment (data not shown). Ricegrass were cultivated under natural light with a day/night average temperature of 33/25 ºC, relative humidity 63±5.0 % and photoperiod 12/12 (day/night) and harvested at day 8.

3.3.3 Growth measurement

The height of shoots throughout the plantation period was determined using a caliper. Defects and plant characteristics were noted. After 8 d of the plantation, plants were harvested, washed, and drained in the sieve for 2 min, and then shoots and roots were separated before being taken to measure their fresh weight.

3.3.4 Nutritional composition

The nutritional composition of young ricegrass, including protein, fat, carbohydrate, ash, and fiber contents, were analyzed following the method of (AOAC, 2000).

3.3.5 Selenium speciation analysis

Ricegrass were dried in the oven at 50ºC until the weight was constant. Total Se content and inorganic Se content in the shoots were determined using induced coupled plasma optical electron spectrophotometer (ICP-OES) (Wang *et al.*, 2013). For the total Se determination, 0.2 g of samples were digested with 3 ml of concentrated HNO_3 and 1 ml of 30% H_2O_2 in a digestive stove heated at 180ºC for 1.5 h. The digested product was reconstituted to 10 ml with Milli-Q water and auto-sampler for total Se content. The inorganic Se content in ricegrass shoots was also determined by initially digesting 2 g samples with 3 ml of 4 M HCl at 100ºC for 10 min followed by the centrifugation at 2500×g

for 10 min at 4ºC to remove debris. The supernatants were reconstituted to 10 ml with Milli-Q water and auto-sampler for inorganic Se content. The organic Se content was calculated as the difference between the total Se and inorganic Se content respectively.

3.3.6 Chlorophyll and carotenoid content

The total chlorophyll content was analyzed using the defined method of Arnon (1949) and carotenoid was calculated according to the Lichtenthaler and Wellburn (1983) equation. One gram of fresh ricegrass was grounded in a mortar and with 0.1 g $CaCO_3$ and sand. Afterward, it was extracted with 80 % acetone then filtrated and re-extracted until the green color disappeared. Then the volume of extract solution with absolute acetone (99.5%) to 100 ml and the absorbance was measured with a spectrophotometer at 470, 645, and 663 nm. The concentration of pigments was calculated from the following equations when A is absorbance (nm):

Total chlorophyll content was 20.2 (A645) + 8.02 (A663)
Carotenoid content was $(1000(A470) – 3.27C_a –104C_b)/229$

3.3.7 Extraction

Ricegrass growing at various concentrations of sodium selenite fortification were extracted with water at a ratio of 1:2 (w/v) using a juicer machine (Hurom DA-900) at room temperature. The samples were filtered with Whatman No. 4 filter paper to get rid of residues and followed by centrifugation at 10,000×g for 20 min. The supernatant was subjected to freeze-drying and then stored at 4ºC in dark bottle.

3.3.8 Ascorbic acid content

The ascorbic acid content of the sample was analyzed using the indo-phenol colorimetric method (AOAC, 2000). Aqueous extracts of the ricegrass were diluted with 2% meta-phosphoric acid to the appropriate concentration. 5 ml of diluted samples were added with 10 ml of 1.7 mM 2, 6 di-chloro-indophenol solutions, mixed well and measured for the absorbance using spectrophotometer at 518 nm. Ascorbic acid (0-400 µg/ml) was used as the standard.

3.3.9 Total extractable phenolic content

The total extractable phenolic content of ricegrass juice extracts was measured using a modified method of Singleton and Rossi (1965). Briefly, 20 µl of the extract was added to 96-well microplate. Then, 100 µl of Folin reagent (10% v/v) and 80 µl of Na_2CO_3 (7.5% w/v) were added and mixed thoroughly. After incubation for 30 min in the dark at ambient temperature, the absorbance was measured at 765 nm using the microplate reader. The total phenolic content was expressed as mg of pyrogallol equivalent (PYE) through the calibration curve.

3.3.10 Phenylalanine ammonia lyase (PAL) assay

Fresh ricegrass was extracted followed the method of Sunohara and Matsumoto (2004) and the supernatant was used as an extract enzyme sample. Phenylalanine ammonia lyase (PAL) activity was determined by the modified method of Cheng and Breen (1991). Briefly, the reaction started from the mixing of 1 ml of 14 mM phenylalanine solution and 2.7 ml of 50 mM borate buffer (pH 8.8). After that 0.3 ml of the extract enzyme sample was added and left for an hour. Thereafter, the reaction was stopped with 0.1 ml of 6N HCl. The production of cinnamate from phenylalanine was measured by change in absorbance at 290 nm (E = 9.63 mM-1cm-1). Enzymes activity was calculated according to the calibration curve of protein content using bovine serum albumin (BSA) as a standard determined by the assay of Bradford (1976).

3.3.11 Anti-oxidative properties

3.3.11.1 DPPH radical scavenging activity

1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was measured using the modified method of Brand-Williams *et al.* (1995). Briefly, 150 µl of the extract was added with 150 µl of 0.2 mM DPPH in 95% ethanol. The mixture was mixed and left to stand for 30 min in the dark. The absorbance was determined at 517 nm using the microplate reader. Standard curves were prepared and reported as mg Trolox equivalent (TE)/g extract (1-10 µg/ml).

3.3.11.2 ABTS radical scavenging activity

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay was determined followed the method of Arnao *et al.* (2001). Stock solutions consisted of 7.4 mM ABTS solution and 2.5 mM $K_2S_2O_8$ solution were prepared and equally mixed to generate ABTS radical (ABTS•). After allowing them to react for 12 h at room temperature in the dark, the radical solution was diluted to obtain an absorbance of 1.1±0.02 at 734 nm before performing the assay. 15 µl of the extract was mixed with 285 µl of ABTS• solution and left at room temperature for 2 h in the dark. The absorbance was measured at 734 nm using the microplate reader. Standard curves were prepared and reported as mg Trolox equivalent (TE)/g extract (10-100 µg/ml).

3.3.11.3 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was performed according to a method of Benzie and Strain (1996). Fresh FRAP solution was prepared from the mixture of 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution at the ratio 10:1:1 (v/v/v) and then warmed at 37 ºC for 30 min before use. 15 µl extract was added with 285 µl of FRAP solution and left to stand for 30 min in the dark. The absorbance was read at 593 nm using microplate reader. Standard curves were prepared and reported as mg Trolox equivalent (TE)/g extract (10-100 µg/ml).

3.3.11.4 Ferrous ion chelating activity (FCA)

Ferrous ion chelating activity was determined using the method of Ebrahimzadeh *et al.* (2009). 277.5 µl of the extract was mixed with 7.5 µl of 2 mM $FeCl_2$ and 15 µl of 5 mM ferrozine for 10 min at room temperature. The absorbance was read at 562 nm using microplate reader. A standard curve was prepared using EDTA. The activity was expressed as mg EDTA equivalent (EDTAE)/g extract (20-40 µg/ml).

3.3.12 Sensory evaluation

Se bio-fortified ricegrass juice at 0, 10, 20, 30 and 40 mg/L were subjected to sensory evaluation employing the attributes of appearance, color, odor, taste and overall with a 9-point hedonic scale from 1 = disliked much to 9 = liked much. The samples were served in random order for 50 panelists consisting of scientists and students in Faculty of Agro-Industry, Prince of Songkla University who familiar with drinking vegetable juice.

3.3.13 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experimental data were presented as the mean ± standard deviation of three replications. Means were analyzed using the analysis of variance (ANOVA). The significant differences among means were determined by Tukey's test ($p<0.05$) using SPSS for Windows (SPSS Inc, Chicago, IL).

3.4 Results and discussion

3.4.1 Growth measurement

The bio-fortification of sodium selenite ranging from 10 to 40 mg Se/L did not affect the growth of ricegrass at day 1 of the plantation (Figure 13). The results revealed that the plant height was plantation time dependent. Se fortified at 10 mg Se/L into the germinated rice grain resulted in a non-significant difference of shoot height to the control with marginally higher in the last 3 days ($p<0.05$). However, the fortification of Se at a higher concentration from 20-40 mg Se/L appeared to reduce the growth rate determined as height particularly at 40 mg Se/L treatment. This might indicate as a sign of the excess amount of Se bio-fortified into ricegrass.

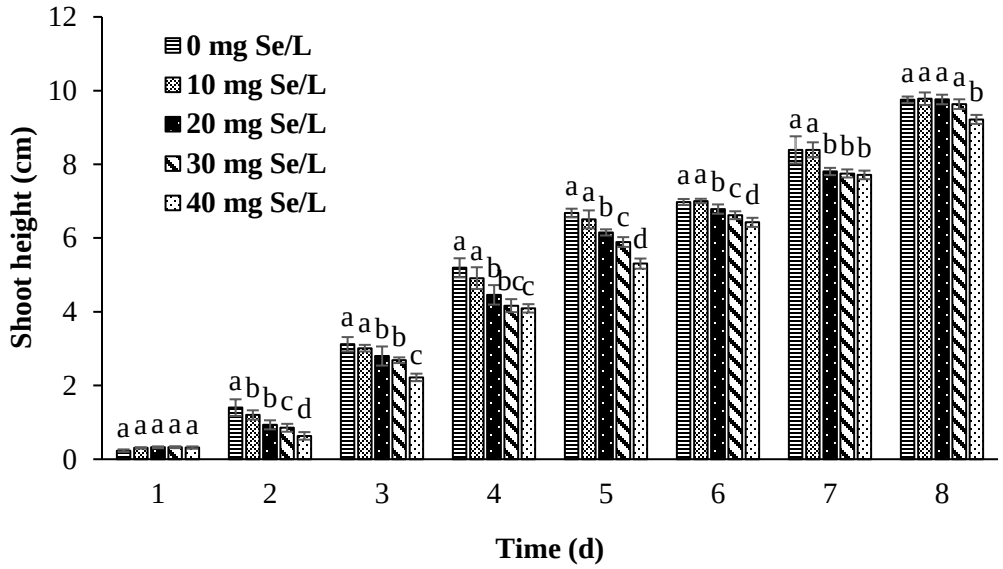

Figure 13. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg Se/L) on the height of ricegrass throughout 8 d of the plantation. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences ($p< 0.05$).

The biomass of ricegrass shoot as affected by different Se bio-fortification were not significantly different ($p<0.05$) from each other as shown in Table 10. Only slightly reduction in treatment 30 and 40 mg Se/L Se bio-fortification were observed. However, these two treatments reduced the biomass of the root part significantly, as root parts were noticed as the first part which directly contacts to Se solution and absorbed a greater amount of them. Moreover, while plantation at day 1 to day 3, these two treatments reflected offending smell which may represent the odor of volatile Se compounds. These may be a signal indicating the stress of plants grown at excess Se concentration. Se toxicity was reported to relate with various metabolic aberrations. As Se and sulfur (S) compounds are inter-related molecules (Germ *et al.*, 2007), Se bio-fortification may promote the substitution to S compounds in protein structure and lead to the loss in its stability. Furthermore, an excess amount of Se, especially in inorganic form, was reported to act as pro-oxidant by reacting with tissue thiols to form seleno-trisulphides which generated the oxygen free radicals when they reacted with other thiols (Seko *et al.*, 1989). However, all living plants continued to struggle and acclimate themselves through the volatilization process to discard excess Se compounds in plant tissue to the environment by transforming

Se into volatile forms called dimethyselenide and dimethydiselenide (Zayed *et al.*, 1998). This may be the reason whether the plant height of using 10-30 mg Se/L was similar to the control at day 8 of the plantation (Figure 13). As a result, all ricegrass Se fortification except for treatment 40 mg Se/L grew and reached the similar range of height with no difference in shoot biomass at day 8.

Table 10. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg Se/L) on biomass of ricegrass shoot and root parts.

Se bio-fortification (mg Se/L)	Root part biomass (g FW/100 g seeds)	Shoot biomass (g FW/100 g seeds)
0	338.85 ± 7.83 [a]	41.55 ± 2.12 [a]
10	337.83 ± 13.52 [a]	41.23 ± 2.25 [a]
20	339.10 ± 15.64 [a]	41.07 ± 1.85 [a]
30	307.41 ± 12.11 [b]	39.78 ± 1.90 [a]
40	274.53 ± 15.82 [c]	37.90 ± 3.40 [a]

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same column indicated significant differences ($p< 0.05$). FW: fresh weight.

3.4.2 Nutritional compositions

The nutritional compositions of Se bio-fortified ricegrass (Table 11) demonstrated an increase in protein and fat content at 10 mg Se/L fortification while higher levels of Se treatment were reduced to less than that of the control. 10 mg Se/L treatment may be the amount limit of Se that does not affect the development of plants. Therefore, Se promotes the accumulation of nitrogen (N) compounds in ricegrass, which is the initial substrate of protein. However, an excess of concentration will probably imbalance the pool of mineral and further affect the production of macronutrients. Moreover, the reduction in protein and fat concentrations due to high Se accumulation may be related to the incorporation of Se in non-protein amino acids, including Se-methyl-selenocysteine by replacing S compounds in cysteine and methionine (Nigam and McConnell, 1973), thereby

making these amino acids partly unavailable for protein synthesis. Furthermore, the reduction in the percentage of fat at high Se fortification treatment may be due to the substitution of Se to S, which leads to the diminishing in activity of fatty acid synthetase and acetyl CoA carboxylase, which were enzymes having SH groups at the active site that played role in fat production (Terry *et al.*, 2000). The percentage of ash between treatments did not show any significant difference in all treatments. This may indicate the ability of the plants to equilibrate minerals status in their tissues as homeostatic phenomena.

Table 11. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg Se/L) on nutritional compositions of ricegrass

Se conc. (mg Se/L)	% Protein	% Fat	% Carb	% Fiber	% Ash
0	23.27 ± 0.52 ^{a}	9.91 ± 0.60 ^{b}	61.24 ± 0.65 ^{c}	22.91 ± 0.59 ^{ab}	5.58 ± 0.22 ^{a}
10	23.88 ± 0.16 ^{a}	12.16 ± 0.37 ^{a}	58.56 ± 0.69 ^{d}	22.86 ± 0.44 ^{b}	5.39 ± 0.19 ^{a}
20	22.65 ± 0.64 ^{b}	9.62 ± 1.06 ^{b}	62.34 ± 0.23 ^{b}	23.96 ± 0.13 ^{a}	5.39 ± 0.35 ^{a}
30	22.53 ± 0.06 ^{b}	8.28 ± 0.61 ^{c}	63.88 ± 0.56 ^{a}	23.66 ± 0.90 ^{ab}	5.31 ± 0.31 ^{a}
40	21.43 ± 0.43 ^{c}	8.82 ± 0.21 ^{bc}	64.28 ± 0.26 ^{a}	23.23 ± 0.28 ^{ab}	5.46 ± 0.20 ^{a}

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same column indicated significant differences ($p< 0.05$).

3.4.3 Selenium content

The total Se content of rice shoots increased steadily, corresponding to an increased amount of exogenous sodium selenite supplementation in every treatment (Figure 14). The bio-fortification of Se can significantly increase Se content in the shoot of rice plant up to 529 % at 40 mg Se/L fortification. Furthermore, the results indicated that Se was naturally found in the form of organic in ricegrass. With inorganic Se supplementation, plants demonstrated the ability to transform the inorganic Se to an organic form, which was supposed to be a safe form that is also highly bioavailable for

human consumption. The fortification of Se at 20 mg Se/L treatment appeared to be a boundary of ricegrass to accumulate Se in organic form since the fortification of a higher dose (30 and 40 mg Se/L), Se in inorganic form was significantly accumulated. This may be due to the fact that protein and S containing compound contents in each plant was the key factor indicating how much Se can be accumulated in organic and inorganic form. Additionally, plants started to express some abnormal symptoms when the inorganic form content was over its homeostatic capacity.

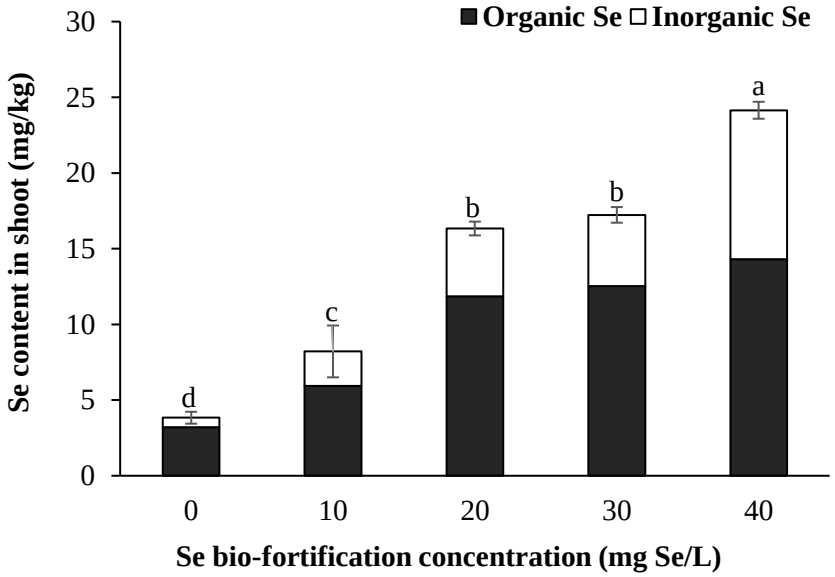

Figure 14. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg Se/L) on Se content of ricegrass. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences ($p< 0.05$).

3.4.4 Bioactive compounds content

When using the bio-fortification as an elicitation technique, the improvement of bioactive compounds content may exert additional benefits to usual plants. The total chlorophyll content of ricegrass as affected by Se bio-fortification significantly decreased to the lowest at 20 mg Se/L bio-fortification treatment and increased afterward (Table 12). Many studies suggested the role of Se was to promote the electron flow in respiratory, which thereby promotes the chlorophyll biosynthesis (Dong *et al.*, 2013,

Saffaryadi *et al.*, 2012). However, the results of this study were not consistent with those studies. The difference of these results may differ from genes and other factors in each plant sample, in addition to the individual path of plant development. The reduction in chlorophyll content in ricegrass may vary not from the direct effect of Se concentration but may from its indirect effect from other compounds. An increase of phenolic compounds may probably be induced by Se as an enhancer and further relate to a reduction in chlorophyll biosynthesis due to the competitive production between polyphenol and chlorophyll biosynthesis from the similar substrate (Meyer *et al.*, 2006). As can be seen in Table 13, the chlorophyll and total phenolic content were negatively significantly correlated ($p<0.01$). However, the level of carotenoids, which is one kind of protective substances in plants, increased to protect plant cell from stress stimuli, including excess Se. As a result, the plant required higher amounts of carotenoids to function via xanthophyll cycle to extenuating those kinds of abnormality (Li *et al.*, 2010).

The total extractable phenolic content (TPC) of ricegrass was slightly increased when fortified with Se at 10 mg Se/L and 20 mg Se/L. However, the reversed effect was observed at a higher dose (Table 12). An increase of the total extractable phenolic content was significantly correlated with an increased level of phenylalanine ammonia lyase (PAL) activity ($p<0.01$) (Figure 15). One possibility is that Se may influence the synthesis of phenolic compounds through a stimulation of this enzyme. PAL is the first and a key enzyme of the phenylpropanoid pathway involving the synthesis of benzoic acid and a variety of other phenol defense-related plant secondary metabolites (Hahlbrock and Scheel, 1989). However, PAL content was the highest at 20 mg Se/L treatment, and the levels of TPC of the plant at 10 mg Se/L and 20 mg Se/L were similar. This may indicate slightly delaying time of enzymes response to the production of phenolic compounds. Additionally, the utilization of phenols may occur significantly for being an antioxidant during high growth condition. As known, the main antioxidant agents in plant induces ascorbic acid and/or vitamin E in addition to phenolic compounds. Another mechanism which can explain an improvement of TPC content may be since S and Se are antagonists at similar routes of absorption by plant roots. Meanwhile, N and S are the major

substrates for protein production. The accumulation of one mineral either S or N leads to the limitation of the other. Therefore, the addition of Se into a plant may exert the accumulation of N in plants by enhancing amino acids and protein synthesis (Barney and Bush, 1985). Phenylalanine is the main substrate for phenolic biosynthesis; consequently, Se supplementation at the proper dose may trigger the production of phenolic compounds, while higher doses may cause toxicity.

Ascorbic acid claims to be one kind of potent antioxidant molecules to defend against radicals in water-soluble phase (Bendich *et al.*, 1986). A reduction in ascorbic acid content was observed when a higher level of Se was fortified. This suggests that ascorbic acid was used by ricegrass as an antioxidant substance to protect, in contradiction to the pro-oxidant molecules induced by Se. Evidence of cactus pear treated with selenoferous soil also revealed the similar results that ascorbic acid content was reduced. Likewise, the level of phenolic compounds tended to higher afterward (Bañuelos *et al.*, 2012). This may propose the roles of ascorbic acid in the plant as primary defensive substances. After the pool of it became low, plants may substitute it with other compounds, namely phenolics.

Table 12. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg Se/L) on bioactive compounds content of ricegrass shoot.

Se conc. (mg Se/L)	**Total Chlorophyll** (mg/g FW)	**Carotenoids** (mg/g FW)	**Ascorbic acid** (mg/g FW)	**TPC** (mg PYE/ g extract)
0	1.174 ± 0.014 ^{a}	0.094 ± 0.018 ^{bc}	0.243 ± 0.002 ^{a}	20.47 ± 0.15 ^{b}
10	0.840 ± 0.018 ^{c}	0.104 ± 0.004 ^{ab}	0.234 ± 0.001 ^{c}	21.82 ± 0.48 ^{a}
20	0.798 ± 0.017 ^{d}	0.107 ± 0.008 ^{a}	0.238 ± 0.001 ^{b}	21.55 ± 0.27 ^{a}
30	0.804 ± 0.051 ^{d}	0.094 ± 0.010 ^{bc}	0.230 ± 0.001 ^{d}	20.72 ± 0.16 ^{b}
40	0.971 ± 0.012 ^{b}	0.090 ± 0.005 ^{c}	0.218 ± 0.001 ^{e}	20.35 ± 0.27 ^{b}

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same column indicated significant differences ($p< 0.05$). TPC: Total extractable phenolic content FW: fresh weight. PYE: pyrogallol equivalent.

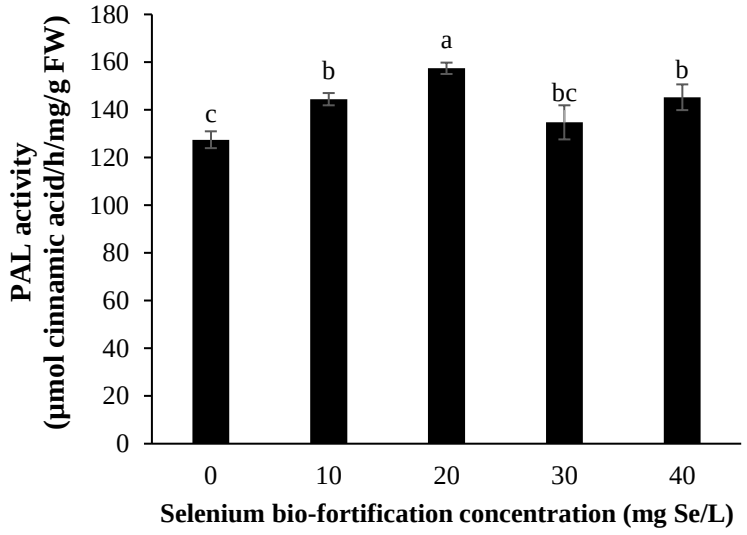

Figure 15. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg Se/L) on phenylalanine ammonia lyase (PAL) of ricegrass. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences ($p< 0.05$).

Table 13. Correlation analysis between bioactive compounds content, PAL activities and anti-oxidative properties of ricegrass

Remark: * and ** means significant different at p<0.05 and *p*<0.01 respectively.

Variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. TPC	1								
2.Chlorophyll	-0.858**	1							
3.Carotenoids	0.483	-0.626*	1						
4. Ascorbic acid	-0.045	-0.168	0.210	1					
5. PAL	0.645*	-0.644*	0.550*	0.219	1				
6. DPPH	0.880**	-0.964**	0.541*	0.064	0.686**	1			
7. ABTS	0.592*	-0.867**	0.690**	0.299	0.056	0.793**	1		
8. FRAP	0.385	-0.411	0.397	-0.052	0.286	0.506	0.460	1	
9. FCA	0.340	0.034	-0.244	-0.851**	-0.027	0.119	-0.227	0.156	1

* indicated significant different at *p*<0.05 and ** indicated significant different at *p*<0.01

3.4.5 *In vitro* anti-oxidative properties

The antioxidant activities of ricegrass were detected using various systems to detect the action of the different chemical structure of antioxidant compounds to the radical, in addition to different routes of antioxidant protection process. Se bio-fortification into germinated rice grain at 10 mg Se/L resulted in the highest DPPH, ABTS, and FRAP activities (Table 14). However, the antioxidant activities of Se bio-fortified at 30 and 40 mg Se/L seemed to be subsequently reduced.

Table 14. Effect of Se bio-fortification (0, 10, 20, 30 and 40 mg/L) on antioxidant activities of ricegrass shoot.

Se conc. (mg/L)	**DPPH** (mg TE/g extract)	**ABTS** (mg TE/g extract)	**FRAP** (mg TE/g extract)	**FCA** (μmol EDTAE/g extract)
0	4.37 ± 0.26 ^{c}	39.16 ± 0.38 ^{a}	29.80 ± 0.52 ^{b}	13.76 ± 0.71 ^{a}
10	5.47 ± 0.12 ^{a}	40.15 ± 0.47 ^{a}	30.92 ± 0.82 ^{a}	14.55 ± 1.29 ^{a}
20	5.63 ± 0.05 ^{a}	39.66 ± 0.95 ^{a}	30.56 ± 0.20 ^{ab}	7.41 ± 0.48 ^{b}
30	5.42 ± 0.10 ^{ab}	38.76 ± 1.24 ^{a}	29.84 ± 0.77 ^{b}	4.98 ± 1.12 ^{c}
40	5.18 ± 0.08 ^{b}	35.22 ± 0.36 ^{b}	30.11 ± 0.57 ^{ab}	4.53 ± 0.95 ^{c}

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same column indicated significant differences (p<0.05). PYE: pyrogallol equivalent. EDTAE: Ethylenediaminetetraacetic acid equivalent.

Plants are a rich source of antioxidant compounds. Various natural substances which provide antioxidant activities included ascorbic acid, tocopherols, and carotenoids. Moreover, the current topic also highlights the potential role of phytochemical components including flavonoids and phenolic acids as important antioxidant compounds in diet. This may be due to the physicochemical of ascorbic acid, which is heat sensitive and easily oxidizes. Thus, only a small amount of ascorbic acid was left in the sample. Furthermore, it was possible that the plants did not much produce and accumulate ascorbic

acid, as rice is not a source of ascorbic acids like fruits are. Therefore, the substances which play roles in anti-oxidative properties of ricegrass was supposed to be phenolic compounds, since the treatment which was high in phenolics was to exhibit the highest ability of H and electron donor to radicals. This can be claimed due to the strong correlation between TPC and DPPH ($p<0.01$), as well as TPC and ABTS ($p<0.05$). Additionally, it was observed that the DPPH value was lower than the ABTS value. This may be due to the stronger steric effect of DPPH radical molecule and result in the difficulty of antioxidant compounds to react with its radical site (Prior and Cao, 2000). The chelating activity of ricegrass, as affected by different Se supplementation, increased at 10 mg Se/L treatment and then dramatically decreased. This may relate to the high level of inorganic Se accumulation in the samples and disturb the chelation between iron and antioxidant molecules. Nevertheless, these *in vitro* anti-oxidative measurements cannot detect the ability of Se as an antioxidant cofactor. Thus, further studies on cell cultures and *in vivo* study are needed to prove beneficial effects of Se on anti-oxidative properties.

3.4.6 Consumer acceptability

The sensory acceptability of ricegrass juice as affected by Se bio-fortification has been checked whether the bio-fortification of Se into ricegrass influences on sensory attributes of ricegrass juice or not because sodium selenite itself had a bitter taste. The results showed that all attributes of ricegrass juice bio-fortified with Se at different concentration did not a significant difference from each other ($p<0.05$) (Table 15). The bio-fortification of Se into ricegrass seem to have an only minor effect on compounds which responsible for the sensory attributes. Se bio-fortification into ricegrass at 10 and 20 mg Se/L appeared to have the highest score on overall liking compared with others. The panelists commented on decent odor from these two treatments. All conditions of ricegrass juice bio-fortified with Se can be accepted by consumers as a functional drink with the moderate score.

Table 15. Sensory acceptability of ricegrass juice as affected by Se bio-fortification

Se conc. (mg/L)	Appearance	Color	Odor	Taste	Overall
0	7.42 ± 1.15 [a]	7.21 ± 1.32 [a]	6.66 ± 1.21 [a]	6.19 ± 0.88 [a]	6.61 ± 1.17 [a]
10	7.36 ± 1.08 [a]	7.24 ± 1.39 [a]	6.78 ± 1.24 [a]	6.18 ± 1.09 [a]	6.71 ± 0.94 [a]
20	7.41 ± 1.01 [a]	7.35 ± 1.18 [a]	6.56 ± 1.16 [a]	6.30 ± 0.95 [a]	6.71 ± 0.78 [a]
30	7.30 ± 1.21 [a]	7.18 ± 1.27 [a]	6.25 ± 1.14 [a]	6.22 ± 0.80 [a]	6.45 ± 0.89 [a]
40	7.21 ± 1.17 [a]	6.97 ± 1.42 [a]	6.47 ± 1.29 [a]	6.07 ± 0.86 [a]	6.65 ± 0.88 [a]

Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same column indicated significant differences (p<0.05).

3.5 Conclusion

The present work demonstrated that the bio-fortification of Se at 10 mg/L treatment was the ideal level to trigger the synthesis of phenolic compounds through phenylpropanoids pathway which provides the best ability oppose the oxidation process. However, the fortification of 40 mg/L Se treatment was significant to produce Se plant foods, since ricegrass can accumulate up to 529% higher level of Se content. Though, further studies in cell cultures and *in vivo* are needed to confirm the actual properties of ricegrass on anti-oxidative properties as well as other bioactivities. Three conditions of the experiment included the control (0 mg Se/L fortification), the one which is the highest in TPC content (10 mg Se/L fortification) and the one which is the highest in Se content (40 mg Se/L fortification) were chosen for the next part of studies conducted on bioactivities in cell lines and microorganisms.

CHAPTER 4

THE ANTIOXIDANT AND INHIBITION OF NITRIC OXIDE SYNTHESIS PROPERTIES OF SELENIUM BIO-FORTIFIED RICEGRASS JUICE IN RAW264.7 MURINE MACROPHAGE CELLS [1]

4.1 Abstract

Ricegrass juice (*Oryza sativa* L.) was introduced as a functional food as the consumption of sprouts or seedlings has been claimed to provide high nutritive value. Selenium (Se) is a trace mineral that plays a key role in the human antioxidation scheme. Supplementation of Se into plants is one strategy to enhance plant bioactivities. In this study, the effect of ricegrass juice extracts bio-fortified with 0, 10 and 40 mg Se/L named as RG0, RG10, and RG40, respectively, were investigated for a cytotoxicity, changes of endogenous antioxidant enzymes, lipid peroxidation and nitric oxide inhibition in RAW264.7 macrophage cells. Flavone glycosides, namely chrysoeriol arabinosyl arabinoside derivatives were found to be the foremost bioactive components in ricegrass juice extract indicated by UHPLC-MS. Results of cell culture assessment revealed that RG40 showed an ability to promote macrophage cell proliferation at low concentration. Ricegrass juice extract in all treatments possessed the ability to reduce MDA content, which may be regarded as the bioactivity of phenolic compounds. Moreover, Se also played a role in this effect since RG40 showed the greatest ability via increasing the level of GPx enzyme. It was also discovered that phenolic compounds in the extracts played a role in inhibiting nitric oxide in LPS induced RAW264.7 cells. Furthermore, RG40 expressed significantly higher NO inhibition properties at IC_{50} 118.76 µg/ml compared with RG0 and RG10, at 147.02 and 147.73 µg/ml, respectively. Se bio-fortified ricegrass juice could be considered as a new potent functional food which can lower the risk of oxidative stress and chronic inflammation diseases.

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[1] The content of this chapter has been published in *Antioxidants*

4.2 Introduction

At present, humans are more simply at risk of getting ill or diseases. Since humans facing various harmful environments as well as behave more a non-healthy lifestyle such as smoking, low physical activity, excessive stress and poor diet consumption. All these factors lead to the progression of various chronic diseases over time (Devasagayam *et al.*, 2004). To fight these damages, the antioxidant protection is needed. Also, in response to oxidative damage, injury, toxins, bacteria or metals, the immune system reacts to dangers by generating the process called inflammation (McGeer and Mcgeer, 2004). It is an important process to diminish all threats by signaling the white blood cells to fight the invaders. Inflammatory cytokines like nitric oxide (NO) are released for host defense response. Under normal conditions, it plays a part in the regulation of vasodilatation and neurological issues. On the other hand, overproduction of NO induces tissue damage and associated with chronic inflammation including heart diseases, cancers, Alzheimer's diseases, autoimmune diseases and neurological diseases (McGeer and Mcgeer, 2004). As many of the synthetic antioxidants and anti-inflammation have been widely used nowadays, the risk of toxicity should be concerned. Accordingly, growing attention has been paid to the development of efficient compounds from natural resources which can naturally modulate the antioxidant protection and inflammation responses.

Plants phytochemicals are the major natural antioxidant compounds. Consequently, the consumption of plant foods in the usual diet may deliver many bioactive components and improve well-being. Ricegrass is a brand- new sprout which was recently produced in Thailand from 8-10 days grown rice sprouts. It was interesting in an economy plant food since it can produce from low-cost rice seeds. Currently, a study on how to produce or improve the functional foods or ingredients quality is of interest since there are many methods available to boost the benefits of ingredients (Baenas *et al.*, 2014). Se bio-fortification has been widely used to supplement Se content in plant foods as well as exerted advantageous effects on plant bioactivities (Chomchan *et al.*, 2017a). Since Se is an essential trace mineral required by a human and has a major function in the antioxidant system by involving in many endogenously antioxidant enzymes (Rayman, 2000). The

addition of Se into ricegrass may possibly improve the ability of typical ricegrass on boosting antioxidant properties as well as benefits on nitric oxide synthesis inhibition. The objective of this study is to identify the type of phenolic compounds and Se content of ricegrass juice extract in relation to the supplementation of sodium selenite into ricegrass. The effect of Se bio-fortified ricegrass juice extracts (RG0, RG10, RG40) were then investigated for a cytotoxicity, changes in antioxidant enzymes activity, lipid peroxidation and the inhibition of nitric oxide in RAW264.7 murine macrophage cells.

4.3 Materials and Methods

4.3.1 Chemicals

Reduced glutathione (GSH), 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB), riboflavin, L-methionine, nitroblue tetrazolium chloride (NBT), bovine serum albumin (BSA), Coomassie brilliant blue G-250 dye, malondialdehyde (MDA), 2-thiobarbituric acid (TBA), formic acid, HPLC grade acetonitrile (ACN), HPLC grade methanol, lipopolysaccharide (LPS) from *E.coli*, L-nitro-arginine (LNA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma Aldrich Co. (St.Louis, MO, USA). Hydrogen peroxide (H_2O_2) and trichloroacetic acid (TCA) were purchased from Thermo Fisher Scientific Co. (San Jose, CA, USA). Reagents and media for cell line included trypan blue dye, trypsin-EDTA, fetal bovine serum (FBS), penicillin, streptomycin and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Gibco BRL, Life Technologies Inc. (Rockville, MD, USA) and Griess reagent was from Merck (Darmstadt, Germany).

4.3.2 Plant extracts

Young ricegrass juice extract (RG0) and Se bio-fortified ricegrass juice extract RG10, RG40) prepared from lyophilized aqueous extracts of 8 days grown ricegrass (*Oryza sativa* L. cv. Chainat 1) according to the extraction method in the previous chapter were used in this study.

4.3.3 Selenium content determination

Total Se content of plant extracts was determined using an inductively coupled plasma optical electron spectrophotometer (ICP-OES). Approximate 0.1 g of extracts were digested with 3 ml of concentrated HNO_3 and 1 ml of 30% H_2O_2 in a digestive stove heated at 180ºC for 1.5 h. The digested product was reconstituted to 10 ml with Milli-Q water and auto-sampler for total Se content (Wang *et al.*, 2013).

4.3.4 Total extrctable phenolic content

The total extractable phenolic content of Se bio-fortified ricegrass juice extracts were measured using a method modified from Singleton and Rossi (1965). Briefly, 20 µl of the extract was added to 96-well microplate. Then, 100 µl of Folin reagent (10% v/v) and 80 µl of Na_2CO_3 (7.5% w/v) were added and mixed thoroughly. After incubation for 30 min in the dark at ambient temperature, the absorbance was measured at 765 nm using a microplate reader and expressed as mg pyrogallol equivalent (PYE)/ g extract.

4.3.5 Phenolic profiles identification using UHPLC-DAD-ESI-MS

Main polyphenol compounds in ricegrass juice extracts were investigated using a Thermo Scientific Dionex Ultimate 3000 Ultra High-Performance Liquid Chromatography (UHPLC) system equipped with Diode Array Absorbance Detector, Electron Spray Ionization and LTQ XL mass detector (DAD-ESI-MS). The extracts 10 mg/mL were dissolved in HPLC water and filtered with a sterile syringe filter of 0.45µm. Separation was carried out using a Purosper STAR (250 mm × 4.6 mm) with LiChrocart, RP-18 column end-capped with 5µm diameter particles (Merck, Germany) as the stationary phase. The mobile phase consisted of H_2O containing 0.5% formic acid (solvent A) and acetonitrile (solvent B), using the following gradient of solvent B: 0.00–10.00 min, 0%–10.0%; 10.00–15.00 min, 10.0%; 15.00–20.00 min, 10.0%–15.0%; 20.00–30.00 min, 15.0%–25.0%; 30.00–35.00 min, 25.0%; 35.00–45.00 min; 25.0%–100% followed by washing with 100% methanol and re-equilibration. The flow rate was 0.8 ml/min and the column temperature was 40 °C with the injection volume of 20 µl. The MS parameters

were as follows for both the negative and positive mode: heater temperature: 250°C; capillary temperature: 330°C; sheath gas flow: 50 arbitrary units; auxiliary gas flow: 10 arbitrary units. The mass data for the molecular ions were processed with Thermo XcaliburTM software version 2.2.44

4.3.6 Cell culture model

RAW264.7, mouse murine macrophage cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells passage between 30-40 were used. Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1.5 % sodium bicarbonate, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin was used for the maintenance of cells at 37^{o}C, 5 % CO_2, in a fully humidified incubator. Phosphate buffer saline (PBS) at pH 7.2 was used to wash the cells throughout the experiment.

4.3.7 Cell viability assay

The MTT assay is a colorimetric assay for assessing cell metabolic activity. The tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide can be reduced to its insoluble formaza by NADPH-dependent cellular oxidoreductase enzymes from the viable cells thus this data reflect the number of viable cells present. The MTT assay was used to determine the percentage of cell viability. Cells grown at 80-90% confluence were harvested with 0.25% trypsin – EDTA and suspended in a fresh medium. Cell counts were determined using a standard haemocytometer based trypan blue cell counting technique (Louis and Siegel, 2011). RAW264.7 cells at the density of 1×10^6 cells/ml were seeded in 96-well tissue culture plates and allow to adhere for 2 h. After washing the cells with PBS (pH 7.2), they were allowed to react with the medium mixed with various concentrations of the extracts (0.25 – 5 mg/ml) followed by 24 h incubation. Cell viability after 24 h was checked by removing 100 µl of supernatant and 10 µl of MTT solution was added. After 2 h, the MTT solution was removed, and 100 µl of 0.04 N HCl in isopropanol was added to dissolve the formazan crystals. Absorbances were recorded at

570 nm using a microplate reader. The percentage of cell viability was calculated by the following equation;

% Cell viability = [Absorbance of sample/ Absorbance of control] × 100

4.3.8 Preparation of endogenous cellular extracts

RAW264.7 cells at the density of 1×10^6 cells/ml were seeded in 60 mm tissue culture plates and incubated for 2 h to allow the cells to adhere to the tissue culture plates. Cells were washed with 2 ml of PBS (pH 7.2) before being treated with 6 ml of the extracts at the concentration of 250 and 1000 µg/ml for 24 h. The endogenous cellular fluid was extracted according to a method modified from Du *et al.* (2016). Supernatants were removed; cells were washed with PBS and harvested with 0.5 mL of 0.25% trypsin-EDTA. 1 ml of culture medium was added to stop the reaction and followed by centrifugation at 1000×g for 10 min. Cell pellets were washed with PBS until clean and re-suspended in 1 ml of cold PBS. Cells were lysed using a probe-type sonicator (Vibra-Cell, Sonics and Materials Inc., Newtown, CT, USA) by pulsing at 15 s on and 10 s off for 5 cycles on ice. The cell extracts were centrifuged at 10,000×g (4 ◦C) to discard the cell debris while supernatants were used for the determination of antioxidant enzymes activity and lipid peroxidation assay.

4.3.9 Determination of endogenous antioxidant enzyme activity

Total superoxide dismutase (SOD) assay was performed using a mixture of 20 µM riboflavin (0.3 ml), 130 mM methionine (0.3 ml), and 100 µM di-sodium ethylenediaminetetraacetic acid (EDTA-Na_2) (0.3 ml), 50 mM potassium phosphate buffer at pH 7.8 (1.5 ml), deionized water (0.25 ml), 750 µmol/L NBT (0.3 ml) and followed by 0.05 mL of the endogenous cellular extracts. The mixture was placed under light at a photon flux density of 78 µmol photons s^{-1} m^{-2} for 20 min. The absorbance was measured under UV light at 560 nm (Giannopolitis and Ries, 1977). The level of glutathione peroxidase (GPx) activity which was the main selenoenzyme involved in antioxidant protection system was measured (Flohé and Günzler, 1984). The mixture of 0.2 ml

endogenous cellular extracts, 0.4 ml of 0.1 mM reduced L-glutathione (GSH) and 0.2 ml of 0.067M $KNaHPO_4$ was pre-heated at 25◦C for 5 min. Afterward, 0.2 ml of 1.3 mM of H_2O_2 was added to initiate the reaction and left for 10 min. 1 ml of 1% of trichloroacetic acid was added to terminate the reaction followed by incubation on an ice bath for 30 min. The mixture was centrifuged at 1000xg for 10 min and the supernatant (0.48 ml) was then mixed with 0.32 M of Na_2HPO_4 (2.2 ml) and 1 mM of DTNB (0.32 ml) in the cuvette. The absorbance at 412 nm was measured under UV light after 5 min (E = 39.4 $mM^{-1}cm^{-1}$). Catalase (CAT) assay was investigated from the mixture of 0.3% H_2O_2 in 50 mM potassium phosphate buffer pH 7.0 (1.9 ml) with the endogenous cellular extracts (0.1 ml) in the cuvette. The activity of CAT was measured from absorbance change in 60s at 240 nm (E = 39.4 $mM^{-1}cm^{-1}$) (Brennan and Frenkel, 1977). All enzyme activities were calculated according to the calibration curve of BSA as a standard determined by the assay of Bradford (1976) and expressed as a percentage of the control.

4.3.10 Determination of lipid peroxidation

Lipid peroxidation assay was measured using thiobarbituric acid reactive substances assay (TBARS) (Chen *et al.*, 2002a). 1 ml of endogenous cellular extracts were mixed with 4 ml of 20% TCA containing 0.8% of TBA (w/v). The mixtures were heated at 95°C for 60 min, then cooled on ice and centrifuged at 3,000xg for 10 min. The absorbance was measured at 532 nm. The amount of red complex was compared with an external standard of MDA. Protein content was determined based on the assay of Bradford (1976). The amount of TBARS was expressed as nmol MDA/mg protein.

4.3.11 Nitric oxide synthesis inhibition determination

Nitric oxide (NO) synthesis inhibition determination was measured as the screening method for anti-inflammatory properties of ricegrass juice extracts. Briefly, RAW 264.7 cells at the density of 1×10^6 cells/ml were seeded into 96-well plates and allowed to adhere for 2 h. Cells were induced to produce NO by treating them with 100 µl of 100 µg/ml lipopolysaccharide (LPS) from *E.coli* as a foreign matter. 100 µl of extracts

at various concentrations from 0.1 – 1 mg/ml were further added and incubated for 24 h. L-nitro arginine was used as a positive control. Pyrogallol, sodium selenite and Se-methionine (Se-Met) from Se-yeast were also used as the sample control. The percentage of NO synthesis inhibition was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The supernatant (100 µl) was transferred to 96-well tissue culture plates and then added with 100 µl of Griess reagent. The mixtures were measured using a microplate reader at 570 nm. The percentage of NO synthesis inhibition (%) was calculated from equation (2). The rest of cells in 96-well plates were rechecked for the number of cell viability with MTT assay to confirm the effectiveness of the extract on the inhibition of NO synthesis.

$$\% \text{ Inhibition of NO synthesis} = \frac{[(\text{Control} - \text{Blank of control}) - (\text{Sample} - \text{Blank of sample})]}{(\text{Control} - \text{Blank of control})} \times 100 \quad (2)$$

4.3.12 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experimental data were presented as the mean ± standard deviation of three replications. Means were analyzed using the analysis of variance (ANOVA). The significant differences among means were determined by Tukey's test (p<0.05) using SPSS for Windows (SPSS Inc, Chicago, IL).

4.4 Results and Discussion

4.4.1 Changes in Se Content and Total Polyphenol Content

Rice has been classified as a moderate Se accumulating plant. According to the preliminary results, ricegrass was revealed to have the limitation of not accumulating Se compounds more than 40 mg Se/L, since higher levels of Se concentration led to toxicity, which resulted in significant growth limitation. The addition of Se at 10 and 40 mg Se/L revealed no significant changes in the physiology and yield of ricegrass collected on the day of harvesting (Chomchan *et al.*, 2017b). In the present experiment, Se compounds were found to be accumulated in ricegrass in a dose-dependent manner. The

supplementation of sodium selenite compounds in the plantation at 10 and 40 mg Se/L resulted in higher levels of Se accumulation than when feeding with water alone. After the aqueous extraction of ricegrass, the highest level of Se compounds that would be achieved in ricegrass juice extract was at the level of 59.76 ± 1.52 µg/g extract at treatment 40 mg Se/L supplementation (Figure 16).

In previous research work, the technique of Se supplementation into plants had been proposed as an alternative path to trigger some of the phytochemical constituents in numerous plants (Chomchan *et al.*, 2017a). For example, an enhancement of naringin chalcone and kaempferol level in tomato fruits (Schiavon *et al.*, 2013), and a promotion of catechin accumulation in Assam tea has been observed (Sae-Lee *et al.*, 2012). However, the supplementation of Se compounds into ricegrass during plantation in this study showed only a minor modification in the total polyphenol content (TPC) of its aqueous extract.

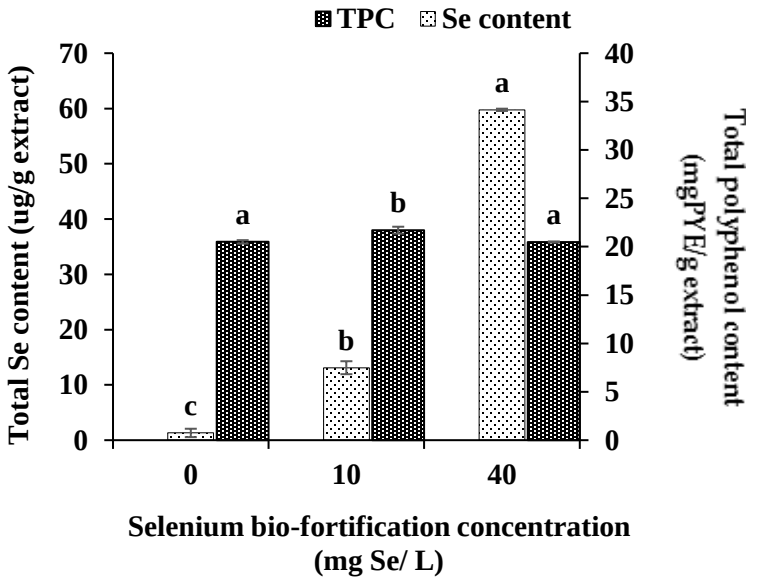


Figure 16. Total Se content (µg/g extract) (left axis) and total polyphenol content (right axis) of ricegrass juice extract after being bio-fortified with various concentrations of sodium selenite (0, 10 and 40 mg Se/L) during plantation. Each value was expressed as the mean ± standard deviation (n=3). Different letters in the same row indicated significant differences ($p< 0.05$). PYE: pyrogallol equivalent.

4.4.2 Phenolic profiles identification using UHPLC-DAD-ESI-MS

In this study, the supplementation of Se compounds into ricegrass appeared to have minimal effects on both the number of phenolic compounds as reported by TPC level and the quality of phenolic compounds. The types of phenolic compounds detected in ricegrass juice extract (RG0) and Se-ricegrass juice extract (RG10, RG40) investigated using HPLC were similar (data not shown). Ricegrass juice extract was identified for the main type of phenolic compounds which can be a major substance exhibited the bioactivities using the reversed-phase UHPLC-DAD-ESI-MS, since there was only limited data on the specific types of phenolic compounds found in the aqueous extract of young ricegrass. The data analyzed using HPLC in part 1 revealed that 4 phenolics included pyrogallol, vanillic acid, syringic acid, and ferulic acid were found in the extract. However, while confirmed the experiment using the mass spectroscopy, the wrong interpretation was detected. Since only similar retention time compares with the external standard was used to indicate the type of phenolic compounds from HPLC.

Figure17 presents the reversed-phase UHPLC-DAD chromatogram of ricegrass juice extract cultivar Chainat 1 obtained using the suitable gradient elution program. In total, 11 compounds were tentatively identified through the ESI-MS in negative mode considering the UV and MS spectral data. Data concerning the identification of the peaks were shown in Table 16, where the retention time, molecular weight and electrospray ionization mass spectrometry of all the compounds detected were also reported. Peak 1 was observed to represent a base molecular anion at m/z 191, thus it was identified as quinic acid. Peaks 3 and 4 were identified as phenolic glycosides according to the m/z of base ions at 315 and 385 resembling the spectrum of protocatechuic glucoside and 1-o-sinapoyl-β-D-glucose, respectively. The ion found at the retention time 23.87 min (peak 5), with the m/z of 367, presented a fragment at m/z 193 which resembles the m/z of the ferulic acid fragment. Thus, this compound was consistent with 3-o-feruloyl-quinic acid. The large group of secondary metabolite compounds in the leaves of *Oryza sativa* have been reported earlier to be flavone glycosides (Besson *et al.*, 1985). The spectra of peaks 6-9 showed a similar base peak of 563 which resembles the spectrum of chrysoeriol

arabinosyl arabinoside derivatives. This compound has been formerly found in the literature investigating the compounds in rice leaves using MS/MS with the same m/z recorded (Yang *et al.*, 2014). Peak 7 was recognized as the main compound in ricegrass juice extract. The ESI-MS on negative ion of this peak is presented in Figure 18. Peaks 2, 10 and 11 were the group of compounds which also tentatively identified as flavone glycosides referred to the literature. The m/z of 325, 445, 491 were detected and they were identified as tricin, swertisin, and tricin-7-o-β-D-glucopyranoside, respectively, which are the compounds existing in the leaves of rice and wheat (Kim *et al.*, 2008, Moheb *et al.*, 2013, Yang *et al.*, 2014).

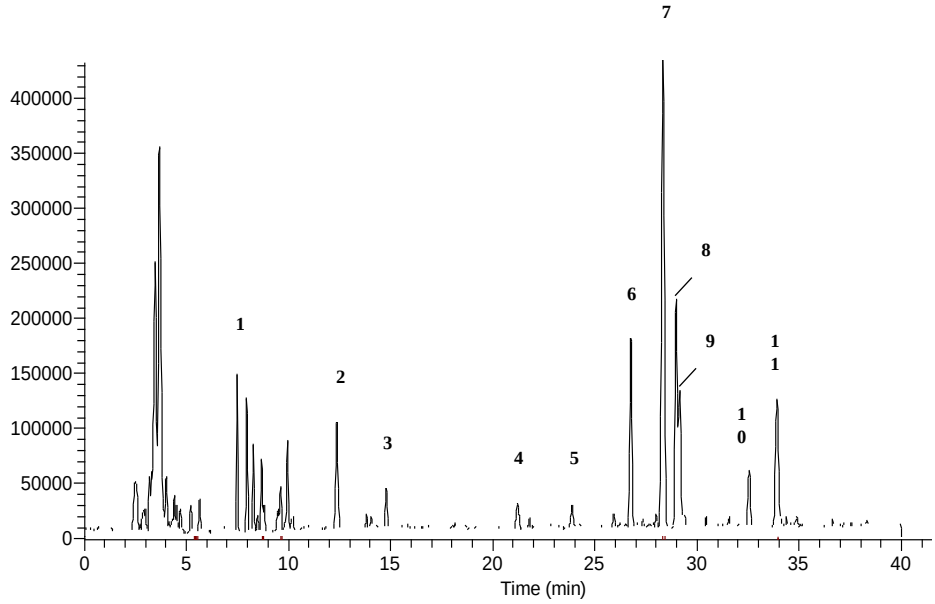


Figure 17. Reversed-phase UHPLC-DAD chromatogram of Thai ricegrass juice extract (*Oryza sativa.*, Chainat1) with identified peak (1-11).

Table 16. Tentative identification of phenolic compounds in ricegrass juice analyzed by UHPLC-DAD-ESI-MS.

Peak no.	RT (min)	Tentative compounds	MW	[M-H]+ (m/z)	Fragment ion	Ref.
1	7.95	Quinic acid	192	191	111, 192, 613	(Zhao *et al.*, 2013)
2	12.38	Tricin	330	329	175, 461	(Yang *et al.*, 2014)
3	14.79	Protocatechuic glucoside	316	315	175, 445, 575	(Fang *et al.*, 2002, Sulaiman *et al.*, 2012)
4	21.20	1-*o*-Sinapoyl-β-D-glucose	386	385	175, 469, 599	(Yang *et al.*, 2014)
5	23.87	3-*o*-Feruloylquinic acid	368	367	193, 305, 497	(Markham *et al.*, 1998, Yang *et al.*, 2014)
6	26.75	Chrysoeriol arabinosyl arabinoside derivatives	564	563	175, 305, 693	(Besson *et al.*, 1985, Yang *et al.*, 2014)
7	28.31	Chrysoeriol arabinosyl arabinoside derivatives	564	563	565, 693	(Besson *et al.*, 1985, Yang *et al.*, 2014)
8	29.00	Chrysoeriol arabinosyl arabinoside derivatives	564	563	693	(Besson *et al.*, 1985, Yang *et al.*, 2014)
9	29.14	Chrysoeriol arabinosyl arabinoside derivatives	564	563	693	(Besson *et al.*, 1985, Yang *et al.*, 2014)
10	32.56	Swertisin	446	445	175, 305	(Yang *et al.*, 2014)
11	33.90	Tricin-7-*o*-β-D-glucopyranoside	492	491	769, 983	(Yang *et al.*, 2014)

[a] Peak numbers and retention times refer to Figure 17. RT: retention time. MW: Molecular weight

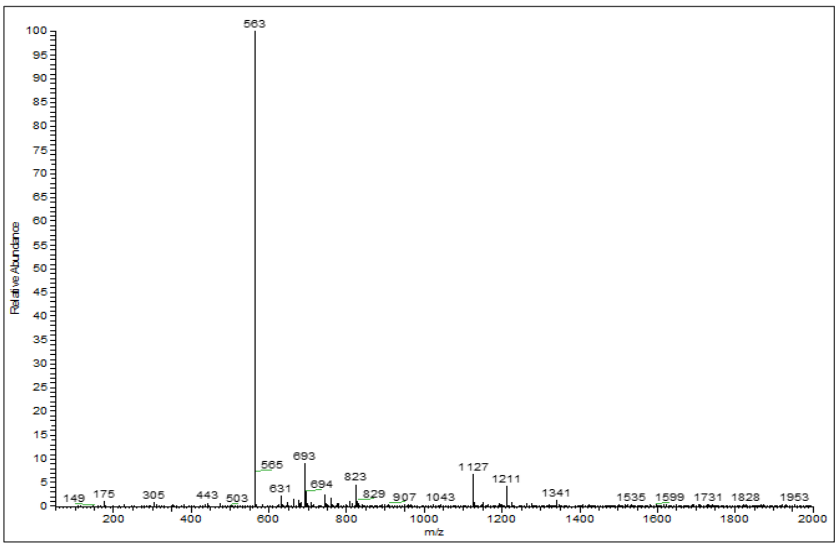
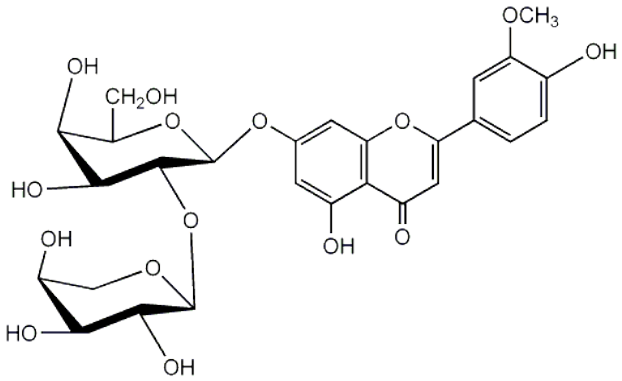
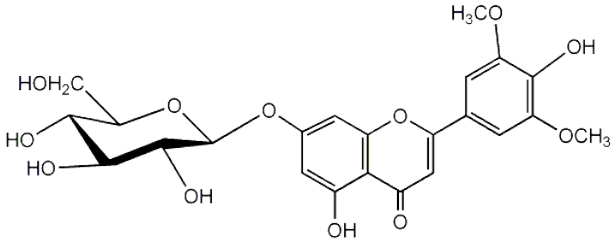


Figure 18. Electro-Spray ionization mass spectrum on negative ion of peak 7 (RT 28.71) in ricegrass juice extract.



Chrysoeriol arabinosyl arabinoside



Tricin-7-o-β-D-glucopyranoside

Figure 19. Structure of main polyphenol components found in ricegrass juice extract

4.4.3 Cell viability

Safety aspect was stated as it is necessary for introducing each functional food product into the market. Reliable evidence is needed to make a claim on the cytotoxicity of the extract on mammalian cells. The cytotoxicity of the extracts on RAW264.7, a murine macrophage cell, was chosen since it can represent the influence of compounds specifically on an essential immune system, which is the first defense step in the human body. Macrophages are phagocytic cells which play a crucial role in the clearance of microorganisms, pathogens, and harmful disturbances. Thus, it is also important for the function of antigen presentation, cytokine production and anti-tumor activity (Cekici *et al.*, 2014). The number of viable cells was measured after incubating the cells with the extracts for 24 h.

Figure 20 reveals the number of cell viability of RAW264.7 cells after incubating with various concentrations of 3 extracts (RG0, RG10, RG40), ranging from 250 – 5,000 µg/ml compared with the control. The macrophage cell number slightly decreased through an increased concentration of extracts because an excessive concentration of phenolic compounds, as well as Se compounds in the extract, can act as pro-oxidant molecules which cause damage to cells and lead to cell death. However, the results still confirmed that all of the extracts had no or low toxicity to the RAW264.7 macrophage cells. According to the system of drug screening for neglected diseases, the 50% cytotoxicity concentration (CC_{50}) of any substance which was higher than 90 µg/ml was classified as no toxicity (Ioset *et al.*, 2009), thus the extracts were confidently claimed as safe (CC_{50} over 5,000 µg/ml). Furthermore, it was found that RG40 greatly promoted the cell number of RAW264.7 cells ($p<0.05$) compared with the control especially at low concentration, while RG0 and RG10 did not. All the extracts caused a reduction in the number of the viable cells while the concentration has been increased. Effect of ricegrass juice extract on cell viability in this study can be stated from both phenolics and Se. Other literature which checked the effect of Se on mouse monocyte-macrophage cell line (TIB69) also revealed similar results on the supplementation Se which influences on cell proliferation (Genuardi *et al.*, 1999).

Both phenolic and Se compounds have been claimed individually for the positive effects over the cell viability at low amount by being involved as a source of nutrition and possibly playing a role in antioxidant protection in the metabolism of the cells, however, the higher level of both Se and phenolic compounds can cause the oxidative damage to cells and result in cell death. It could be assumed that RG40 at low concentration (250 µg/ml) is the most suitable condition for RAW264.7 cells to grow while RG10 at about 1000 or 2000 µg/ml, though provide similar level of Se compounds to RG40 at 250 µg/ml, it provides higher level of phenolic compounds and may indicate in the reduction of cell viability. Moreover, the bio-fortification of Se into ricegrass may propose changes in various components not only phenolic compounds and selenium content. The LCMS analysis may only visible some compounds contained in the extracts which do not differ from each other. The other compounds may play some role in the cell proliferation as well as cell reduction.

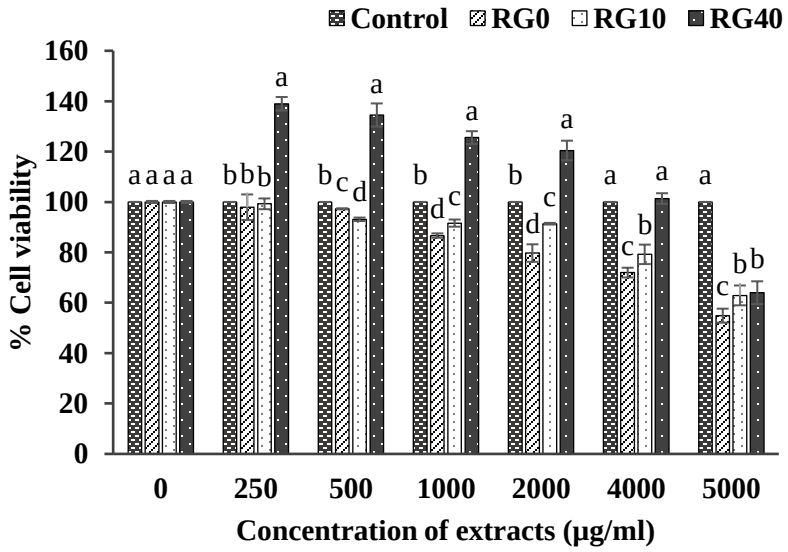


Figure 20. Cell viability and cell proliferation of RAW264.7 murine macrophage cells after incubating with various concentrations of Se bio-fortified ricegrass juie extracts from 250-5,000 µg/ml for 24 h. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration ($p< 0.05$).

4.4.4 Lipid peroxidation and antioxidant enzymes level

Oxygen is the primary agent used for the respiration and the metabolism of living organisms. ROS are a natural byproduct of the normal metabolism of oxygen. Generally, it has had an important role in cellular signaling and defense against pathogens (Halliwell, 1991). However, during times of environmental stress, ROS levels can increase dramatically and the elevated amounts include superoxide anion ($O_2^{\bullet-}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) and nitric oxide (NO), resulting in significant damage to cell structures. Living organisms contain lipid as the main structure of cellular membranes (Ray Halder and Bhattacharyya, 2014). Thus, lipid peroxidation can be stated as a crucial step in the pathogenesis of several disease states in humans such as atherosclerosis, Alzheimer's disease, and cancer. Therefore, the extent of lipid peroxidation by-products produced like malondialdehyde (MDA) can reflect the extent of oxidative damage to cells (Ayala *et al.*, 2014) (Figure 21).

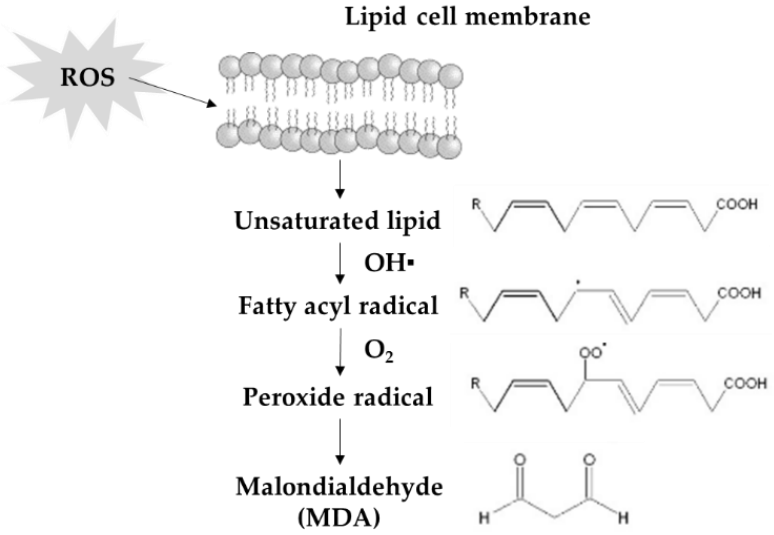


Figure 21. Production of MDA from lipid peroxidation process

The level of MDA detected from the cellular extracts of RAW264.7 cells after treatment with the extracts, therefore, revealed the effect of Se bio-fortified ricegrass juice extract and on the role of oxidative stress protection (Figure 22). Outcomes indicated

that ricegrass juice extracts of all treatments can significantly reduce the level of MDA detected from RAW264.7 cellular compared with the normal cells. The role of phenolic compounds in the extracts was considered as having the major effects. Phenolic compounds possess a mechanism to lower the MDA level through the ability as an electron donor, which can stabilize the hydroxyl radicals and lipid peroxyl radicals, thereby lowering the extent of oxidative damage to the lipid cell membrane. In addition, an increased level of Se content in the RG10 and RG40 showed more reduction in MDA content compared with the RG0 at dose 1000 µg/ml.

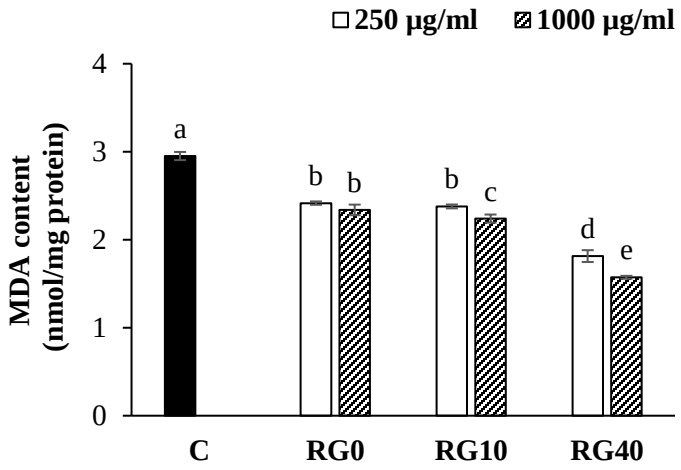


Figure 22. Level of malondialdehyde (MDA) in RAW264.7 cell line treated with Se bio-fortified ricegrass juice extract (RG0, RG10, RG40) at level 250 and 1000 µg/ml compared with control (Media alone). Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences ($p< 0.05$).

In order to clarify the conceivable mechanism of the extracts in lowering the level of lipid peroxidation, their ability to regulate three main endogenous antioxidant enzymes including SOD, CAT, and GPx was also studied. SOD is the defense enzyme that deals with superoxide radicals ($O_2^{\bullet-}$) which are the first radicals generated from oxygen metabolism and convert them to hydrogen peroxide (H_2O_2) which CAT and GPx are occupied and further transformation to harmless water (H_2O) which terminates the damaging chain reaction (Mates, 2000).

All the extracts at low concentration seemed to have no effect on the level of total SOD, Yet at a higher concentration, there was a slight increase total SOD. No effect was detected from the different concentration of Se in the extracts (Figure 23a). The addition of extracts to RAW264.7 cells in all treatments was able to promote the activity of GPx which are classified as the seleno-enzymes. It appeared that the RG40 at 1000 µg/mL can increase the highest proportion of GPX activity, up to 73% above the control (Figure 23b). Since Se is the main cofactor of this enzyme, extracts which contain a high level of Se can then logically promote a greater level of GPx activity. In the CAT assay, low level of the extracts at RG10 and RG40 treatment showed an increasing CAT activity above the level of normal cells (C) while the level of CAT in RG0 showed no change. However, a higher level of the extracts at 1000 µg/mL suppressed CAT activity (Figure 23c). This phenomenon can be explained by the fact that GPx and CAT had grossing similar roles in converting H_2O_2 to H_2O. While GPx was high enough to reduce the level of the substrate, the CAT was probably sparing in the cells to persist the homeostasis. According to the results, ricegrass juice extracts revealed the supportive activity on all three antioxidant enzymes above the regular one. Animal studies on the effect of dietary supplementation of phenolic compounds on antioxidant enzymes levels in rat suggested that phenolic compounds selectively induced the mRNA expression through the upregulation of gene transcription and Nrf2 transcription factor (Yeh and Yen, 2006). Moreover, it was noticed that the tested extracts provided larger effects on CAT and GPx enzymes compared with total SOD. The reduction in MDA content from the cellular extracts of cells treated with the test samples especially in RG40 treatment, thus indicating that the proposed role of these two enzymes is via both Se element and phenolics function.

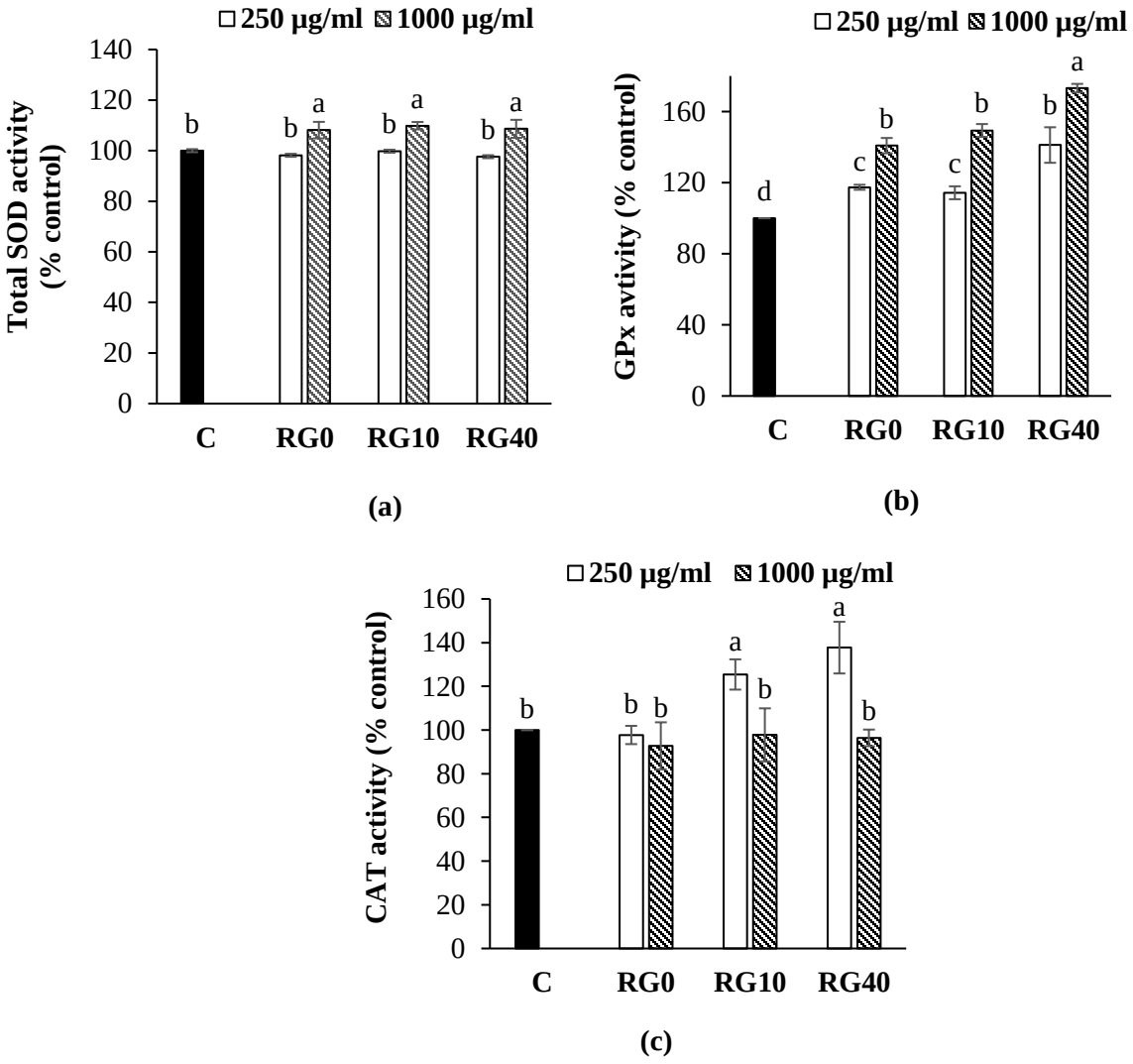


Figure 23. Level of (a) SOD, (b) GPx and (c) CAT in RAW264.7 cells treated with Se bio-fortified ricegrass juice extract (RG0, RG10, RG40) at level 250 and 1000 µg/ml compared with control. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences ($p< 0.05$).

4.4.5 Nitric oxide inhibition

Nitric oxide (NO) is a major mediator produced by macrophages during inflammation responses. It is produced by the inducible nitric oxide synthase (iNOS) from the substrate, arginine (Sharma *et al.*, 2007). NO is vital for many physiological functions such as being a neurotransmitter and affecting blood flow and synaptic plasticity. On the

other hand, an excess amount of NO can destroy and induce dysfunction in the macrophages themselves as well as in surrounding normal cells (Kim and Park, 2016). Therefore, the study on plant foods which can modulate the level of NO produced by its homeostasis is an important issue because they are safer, limited side effects and relatively low cost compared with medication. In this study, LPS from E. coli as a foreign matter were applied to RAW264.7 cells to initiate the production of NO. Then, RG0, RG10, and RG40 were co-treated to analyze their NO production inhibitory effects. The concentration of ricegrass juice extracts which did not affect the percentage of cell viability to be lower than 80% was 2,000 µg/ml. Thus, this range was the maximum concentration used for the determination of nitric oxide inhibition properties to verify that NO was truly produced. The level of LPS which did not affect cell viability and could stimulate the highest level of NO was scanned and the level of LPS at 0.5 µg/ml was chosen. The percentage of NO production inhibition was measured using the Griess assay and it was shown as the percentage of negative control (Figure 24). The co-incubation experiment of LPS and the extracts at various concentration showed a dose-dependent inhibition of NO production. Results revealed that NO from LPS-induced RAW264.7 was significantly inhibited by ricegrass juice extracts. The Se biofortified one, RG40, showed the greatest ability to inhibit NO compared with other treatments. The IC50 of RG0, RG10, and RG40 were 147.02, 147.73 and 118.76 µg/ml, respectively. Although the IC_{50} values were still higher compared with the positive control, L-nitro-arginine (LNA), which is known as the enzyme iNOS inhibitor (IC_{50} = 30.05 µg/ml). The cell number after tested the NO inhibition were also confirmed no toxicity of extracts (Figure 25). Therefore, ricegrass juice in all treatments still on a considerable amount that can be classified as functional food.

The NO inhibition of ricegrass juice extract can be initially attributed to the role of phenolic compounds. A correlation between high intake of phenolic compound rich food and the ability to downregulate the inflammatory responses *in vitro* and *in vivo* has been previously reported (Panico *et al.*, 2006, Terra *et al.*, 2009). It has been hypothesized that phenolic compounds exert anti-inflammatory activity by inhibiting the synthesis of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of activated

immune cells, or inhibition of iNOS and cyclooxygenase-2 via inhibitory effects on nuclear factor NF-κβ (Chuang and McIntosh, 2011). Main components of phenolic compounds found in ricegrass juice extract were in the group of flavone glycosides. The previous study has reported on the effect of flavonoid compounds like apigenin and quercetin on NO; the results indicated that the inhibition of NO was due to the reduction of iNOS expression (Chen *et al.*, 2001). Moreover, flavone compounds may possess scavenging ability since NO is one of the reactive oxygen species (ROS) which is a by-product formed during the mitochondrial respiratory process (Yeh and Yen, 2006). Phenolic compounds can effectively scavenge the radicals as well as NO radical through the antioxidation process by stabilizing the radicals through the active site of -OH group and the planar ring. Thus, a reduction in the NO level in cell surrounding was found.

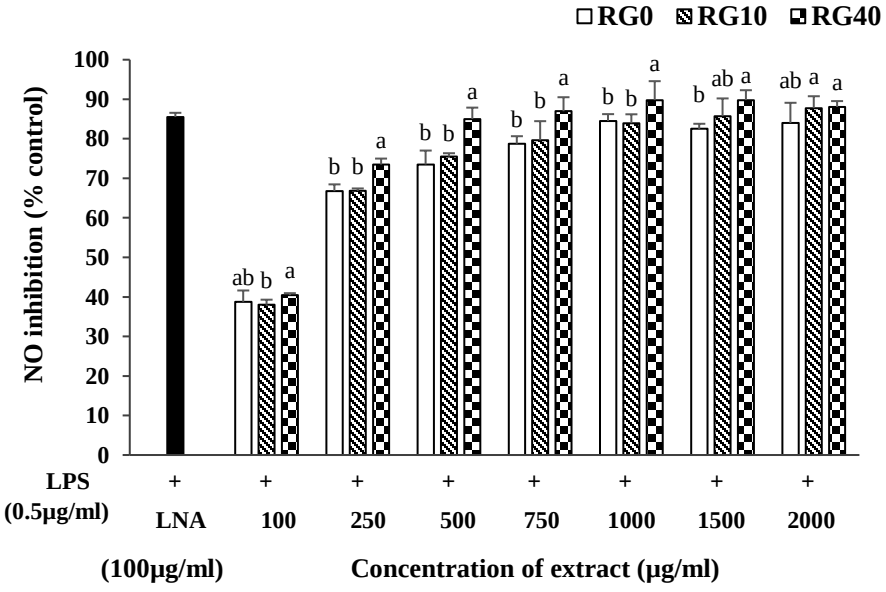


Figure 24. Effect of Se bio-fortified ricegrass juice extracts from 100-1,000 µg/ml on the inhibition of nitric oxide (NO) in LPS-induced RAW264.7 murine macrophage cells for 24 h. LNA was used as a positive control (100 µg/ml). Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration ($p< 0.05$).

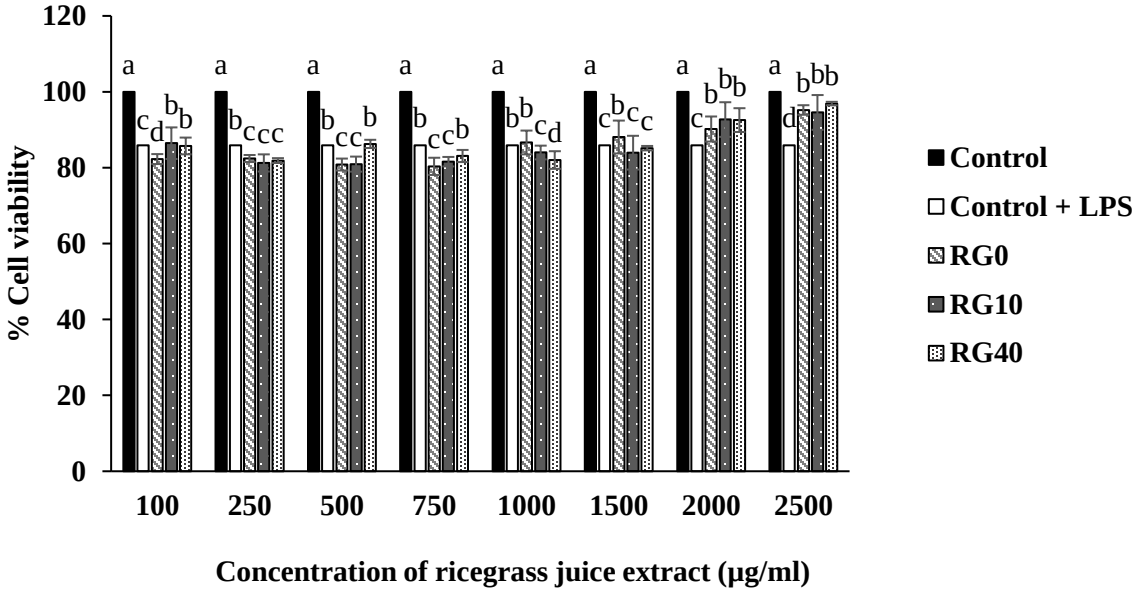


Figure 25. Effect of Se bio-fortified ricegrass juice extract on the cell viability after determination of NO inhibition of RAW264.7 murine macrophage cells. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration ($p< 0.05$).

Another segment which influences the reduction of NO production may relate to the activity of Se. Since RG40 exerted approximately an extra 5% on NO inhibition activity compared with RG0 and RG10, the effect of Se itself on NO inhibition was then studied to confirm the additional benefit (Figure 26). The experiment was designed to use two forms of Se compounds including sodium selenite as inorganic Se supplement and selenomethionine (Se-Met) from Se yeast as organic Se supplement. Se in both forms exerted NO inhibition properties at a low dose, which less than 0.15 µg/ml, while a higher dose of the pure compounds was toxic to the cells. Furthermore, Se-Met exerted higher ability than sodium selenite to reduce the level of NO. The underlying mechanisms of Se to inhibit the production of NO might be that Se as an essential part of the enzyme glutathione peroxidase (GPx), Se-GPx active site may exert its chemo-preventive effect by inhibiting the expression of iNOS and subsequently inhibiting NO production (Prabhu *et al.*, 2002). Moreover, the organic form of Se provided higher ability could be due to the bioavailability of the organic Se in which the element was already

bound to amino acid and could be utilized straightforwardly as a part of the enzyme while inorganic form like selenite needs to initially be transformed to the organic form before being utilized, so an extra stage is required (Verma *et al.*, 2012). Since the majority of Se in the ricegrass juice extract was supposed to be organic form, the RG40 treatment which contained 5 times of Se content than the control, may provide more advantages from the higher level of organic Se. These outcomes provided evidence for the beneficial effects of dietary Se supplementation in the prevention and or treatment of oxidative-stress-mediated inflammatory diseases.

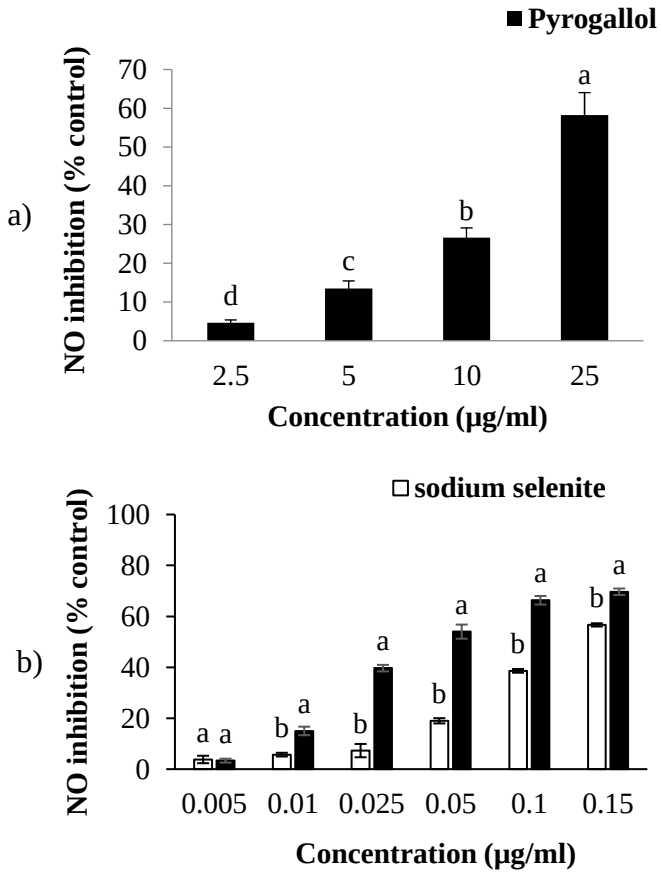


Figure 26. Effect of (a) pyrogallol and (b) sodium selenite and seleno-methionine (se-Met) from Se-yeast of extracts on the inhibition of nitric oxide (NO) in LPS-induced RAW264.7 murine macrophage cells. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration ($p< 0.05$).

4.5 Conclusion

Se-rich ricegrass juice extract can be classified as safe since it had no toxicity on RAW264.7 murine macrophage cells, additionally RG40 promoted proliferation of the cells. Flavone glycosides were identified as the main phenolic compounds in ricegrass juice extracts and possessed biological properties on the reduction of oxidative stress and NO inhibition. This finding was the first work to report the ability of ricegrass juice to play a part in the treatment of anti-inflammation. Moreover, Se supplemented treatment (RG40) reduced the greatest level of MDA content and inhibited NO production in which the mechanism was related to an increase in CAT and GPx activities. Therefore, Se-rich ricegrass juice could be considered for production as a value-added functional drink from rice that can lower risk of oxidative stress and chronic inflammatory disease and promote human well-being.

CHAPTER 5

PROBIOTIC GROWTH ENHANCER AND ANTI-COLON CANCER PROPERTIES OF SELENIUM BIO-FORTIFIED RICEGRASS JUICE [1]

5.1 Abstract

Gut microbiota play roles in maintaining good health and wellness to human. Exogenous plant food or ingredients which can promote advantage role to those beneficial bacteria are concerned. Young ricegrass was introduced shortly as a new functional food. The enrichment of selenium (Se) into the plant is one strategy to enhance the bioactivity of those plants. This work aimed to investigate the effect of Se bio-fortified ricegrass juice extract (RG0, RG10, RG40) on the ability of probiotic growth enhancer properties and the possibility of them to inhibit colon cancer cells. *Lactobacillus plantarum* was used as a represent probiotic strain. MRS broth was extra added with the extracts and microbial numbers were count every 12 h until 60 h. The extracts were treated to HT-29, human colon adenocarcinoma cells and the % cell viability was checked using MTT assay. Results revealed that ricegrass juice extract can support the growth of *Lactobacillus plantarum* and prolong their life cycle. It also showed the ability to inhibit colon cancer cells. Moreover, the treatment enriched with Se shows superior ability to that without Se. Those results were supported by the mechanisms of phenolic compounds in ricegrass juice and Se which possibly exerted the reduction of ROS, managing the oxidative stress and inducing cancer cell death. Se-enriched ricegrass juice extract can be used as a functional food product to promote good gut health.

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[1] The content of this chapter has been published in the proceeding of *Food and Applied Bioscience*

5.2 Introduction

Human colon is the main habitation of more than 500 species of microbial flora which play multiple roles in human health (Fuller and Peridigón, 2008). It is now generally accepted that the composition of the human intestinal microbiota has an important role in maintaining good health and the prevention of some diseases (Guarner and Malagelada, 2003). Probiotics were claimed on their mechanisms of action on the immunomodulation, suppression of the pathogens, supply the production of short chain fatty acids which can reduce the regression of tumors and carcinogenic compounds (Charalampopoulos and Rastall, 2009). The supplementation of live probiotic bacteria exogenously from food product can increase the number of gut microbiota, however, a high percentage of ingested lactic acid bacteria may lose while facing with stomach acid and bile during the delivery through the gastrointestinal tract (Marteau *et al.*, 1997). Accordingly, the additional consumption of diet exogenously which contains the ingredients or substances that can promote the positive environments to those live bacteria, prolong their period of living and enhance the proliferation of them in the gut become an important subject in nutritional sciences area.

Plants are the most abundant food sources for a human. Young ricegrass are currently introduced as new functional foods as it is not well-known as edible food for human earlier. However, recent literature was attentive on the production of ricegrass as a substitution of wheatgrass which was recognized to contains many beneficial compounds (Padalia *et al.*, 2010). Since rice and wheat were the plants of the same species. Correspondingly, rice was a plentiful raw material in the tropical area. Thus, it could be produced similarly to wheatgrass. Moreover, some researchers indicated that ricegrass juice extract was associated with many phenolic compounds and possess beneficial biological properties included antioxidant activities (Rattanapon *et al.*, 2016, Chomchan *et al.*, 2016) and DNA protective properties (Khanthapoka *et al*., 2015).

Selenium (Se) is a trace mineral which works as a chief component of antioxidant enzymes in the human body. Se has been known to manage various reactive oxygen species-triggered diseases (Brenneisen *et al.*, 2005). Moreover, it was proved by

plenty of clinical studies that Se is an active anti-cancer substance that can reduce the incidence of all causes cancer (Rayman, 2005). The enrichment of Se into the plant is therefore becoming a useful tool to provide Se-plant foods which can promote superfluous advantages to human health. The present study aimed to investigate the possibility of Se enriched ricegrass juice extract on gut health promoting effect to better support the use of them in functional food products by investigating the effect of them on the proliferation of probiotic bacteria namely *Lactobacillus plantarum.* Moreover, the effect on HT-29 human colon cancer cell viability was also studied.

5.3 Materials and Methods

5.3.1 Plant materials

Young ricegrass juice extract (RG0) and Se bio-fortified ricegrass juice extract (RG10, RG40) prepared from lyophilized aqueous extracts of 8 days grown ricegrass (*Oryza sativa* L. cv. Chainat 1) were used in this study.

5.3.2 Probiotic growth enhancer properties

A pure culture of *Lactobacillus plantatum* TISTR1465 was obtained from Thailand Institute of Scientific and Technological Research, Thailand. Briefly, 10% (v/v) of ricegrass juice extracts (RG0, RG10, and RG40) were initially filtered sterilized and supplemented to fresh MRS broth at the concentration of 10 mg/L and 20 mg/L. Pyrogallol, and sodium selenite at various concentration were used as the sample control. The inoculum of 10% (v/v) *L. plantarum* culture at late-log phase was introduced to a total 5 ml of MRS broth containing the extracts. Sterilie distilled water was used instead of the extracts for the positive control and medium alone represented the negative control. The tubes were incubated anaerobically at 37◦C for 12, 24, 36, 48 and 60 h. At each incubation period, 100 µl aliquot of each dilution was spread in duplicate on the surface of the plates containing MRS agar supplemented with 0.001% bromocresol blue. The plates were incubated anaerobically at 37◦C for another 48 h and the number of viable bacterial cells

were recorded. The experiment was referred to the modified method of Molan *et al.*, (2009).

5.3.3 Anti-cancer properties

HT-29 human colon adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin was used for the maintenance of cells at 37◦C, 5 % CO_2, in a fully humidified incubator. Phosphate buffer saline (PBS) at pH 7.2 was used to wash the cells and clean the surrounding through the experiment. The MTT assay was used to determine anti-cancer properties. Cells grown at 80-90% confluent were harvested with 0.25% trypsin – EDTA and suspended in a fresh medium. Cell counts were measured using a standard haemocytometer based trypan blue cell counting technique. HT-29 cells at the density of 1 × 10^5 cells/ml were seeded in 96-well tissue culture plates and allow them to adhere for 24 h. After washing the cells with PBS (pH 7.2), they could react with the media mixed with a various concentration of the extracts (0.25 – 10 mg/ml) followed by 24 h incubation. Pyrogallol, sodium selenite and Se-methionine (Se-Met) from Se-yeast were also used as the sample control. The cell viability after 24 h was checked by removing 100 µl of supernatant and 10 µl of MTT solution was added. After 2 h, MTT solution was removed, and 100 µl of 0.04 N HCl in isopropanol was added to dissolve the formazan crystals. Absorbances were recorded at 570 nm using a microplate reader. The percentage of cell viability was calculated as a followed equation;

$$\% \text{ Cell viability} = [\text{Absorbance of sample} / \text{Absorbance of control}] \times 100$$

5.3.4 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experimental data were presented as the mean ± standard deviation of three replications. Means were analyzed using the analysis of variance (ANOVA). The significant differences

among means were determined by Tukey's test (p<0.05) using SPSS for Windows (SPSS Inc, Chicago, IL).

5.4 Results & Discussion

5.4.1 Probiotic growth enhancer properties

The growth curve of *Lactobacillus plantarum* TISTR1465 was initially studied to observe the regular growing phase cycle of this bacterial strain (Figure 27). Log phase of every living cells has been specified as the most vigorous stage and the late-log phase indicated the maturity of the cells. The number of colonies measured by total viable plate count technique indicated that the late log phase of this strain was at 12 h. The stationary phase of the *L. plantarum* remained for another 24 h and slightly turned to the death phase afterward.

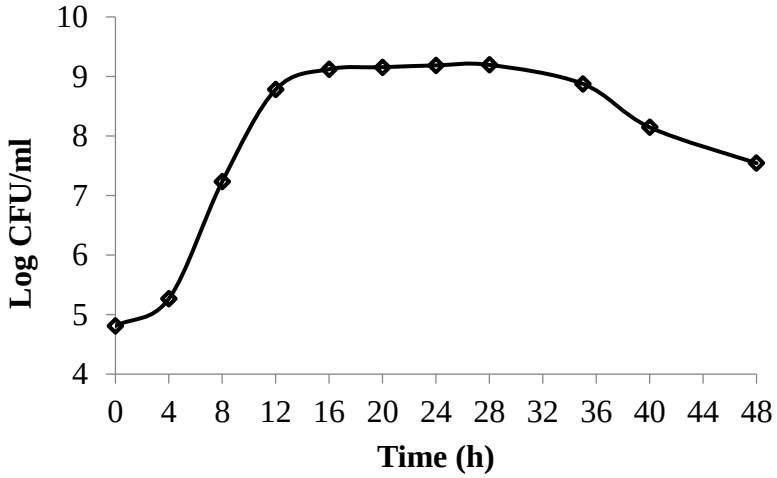

Figure 27. Growth curve of *Lactobacillus plantarum* TISTR1465.

Addition of all extracts from ricegrass juice (RG0, RG10, RG40) to MRS broth influences on higher number in the bacterial population. The fortified of RG40 into MRS media presented the most effective capability on promoting the growth of *L. plantarum* pure cultures than the normal ricegrass juice extract at the late log phase (12 h). Furthermore, it also prolongs the life cycle of bacteria by retaining a high number of survival of cells after late log phase until the early death phase, especially the most

significant difference number from the control have been observed at 60 h. Different doses of each extract (10 and 20 mg/ml) were also performed on this activity, a higher concentration of all the extracts at 20 mg/ml addition of RG treated to the bacteria exerted a better effect on the protectant of cell death than the dose of 10 mg/ml. The main finding of this experiment was ricegrass juice extract showed an improvement of the microbial population better than fed with MRS alone. This result can be explained that polyphenol compounds showed an ability to improve the growth of bacteria by modulate the incidence of oxidative stress in the medium and provide a better environment for the multiplication of probiotics. The examining effect of pyrogallol as a represent of phenolic compounds in this study also confirmed this fact. (Figure 29). The minor effect from different TPC content in each treatment of Se bio-fortified ricegrass extracts (RG0, RG10, RG40) can be counted since the addition of Se compounds exposed only minor changes. The major effects involved in an increasing number of this bacteria from RG40 may propose to a high level of Se compounds. Effect of Se enriched plants on promoting the growth of probiotic strain in this study was inconsistent to the previous work which stated that Se fortified green tea contain prebiotic effects by promoting the growth of *Lactobacillus rhamnosus* and *Bifidobacterium breve* when added to MRS broth (p = 0.0001) compared with the regular one (Molan *et al.*, 2009). This work attempted to explain that the combination of polyphenol compounds and Se compounds which can act as the cofactor of the endogenous antioxidant enzymes, thus promote the action as an antioxidant to modulate the oxidative stress generated by the metabolic activities and consequently provided a better environment for the survival of these bacteria.

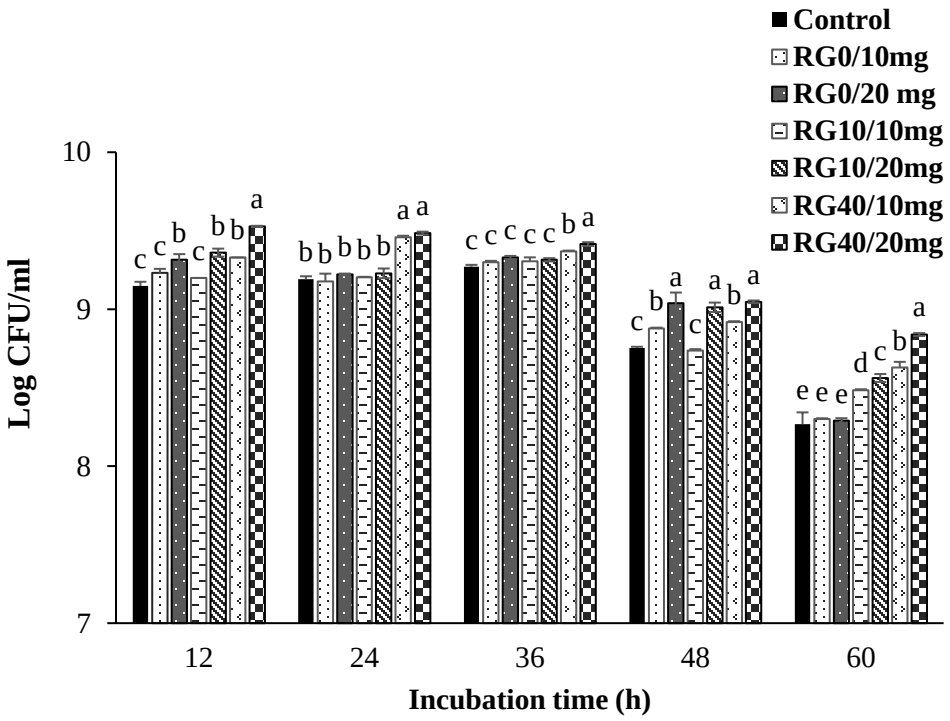

Figure 28. Effect of Se bio-fortified ricegrass juice extract (RG0, RG10, RG40) on the survival of *Lactobacillus plantarum* TISTR1465 compared with the control (MRS alone) at different concentration. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences (p<0.05).

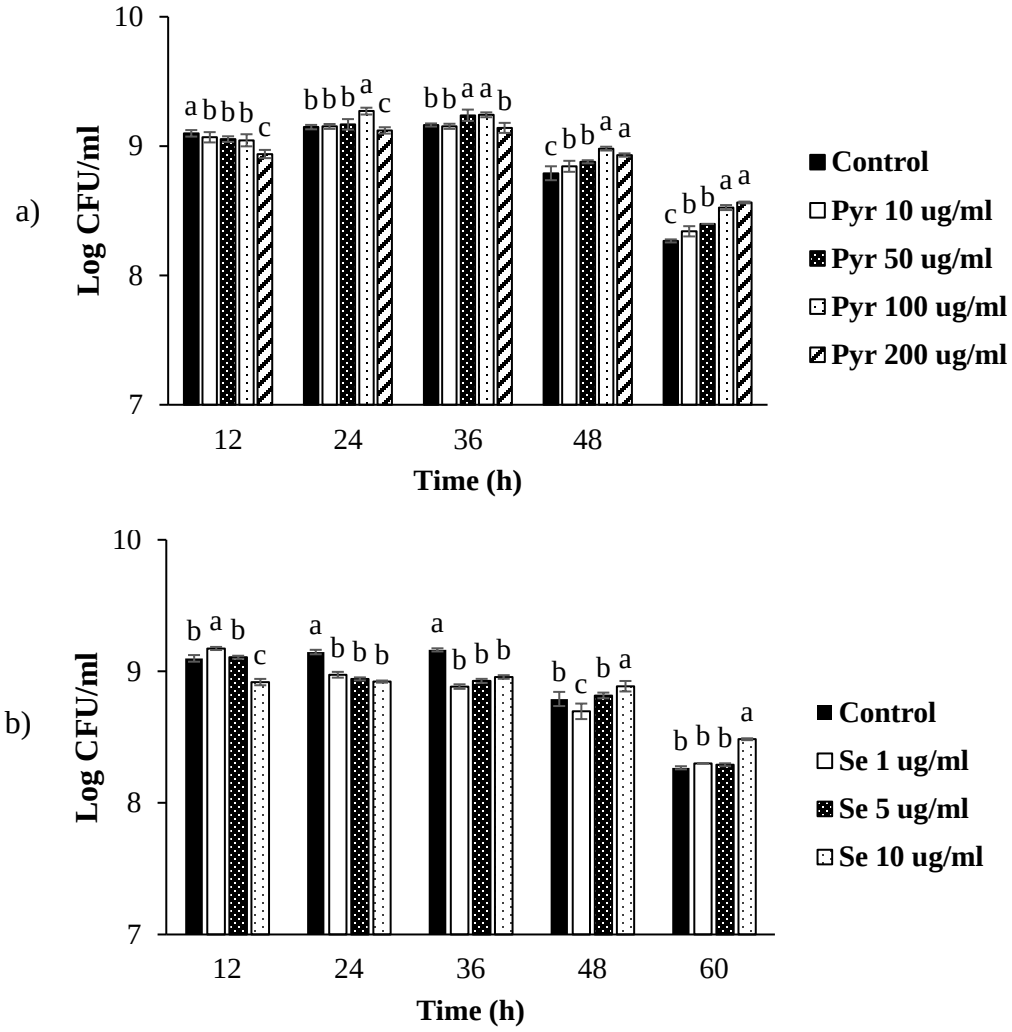

Figure 29. Effect of a) pyrogallol and b) sodium selelnite on the survival of *Lactobacillus plantarum* TISTR1465 compared with the control (MRS alone) at different concentration. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration (p<0.05).

5.4.2 Anti-colon cancer properties

Colon cancer is the fourth most common cause of cancer mortality worldwide (Rosa *et al.*, 2016). Oxidative stress has been stated to activate the inflammation pathways which lead to the transformation of normal cells to cancer cells (Reuter *et al.*, 2010). Plants which contain a wide variety of free radical scavenging molecules may possess as good anti-cancer substances since various epidemiological studies have shown

the benefits of natural antioxidants intake are associated with reduced risks of cancer (Kuo, 1997). The anti-cancer properties of Se bio-fortified ricegrass juice extract on colon cancer HT-29 cells were then investigated while incubated for 24 h using MTT assay. Results revealed an inhibition in the growth of cancer cell with dose-dependent when treated the cells with all treatments of Se bio-fortified ricegrass juice extracts. A higher level of all extracts can inhibit a higher number of cells while the RG40 demonstrated the greatest efficiency in killing the cancer cells (Figure 30). The half maximal inhibitory concentration (IC_{50} value) of the three extracts RG0, RG10 and RG40 on inhibiting HT-29 cells were 10.97, 9.27 and 7.88 mg/ml, respectively. According to the standard of the National Cancer Institute (NCI), an extract is considered to have significant anti-cancer properties if the IC_{50} value is less than 20 µg/ml (Cordell, 1993). Consequently, ricegrass juice extracts were not classified as strong anti-cancer substances or drugs. On the other hand, their properties on fighting with the cancer cells still useful on the use of them as a functional food for natural cancer treatment which can consume in everyday life.

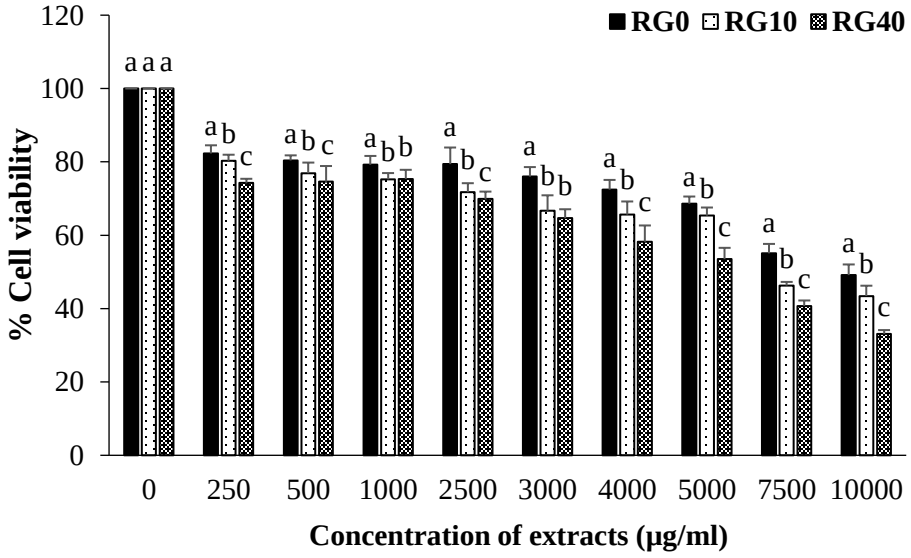

Figure 30. Effect of Se bio-fortified ricegrass juice extract (RG0, RG10, RG40) on the % cell viability of HT-29 colon cancer cells. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration (p<0.05).

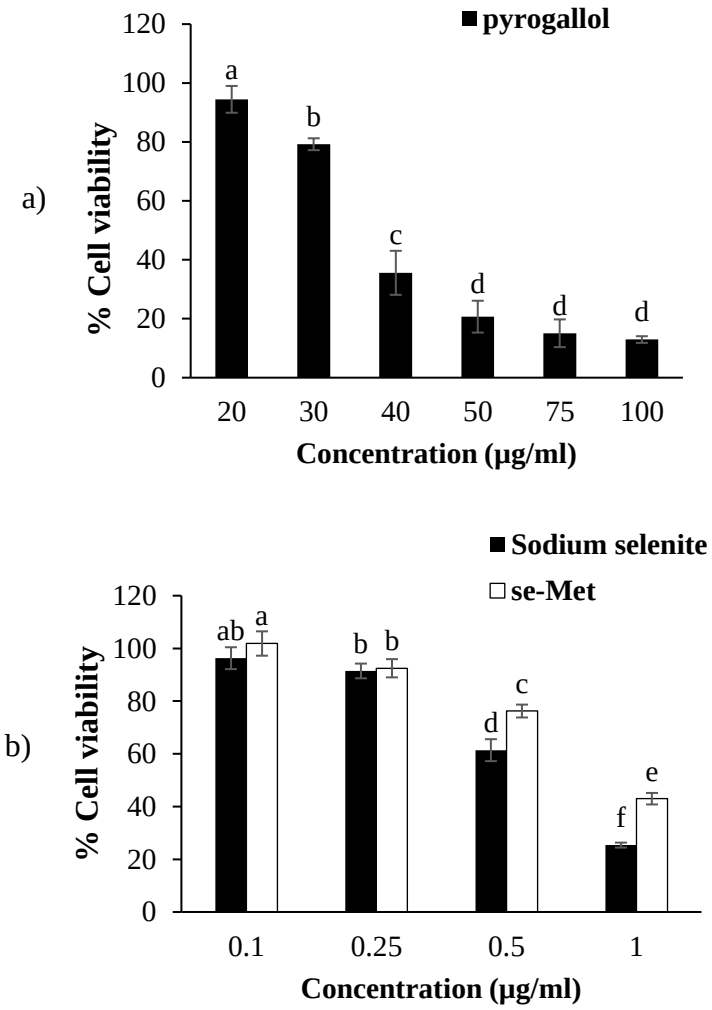

Figure 31. Effect of a) pyrogallol and b) sodium selelnite ans Se-Met from Se-yeast on the % cell viability of HT-29 colon cancer cells. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration (p<0.05).

The underlying mechanism of treatment RG40, Se bio-fortified ricegrass juice extract on the greatest ability of inhibiting cancer cells may propose from numerous points (1) phenolic compounds which play roles in scavenging free radicals and balance the homeostasis of the normal cells. They also effect on the modulation of cellular signaling pathways including those involved in DNA damage repair, cell proliferation, apoptosis and invasion and alteration of the tumor differentiation processes (Rosa *et al.*, 2016).

Pyorogallol as a representative of phenolics showed a reduction in cancer cells viability (Figure 31a); (2) Se is the well- known substance inhibiting cancer cells. The bio-fortification of Se compounds in ricegrass may affect an enhancement of immune system response by increasing the number of cytotoxic lymphocytes and natural killer cells (Kiremidjian-Schumacher *et al.*, 1996). Moreover, nutrigenetic researchers discovered that seleno- methionine, organic Se can activate tumor suppressor protein p53 gene which can inhibit the proliferation and induce the early apoptosis of cancer cells (Smith *et al.*, 2004). An excellent reduction in cancer cells viability was also found when incubated with both sodium selenite and se-Met from Se yeast (Figure 31b) while sodium selenite which is the inorganic form exerted better ability.

5.5 Conclusion

Se bio-fortified ricegrass juice, the RG40, exerted great ability on promoting probiotic bacteria number and prolongs their life cycles which provided magnificent benefits for gut health via antioxidant protection. Additionally, its ability on inhibiting the colon cancer also provided significant outcomes to prove their function on health protection. These accumulated results are important on the growing interest in the use of Se bio-fortified ricegrass juice extract as an ingredient of functional food for improving gut health and modulate incidence of colon cancer.

CHAPTER 6

ANTI-CADMIUM TOXICITY AND DNA PROTECTIVE PROPERTIES OF SELENIUM BIO-FORTIFIED RICEGRASS JUICE IN HEK293 EMBRYONIC KIDNEY CELLS

6.1 Abstract

Cadmium (Cd) contamination in food is a dangerous problem endangering human health. Cd detoxication is interesting topic particularly the food which providing no undesirable side effects. Ricegrass juice is a squeezed juice from young age rice plants which introduced as a functional drink rich in polyphenol components. Se-enrichment into ricegrass is initiated to provide extra advantages on their functional properties. This study aimed to evaluate the cytotoxicity of Se bio-fortified ricegrass juice (RG0, RG10, RG40) on HEK293 kidney cells as well as the possibility to protect against cadmium toxicity during pre, co and post-treatment with $CdCl_2$, besides their role on DNA protective properties were examined. Results confirmed that the extracts had very low toxicity to kidney cells. All extracts showed a protective role during pre-treatment and co-treatment against Cd toxicity by exerted the reduction in MDA content and exhibited a DNA protective role by decreasing the percentage of DNA damage in tail and tail length of the comets over the Cd-treated cells. Yet, the RG40 indicated additional benefits on all properties over the RG0 and RG10. High Se content in RG40 resulted in providing more protective effects to the regular ricegrass juice. In summary, this study provided clear evidence that Se bio-fortified ricegrass juice was a candidate functional food to protect cells against Cd toxicity via reduction of oxidative stress and DNA damage.

6.2 Introduction

Currently, daily food consumption could lead to the unexpected exposure of contaminated compounds into the human body. Heavy metals, known as harmful agents, enter the food chain excessively via industrial operations, mining, sewage sludge and waste disposal from households into agricultural lands and water resources (Tchounwou *et al.*, 2012). Accumulation of heavy metals in the environment has been reported as increasing substantially over the past decades (Elinder and Järup, 1996). Due to the highly soluble ability of cadmium (Cd) compounds when compared with other metals, Cd is readily taken up by plants resulting in food and feed accumulation. Cd contamination from the environment is a subject of serious health complication affecting cellular organelles and components such as cell membrane, mitochondrial, lysosome as well as genetic DNA (Wang *et al.*, 2001). The kidney is a critical target organ where Cd is bioaccumulated. The exposed level of Cd can cause chronic difficulties, thus leading to damage of kidney filtering mechanisms and kidney dysfunction as well as liver damage (Järup, 2003). The mechanism of Cd toxicity is related to the generate of reactive oxygen species (ROS) such as superoxide ions, hydrogen peroxides and hydroxyls radicals, and therefore inducing oxidative stress and DNA damage by initiation of the lipid peroxidation (Liu *et al.*, 2009).

Recently, several studies have been reported that antioxidant molecules have protective effects against renal and hepatic cadmium toxicity via a function of free radical scavenger (Shaikh *et al.*, 1999). Plants are the foremost source of natural antioxidant molecules such as vitamin C, vitamin E, and polyphenols which have been hypothesized as effective anti-cadmium toxicity materials. The discovery of functional plant food rich in antioxidant compounds is now being considered. Sprouts or young plant of cereals, grains or legumes are currently interested since plants at the beginning of the growing stage are associated with large amounts of quality bioactive compounds and antioxidant molecules like polyphenols. Ricegrass is a brand-new sprout which was recently introduced as a substitute to wheatgrass particularly in tropical areas as a low-cost ingredient and the place where wheat hypoallergic reported. It is rich in polyphenol

compounds and has been previously investigated on its ability to scavenge free radicals *in vitro* effectively (Chomchan *et al.*, 2016)

There was proposed that human body attempt to reduce the heavy metals toxicity via some antioxidant mechanisms such as metal chelation or degradation of free radicals (Sandbichler & Höckner, 2016). It has been stated that Se can be used as antidote agent to mercury (Hg), cadmium (Cd) and silver (Ag) (Mukherjee and Sharma, 1988). The enrichment of Se into ricegrass, which can increase the level of Se content in plants, may possibly propose an extra role on the anti-cadmium toxicity. This study investigated the effect of Se bio-fortified ricegrass juice extract (RG0, RG10, RG40) on *in vitro* anti-cadmium toxicity in HEK293 kidney cells. Its effect on lipid peroxidation and DNA protective properties were also investigated. If this works practically, this study may trigger interest in the use of Se bio-fortified ricegrass juice as a useful tool in protecting the human body from the deleterious effects of cadmium and could lead to the development of new therapies to treat against cadmium toxicity.

6.3 Materials and methods

6.3.1 Chemicals

Di-sodium ethylenediaminetetraacetic acid (EDTA-Na_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma Aldrich Co. (St. Louis, MO, USA). SYBR gold nucleic acid stain and trichloroacetic acid (TCA) were purchased from Thermo Fisher Scientific Co. (San Jose, CA, USA). Reagents and media for cell line including trypan blue dye, trypsin-EDTA, fetal bovine serum (FBS), penicillin-streptomycin and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco BRL, Life Technologies Inc. (Rockville, MD, USA). Low melting point agarose (LMA), dimethyl sulfoxide (DMSO) and Triton-X were purchased from Amresco Inc. (Solon, OH, USA).

6.3.2 Plant materials

Young ricegrass juice extract (RG0) and Se bio-fortified ricegrass juice extract (RG10, RG40) prepared from lyophilized aqueous extracts of 8 days grown ricegrass (*Oryza sativa* L. cv. Chainat 1) were used in this study.

6.3.3 Cell culture model

HEK293, human embryonic kidney cells, were purchased from the American Type Culture Collection (Manassas, VA, USA). DMEM supplemented with 1.5 % sodium bicarbonate, 10% FBS, 1% penicillin- streptomycin was used for the maintenance of cells at 37ºC, 5 % CO_2, in a fully humidified incubator. Phosphate buffer saline (PBS) at pH 7.2 was used to wash the cells and clean them throughout the experiment.

6.3.4 Cell viability assay

The MTT assay was used to determine the cell viability. Cells grown at 80-90% confluent were harvested with 0.25% trypsin – EDTA and suspended in a fresh medium. Cell counts were measured using a standard haemocytometer based trypan blue cell counting technique (Louis & Siegel, 2011). HEK293 cells at the density of 1×10^6 cells/ml were seeded in 96-well tissue culture plates and allowed to adhere for 24 h. After the cells were washed with PBS (pH 7.2), the media mixed with a various concentration of the extracts (250 – 10,000 µg/ml), were treated to the cells. This was followed by a 24 h incubation. After 24 h, the cell viability was evaluated by adding 20 µl of MTT solution and 2 h incubation. Afterward, the MTT solution was removed, and 100 µl of 0.04 N HCl in isopropanol was added to dissolve the formazan crystals. Absorbances were recorded at 570 nm using a microplate reader. The percentage of cell viability was calculated with the equation (1);

$$\% \text{ Cell viability} = [\text{Absorbance of sample}/ \text{Absorbance of control}] \times 100 \quad (1)$$

6.3.5 Anti-cadmium toxicity properties

To examine the anti-cadmium toxicity properties of RG0, RG10, and RG40 in HEK293 cells, initially, the half maximal cytotoxicity concentration (CC_{50}) of $CdCl_2$ was investigated to be used as the established dose to induce the toxicity to the cells. After seeding HEK293 cells into 96-well plates at a density of 1×10^6 cells/ml, the cells were left to attach for 24 h before being treated with either $CdCl_2$ or extracts. The experiment has been divided into three groups separated by different time order of treating the extracts to cells; these groups were pre-treatment, co-treatment and post-treatment groups. The detail of each group is briefly indicated in Table 1. The extract was fixed to have a contact time of 24 h on cells in all treatments. The percentage of cell viability was detected by MTT cytotoxicity assay and calculated as equation (1). The morphology of the cells in each treatment was also observed and captured using a microscope.

Table 17. The experimental treatment group on anti-cadmium toxicity properties

	Control		**Negative control**		**Sample**	
Time	24 h	24 h	24 h	24 h	24 h	24h
Pre-incubation	Media	Media	Media	$CdCl_2$	Extracts	$CdCl_2$
Co-incubation	Media	-	Media + $CdCl_2$	-	Extracts + $CdCl_2$	-
Post-incubation	Media	Media	$CdCl_2$	Media	$CdCl_2$	Extracts

6.3.6 Determination of lipid peroxidation

TBARS (Thiobarbituric acid reactive substances) assay was used to determine the level of lipid peroxidation. The endogenous cellular fluid was extracted according to the modified method of Du *et al.* (2016). The cells treated with the previous condition defined in 6.3.5 were harvested with 0.25% trypsin-EDTA and followed by centrifugation at 1000×g for 10 min. Cell pellets were washed with cold PBS until clean and re-suspended in 1 ml of cold PBS. Cells were lysed using a probe-type sonicator (Vibra-Cell, Sonics and Materials Inc., Newtown, CT, USA) by pulsing at 15s on and 10s

off for 5 cycles on ice. The cell extracts were centrifuged at 10,000×g (4 ◦C) to discard the cell debris while supernatants were used for the determination of MDA content and protein levels. Protein content was examined using bovine serum albumin (BSA) as standard (Bradford, 1976). The modified method of Chen *et al.* (2002a) was used to determine the MDA content. 1 ml of cellular extracts were mixed with 4 mL of 20% TCA containing 0.8% of TBA (w/v). The mixtures were heated at 95 °C for 60 min, then cooled in ice and centrifuged at 3,000xg for 10 min. The absorbance was measured at 532 nm. The amount of MDA–TBA red complexes were compared with an external standard of MDA. The amount of TBARS was expressed as nmol MDA/mg protein.

6.3.7 DNA protective properties using comet assay

The alkaline single cell gel electrophoresis assay or comet assay was used to evaluate the DNA damage (Singh, 2000). The DNA protective properties of the extracts on HEK293 cells towards the exposure to $CdCl_2$ were investigated. Briefly, HEK293 cells were seeded at 1×10^6 cells/ml in 12-wells plates and incubated at 37°C for 24 h. The cells were treated by the following condition stated above (Table 17). Then, cells were harvested and fixed into slides which had been covered with 150 µl of 1.5% LMA as the first layer. After solidification, 20 µl of freshly prepared cell suspension with 180 µl of 0.5% LMA (ratio 1:10) was rapidly mixed by pipetting, and 80 µl of the mixture was loaded as the second layer. Then, 70 µl of 1.0% LMA was added on to the cell layer as the third layer. Once the gel was solidified, the slides were placed in a chilled lysis buffer containing 2.5M NaCl, 100mM EDTA, 100mM Tris–HCl at pH 10 and 1% DMSO, 1% Triton X-100 for at least 2 h at 4°C. The slides were then removed and placed in an electrophoresis tank (Model CSL-COM20, Cleaver Scientific, UK) filled with freshly prepared alkaline buffer at 4°C (300mM NaOH, 1mM Na_2EDTA, pH≥13) for 15 min to unwind the DNA and the electrophoresis was carried out at 25 V and 300 mA for 45 min. Afterward, the slides were rinsed with deionized water and neutralized gently with 0.4 M Tris–HCl buffer, pH 7.5 for 5 min. Finally, the slides were soaked in ethanol for 5 min and left at room temperature until they were completely dried. The cellular DNA was stained using SYBR gold nucleic acid stain in the dark for 20 min and visualized using a fluorescent microscope (Eclipse

80i, Nikon, Japan). The comet images (45–60 cells/slide) were captured and analyzed. The quantification of the DNA strand breaks was done using CometScore 2.0.0.38 software (Tritek, USA). The % DNA in tail and tail length were obtained.

6.3.8 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experimental data were presented as the mean ± standard deviation of three replications. Means were analyzed using the analysis of variance (ANOVA). The significant differences among means were determined by Tukey's test ($p<0.05$) using SPSS for Windows (SPSS Inc, Chicago, IL).

6.4 Results and discussion

6.4.1 Cytotoxicity

Functional food products which can be claimed as safe need to be confirmed on their viability effect of mammalian cells. The experiment was operated on human embryonic kidney cells, HEK293, by measuring the cytotoxicity after incubating the healthy cells with extracts for 24 h. The dose of extracts which was indicated as safe to the cells will be used for the anti-cadmium toxicity test. Figure 32 revealed that the cell number of HEK293 slightly decreases while treated with all treatments of ricegrass juice extracts and remained constant while the concentration of the extracts was increased up to the dose of 10,000 µg/ml. Rich Se extracts (RG10, RG40) displayed no significantly differing effect on the reduction of cell numbers compared with the RG0. There was a minor reduction in cell number of all treatments, however, the cell numbers remained higher than 80% of the control which was indicated as the acceptable range to be classified as safe (Langdon *et al.*, 2010). Although the alteration of cell morphology after treatment with the extracts has been detected, it was regarded as a result of the sensitivity of cells when they had been encountered the foreign matter and attempted to adapt themselves to the new environment. Therefore, this result can be assumed that extracts had no or low toxicity to the kidney cells

and the dose of extracts at the highest concentration (10,000 µg/ml) could be used for the next experiment.

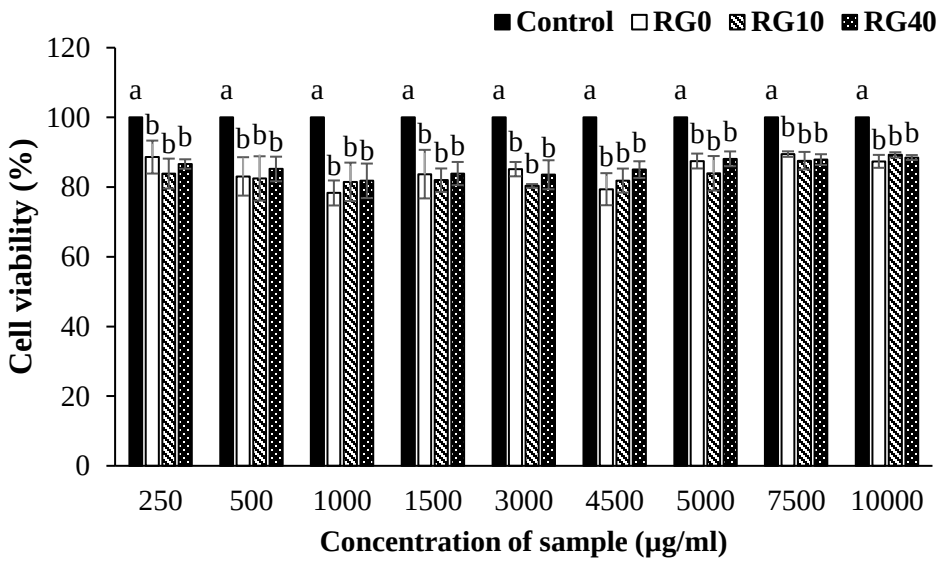

Figure 32. Effect of Se bio-fortified ricegrass juice extract (RG, RG10, RG40) on the cell viability of HEK293 kidney cells and expressed as a percentage of control. Each value was expressed as the mean ± standard deviation (n=3). Different letters indicated significant differences between samples at the same concentration (p<0.05).

6.4.2 Anti-Cd toxicity properties

The kidney is the critical organ affected by chronic Cd exposure and toxicity. It is responsible for the processing of blood supplied via filtration, reabsorption, excretion of the excess amount of acid-base, electrolyte and extracellular fluid through the urine (Bucking *et al.*, 2010). Cd accumulates in the kidney because of its preferential uptake by the receptor in the renal proximal tubule (Johri *et al.*, 2010). It is known to accumulate in the human kidney for a relatively long time, from 20 to 30 years. The exposed level of Cd can cause chronic difficulties, thus leading to damage of kidney filtering mechanisms and kidney dysfunction (Järup, 2003) . HEK293 cells, human embryonic kidney cells, were used as a demonstrative model to examine the effects of $CdCl_2$ toxicity and the protective role of extracts against $CdCl_2$. The level of $CdCl_2$, which

could be used to induce the cytotoxicity, was examined. Figure 33 showed that the viability of HEK293 cells was significantly decreased while exposed to higher level of $CdCl_2$. Cd is not able to generate radical itself but the toxicity was related to the generation of ROS such as superoxide ions, hydrogen peroxides and hydroxyls radicals and therefore they induced oxidative stress and DNA damage by initiation of the lipid peroxidation (Liu *et al.*, 2009). The estimated half maximal concentration (CC_{50}) dose of $CdCl_2$ in HEK293 was indicated as 70 µmol/L and this level could be used as a suitable dose for the evaluation of anti-cadmium toxicity properties of the extracts.

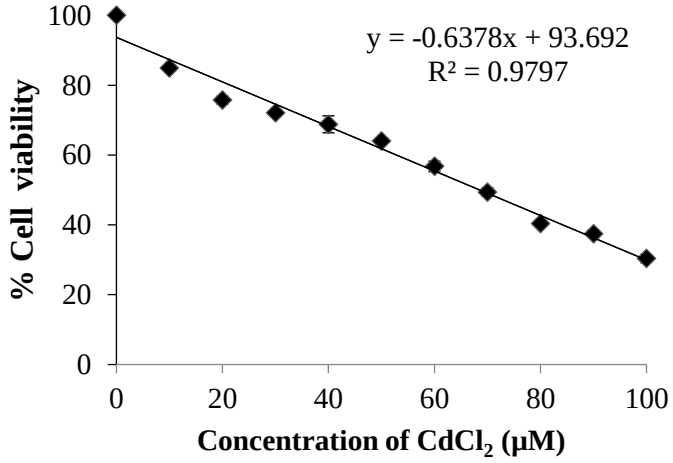

Figure 33. Effect of different concentrations of $CdCl_2$ on the cell viability of HEK293 kidney cells. The cell viability was expressed as a percentage of control. Each value was expressed as mean ± SD (n=3).

The experiment on anti-cadmium toxicity properties was designed to assess the effect of Se bio-fortified ricegrass juice against $CdCl_2$ exposure on the cell viability and lipid peroxidation at different time orders, as each substance may alleviate the toxicity from Cd induction differently (Sandbichler and Höckner, 2016). The first treatment group was the extracts pre-treatment; the ideal substances which can reduce the toxicity from this treatment could represent the role of the protective substances. Secondly, the group of co-treatment was examined to check if the extracts could provide the protective role to cells

during they directly reacted to Cd. Lastly, the group of post-treatment was designed to indicate the role of the extracts as therapeutic agents.

Results revealed that ricegrass juice significantly increased the percentage of cell viability during pre-treatment and co-treatment but not during post-treatment with Cd compared with the cells treated with Cd at CC_{50} level alone (Figure 34). The highest concentration of all extracts (10,000 µg/mL) exerted the highest ability to protect HEK293 cells against Cd toxicity. Thus, the pathological evaluation of cell morphology treated with the extracts at this concentration was observed as shown in Figure 35. The morphological changes while treating the cells with Cd were detected. Majority of the cells were broken and floated into the media while the rest were weakened and lost their cell structure. Although the cells in the condition of extracts pre-treatment illustrated some lost and unusual cells morphology, the cells remained strengthened in their frame similarly to web shape. The changes of cells in co-treatment conditions were also detected as they were swollen and changed to circle-like shapes, but they preserved their structure. These data suggested that Se bio-fortified ricegrass juice could improve the Cd-induced pathological damage of kidney cells better than without the extracts. Therefore, the extracts can be a potential protectant of kidney cell damage.

Living organisms contain lipid as the main structure of cellular membranes. Cd could induce the damaging effects to the cells from the lipid peroxidation process (Eneman *et al.*, 2000). Therefore, the extent of lipid peroxidation by-products produced like malondialdehyde (MDA) can imitate the extent of cells oxidative damage initiated by Cd (Ayala *et al.*, 2014). TBARS assay is a well-established method used as an index of lipid peroxidation and lipid hydroperoxides. When the cells exposed to Cd, the MDA content was markedly increased, thus suggesting the increase in oxidative stress of kidney cells (Figure 36). However, outcomes indicated that during pre-treatment and co-treatment of Se bio-fortified ricegrass juice extracts, the level of MDA in HEK293 was significantly reduced compared with Cd-treated cells ($p<0.05$). It confirmed that Cd is very toxic to the living cells and difficult to get rid of when entering to the cells for certain time. Therefore, it must be great to avoid Cd intake, otherwise protecting procedure needs to be stricted.

The role of phenolic compounds in the extracts was considered as having the major effects on the protective role against Cd-induced damage. It could be explained that the extracts rich in polyphenols compounds possess the inhibition of lipid peroxidation chain reaction by stabilizing the hydroxyl radicals and lipid peroxyl radicals, thereby lowering the extent of oxidative damage to the lipid cell membrane and lower level of MDA. Moreover, phenolic compounds as antioxidant molecules could propose the role on upregulating the antioxidant protection system by stimulating the production of antioxidant enzymes including SOD, CAT, and GPx. As a result, strengthening the immunity and lowering the damage caused by Cd during pre-treatment and co-treatment to the cells (Sandbichler and Höckner, 2016). Ricegrass juice contained abundant polyphenols like flavone glycosides. Therefore, the protective role of the extracts could be related mainly to these groups of compounds. Similar results also indicated the protective effects of bioflavonoids, for example, quercetin against Cd-induced oxidative stress-related renal dysfunction in rats by attenuating the Cd-induced biochemical alterations in serum, urine and tissue pathological changes via a decrease in lipid peroxidation rate (Renugadevi and Prabu, 2010). Flavonoid, namely catechin from green tea, has also been proved to protect against bone metabolic disorders in cadmium-poisoned rats (Choi *et al.*, 2003).

While focusing on the effect of high Se, RG10 did not show a significantly different effect from RG0 while RG40 revealed marginally higher protective properties against Cd toxicity in pre-treatment and co-treatment conditions. Se was used as antidote agent to a range of heavy metal toxicities including Cd, Hg, and Ag (Mukherjee and Sharma, 1988). Generally, studies have indicated the beneficial effect of Se on antioxidant status and lipid peroxidation when pre-exposed and co-exposed to Cd (El-Sharaky *et al.*, 2007, Liu *et al.*, 2015, Ognjanovic *et al.*, 2008). Lipid peroxidation occurred because of Cd-exposure; moreover, a significant decrease in the antioxidant composition factors, such as GSH levels, the activities of GPx and thioredoxin reductase (TrxR), was also stated (El-Sharaky *et al.*, 2007). Se compounds have been generally known as a major cofactor of GPx and TrxR, thus, Se could logically promote the greater level of the antioxidant enzymes activity and play a role in managing the radicals occurring in the cells. Se could

also present protective effects on mitochondria dysfunction by blocking the ROS generation, a possible inhibition of Cd-induced mitochondrial membrane collapse (Zhou *et al.*, 2009).

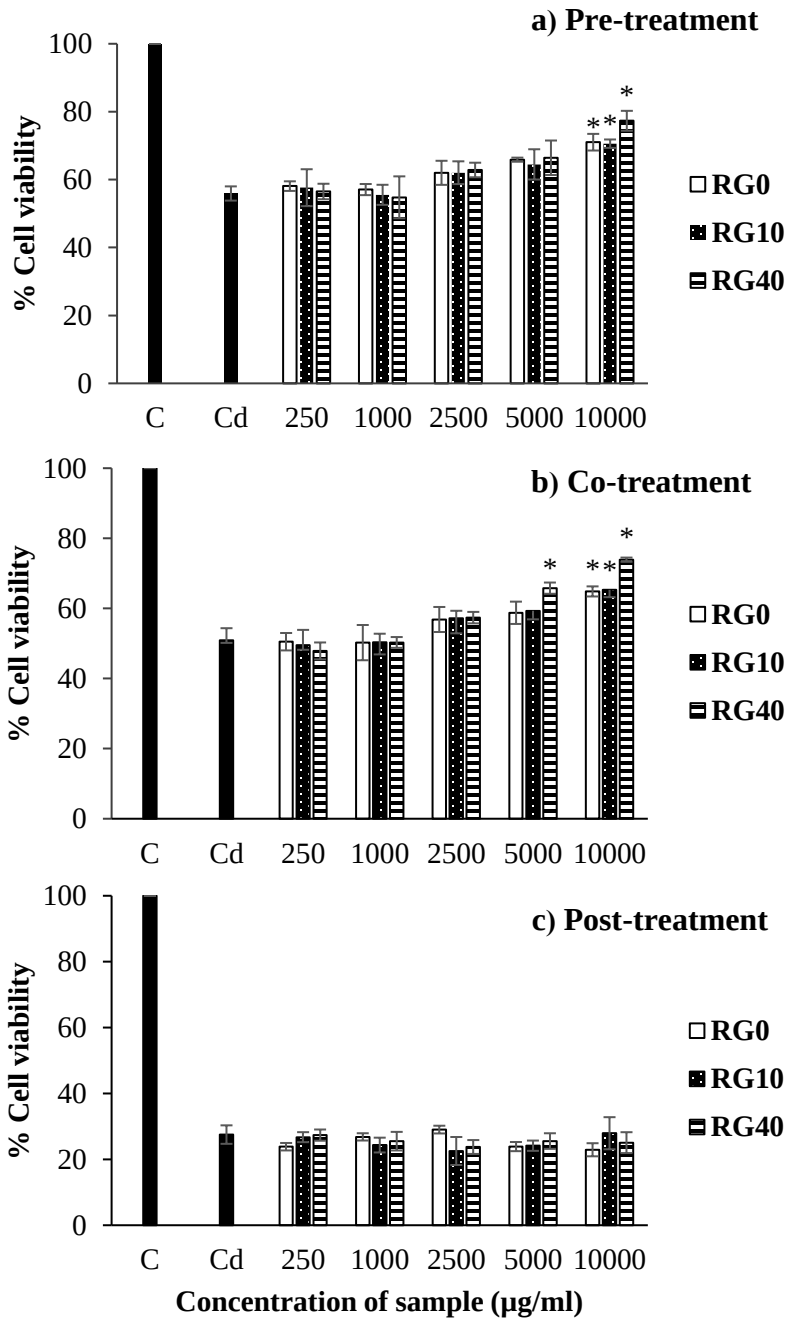

Figure 34. Anti-Cd toxicity properties of Se bio-fortified riecgrass juice on HEK293 kidney cells during exposure to $CdCl_2$ at different time order (a) pre-incubation (b) co-incubation (c) post-incubation. C means blank control; Cd means $CdCl_2$ at CC_{50} level (70 µg/ml). The cell viability was expressed as a percentage of control. Each value was expressed as mean ± SD (n=3). * indicated significant different from Cd at $p<0.001$.

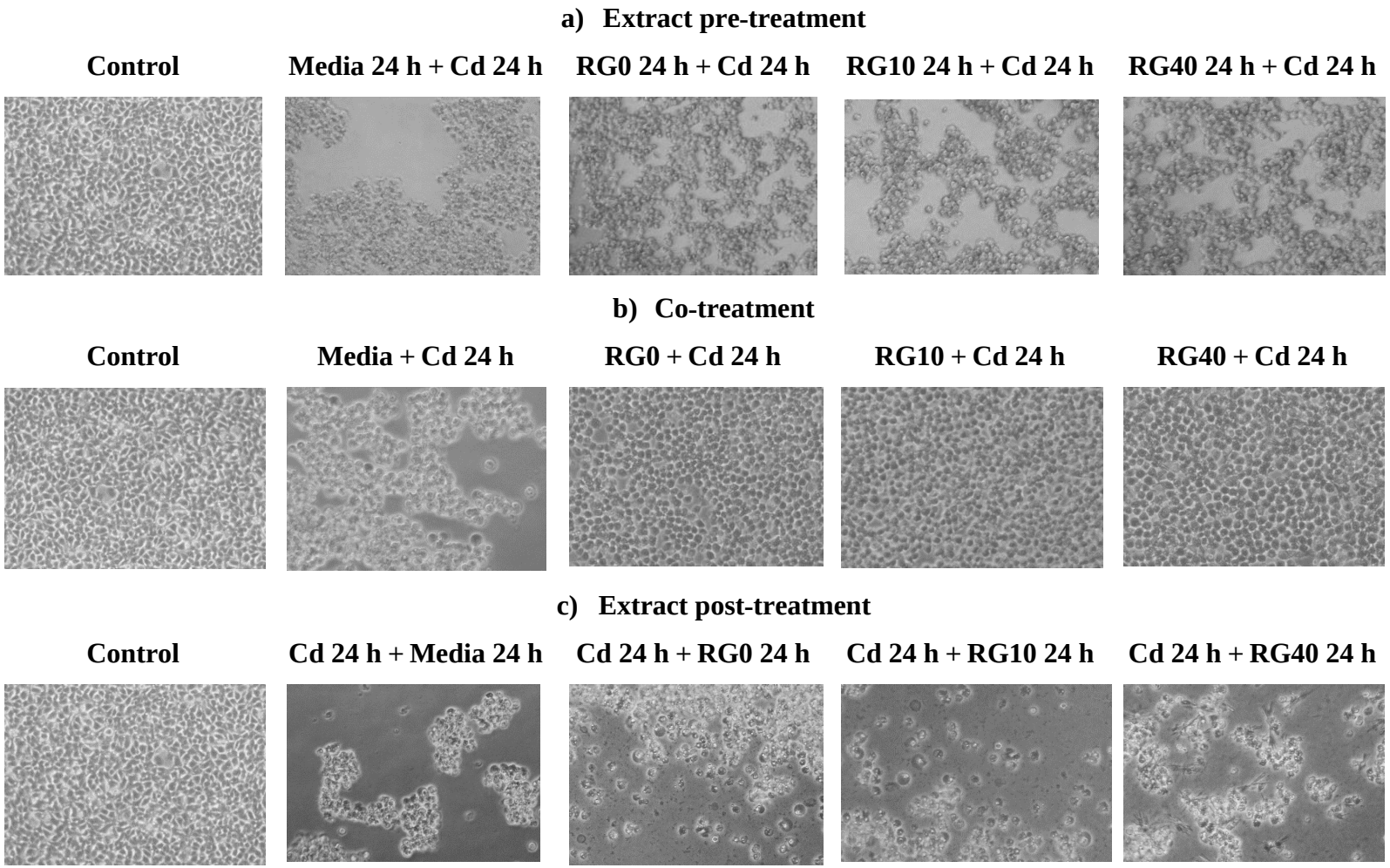

Figure 35. Morphology of cells of HEK293 kidney cells while incubated with Se bio-fortified ricegrass juice and $CdCl_2$ at different time order of treating the extracts (a) pre-incubation (b) co-incubation (c) post-incubation. C means control; Cd means $CdCl_2$ at CC_{50} level (70 µg/ml).

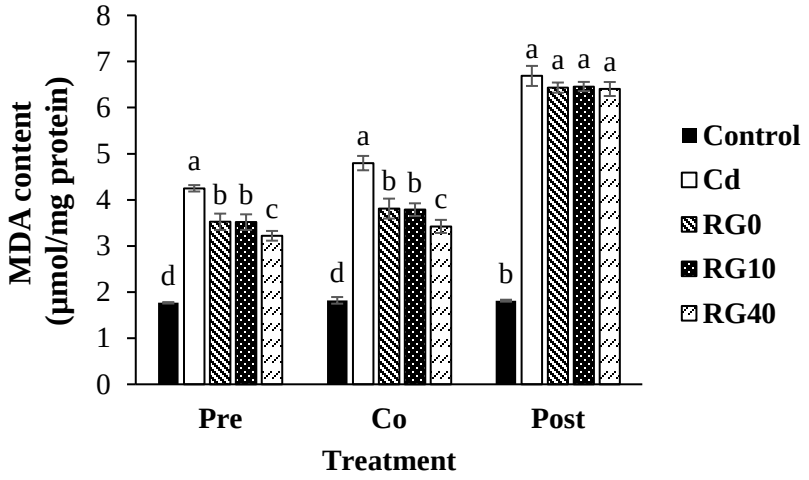

Figure 36. Level of malondialdehyde (MDA) in HEK293 cells treated with RG and Se-RG at the level 10,000 µg/ml compared with control and Cd-treated one. The MDA content was determined using TBARS assay. Each value was expressed as mean ± SD (n=3). Different letters indicated significant differences between samples at the same treatment condition (p<0.05).

6.4.3 DNA protective properties

DNA damage can be induced by exogenous agents such as heavy metals, polycyclic aromatic hydrocarbon from pollution, endogenous chemical genotoxic agents such as reactive oxygen species (ROS) and natural chemical reactions (Ercal *et al.*, 2001). The damaging in the cellular genome can generate errors in the transcription of DNA and protein translation which impair the signaling and cellular function and could result in the development of diseases (Lindahl and Barnes, 2000). Comet assay or single cell gel electrophoresis is a standard rapid method for detecting DNA damage in individual cells. The term “comet” is used to identify the DNA migration pattern of the cells after passing through electrophoresis. Comets can be divided into two parts as head and tail. The head representd nuclei and unbroken DNA of cells and the tail indicated as damaged DNA fragments (Tice *et al.*, 2000). The percentage of DNA in the tail and tail length was analyzed as the measure of primary DNA damage (Pourrut *et al.*, 2015). Therefore, comet assay was used for this study to evaluate the DNA protective role of Se bio-fortified ricegrass juice extracts. The only RG0 and RG40 extracts were chosen since the RG10 did

not showed significant difference effect on protecting against Cd damage from the RG0. The highest concentration of both extracts (RG0 and RG40) were used (10,000 µg/ml) as they protect the highest number of percent cell viability. Figure 37 showed the parameters of the comets evaluation included % DNA in tail and tail length. Figure 38 illustrated the capture of comet cells of each treatment during pre-treatment, co-treatment and post-treatment with RG0 and RG40 compared with the Cd-treated condition and control.

Comet cells of the control of every treatment displayed circle-like shapes which the whole nuclei and DNA were beautifully stained with fluorescent color. The condition of Cd-treated cells indicated the presence of a clouded comet tail which the % DNA in tail and tail length were increased significantly ($p<0.05$) because of Cd-induced oxidative damage. In both pre-treatment and co-treatment conditions, Se bio-fortified ricegrass juice treated to cells significantly exhibited the reduction in the % DNA in tail and tail length compared with the Cd-treated group (negative control), thus illustrated the DNA protective effect. The results on comet assay parameters of each treatment were correlated to the content of MDA production. The damaging of DNA could be a subsequent effect from the production of high ROS and lipid radicals. The role of flavone glycosides as a natural antioxidant in the Se bio-fortified ricegrass juice may influence this protective property by possibly up-regulating the level of the antioxidant defense system and abolishing oxidative DNA damage via the donation of electrons to reactive metabolites, rendering them inactive to prevent the interaction to the DNA. The experiment on the protective role of flavonoid compounds as an excellent radical scavenging to reduce the DNA damage in human blood lymphocyte is also consistent with this result (Devipriya *et al.*, 2008). RG40 showed higher ability on the reduction of the tail length and % DNA in tail of the comets compared with the RG0. This indicated that Se in combination with the polyphenols could provide an extra protection and promote protective role to kidney cells. Se as the cofactor of various endogenous enzymes works in an antioxidant system could support the activity on the destruction of ROS. Fischer *et al.* (2006) suggested another possible role of Se, especially in the organic form, on the protecting of DNA damage via induction of p53 DNA repair pathway and transactivation of p53-regulated effector genes.

In the post-treatment condition, though the addition of the extracts showed no significant effect on an improvement in cell viability of HEK293 cells, minor role on DNA protective can visibly be seen. In Cd-treated condition, most of DNA in cells were broken down as indicated by the size of comet head in were obviously decreased from the control. DNA fragments were spread into the surrounding area as detected from the blurred green background. However, the addition of the extracts, though could not save the cell viability from Cd exposure, the intensive black background remained to observe. This might indicate a slight reduction in the number of DNA fragments in the surrounding area as ricegrass juice indicated a reduction in % DNA in the tail of comets compared with Cd-treated cells. Moreover, the addition of RG40 indicated significant reduction in the tail length ($p<0.05$) thus showed a better DNA protective property.

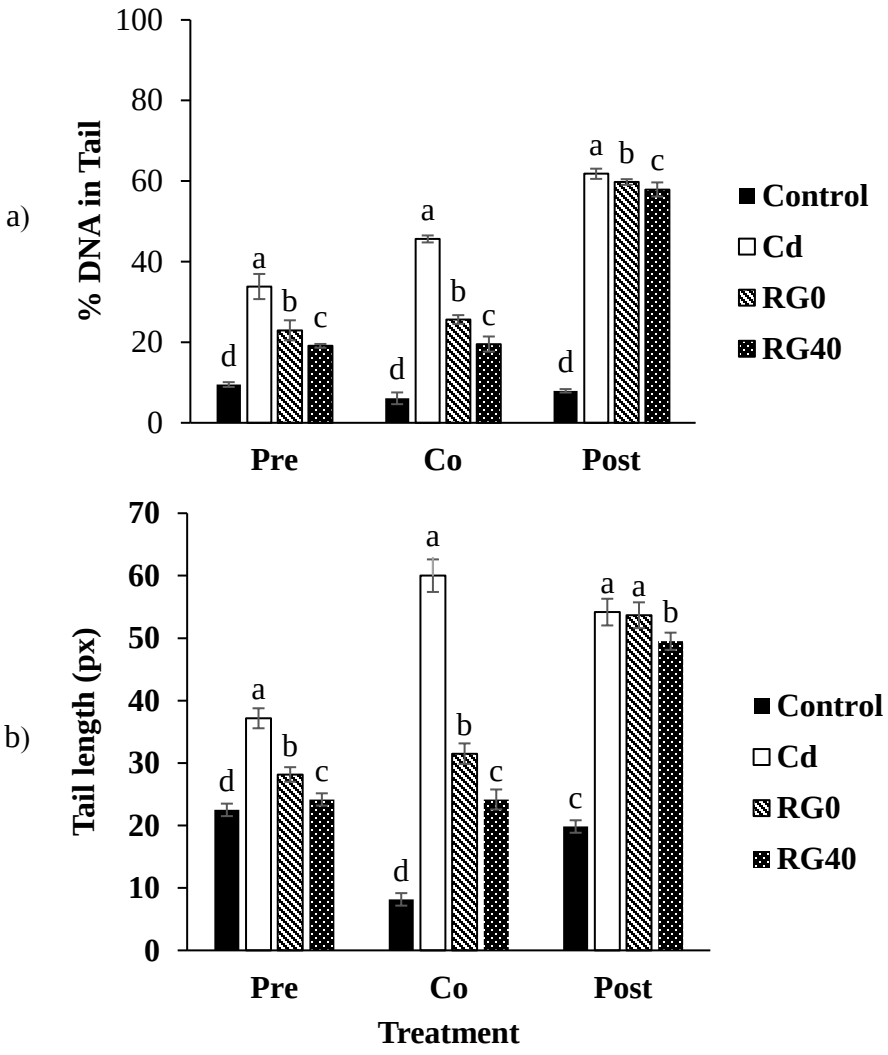

Figure 37. Assessment of DNA damage parameters in HEK293 cells treated with RG0 and RG40 at the level 10,000 µg/ml compare with control and Cd-treated one evaluated using the CometScore software. (a) Changes in % DNA in tail; (b) Changes in tail length. Each value is expressed as mean ± SD of 50 comets. Different letters indicated significant differences between samples at the same treatment condition (p<0.05).

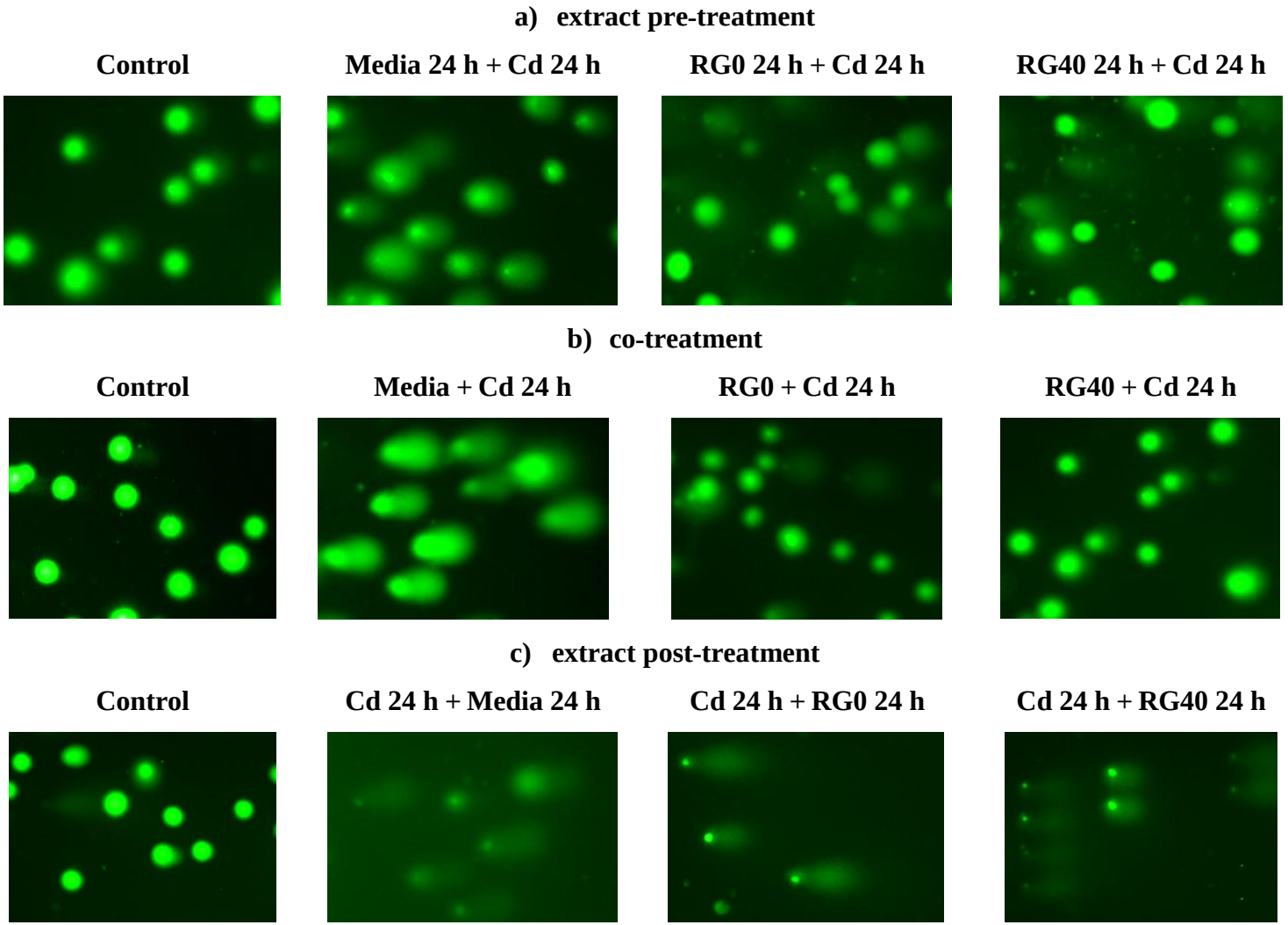

Figure 38. DNA protective effect of RG0 and RG40 against $CdCl_2$ induced DNA break in HEK293 kidney cells while incubated the cells with RG0/RG40 and $CdCl_2$ at different time order of treating the extracts evaluated by comet assay (a) extract pre-incubation (b) extract and $CdCl_2$ co-incubation (c) extract post-incubation. C means blank control; Cd means $CdCl_2$ at CC_{50} level (70 µg/ml)

6.5 Conclusion

In conclusion, our results showed the effectiveness of Se bio-fortified ricegrass juice in counteracting the Cd-induced damage in HEK293 kidney cells during pre-treatment and co-treatment to Cd were approached. The results could support the hypothesis that polyphenols in combination with Se compounds may help in the reduction of oxidative stress, lipid peroxidation rate, morphological impairments and DNA damage to kidney cells. Flavone glycosides as the major polyphenols in the extracts should contribute to these beneficial effects. Se bio-fortification into ricegrass could promote additional benefits over the typical ricegrass through the upregulation of GPx enzyme. RG40 should have potential to be produced and consumed as a functional food to protect the human body from Cd contamination.

CHAPTER 7

CONCLUSION AND SUGGESTION

7.1 Conclusion

1. Ricegrass juice produced from Chainat1 rice grain, contained lower level of chlorophyll and ascorbic acid compared with wheatgrass juice. However, it was found to contain higher level of phenolics detected by HPLC and the comparable antioxidant activities to wheatgrass juice. When dedicate the cost of ingredients and the probable benefits achievement, ricegrass juice could be a potential candidate to produce as new functional food.

2. Selenium bio-fortification is a useful technique to increase level of organic Se compounds into plants. However, the species of plants are seriously concerned. The fortification of Se into ricegrass had a limitation at 40 mg Se/L.

3. Selenium bio-fortification revealed minor influence on the trigger of phenolic compounds in ricegrass. The addition of 10 mg Se/L could only slightly increase the level of total phenolic content and antioxidant activities but not to the large extent.

4. Flavone glycosides included Chrysoeriol arabinosyl arabinoside derivatives, Tricin, Tricin-7-*o*-β-D-glucopyranoside, and Swerticin were found to be the foremost group of polyphenols in ricegrass juice.

5. Se bio-fortified ricegrass juice provided the effect of enhancing probiotic bacteria number namely, *Lactobacillus plantarum*, on its late-log phase and can prolong their life-cycle

6. Se bio-fortified ricegrass juice proposed role in reducing lipid peroxidation and nitric oxide production in RAW264.7 murine macrophage cells and inhibited the HT-29 colon cancer cells.

7. Se bio-fortified ricegrass juice could reduce the number of cell death during pre-incubation and co-incubation with $CdCl_2$ by reducing the lipid peroxidation rate as well as reducing the DNA damage determined by comet assay.

8. The extract RG40 exhibited the greatest ability on all activities because it contained an extra amount of Se which is the cofactor of GPx, the antioxidant enzymes in the human body, in combination with polyphenols compounds in ricegrass. Therefore, strengthened the antioxidant protection to cells.

9. Se bio-fortified ricegrass juice at level 40 mg Se/L produced from Chainat1 has potential to produce as new functional food which provides tons of benefits and offers the added value to rice in the future.

7.2 Suggestion

1. The development of functional food products from Se bio-fortified ricegrass juice for commercial production should be further examined.

2. The biological properties of Se bio-fortified ricegrass juice in *in vivo* models and clinical studies should be investigated.

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List of Publication

Chomchan R., Puttarak P., Brantner A. and Siripongvutikorn S.* 2018. Selenium-rich ricegrass juice improves antioxidant properties and nitric oxide inhibition in macrophage cells. Antioxidants. 7(4): 57.

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List of presentation

Chomchan R., Siripongvutikorn S. and Puttarak P. 2016. Improvement of total phenolic contents and anti-oxidative properties of ricegrass (*Oryza sativa* L.) using selenium bio-fortification. The 18th International Conference on Food Security and Nutrition (ICFSN 2016). Mercure Kuta, Bali, Indonesia, 13th-14th October 2016. Oral presentation.

List of petty patents

รัตนามณี ชมชาญ สุนิสา ศิริพงศ์วุฒิกร และภาณุพงศ์ พุทธรักษ์. 2560. สูตรเครื่องดื่มเจลลี่จากน้ำคั้นต้นอ่อนข้าวและกรรมวิธีการผลิตผลิตภัณฑ์. เลขที่คำขอ 1703002494 ลงวันที่ 15 ธันวาคม 2560.

รัตนามณี ชมชาญ สุนิสา ศิริพงศ์วุฒิกร และภาณุพงศ์ พุทธรักษ์. 2559. กรรมวิธีการผลิตเครื่องดื่มผงจากต้นอ่อนข้าวเสริมแร่ธาตุซีลีเนียมขณะปลูก. เลขที่คำขอ 1603000793 ลงวันที่ 12 พฤษภาคม 2559.

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