BIOACTIVE PHENOLIC COMPOUNDS FROM BLACK RICE BRAN

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งานวิจัยชิ้นนี้มีวัตถุประสงค์เพื่อศึกษาสภาวะที่เหมาะสมในการสกัดสารประกอบฟีนอลิกจากรำข้าวดำด้วยเทคนิคอัลตราโซนิกโดยใช้การออกแบบการทดลองทางสถิติ (Box-Behnken-BBD) และใช้วิธีพื้นที่ผิวตอบสนอง (Response surface method: RSM) โดยในการศึกษาครั้งนี้ได้ทำการศึกษาผลของความเข้มข้นของตัวทำละลายเอทานอล อุณภูมิ และค่ากรด-ด่างของตัวทำละลายที่ใช้ในการสกัดสารประกอบฟินอลิก (กลุ่มแอนโทไซยานิน และฟลาโวนอยด์) จากรำข้าวดำ ผลการทดลองพบว่าเมื่อใช้ตัวทำละลายเอทานอล 57 เปอร์เซ็นต์ ที่มีค่าพีเอช 3.1 ที่อุณหภูมิ 50 องศาเซลเซียส สามารถสกัดสารประกอบฟีนอลิกได้ในปริมาณสูงสุดที่ความเข้มข้น 136.2 มิลลิกรัม (gallic acid equivalent/g dry weight), แอนโทไซยานิน 2.3 มิลลิกรัม (cyanidin-3-glucoside equivalent/g dry weight) และฟลาโวนอยด์ 38.6 มิลลิกรัม (quercetin equivalent/g dry weight) จากการวิเคราะห์องค์ประกอบของสารสกัดด้วยเทคนิค Ultra-High Pressure Liquid Chromatography (UHPLC) พบว่าสารสกัดฟีนอลิกประกอบด้วยสารสำคัญ 6 ชนิด ได้แก่ gallic acid (1.8 µg/mL), protocatechuic acid (4.9 µg/mL), vanillic acid (0.5 µg/mL), syringic acid (91.5 µg/mL), p-coumaric acid (2.3 µg/mL) and ferulic acid (2.4 µg/mL) เมื่อนำสารสกัดมาศึกษาฤทธิ์ทางชีวภาพในแง่การต้านอนุมูลอิสระด้วยวิธี $ABTS^{+}$ และ DPPH พบว่าสารสกัดจากรำข้าวดำมีประสิทธิภาพในการยับยั้งอนุมูลอิสระได้ดี นอกจากนี้ยังได้ศึกษาผลการยับยั้งรังสียูวี (UVB) โดยใช้เซลล์ผิวหนังมนุษย์ชนิดเคอราติโนไซต์ (HaCaT) พบว่าสารสกัดจากรำข้าวดำมีประสิทธิภาพในการยับยั้งการสร้าง ROS ที่เกิดจากแสงยูวีในเซลล์ HaCaT ได้อย่างมีประสิทธิภาพ และเมื่อศึกษากลไกลการยับยั้งแสงยูวีพบว่าสารสกัดจากรำข้าวดำสามารถลด MMP-1, MMP-2 ที่เกิดจากรังสี UV-B และเพิ่มการแสดงออกของคอลลาเจนและอีลาสตินได้ดีตามความเข้มข้นของสารสกัดที่ใช้ทดสอบ ผลการศึกษาวิจัยนี้ประสบความสำเร็จในการศึกษาสภาวะที่เหมาะสมเพื่อสกัดสารประกอบฟีนอลิกจากรำข้าวดำด้วยเทคนิค RSM ซึ่งทำให้ได้สารสกัดที่มีฤทธิ์ทางชีวภาพในแง่ของการต้านอนุมูลอิสระ และปกป้องเซลล์จากรังสียูวีได้อย่างมีประสิทธิภาพ

โดยคาดว่าจะสามารถเพิ่มมูลค่าสารสกัดจากรำข้าวดำเพื่อพัฒนาเป็นผลิตภัณฑ์เวชสำอางสำหรับดูแลผิวในอนาคต

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In this study, optimization of the ultrasonic-assisted extraction of phenolic compounds using statistical experimental design (Box-Behnken Design - BBD) followed by respond surface methodology (RSM) was performed. To improve the extraction efficiency of phenolic compounds, monomeric anthocyanin, and flavonoid from black rice bran (BRB), the effect of ethanol concentration, temperature, and pH were examined. Extraction at 50°C using 57% ethanol (pH 3.1) rendered BRB extract with the highest yields of 136.2 mg gallic acid equivalents/ g dry weight of total phenolic, 2.3 mg cyanidin-3-O-glucoside equivalents / g dry weight of monomeric anthocyanin, and 38.6 mg quercetin equivalents/ g dry weight of total flavonoid. The UHPLC analysis of the extract showed the presence of six compounds: gallic acid (1.8 µg/mL), protocatechuic acid (4.9 µg/mL), vanillic acid (0.5 µg/mL), syringic acid (91.5 µg/mL), p-coumaric acid (2.3 µg/mL) and ferulic acid (2.4 µg/mL) of dry weight. Moreover, optimized extraction conditions improved the $ABTS^{+}$ and DPPH antioxidant activity of the extract. Besides, the anti-photoaging properties of the extract on human keratinocyte cells were explored. The black rice bran extract contained activity attenuating UVB-induced ROS generation. Moreover, the extract was able to diminish UVB-induced MMP-1 and -2 expression and increased the type I collagen and elastin expression in a dose-dependent manner. These findings suggest that the total phenolic content of black rice bran extract can be enhanced by RSM-optimized conditions and can improve the value of black rice bran as a valuable skincare product formulation.

School of Biotechnology Student's Signature _____

Academic Year 2023 Advisor's Signature _____

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LIST OF ABBREVIATIONS

%	=	Percentage
(m, μ) g	=	(Milli, micro) Gram
(m, μ) M	=	(milli, micro) Molar
$\mu W/cm^2$	=	Micro Watt Per Centimeter Square
°C	=	Degree Celsius
$ABTS^+$	=	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
ADP	=	Adenosine diphosphate
AMP	=	Adenosine monophosphate
ANOVA	=	Analysis of Variance
AP-1	=	Activator Protein 1
ATP	=	Adenosine triphosphate
bp	=	Base Pair
BBD	=	Box-Behnken Design
BRB	=	Black Rice Bran
C3GE	=	Cyanidin-3-O-Glucoside (C3GE) Equivalent
CAT	=	Catalase
CCD	=	Central Composite design
cDNA	=	Complementary deoxyribonucleic acid
CO_2	=	Carbon Dioxide
COL1A1	=	Collagen type I alpha 1 chain
COX-2	=	Cyclooxygenase 2
DCF	=	2'-7'dichlorofluorescein
DCFH-DA	=	Dichloro-dihydro-fluorescein diacetate
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
ECM	=	Extracellular Matrix
GAE	=	Gallic Acid Equivalent
ERK	=	Extracellular signal-regulated kinase

LIST OF ABBREVIATIONS (continued)

ECM	=	Extracellular Matrix
FBS	=	Fetal bovine serum
F-C	=	Folin-Ciocalteu
Fe	=	Iron
FRAP	=	Ferric Reducing Antioxidant Power
G-6-P	=	Glucose-6-phosphate
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GMP	=	Guanosine monophosphate
GPX	=	Glutathione peroxidase
GRAS	=	Generally Recognized As Safe
h	=	Hour
H_2O_2	=	Hydrogen peroxide
HaCaT	=	Nontumorigenic immortalized keratinocyte cell line
HDF	=	Human dermal fibroblast
IFN-γ	=	Interferon gamma
IL-1 β	=	Interleukin 1 beta
IL-6	=	Interleukin 6
IL-8	=	Interleukin 8
JNK	=	c-Jun N-terminal kinase
LLE	=	Liquid-liquid extraction
Lx	=	Lux
MAE	=	Microwave-assisted extraction
MAPK	=	Mitogen-activated kinase
$MgSO_4$	=	Magnesium sulfate
mm	=	Millimeter
MMP	=	Matrix metalloproteinase
mRNA	=	Messenger ribonucleic acid
MTT	=	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NF-κB	=	Nuclear factor kappa B

LIST OF ABBREVIATIONS (continued)

nm	=	Nanometer
NOx	=	NADPH oxidase
Nrf2	=	Nuclear factor erythroid-derived 2-like 2
NK-92 cell	=	Interleukin-2 dependent natural killer cell line
O.D.	=	Optical density
SFE	=	Supercritical fluid extraction
SPF	=	Sun protection factor
PBS	=	Phosphate buffer saline
QE	=	Quercetin Equivalent
RNA	=	Ribonucleic acid
RNR	=	Ribonucleotide reductase
ROS	=	Reactive oxygen species
RT-PCR	=	Reverse transcription polymerase chain reaction
RSM	=	Response Surface Methodology
SOD	=	Superoxide dismutase
SD	=	Standard deviation
THP-1	=	Human monocytic leukemia cell line
TGF-β	=	Transforming growth factor beta
TNF-α	=	Tumor necrosis factor alpha
TAC	=	Total Anthocyanin Content
TFC	=	Total Flavonoid Content
TPC	=	Total Phenolic Content
UHPLC	=	Ultra High Purification Liquid Chromatography
UAE	=	Ultrasonic-Assisted Extraction
UV	=	Ultraviolet
UVA	=	Ultraviolet A-rays
UVB	=	Ultraviolet B-rays
UVC	=	Ultraviolet C-rays
v/v	=	volume/volume
W	=	Watt

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Significance of the background

In the several past years, the manipulation of natural products has promoted great interest in the utilization of renewable resources and designing the extraction processes. Among numerous resources, black rice bran (BRB) is one of the byproducts of rice processing. BRB contains biologically active compounds such as anthocyanins, ferulic acid, and phytosterols, which have been shown to have potent antioxidant and anti-inflammatory properties (Halee et al., 2018). It is becoming popular in the cosmetic and skin care industry (Choi et al., 2010; Jun et al., 2012). Since it is a valuable source of phenolic compounds and other bioactive components, the extraction of phenolic compounds from BRB can be challenging due to the high concentration of active components, leading to a need for efficient extraction methods that preserve the active components in the extract. The concentration of phenolic compounds can vary depending on the rice variety, growing conditions, and processing methods.

For phenolic compound extraction, different extraction methods, such as Soxhlet, maceration, and heat-reflux are employed. Although these conventional extraction techniques are still used, processes including oxidizing, hydrolysis, and thermal degradation that take place at high extraction temperatures often result in the loss of active ingredients (Shadab Gharaati, 2019). Moreover, some of the techniques require expensive equipment for production on a small scale and suffer their drawbacks such as the cost of the high amount of organic solvent, longer time of extraction, and lower recovery yield. One of the unconventional extraction techniques, Ultrasonic-assisted extraction (UAE) has been demonstrated to be a very efficient extraction technology that can minimize the operating expenses and volume of solvent and decrease the duration of extraction. Therefore, UAE has been extensively employed in various research for the recovery of biologically valuable

compounds comprising the isolation of phenolic compounds from various samples due to its simplicity, easy of handling, low cost, and high efficiency (Olmo-García et al., 2018).

During the extraction processes, the selection of solvent is also essential. Ethanol and methanol are universal solvents in solid-liquid extraction. Selection of solvent can be considered based on the criteria of their solubility, cost, and safety (Olmo-García et al., 2018). Due to the efficiency and ability to preserve the active components in the extract, the use of ethanol as a solvent for the phenolic compound extraction with UAE has become a popular method for extracting BRB extract. In our preliminary studies, ethanol/water v/v (20%, 40%, 60%, 80%), absolute ethanol, and methanol were employed to explore the efficiency of UAE. Our preliminary study demonstrated that 40% and 60% ethanol extract for total phenolic compound (TPC), 40% ethanol extract for total anthocyanin compound (TAC) and total flavonoid compound (TFC) content were obviously higher than 100% methanol extract. These findings agreed with the literature where 40-60% of ethanol extract has been revealed to enhance the phenolic extraction from grape seeds, correspondingly (Ghafoor et al., 2009). Additionally, the use of ethanol as a solvent in ultrasonic-assisted extraction was remarked as a generally recognized safe (GRAS) solvent in the literature (Turrini et al., 2018). Furthermore, ultrasonic energy helps to minimize the ethanol percentage to 50% used in the extraction process as reported above. To sum up, we chose 50% ethanol as the center point for the optimization process in Response Surface Design.

BRB has been proven to have a preventive effect against UVB-induced deterioration, making it a promising ingredient for skin care products (Haryanto et al., 2020). UVB exposure is a major contributor to skin aging and the development of skin diseases. UVB radiation exposure causes oxidative stress and inflammation, resulting in the synthesis of reactive oxygen species (ROS) and the release of pro-inflammatory cytokines. This results in the damaging of DNA, proteins, and lipids, leading to cellular dysfunction and death (Piao et al., 2014). The use of BRB extract as a potential protective agent preventing UVB-induced keratinocyte damage in humans has been the subject of numerous studies. Keratinocytes are the predominant cell type in the

epidermis and play a crucial role in the skin's protective barrier function. The extract has been shown to reduce ROS formation and the production of pro-inflammatory cytokines in response to UVB exposure (Kaneda et al., 2006; Widjaja et al., 2019).

Ozone depletion resulted in an increase of UVB components and has raised concerns about the usage of tropical UV protective products. Moreover, the use of BRB extract has become an active ingredient for rice bran-based skin care products (Vardhani et al., 2020). The extract has shown potential as a protective agent against UVB-induced damage in human keratinocytes. For rice-bran based products, the use of ultrasonic-assisted extraction in combination with ethanol as a solvent has several benefits, including a higher yield of active components in the extract and reduced extraction running costs. Moreover, this study investigates the capability of ultrasonic-assisted extraction techniques for the extraction of TPC from BRB. Here, we highlighted the optimization of extraction parameters, potential benefits of BRB extract on UVB induced human keratinocyte cells and determined the effectiveness and safety for extracting BRB extract for topical use. Ethanol solvent usage will also be minimized and increasing phytochemical compounds can improve the low value commodity of black rice bran to higher value.

1.2 Research objectives

The objectives of this research were:

I. To identify the optimal conditions for phenolic compound extraction from black rice bran by using UAE integrated with Response Surface Methodology (RSM) design.

II. To evaluate the skin anti-photooxidative damage effect of BRB extract on UVB induced human keratinocytes cells.

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CHAPTER II
LITERATURE REVIEW

2.1 Black Rice Bran: Health Benefits and Phenolic Compounds

2.1.1 Rice phenolic compounds overview

Rice is a staple food, and it is one of the most consumed cereals in Asia (Papademetriou, 2000). Rice bran is a by-product and most of the phenolic compounds remain in the rice bran due to the grain polishing step in the rice milling process. Rice bran represents about it accounts for 12% of the total kernel weight and is made up of the seed's exterior layers (pericarp and aleurone layer). These outer kernel layers are richer in bioactive compounds than the core endosperm (Fritsch et al., 2017; Kahlon, 2009). Rice bran is regarded as nutritionally abundant as it is composed of approximately 20% of lipids, 40% of carbohydrates, 9% of fiber, 8% of ash, and 13% of protein (Kao & Luh, 1991). Hydroxycinnamic acids and hydroxybenzoic acid derivatives are mainly present as phenolic compounds in the free form of soluble and insoluble bound form of phenolic acids in the rice bran layer (Sohail et al., 2017).

In the pigmented rice bran, the total phenolic contents in gallic acid equivalent to 100 g is six times higher than non-pigmented rice bran stated in the amount of 3,500 mg for pigmented varieties such as black, purple, and red while non-pigmented varieties were 600 mg. Moreover, the antioxidant activities of the husk, whole grain, and endosperm fractions exhibited lower antioxidant activity than the bran fractions. This finding is consistent with the other literature (Goufo & Trindade, 2014) which stated rice bran is the most abundant source of phenolic compounds in rice. Another study stated that black rice bran has higher phenolic content and antioxidant activity when compared to other rice bran (Zhang et al., 2010).

For instance, the total flavonoid content of pigmented rice bran is 1,400 mg GAE/100g while non-pigmented rice bran is 577 mg GAE/100g. Among the pigmented rice bran, significant differences were found as black rice > red rice >

purple rice (Shen et al., 2009). For total anthocyanin, content in black rice bran is mainly higher than red and purple rice, since pigments in black rice varieties are mainly composed of anthocyanin. Depending on the total amount of phenolic acids and anthocyanin, the sensitivity of the antioxidant assays (for example $ABTS^{+}$, and DPPH) are altered.

2.1.2 Benefits of Ultrasonic Assisted Extraction (UAE)

Different extraction techniques have been applied for phenolic compounds extraction due to its significance in human health. Among the various extraction techniques, conventional extraction methods called Liquid-liquid extraction (LLE) such as Soxhlet, maceration, and hydrodistillation methods have been utilized to extract phenolic compounds from various plant sources (Garcia-Salas et al., 2010). The most used extraction techniques: Soxhlet and maceration take extraction times more than 6 to 12 hours, depending on the sample types, and solvent consumption of Soxhlet extraction is quite high. Although Soxhlet extraction does not require filtration of the extract, longer extraction time and higher temperatures diminish the bioactivity of the extracts as a disadvantage (Azmir et al., 2013). Maceration is not expensive and complicated. However, longer extraction time and lower yield weaken the conventional extraction as compared to unconventional (Ji et al., 2018).

Alternative extraction methods such as the application of microwave, carbon dioxide compression, and ultrasound waves have been used widely nowadays. Generally, MAE can increase the extraction yields after increasing the microwave power which localized heating and contributes to rupture of the matrix. However, increasing microwave power to more than 500W generates the degradation of phenolic compounds due to overheating and overexposure to microwave radiation. Phenolic compounds extracted by microwave-assisted extraction methods had higher antioxidant and anti-diabetic activity than those extracted by the LLE method in Vernoniacinerea leaves (Alara et al., 2018).

Moreover, MAE has an explosion risk during microwave extraction due to the solvent can absorb microwave power (Gharaati Jahromi, 2019). Carbon dioxide is used in SFE (supercritical fluid extraction) because it is stable, nontoxic,

environmentally safe, and cheap. Many studies have used carbon dioxide as a solvent for the recovery of numerous phenolic compounds (Vatai et al., 2009). The extraction process involves cooled CO_2 that is pressurized by a liquid pump. When CO_2 reaches the supercritical point after being heated, it is passed through the extractor vessel, and co-solvent methanol or ethanol may be injected in the vessel to adjust the polarity of the CO_2 since CO_2 is a non-polar solvent. In addition, CO_2 and extract can be efficiently recovered by adjusting the pressure and temperature (Hatami et al., 2018). Marques et al., (2016) investigated phenolic compounds extracted with SFE extraction methods had an antioxidant activity higher than the LLE method. Moreover, Roselló-Soto et al., (2019) reported extraction with SFE using optimized conditions of CO_2, ethanol, pressure, and temperature, increased the extraction yields. As a disadvantage, the equipment necessary is more expensive, and power consumption is extremely high on an industrial scale.

Ultrasound is used in the process of degassing, sterilization, and extraction of various bioactive compounds. In it, cavitation creates compression in the liquid medium by causing pressure changes and the formation of collapsing bubbles (Tiwari, 2015). Due to the cavitation force, the cell wall of the plant matrix is disrupted, and extraction solvent penetrates the matrix which triggers the greater extraction yields. Besides, ultrasonic parameters such as amplitude, frequency and wavelength have a lot of effect on the phenolic compounds' extraction process. The highest yield is achieved after increasing the ultrasound power (Rutkowska et al., 2017). Not only ultrasonic power, design and ultrasound wave systems highly impact the extraction process (Wang et al., 2018). Unlike conventional methods, the UAE method is easy to handle because of its simplicity, low cost, high efficiency, and lower consumption of organic solvents for phenolic compounds extraction. Not only high efficiency but also reduction of extraction time and less electricity consumption have attracted a lot of attention for the large-scale level and industries among a lot of extraction techniques (Olmo-García et al., 2018). On the other hand, less energy consumption makes the world greener and extensively benefits the ecological system. As a single extraction of phenolic compounds from plant materials is not enough, we hypothesized the integration of microbial fermentation and phenolic

compound extraction from fermented biomass with UAE could improve the phenolic profiles of BRB extract.

Several researchers reported that rice bran fermented solution can improve cosmetic properties, such as whitening, anti-wrinkle, and anti-aging (Abd Razak et al., 2019; Chen et al., 2018). In our studies, we proposed steaming with **α**-amylase as a pretreatment method which could improve the fermentation process by providing a nutrient source for lactic acid bacteria based on previous literature. Moreover, the addition of thermostable **α**-amylase can gelatinize and liquefy the starch in rice bran and can provide the sugars for lactic acid bacteria growth in the fermentation stage. This method is an alternative approach to enzymatic treatment which is limited by the high cost of enzymes and the unavailability of phenolic esterase on a large scale. For the lactobacillus bacterial strain selection, black rice bran was fermented with *L. acidophilus, L. brevis, L. fermentum*, and *L. plantarum*. Details of experimental protocols and results were discussed in Appendix A. Our studies indicated that the integration of **α**-amylase pretreatment, lactic acid bacteria fermentation, and UAE did not significantly increase the TPC when compared to non-fermented solution and non-fermented biomass. The enzymatic treatment effectively cleavages the lignin from the plant cell walls and a range of enzymes from Lactobacillus degraded phenol rings, causing polymerization/depolymerization and not significant increase of TPC observed. Hence, we removed the fermentation and pretreatment parts from our study and continued to optimize the extraction parameters such as ethanol concentration, temperature, and pH.

2.1.3 Importance Extraction Parameters in UAE

UAE extraction yield and antioxidant activity depend not only on the extraction method but also solvent used for extraction (Do et al., 2014). Polar solvents such as ethanol, methanol, acetone, and ethyl acetate are frequently used for phenolic compound extraction. Among these polar solvents, Turrini et al., (2018) evaluated ethanol by UAE as an eco-friendly and non-toxic solvent that is considered as GRAS (Generally Recognized as Safe) according to the United States Food and Drug Administration designation. Their aim was to reduce solvent consumption and costs. They revealed that the employment of UAE significantly

reduced both the time of extraction and the solvent volume (24 mL/g rice). When the extraction process is underway at a large-scale level in the industry, ultrasound power, amplitude, temperature, time, and type of solvent can be efficiently optimized not only for extraction efficacy but also for extract composition. Lower energy consumption is also very important at a large-scale level in industry, and it can be used as a simple and reliable extraction process (Gharaati Jahromi, 2019).

Although the type and qualities of the raw materials, including rice variety, cultivar, and phase of maturity substantially impact the extraction of phenolic compounds, all extraction procedures share several important factors. For example, extraction solvent, extraction temperature, and extraction time can exhibit distinct amounts of phenolic compounds in all chosen extraction processes. Depending on the selection of extraction solvent and concentration, phenolic compounds are likely to be highly or mildly dissolved. Mostly phenolic compounds are favoured in polar solvents in the mixture of water and ethanol since they can increase the rate of diffusion and mass transfer. Moreover, the extraction process can also be enhanced by adjustment of extraction temperature and times that will allow for a more intimate and effective contact between solvent and matrix. Moreover, the nature of raw materials influences the extraction of phenolic compounds, and maturity stage, storage condition, and cultivar, affecting the extraction process, seen as with the other extraction methods (Osorio-Tobón, 2020).

All extraction processes share crucial parameters, such as temperature, extraction time, and solvent types. Although increased temperature enhances the maximum extraction yields, heat-sensitive bioactive compounds can decrease their antioxidant activity. Goltz et al., (2018) reported the change in total phenolic content was negative during the extraction of phenolic compound from *A. satureioides* inflorescences after the temperature increased from 25 to 50 °C. In ultrasonic power studies, increasing ultrasonic power from 150 to 250 watts, higher levels of total phenolic content and antioxidant activity were achieved, and it was found that the increase in the ultrasonic power caused major alterations in plant cell matrix due to the cavitation (Goldsmith et al., 2018). Therefore, the selection of suitable solvents,

extraction technique, and optimization of extraction parameters, could achieve the highest content of bioactive compounds.

2.1.4 Phenolic Compounds Profiling and Antioxidant Activity

Folin-Ciocalteu (F-C) Assay was developed for tyrosine measurement and has been used for many years to determine the phenolic content of plant bioactive compounds. This assay was modified and called the Folin-Lowry Assay which was developed to the protein quantitation. Over the years, the Folin and Ciocalteu reagent react not only with phenols but also with other antioxidants (Prior et al., 2005). According to the extensive study of Ikawa et al., (2003) several classes of nitrogenous compounds showed significant reactivity towards the F-C reagent. This could possibly lead to high apparent total phenolic content. Due to this fact, Everette et al., (2010) studied the reactivity of the Folin-Ciocalteu (F-C) reagent towards various compound classes and their study revealed that compound classes including phenols, proteins, vitamins, and thiols were reactive towards the reagent. Their findings also suggested that earlier statements of the reactivity of several amino acids and sugars were not substantialized in their study. Their finding alerted all researchers who have been working on the extraction of phenolic compounds that the F-C assay alone is not enough for a quantitative study of total phenolic content. Analyzing with other different methods might be helpful in quantifying total phenolic content and their antioxidant activity. Moreover, reliability of data can be questioned, since the validation protocol is often completely absent and makes data comparison from different studies challenging.

Due to the importance of the relationship between individual phenolic compounds to the antioxidant properties, quantifying different specific classes of polyphenol and antioxidant assays can be helpful for the evaluation of antioxidant properties by improving the precision and accuracy of the data. For example, pH differential assay for the quantification of total anthocyanin, which is based on the structural changes of anthocyanin chromophore between pH 1 and pH 4.5 can be used. TAC calculates the absorbance difference between 515 nm and 700 nm (Ti et al., 2015). For the antioxidant assays, two decolorization-based assays $ABTS^{+}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-

picrylhydrazyl) are widely used and $ABTS^{+}$ can be applicable to both lipophilic and hydrophilic antioxidants (Re et al., 1999). As a conclusion, all the above antioxidant assays are extremely useful, but the reaction time should be considered because the results are variable depending on the reaction time.

2.1.5 Keratinocytes and UVB radiation

Skin aging is an impairment of skin integrity and mainly categorized into two types: chronological aging (intrinsic) and photoaging (extrinsic). Ultraviolet (UV) radiation corresponds to a considerable environmental factor for human skin health. Because of chronic exposure to UV, photoaging occurs and often can lead to formation of sunburn, wrinkles, uneven-pigmentation, and induction of inflammation. Moreover, photoaging involves disorganization of the dermal matrix and changes of cellular biosynthesis activity (Jenkins, 2002). Depending on the wavelength ranges, UV radiation can be classified into UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm) (Elwood & Diffey, 1993). Recent studies implied that UVB is the most damaging and genotoxic and responsible for sun-induced skin disorders and longer exposure of UVB can increase the risk of premature aging and skin cancer (Piao et al., 2014). Another study reported that UVB is more genotoxic than UVA and its ability to reach in the epidermal basal cell-layer induced direct and indirect adverse biological effects with the formation of photoproducts (de Gruijl, 2002; Trautinger, 2001).

UVB-induced processes can trigger DNA damage and result in the formation of 6-4 photoproducts and cyclobutane-pyrimidine dimer that cause mutations (Cadet et al., 2005). The formation of pyrimidine dimers, often caused by exposure to UV radiation, triggers an elevated production of reactive oxygen species (ROS). These ROS, in turn, induce oxidative stress, leading to cellular and extracellular matrix damage due to their highly reactive nature. This process ultimately contributes to the impairment and deterioration of cells and their surrounding environment, posing significant challenges to cellular function and tissue integrity. To maintain the natural redox homeostasis, ROS are usually neutralized by enzymes (such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase) and non-enzymatic antioxidants (i.e., ascorbic acid) (Tyrrell, 2012). Excessive generation of ROS induces a variety of transcription factors

such as nuclear factor kappa B (NF-kB), activator protein 1 (AP-1), nuclear factor erythroid-derived 2-like 2 (Nrf2), and mitogen-activated protein kinase (MAPK) pathway (Figure 1). When NF-**K**B and AP-1 are activated by specific stimuli (such as cytokines, stress, or inflammatory signals), they bind to specific DNA sequences within the promoter regions of MMP genes, thereby stimulating their transcription. This activation results in the increased synthesis and production of MMPs, which play crucial roles in various physiological and pathological processes, including tissue remodeling, immune responses, and inflammatory conditions. In addition, degradation of ECM may contribute to the invasion and metastasis of cancer cells (Figure 2.1) (Dunaway et al., 2018). The group of metalloproteinases (MMPs) proteins play a critical role in UVB-induced skin damage by controlling DNA replication, cell cycle progression and normal tissue development (López-Otín & Hunter, 2010). Moreover, MMPs are involved in tissue remodeling of collagens, enzymes, and glycoproteins in the extracellular matrix (ECM) of the cells by providing structural and biochemical support to the surrounding cells (Pucci-Minafra et al., 2008).

Keratinocytes are the predominant cell type in the epidermis and play a pivotal role in protecting the skin from UVB-induced damage. Keratinocyte damage is correlated with various skin disorders, including sunburn, photoaging, and photo-carcinogenesis (Luang Pradit Kun et al., 2021). Keratinocytes, as revealed in the study by Brash et al. (1996), employ different mechanisms to keep their cellular function and protect against UVB-induced stress. These mechanisms involve the release of signaling molecules aimed at directing defensive reactions. Additionally, keratinocytes initiate mechanisms such as allowing cell-cycle progression to manage possible damage, starting DNA repair processes to correct UVB-induced DNA defects, and engaging in controlled cell death (apoptosis) as a defense against permanent harm caused by UVB exposure. Despite their protective role, keratinocytes can also suffer damage in the process of defending the skin against UVB radiation. This can lead to cell death or alterations in their functions, ultimately contributing to skin damage and the aging process. Therefore, understanding how keratinocytes respond to UVB radiation and identifying ways to enhance their resilience is of great importance in the field of skincare.

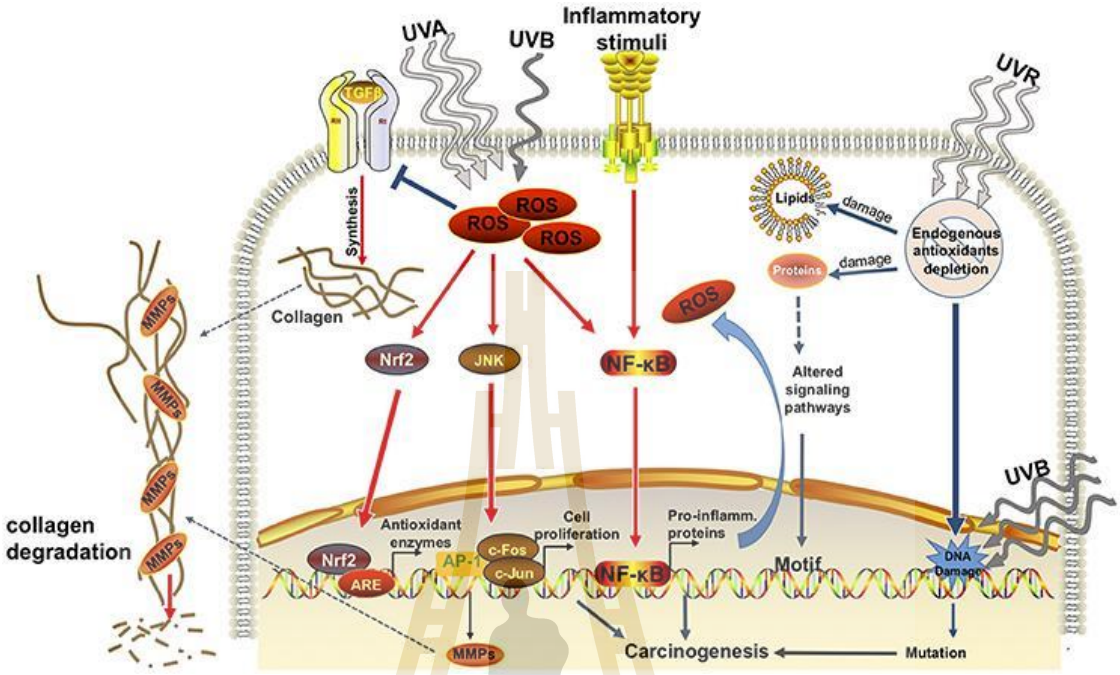

Figure 2.1 UV radiation effect in Skin: Figure represents the effects of UV Radiation (UVA+UVB) in epidermal keratinocytes. Exposure to UVR induces the formation of reactive oxygen species (ROS) to increase ROS causing an imbalance between pro-oxidants and antioxidants. Oxidative stress also leads to DNA damage by direct DNA damage. Moreover, ROS-activated transcription factors such as Nrf2, JNK, and NF-**K**B. They will bind to their specific DNA sequences, antioxidant responsive element (ARE), AP-1 (c-Fos/c-Jun), and NF-**K**B, respectively. Alteration to lipids and proteins that happens by induction of ROS promotes carcinogenesis. In addition, stress synthesizes and releases matrix metalloproteinases (MMPs) that can degrade collagen, a biomarker of skin aging. (Dunaway et al., 2018).

2.1.6 UV protective properties of natural phenolic compounds

Reducing ozone levels decreases protection from the sun's rays and reaches the UVB radiation at the Earth's surface. Repetitive exposures to solar UV radiation result in a dramatic increase in the production of reactive oxygen species (ROS) (Egert et al., 2017). Due to the higher production of ROS than elimination, ROS

accumulation increases and results in oxidative stress. Utilization of antioxidant-containing products can be beneficial to human skin and health to neutralize ROS. Research from Afaq & Mukhtar (2001) and Velioglu et al. (1998) implies that natural antioxidants may have a better safety profile regarding toxicity compared to synthetic antioxidants. Moreover, increasing UV-associated skin disorders has resulted in a high demand for the scientific research community to develop alternative approaches for natural antioxidant compounds extraction and application.

In recent years, bioactive molecules phenolic compounds used in industries are of increased interest, because of their bioactivities such as antioxidant, antimicrobial, anti-inflammatory and antiproliferative properties. Phenolic compounds can reduce solar radiation penetration into the skin by absorbing UV radiation ranging from sun protection factor (SPF) 7-10 because of the presence of chromophores in their structure (de Lima Cherubim et al., 2020). Phenolic compounds possess one common structure called phenol and it has one aromatic ring having at least one hydroxyl substituent. One group of secondary metabolites of plants, phenolic compounds are mostly found in herbs, crops, fruits, vegetables, grains, tea, coffee beans and red wine by enhancing the colors and flavors as an essential part of the human diet (Rice-Evans et al., 2000).

Among several phenolic compounds, flavonoids and phenolic acids activities of reactive oxygen species scavenging and chelating the transitional metal ions which are responsible for the initiation of free radicals. Moreover, several studies have shown that the scavenging activity of both flavonoids and phenolic acids on superoxide anions, singlet oxygen, hydroxyl-radicals, and lipid peroxyl-radicals (Robak & Gryglewski, 1988; Torel et al., 1986; Zhou & Zheng, 1991). Amongst the natural phenolic compounds obtained from the plants, evidence for the UVB protective properties of black rice bran is considerably still less when compared to other parts of the plant (Table 2.1). It has been evaluated that pigmented rice varieties are richer in phenolic acid compared to non-pigmented rice varieties. Therefore, natural antioxidants from black rice bran could be beneficial in UVB protection as other natural antioxidants and could be used as additives in the formulation of skin care products.

Table 2.1 Phenolic compounds in plant extracts and protective effects on skin

No	Types of Extract	Phenolic Compounds	Models	Targets	Cytokines and Pathways	Targeted Genes	References
1	Cocoa extract	Flavan-3-ols and Procyanidins	Ex-vivo on UV exposure (Oral Administration)	Inflammatory responses	TNF-α, IL-6, IL-1β	-	(Cádiz-Gurrea et al., 2017; Calderón-Garcidueñas et al., 2016)
2	Green Tea extract	Catechins with galloyl residues Epigallocatechin gallate	-Human epidermal Keratinocytes -Dermal fibroblasts	Oxidative stress and inflammatory responses	Nf-kB, AP-1, MAPK pathways	NOX-1, NOX-2, TNF-α, IL-1β, IL-6, IL-8 Metalloproteinases	(Seok et al., 2018; Wang et al., 2019)

Table 2.1 (continued)

No	Types of Extract	Phenolic Compounds	Models	Targets	Cytokines and Pathways	Targeted Genes	References
3	Grapes	Stilbenoids and proanthocyanidins	Human epidermal Keratinocytes Dermal fibroblasts	Antioxidant and anti-inflammatory	Nf-kB, Akt, ERK1/2, and p38 MAPK	COX-2, TNF-α, IL-1β, IL-8	(Choi et al., 2018; Tsai et al., 2017)
4	Pomegranate	Ellagitannins	THP-1 human acute monocytic leukemia cells	Oxidative stress and inflammatory responses	TNF-α, IL-1β and MCP-1	ICAM-1, NOX-1, NOX-2, TNF-α, IL-1β, IL-6, IL-8	(Jean-Gilles et al., 2013; Seok et al., 2018)
5	Black Rice	Cyanidin-3-O-β-D-glycoside (C3G), and taxifolin-7-O-glucoside (T7G)	Human epidermal Keratinocytes	Oxidative stress and inflammatory responses	Phospho-ERK1/2, JNK, p38 MAPK, and c-Jun, and ERK1/2, JNK, p38 MAPK, and c-Jun	MMP-1, MMP-3, Type I procollagen	(Han et al., 2018)

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CHAPTER III

ULTRASONIC-ASSISTED EXTRACTION OF BIOACTIVE COMPOUNDS FROM BLACK RICE BRAN BY RESPONSE SURFACE METHODOLOGY

3.1 Abstract

This work is intended to enhance the extraction conditions for phenolic compounds from black rice bran. Ultrasonic-assisted extraction (UAE) parameters were optimized by Response Surface Methodology. Extraction parameters of 57% ethanol at 50°C, and pH 3.1 yielded black rice bran extract with a total phenolic content of 136.2 mg gallic acid equivalents/ g dry weight, total anthocyanin content of 2.3 mg cyanidin-3-O-glucoside equivalents/ g dry weight, and total flavonoid components 38.6 mg quercetin equivalents/ g dry weight. *In vitro* antioxidant experiments revealed free radical-scavenging activity for $ABTS^{+}$ and DPPH. Constituents of phenolic compounds were investigated by UHPLC, and six phenolic components were putatively identified in black rice bran extract, with syringic acid being the most abundant.

3.2 Introduction

Black rice has been traditionally grown in many Southeast Asian countries. Research has demonstrated that black rice has various pharmacological properties, including antioxidants (Sangkitikomol et al., 2010; Zhang et al., 2006), anti-inflammatory, and anticancer effects (Chen et al., 2006). One of the most prevalent and varied classes of active chemicals in plants are phenolic compounds, which include one or more hydroxyl groups and at least one aromatic ring in their structures. These phytochemicals are bioactive compounds that can help improve human health and immunity (Farnsworth, 1966). Among the rice varieties, pigmented

rice has bran with the strongest antioxidant activities based on the diversity, concentration, and structural features of phenolic compounds.

Black rice bran is recognized for its high phenolic content, which is divided into two groups: soluble and insoluble compounds. In terms of natural antioxidants, the recovery of phenolic compounds from colored rice bran may have economic advantages. For the recovery, selecting the right extraction technique is a critical step of isolating and extracting phenolic chemicals from natural plant sources. Due to the importance of phenolic compounds in human health, numerous investigations have been done so far to extract phenolic compounds utilizing different extraction procedures and solvents (Farnsworth, 1966). Black rice includes a spectrum of micro and macro nutrients, amino acids, phytochemicals, and secondary metabolites. Traditional extraction processes require a significant volume of organic solvents and energy and occasionally result in loss of phenolic compounds owing to oxidization and hydrolysis throughout the process (Ivanovic et al., 2014).

Common extraction procedures for phenolic compounds from rice bran, particularly black rice bran, include solid-liquid extraction (SLE): commonly a mixture of water and organic solvent is used to extract phenolic chemicals from the solid matrix. microwave assisted extraction (MAE): utilizes microwave radiation to extract phenolic chemicals. This approach helps speed up the extraction process (Shadab Gharaati, 2019). Ultrasound-Assisted Extraction (UAE): utilized ultrasound waves and have special characteristics such as higher diffusion and mass transfer, which increased the solvent penetration. Moreover, ultrasound waves disrupt the plant cell walls and increase the extraction efficiency (Mason et al., 2011). UAE factors, such as extraction solvent concentration, temperature, and duration, solid to solvent proportion and pH have influences on the extraction process (Pinelo et al., 2005). Methanol, ethanol, and acetone are widely employed as extraction solvents. However, ethanol is the most used solvent due to its low toxicity (Turrini et al., 2018).

The response surface methodology (RSM) is a useful experimental statistical technique for building and optimizing processes, and it can be used to estimate the optimal conditions (Box & Wilson, 1951). RSM uses a sequential order of design of trials to attain optimum response. In RSM, central composite design (CCD) and Box-Behnken design (BBD) are excellent designs for fitting quadratic model. BBD has certain advantages over CCD and requires fewer runs than CCD, and it has a uniform variance over the design area. Hence, BBD can be used to forecast the lower and higher limits of three level points (Box et al., 1978).

In this study, black rice bran was used as raw material to extract phenolic compounds by ultrasonic-assisted extraction (UAE) and RSM. Moreover, monomeric anthocyanin and flavonoid compounds were explored. The extraction variables, like ethanol concentration, temperature, and pH, were examined and optimized for the highest extraction yield. $ABTS^{+}$ and DPPH free radicals test antioxidant capacity of the black rice bran (BRB) extract. UHPLC analysis was also performed to analyze the constituents of the extract.

3.3 Materials and Methods

3.3.1 Materials

Sigma-Aldrich was the source of p-coumaric acid, ferulic acid, syringic acid, vanillic acid, gallic acid, Folin-Ciocalteu reagent, and other substances that were used without additional purification. Every chemical was of the analytical grade. All trials utilized deionized water.

3.3.2 Sample Preparation

Thai black rice bran (*Oryza sativa* cv. Niew Dam 3) was gifted from Assistant Professor Dr. Theera Thummavongsa, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University. The bran was sieved through the 60 mesh to remove broken rice and impurities. The sieved black rice bran was stored at -40 °C until the extraction process. Rice bran (20 g) was added to 100 mL of ethanol,

thoroughly stirred at room temperature, and sonicated for 30 minutes in an ultrasonic water bath.

3.3.3 Extraction of phenolic compounds using RSM design

The experimental design of extraction process was performed by using a Box-Behnken design. The process was done in an ultrasonic water-bath (Bandelin electronic, DT 514 BH, Germany). The effects of three independent variables, namely ethanol concentration (X_1), temperature (X_2), and pH (X_3) were evaluated on the total phenolic compound extracted. The values for the variability are shown in Table 3.1. The pH of the solvent (extractant) was adjusted using 10 M hydrochloric acid. These values were established from preliminary studies (data not shown). The experimental design involved 12 factorial and 6 center-point experiments. The quadratic polynomial equation was obtained from the experimental results to predict the total phenolic compounds, anthocyanin, and flavonoid compounds. The quadratic polynomial equation is as follows.

$$y = A_0 + \sum_{i=1}^{n} A_i X_i + \sum_{i=1}^{n} A_{ii} X_i^2 \sum_{i=1}^{n} \sum_{j=1}^{n} A_{ij} X_{ij} \quad (1)$$

where the dependent and independent variables, respectively, are y and x. The regression coefficients are A_0, A_i, A_{ii}, and A_{ij}. X_i and X_j reflect the linear correlation, whereas Aij and Xij, respectively, represent the non-linear correlation and interaction relationship between independent variables. After UAE extraction, the sample was filtered through Whatman No.1 filter paper. To remove ethanol, 50% and 70% of ethanolic extracts were evaporated under a vacuum in a rotatory evaporator (Heidoph Hei-VAP Rotary Evaporator, Germany). The concentrated extracts were stored at – 80 °C lyophilized and kept at – 80 °C until further analysis.

Table 3.1 Coded values of independent variable.

Variables	Symbol	Levels		
		-1	0	1
Ethanol conc. (%)	X1	30	50	70
Temperature (°C)	X2	30	50	70
pH	X3	1	3	5

3.3.4 Total Phenolic Content (TPC)

The total phenolic content of black rice bran extract was measured by using the Folin-Ciocalteu technique of Singleton, (1999). Gallic acid was utilized in order to generate the standard curve. The mixture was incubated in the dark for 120 min and then the absorbance at 760 nm was measured in a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA). The results were represented as mg gallic acid equivalents/ gram dry weight of extract (mg GAE/g DW).

3.3.5 Monomeric Total Anthocyanin Content (TAC)

The monomeric anthocyanin content of the extract was tested using pH-differential technique reported by (Ti et al., 2015). The absorbance of the samples was measured at 515 and 700 nm at pH 1.0 and 4.5 using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA). Anthocyanin content was evaluated in terms of cyanidin-3-glucoside (C3GE) equivalents using a molar extinction coefficient of 26,900 and a molecular weight of 449.2 g/mol. Following equation (2) and (3) where, DF is dilution factor, MW is the molecular weight of cyanidin-3-glucoside, ε is the molar absorptivity of the reference anthocyanin and C is the concentration in (mg/mL). The results were represented as mg cyanidin-3-O-glucoside equivalents/ gram dry weight of extract (mg C3GE/g DW).

$$A = (A_{515} - A_{700})_{pH1.0} - (A_{515} - A_{700})_{pH4.5} \quad (2)$$

$$\text{Anthocyanin}\left(\frac{mg}{g}\right) = \frac{AxMWxDFx1000}{\varepsilon xC} \quad (3)$$

3.3.6 Total Flavonoid Content (TFC)

The aluminum chloride calorimetric method of Woisky, 1998 (Woisky & Salatino, 1998) was used for the quantification of the total flavonoid content of the black rice bran extract. Quercetin was used to generate the standard curve. The mixture was incubated in the dark for 40 min. The absorbance of the reaction mixture was measured at 415 nm in a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA). The results were represented as quercetin equivalents/ gram dry weight of extract (mg QE/g DW).

3.3.7 Antioxidant Assays

Radical scavenging ability of black rice bran extract against ABTS radical was evaluated using the methodology of (Re er al., 1999). Briefly, 7 mM ABTS solution was combined with 2.45 mM potassium persulfate and incubated in the dark at room temperature for 12-16 h before use. The $ABTS^+$ solution was diluted with water to an absorbance of 0.7 ± 0.02 units at 734 nm. After the addition of 20 µl black rice bran extract to 180 µl of diluted $ABTS^+$ solution, the absorbance was measured against a blank at 734 nm after 5 min. The inhibition of ABTS radical cation (%) was estimated by applying the following equation:

$$ABTS^+ \text{ Radical Scavenging Activity (\%)} = ((A_0 - A_s)/A_0) \times 100\%, \quad (4)$$

where A_s is the sample's absorbance at five minutes, and A_0 is the absorbance of the blank sample.

Black rice bran extract's DPPH radical scavenging ability was assessed by the Brand-Williams et al., (1995) technique. 180 µl of 120 mM DPPH in ethanol received 20 µl of black rice bran extract. The mixture was vigorously mixed and left at room temperature in the dark for 30 minutes. At 515 nm, the reaction mixture's absorbance was measured. The following equation was used to determine the DPPH radical's scavenging capacity:

$$\text{DPPH Radical Scavenging Activity (\%)} = ((A_0 - A_s)/A_0) \times 100\%, \quad (5)$$

where A_0 is the absorbance of the blank sample and A_s is the absorbance of the sample at 30 min. Trolox was used as a standard for both antioxidant assays.

3.3.8 UHPLC analysis of bioactive compounds

UHPLC analysis was performed with an Agilent 1290 Infinity II UHPLC (Agilent Technology, Palo Alto, CA, USA) equipped with a C18 analytical column ZORBAX C18, 5 µm, 4,6 x 250 mm (Shimadzu Co., Ltd., Kyoto, Japan). The mobile phase is composed of 0.1% trifluoroacetic acidified water (A) and 0.1% trifluoroacetic acidified acetonitrile (B). The gradients were 5% B at 0-8 min, 25% B at 13-18 min, 50% B at 18-22 min, 75% B at 22-26 min and 5% B at 26-30 min. The flow was set as 0.2 ml/min for 30 min. The injection volume was at 5 µl and the measurements were carried out at the wavelength of 280 nm. The constituents were quantified by comparing them to the calibration curves of standards (ferulic acid, gallic acid, p-coumaric acid, protocatechuic acid, syringic acid and vanillic acid). In all calibration curves, linearity was sufficient to give an $R^2 > 0.99$.

3.3.9 Analytical Statistics

Three duplicates of each experiment were carried out. Graph-Pad.Prism.v.5.0 was used for additional statistical analysis, while Design-Expert V13.0.5.0 software from State-Ease Inc. in Minneapolis, Minnesota, was used for the response surface approach. To establish the statistical significance, one-way analysis of variance (ANOVA) and Turkey HSD post hoc test were used; $p<0.05$ was regarded as significant.

3.4 Results and Discussion

3.4.1 UAE extraction process modelling

In the current research, pH values of 1, 3, and 5 as well as ethanol concentrations of 30%, 50%, and 70% were tested while temperature values were kept at 30 °C, 50 °C, and 70 °C. Twelve factorial and six center point tests were among the 18 solutions rendered by the experimental design (Table 3.2). An equation of second-order polynomial was fit to the experimental results (Equation 6-8):

Table 3.2 Box-bhen design and responses

Run	X_1	X_2	X_3	TPC mg GAE/ g (DW)	TAC mg C3GE/ g (DW)	TF mg QE/ g (DW)
1	50	50	3	136.47	2.14	42.09
2	30	70	3	96.34	1.35	32.11
3	50	70	1	111.19	1.34	49.18
4	30	50	5	117.13	1.53	55.62
5	70	30	3	119.22	2.11	40.32
6	50	50	3	134.78	2.16	40.83
7	70	70	3	133.81	2.01	42.84
8	50	50	3	133.15	2.14	40.03
9	70	50	1	112.25	1.61	52.01
10	50	50	3	134.89	2.12	43.41
11	30	50	1	64.12	0.81	18.86
12	50	30	1	95.16	0.91	27.03
13	30	30	3	113.29	1.53	43.02
14	50	30	5	138.09	2.04	59.63
15	50	70	5	118.05	1.31	26.03
16	50	50	3	133.89	2.26	44.81
17	70	50	5	111.62	2.03	32.48
18	50	50	3	133.06	2.21	40.04

*Data as a mean of triplicate

X_1: Ethanol Concentration, X_2: Temperature, X_3: pH

Table 3.3 ANOVA (Analysis of variance) for the fitted quadratic polynomial model.

Source	p-value			Remark
	TPC	TAC	TFC	
Model	< 0.0001[a]	< 0.0001[a]	< 0.0001[a]	Significant
X_1-EtOH	< 0.0001[a]	< 0.0001[a]	0.0092	
X_2-Temp	0.0803[b]	0.0015	0.0053	
X_3-pH	< 0.0001[a]	< 0.0001[a]	0.0007	
X_1X_2	< 0.0001[a]	0.3820[b]	0.0069	
X_1X_3	< 0.0001[a]	0.0087	< 0.0001[a]	
X_2X_3	< 0.0001[a]	< 0.0001[a]	< 0.0001[a]	
X_1^2	< 0.0001[a]	< 0.0001[a]		
X_2^2	0.0029	< 0.0001[a]		
X_3^2	< 0.0001[a]	< 0.0001[a]		
Lack of Fit	0.863	0.9701	0.4539	Not significant
R^2	0.9985	0.996	0.9755	
Adjusted R^2	0.9968	0.9914	0.9622	
Coefficient of Variation (%)	0.9192	2.4423	4.9859	

[a] Means significance (*p<0.05*). [b] Means not significance (*p>0.05*). TPC – Total Phenolic Content, TAC – Total Anthocyanin Content, TFC – Total Flavonoid Content.

Results of the Box-Behnken model's ANOVA revealed that the coefficient of determination (R^2) for the quadratic polynomial model was 97% for the TFC and 99% for both TPC and TAC, indicating that the actual levels may be consistent with the anticipated model levels. (Table 3.3). For the lack of fit analysis, p-values were 0.863 for TPC, 0.9701 for TAC, and 0.4593 for TFC. The results denoted no significant lack of fit. In theory, a lack of fit test corresponds to pure experimental

error (either pure error or replication error) throughout the replication of the center point experiment, and a non-significant lack of fit means that the model is well fitted. In addition, the coefficient of variation showed that the models were reproducible. Moreover, the p-values of prediction for the three models were significant. Therefore, adequate mathematical equations for each model were applied for predicting TPC, TAC, and TFC, and experimental data were fitted into second-order regression equations Eq (6-8).

$$Y_1 = 134.373 + 10.7672 * X_1 - 0.772176 * X_2 + 12.7884 * X_3 + 7.88673 * X_1X_2 - 13.4409 * X_1X_3 - 9.04763 * X_2X_3 - 16.5036 * X_{12} - 2.20554 * X_{22} - 16.6196 * X_{32} \quad (6)$$

$$Y_2 = 2.17016 + 0.317359 * X_1 - 0.0714909 * X_2 + 0.278757 * X_3 + 0.0198299 * X_1X_2 - 0.0739147 * X_1X_3 - 0.288489 * X_2X_3 - 0.162343 * X_{22} - 0.256869 * X_{22} - 0.513801 * X_{32} \quad (7)$$

$$Y_3 = 40.5662 + 2.25619 * X_1 - 2.47727 * X_2 + 3.33647 * X_3 + 3.35178 * X_1X_2 - 14.0682 * X_1X_3 - 13.9545 * X_2X_3 \quad (8)$$

One may anticipate the response for certain amounts of each parameter using the response Y equation, where Y_1, Y_2, and Y_3 (TPC, TAC, and TFC) in terms of coded factors. By default, the factors' high values are written as +1 and their low levels as -1. By contrasting the factor coefficients, the coded equation may be used to determine the relative importance of the parameters.

3.4.2 Optimization and validation

For assigning the optimization parameters, all parameters were set in range while total phenolic contents maximize in which value of one represents the case where all goals are met perfectly (Myers et al., 2016). A value of 0.99 desirability of predicted condition was selected which extraction condition was as follows: 57% ethanol, extraction temperature 50 ℃ and pH 3.1. In order to verify the prediction, laboratory experiments were conducted using optimum conditions.

TPC, TAC, and TFC amounts were not significantly differed from predicted (Table 3.4) which indicated that the formulated extraction parameters

were reliable and accurate. Therefore, the RSM model was used to optimize the total phenolic compounds extraction from black rice bran using UAE.

Table 3.4 Optimal extraction condition of predicted Vs experimental.

	TPC mg GAE/g	TAC mg C3G/L	TFC mg QE/g
Predicted	136.3	2.3	41.3
Experimental	136.2	2.3	38.6

3.4.3 Analysis of the total phenolic content using response surfaces

The effects of extraction parameters; ethanol percentage (%), temperature (°C) and pH on the total phenolic content were studied. Ethanol content also had a large effect (p-0.001) on the extraction rate. The amount of TPC in the extract rose with the increase of solvent concentration up to 60%. However, a small reversing trend was noticed after achieving the peak percentage of 60% of ethanol (Figure 3.1c). Polarity modifications enhanced solubility and improved dissolution of phenolic compounds (Ammar Altemimi et al., 2015; Ghafoor et al., 2009). It has been described that an enhancement of mass transfer phenomenon improved the extraction efficacy and that statement is consistent with the earlier finding on the extraction of phenolic chemicals from grape seeds (Ghafoor et al., 2009).

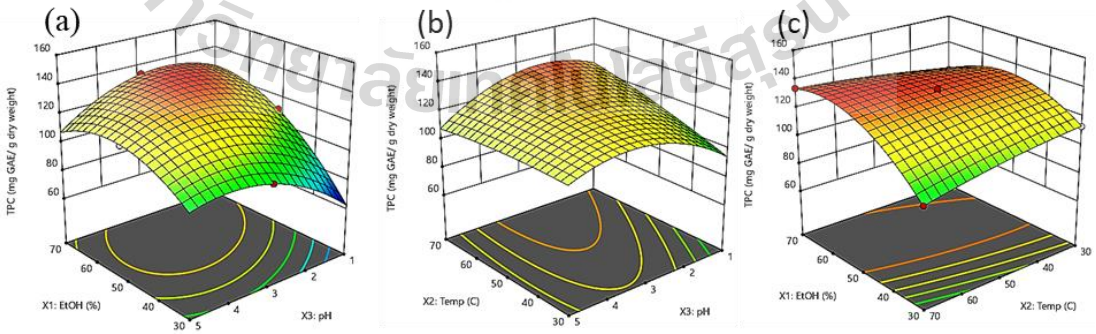

Figure 3.1 Contour Plots: Effect of extraction ethanol percentage, temperature, and pH on Phenolic compounds (a - c).

Figure 3.1b and 3.1c shows the effect of temperature on TPC of black rice bran. The extraction temperature was another critical variable (p-0.001). The yield of phenolic compounds slightly decreased with an increase of temperature from 30°C to 50 °C. Increasing the temperature resulted in the destruction of plant cell walls and releasing phenolic compounds. However, higher temperatures could destroy unstable phenolic compounds, which led to decrease the yield of extraction (Ammar Altemimi et al., 2015). Besides, degradation of phenolic compounds in the extract may be attributed to the oxidative process and similar behavior was investigated by other researchers (Wang et al., 2008). Figure 3.1a and 3.1b illustrated the effect of pH on the total phenolic content of rice bran extract. The pH had a significant effect (p-0.001) on the extraction of TPC. TPC of black rice bran extracts was found to be highest at pH 3.1. Most phenolic compound structures are stable at low pH and change hydrogen-bonding, hydrated structure, and resonance at high pH. The pH strongly influences and affects the absorption spectra changes of phenolic compounds along with the complex formation and often can lead to oxidative degradation at high pH (Friedman & Jürgens, 2000).

3.4.4 Analysis of total anthocyanins and flavonoids using Response surface

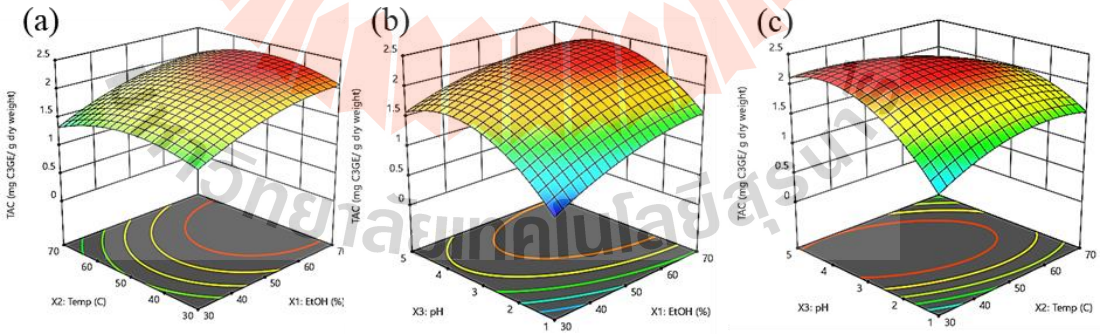

Figure 3.2 Contour Plots: Effect of extraction ethanol percentage, temperature, and pH on anthocyanin compounds (a - c).

Figure 3.2 represented the effect of ethanol concentration, temperature, and pH on the extraction of anthocyanin. Figure 3.2a and 3.2b showed the effect

ethanol concentration and there was a slightly increase of anthocyanin up to 70% and the results are relevant to other studies (Jaberi et al., 2022; Pham et al., 2019). Ethanol has a weak polar property, and this is more effective in separation of nonpolar compounds present in plant cells wall (Lapornik et al., 2005). Higher ethanol concentrations decrease water-soluble components such as polysaccharide and pectin which tightly attach to anthocyanin compounds (Hutabarat et al., 2019). Temperature is another important aspect during the extraction process. In addition, temperature enhances the diffusion of ethanol and increases solubility. The highest anthocyanin content was found at 50 °C while the lowest anthocyanin content was at 30 and 70 °C. The results indicated that high temperatures resulted in unstability and degradation of TAC. Similar results reported that increase of temperature led to degradation of anthocyanin (Wang et al., 2010). For the effect of pH on anthocyanin content, study showed that the yield can be increased slightly with the increase of pH. In other studies, color stability induced the formation of quinoidal bases and cation hydration of red flavylium transformed to colorless carbinol at pH 3.4-6.2 (Hurtado et al., 2009). According to further research, the maximum anthocyanin content was found when 50% ethanol and 2% HCl were combined, and the anthocyanin content declined at greater acidic concentrations. (Jaberi et al., 2022; Mai & Le Van, 2013).

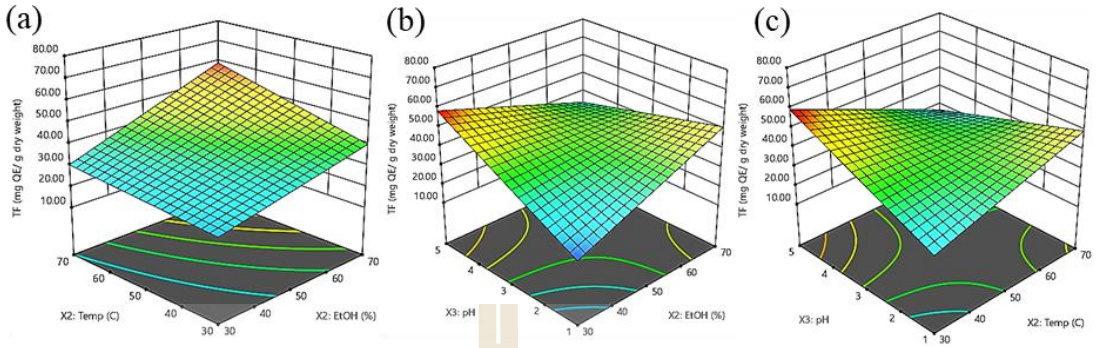

Figure 3.3 Contour Plots: Effect of extraction ethanol percentage, temperature, and pH on flavonoids compounds (a - c).

In our study, the combined effects of solvent concentration with pH and temperature had a significant effect on flavonoid content. Employing ethanol concentration at 30-70% for the extraction provided the linearity increase on flavonoid content (Figure 3.3b). The results agreed with the other studies that 50-70% ethanol concentration was used for extraction of TPC and TFC, because of the infiltration capability into plant cells more than water and other extraction solvents (A. Altemimi et al., 2015; Y. Liu et al., 2017). The effect pH revealed a positive linear effect in Figure 3.3b and 3.3c and demonstrated that with increase in pH from 1-5. In addition, it may be attributed to the polarity changes and diffusion capacity to the black rice bran. Moreover, acidity environments disrupted the chemical bonds of flavonoid and the cellulosic cell walls (Olalere et al., 2018).

3.4.5 Antioxidant Activity

$ABTS^{+}$ and DPPH free radical scavenging assays a widely used to observe the antioxidant activity of bioactive compounds and to explore in ability for free radical reduction (Katalinic et al., 2006; Li et al., 2008). Scavenging activity may vary from 0% to 100%, and factors such as the content of the antioxidant and oxidant (radicals), the ratios of the solvent and reagents utilized, the incubation length, and ambient temperature, as well as the presence or absence of metal, the element hydrogen, and the water in the reaction, all have a significant influence on

scavenging activity. (Dawidowicz & Olszowy, 2012; Dawidowicz et al., 2012). When the extract concentration was raised from 62.5 µg/mL to 500 µg/mL, the BRB's ABTS+ scavenging activity rose from 45% to 92% (Figure 3.4).

In the DPPH radical scavenging assay, the antioxidant capacity of the extract was increased from 30% to 50% and tended to lack of linear relationship when the concentration of BRB increased above 2,000 µg/ml. The lack of linearity can be caused by error due to increasing the concentration of extract. In both assays, the scavenging activities increased with the increasing concentrations of BRB, which indicated that these extracts scavenged radical $ABTS^{+}$ and DPPH in a dose dependent manner. Moreover, to quantify the antioxidant activity, the half maximal effective concentration (EC50) of BRB extract was calculated, and lower (EC50) values indicate greater free radical scavenging activity. BRB extract exhibited $ABTS^{+}$ and DPPH free radical scavenging activity with EC50 of 56 µg/ml and 872 µg/ml while Trolox demonstrated 4.7 µg/ml and 4.1 µg/ml. The antioxidant activity of phenolic compounds largely depends on chemical structures, relative orientation, and attachment of hydroxyl groups to the aromatic rings (Sánchez-Moreno et al., 1998).

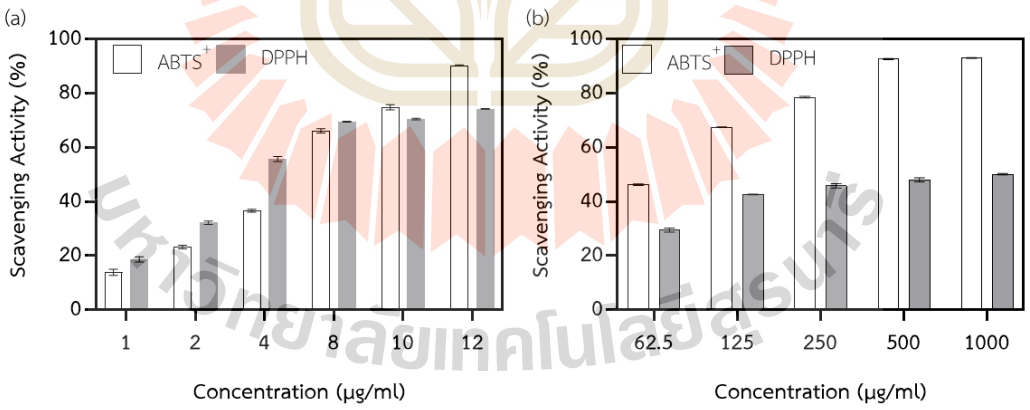

Figure 3.4 $ABTS^{+}$ and DPPH scavenging activity of (a) Trolox (Standard) and (b) black rice bran extract.

3.4.6 Phenolic Acids

UHPLC was used to analyze the phenolic compounds in BRB extract and six phenolic acids; gallic acid, protocatechuic acid, syringic acid, ferulic acid, vanillic acid and p-coumaric acid were detected (Figure 3.5 and Table 3.5). Previous study reported phenolic compounds (protocatechuic acid, syringic acid, ferulic acid, and p-coumaric acid) were observed in free and bound fractions of black rice bran (Ghasemzadeh et al., 2018). In another research study, the black, red, and white rice bran contained various types of phenolic acids (Laokuldilok et al., 2011). Syringic acid was the dominant acid in BRB extract, while vanillic acid had the lowest amount of these detected. Previous study found that the syringic acid was the most abundant phenolic acid in cultivars of four Thai black rice bran (Hom Nin, Khao Niaw Dum, Khao Niaw Dum Mong, and Maled Pai), while ferulic acid and vanillic acid were the lowest (Kapcum et al., 2016) which was fairly comparable to the present study.

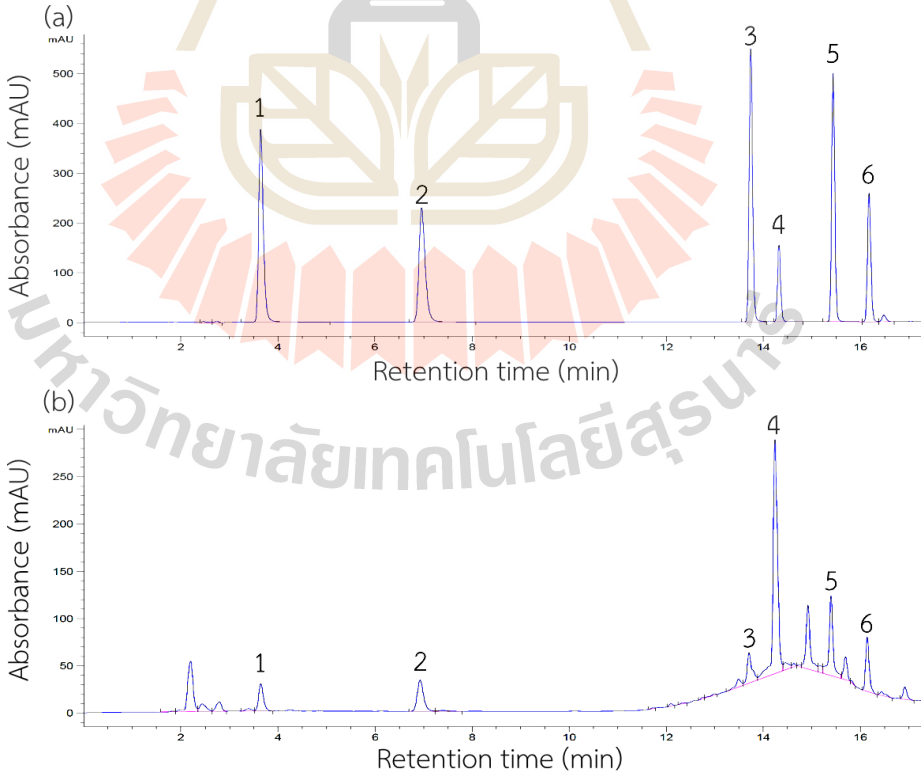

Figure 3.5 Chromatograms of Standards and Black Rice Bran Extract (A) Standard and (B) BRB extract.

Table 3.5 Compounds found in BRB extract by UHPLC.

Peak No	Phenolic Acids	Retention Time	Black Rice Bran Extract (µg/ml)
1	Gallic Acid	3.56	1.8
2	Protocatechuic Acid	6.7	4.91
3	Vanillic Acid	13.5	0.49
4	Syringic Acid	14.2	91.45
5	P-coumaric Acid	15.2	2.25
6	Ferulic Acid	16	2.43

3.5 Conclusions

In conclusion, the response surface methodology approach was used to optimize the extraction of phenolic components from black rice bran. Box-Behnken design was successfully applied to study the effect of ethanol concentrations, temperature, and pH on UAE. The results showed that the optimum condition was effective for maximizing the total phenolic compounds extracted. Among phenolic acids, the most concentrated was syringic acid. As demonstrated, black rice bran extract obtained in these optimum condition was high in syringic acid and could serve as a natural antioxidant. UAE is an environmentally friendly extraction method that can be used.

3.6 References

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CHAPTER IV

UNLOCKING THE POTENTIAL OF BLACK RICE BRAN TO ENHANCE COLLAGEN PRODUCTION AND MMP SUPPRESS EXPRESSION

4.1 Abstract

As there is demand for the replacement of synthetic compounds for the purpose of UV protection, the potential of black rice bran was investigated to see whether it could hinder or repair UV-induced skin damage. The phytochemical constituent and antioxidant potential of BRB extract contained phenolic acids and had free radical scavenging activity. The results revealed that the extract had no cytotoxicity on HaCaT cells and was able to restore the morphology and viability upon the stress of UVB on HaCaT keratinocytes. Moreover, the extract possessed a scavenging activity on intracellular reactive oxygen species in a concentration-dependent manner. BRB extract significantly decreased the level of MMP-1 and MMP-2 expression in UVB-irradiated HaCaT cells. In addition, UVB irradiated HaCaT cells treated with BRB extract had enhanced production of Col1A and elastin gene expression. These findings suggest the anti-photoaging activity of BRB extract and can be a candidate for the potential of sunscreen formulation.

4.2 Introduction

Ultraviolet (UV) radiation is electromagnetic radiation and is classified as a photoaging agent for the skin. Skin aging occurs through two independent biological mechanisms: intrinsic (chronologic) and extrinsic (photoaging). Unlike intrinsic aging, photoaging is caused by environmental factors, especially UV radiation. Depending on the wavelength, three kinds of UV rays are divided into UVA, UVB, and UVC. UVB is notably responsible for sun-induced dermatological conditions including sunburn, oxidative damage, and skin malignancies (Hseu et al., 2012). UVC is nearly fully

absorbed by the ozone layer, UVB predominantly affects the epidermis (the skin's outermost layer), and UVA penetrates deeper into the dermis. (Piao et al., 2014). The dermis rests beneath the epidermis and provides mechanical support for the outer layer of the skin. The extracellular matrix of the dermis is primarily comprised of type I collagen, elastin, fibronectin, and collagen fibrils (Fitzpatrick et al., 1993).

UVB exposure causes DNA damage, and the extracellular matrix (ECM) deteriorates by producing large amounts of reactive oxygen species. (ROS). ROS is predominantly triggered by the oxidative imbalance between biological molecules such as proteins, lipids, and DNA (Cadet et al., 2005). Additionally, UVB induced the skin's metalloproteinases (MMPs) to be produced. MMPs are matrix-degrading enzymes and mainly decompose the collagens, elastin, and other proteins in the skin cells matrix (Scharffetter-Kochanek et al., 2000). Moreover, they crucially play a role in skin tissue repair and remodeling (Varani et al., 2000). In contrast, the production of skin connective tissues decreases and biosynthesis of MMPs increases, accelerating the skin aging process, and deteriorating the skin dryness, color tone, etc.

To retain the redox balance within the cells, oxidative stress needs to be counteracted by antioxidants (Shindo et al., 1994). Among the antioxidants, plant phenolic compounds are well-known as non-enzymatic antioxidant molecules (Kasote et al., 2015). Moreover, several natural antioxidants from plants have demonstrated the ability to eliminate free radicals through reduction or elimination and their photoprotective properties. Additionally, several studies were focused on the phenolic extracts of black rice and reported that extract had antioxidant activity, anti-inflammatory (Wang et al., 2007) and cancer cells suppression activity (Chen et al., 2006). However, there have only been a few studies of BRB extract as a bioactive compound with photoprotective potential on UVB stress HaCaT cells. Hence, this study focuses on the intracellular antioxidant activity and photoprotective properties of BRB extract as well as correlation between MMPs and collagen synthesis in UVB

irradiated HaCaT cells. This finding might broaden the application of BRB extract as an additive of cosmetic products.

4.3 Materials and Methods

4.3.1 Cell culture

A human immortalized HaCaT keratinocyte cell line (CLS Cell Lines Service, 30049) was grown in modified Dulbecco's Modified Eagle's Medium (DMEM) containing heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin-streptomycin, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in the ratio of (10%, 1% and 1.5%) at 37 °C in a humidified environment with 5% CO2.

4.3.2 Cytotoxicity assay

Cell viability was determined using an MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay based on metabolic activity (Freshney, 2015; Jean-Gilles et al., 2013). In 96-well cell culture plates, 1.5x104 density of keratinocytes HaCaT cells were seeded, and the cells were then incubated for 24 hours at 37 °C. After they achieved 80% confluence, 5 different concentrations of black rice bran (BRB) extract were added to the culture plate and incubated for 24 h. Different concentrations of BRB extracts (120, 240 and 500 µg/mL) were prepared in incomplete DMEM media at the final concentration of 0.2% DMSO. After 24 h incubation, the culture medium was collected and rinsed with 1X phosphate-buffered saline (PBS-pH 7.4). 100 µL of freshly prepared MTT reagent (0.5 mg/mL) was transferred in each well and incubated at 37 °C for 4 h. The MTT reagent was taken out after 4 hours of incubation. After fully air dried at room temperature, 50 µL of 100% DMSO was added to solubilize the formazan crystal and another 100 µL of 100% DMSO. The quantity of formazan, formed by the reduction of MTT by the mitochondria of live cells, was quantitated by measuring absorbance at 540 nm (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The following equation was used to determine the cell viability:

$$\text{Cell viability (\%)} = (A_0 - A_t) / A_0 \times 100$$

where A_0 represents the untreated cells absorbance and A_t represents absorbance of the treated and UVB induced HaCaT cells.

4.3.3 UVB irradiation and Optimization of Exposure time

As a UVB source, an ultraviolet-B (UVB) lamp (peak, 306 nm; model G8T5E, 8W, Sankyo Denki, Japan) was used and HaCaT keratinocyte culture was irradiated without removing the lid to induce a photooxidative stress. The distance between the cell culture plate and UVB lamp was 15 cm (Figure 1). To observe the effect of UVB exposure time on HaCaT cells, different exposure time points were set at 30s, 60s and 120s, and cells viability was explored by cytotoxicity assay. To avoid the interference of BRB extract containing medium, the medium was replaced with saline solution in prior to UVB irradiation. Briefly, above 80% confluence HaCaT cells were washed with 1X PBS before exposed to UVB and replaced with serum free DMEM after exposed to UVB. UVB intensity was monitored by UVB meter (model ST513, Sentry, Taiwan R.O.C).

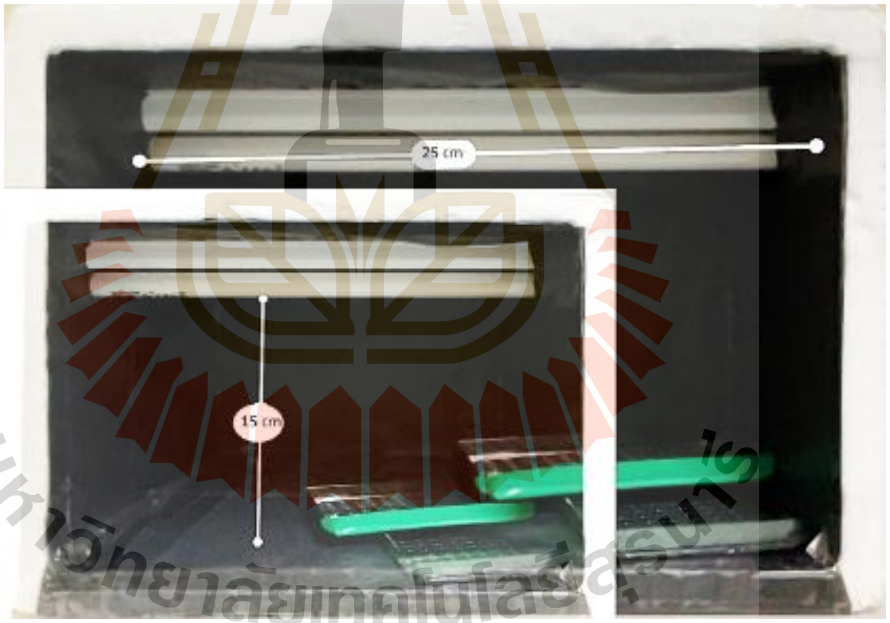

Figure 4.1 Manual Set up for UVB stress.

4.3.4 Quantitation of intracellular Reactive Oxygen Species (ROS)

A redox-sensitive dye probe called DCFH-DA, that emits the fluorescent DCF (2′,7′-dichlorofluorescein) at the λ excitation of 485 nm and λ emission of 530 nm during enzymatic reduction and subsequent oxidation by ROS, was applied to detect intracellular oxidative stress (ROS). After HaCaT keratinocytes were pretreated

with BRB extract for an hour, media was replaced with 1X PBS, and the cells were induced with UVB at 30 sec. Afterward, fresh medium was added and incubated for 24 h. Thereafter, media was removed and washed twice with 1X PBS. 20 mM DCFH-DA was added and incubated for 30 min at 37 °C. Fluorescence visualizations were acquired with a fluorescence microscope (DMIL, Leica Microsystems GmbH, Wetzlar, Germany). The intracellular ROS levels was determined by measuring the DCF fluorescence using a multi-mode microplate reader (Varioskan™ LUX, Thermo Scientific, Massachusetts, USA) (Shin et al., 2018).

4.3.5 RNA isolation, reverse transcription, and RT-PCR analysis

HaCaT cells were induced with UVB for 30 second and treated with BRB at various concentrations (0, 30, 60 and 240 µg/mL) and Protocatechuic acid (150 µg/mL) for 24 h. RNA was extracted by using a commercial total RNA extraction kit (GF-1 Total RNA Extraction kit, Vivantis, Malaysia). The complementary DNA (cDNA) was converted using a cDNA synthesis kit (cDSK01-050, Vivantis, Malaysia). Real-time quantitative PCR was accomplished with qPCR Green Premix kit (Capital qPCR Green Mix, Biotech rabbit, Germany). The cycling conditions for qPCR were 95 °C for 2 min and followed by 40 cycles at 95°C for 15 sec, 60 °C for 20 sec and 72°C for 30 sec. Targeted genes were as follows: Housekeeping gene GAPDH forward, 5′-GTCTCCTCTGACTTCAACAGCG-3′ and reverse, 5′- ACCACCCTGTTGCTGTAGCCAA-3′,MMP-1 forward, 5′- CCTAGCTACACCTTCAGTGG -3′ and reverse, 5′-GCCCAGTACTTATTCCCTTT -3′, MMP-2 forward, 5′- GATACCCCTTTGACGGTAAGGA 3′ and reverse, 5′- CCTTCTCCCAAGGTCCATAGC -3′, type I collagen COL1A forward, 5′-GAGGGCCAAGACGAAGACATC -3′ and reverse, 5′- CAGATCACGTCATCGCACAAC -3′ Elastin forward, 5′- CAAGGCTGGTTACCCAACAG -3′ and reverse, 5′-CACCTGGGACAACTGGAATC -3′. Quantitation of PCR reactions was performed by Bio-Rad Real-time PCR System (CFX Maestro Software, Version 2.3, Bio-Rad, USA). The mRNA relative expression of targeted genes was analyzed by $2^{-\Delta\Delta Ct}$ methods with the normalization of GAPDH housekeeping gene.

4.3.6 Statistical analysis

All experiments were done in triplicate. Statistical analysis was accomplished using Graph-Pad.Prism.v.5.0 (GraphPad Software, USA) and indicated as

the mean ± standard error of the mean (SEM) to evaluate the statistical significance; $p < 0.05$ was determined significant.

4.4 Results and Discussion

4.4.1 Cytotoxicity properties of BRB and effect of UVB exposure time on HaCaT cells

The cytotoxic properties of BRB on HaCaT cells were measured (Figure 4.2). No significant effect on cell viability was seen with 120 and 240 µg/mL BRB. The viability of HaCaT cells increased significantly when the cells were treated at 500 µg/mL BRB extract. Similar results also reported in the cytotoxic properties of BRB on fibroblast cell line NIH3T3 (Oktavya et al., 2023). Other studies showed that Thai pigmented rice extract (Luem-Pua) increased the human fibroblast cell viability in a dose-dependent manner. The results indicated that the BRB extract had no toxic effect to the HaCaT cells. Therefore, doses the same or lower than the tested dose of BRB extract can be used for the further experiments (Teeranachaideekul et al., 2018).

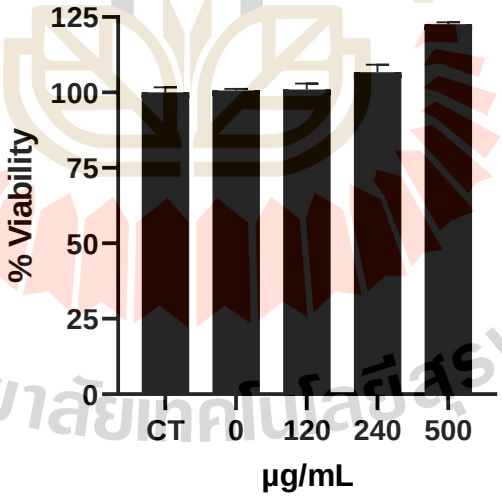

Figure 4.2 Cytotoxic effect of BRB extract on HaCaT cells. The error bar represents the mean ± SD (standard deviation) in triplicates.

4.4.2 UVB irradiation effect on HaCaT cells

To explore the effect of UVB exposure time on HaCaT cells, cells were exposed with various time and incubated for 24 h. Subsequently, cell viability was

measured using MTT assays. UVB inhibited the cells viability significantly in an exposure time dependent manner. Longer exposure time of UVB severely affected the cell viability more than shorter exposure time. Hence, the lowest exposure time of 30 seconds (approx. 18.6 mJ/cm2) was used for the evaluation of reactive oxygen species experiments. However, UVB protective potential of BRB requires longer exposure time to observe the morphological and confluence changes in HaCaT cells.

To evaluate photoprotective potential, HaCaT cells were pretreated with BRB extract for 60 min and exposed to UVB for 120 sec (approx. 74.5 mJ/cm2). Moreover, UVB induced noticeable loss of confluence, cell rounding and shrinkage in morphology and higher apoptosis (Figure 4.3). This indicated enucleation, polarization, and eviction of HaCaT cell nuclei. In addition, these findings were consistent with previous studies (Gag et al., 2023; Hu et al., 2016; Paz et al., 2008). In the literature, UVB irradiation is primarily absorbed by DNA, leading to the formation of photoproducts. Among the photoproducts, cyclobutane pyrimidine dimers (CPDs) were the predominant and the primary contributors to the incidence of 85% of DNA lesions (Dunaway et al., 2018). Moreover, DNA damage is associated and connected with apoptotic cell death. Cells containing unrepairable damage to their DNA undergo apoptosis. It is a highly regulated method of cell death that involves several factors (Vostálová et al., 2010). As shown in Figure 4.4, significant alteration of HaCaT cells morphology and cells number were observed and restored after treated with BRB extract. However, in UVB exposed cells without the BRB, the morphology and cell number were altered. Therefore, BRB has the potential of photoprotective properties and capacity to prevent apoptosis.

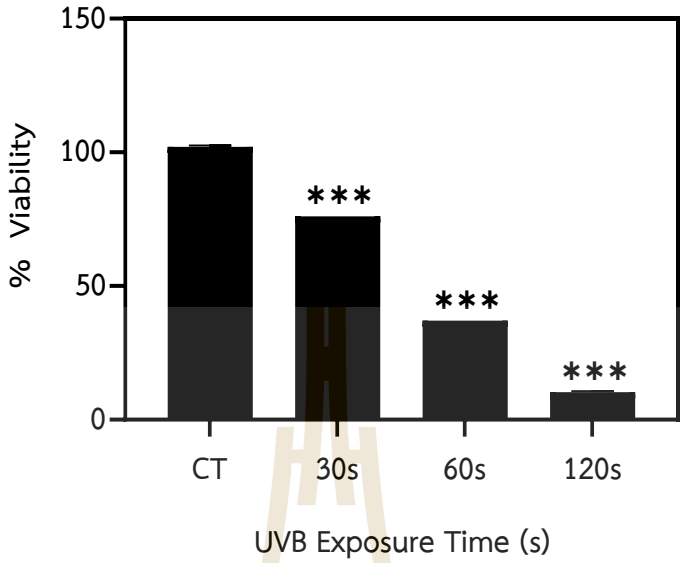

Figure 4.3 The impact of UVB exposure interval on HaCaT cell activity. The results are given as the mean ± standard error of the mean (%), n = 5; ***p < 0.001.

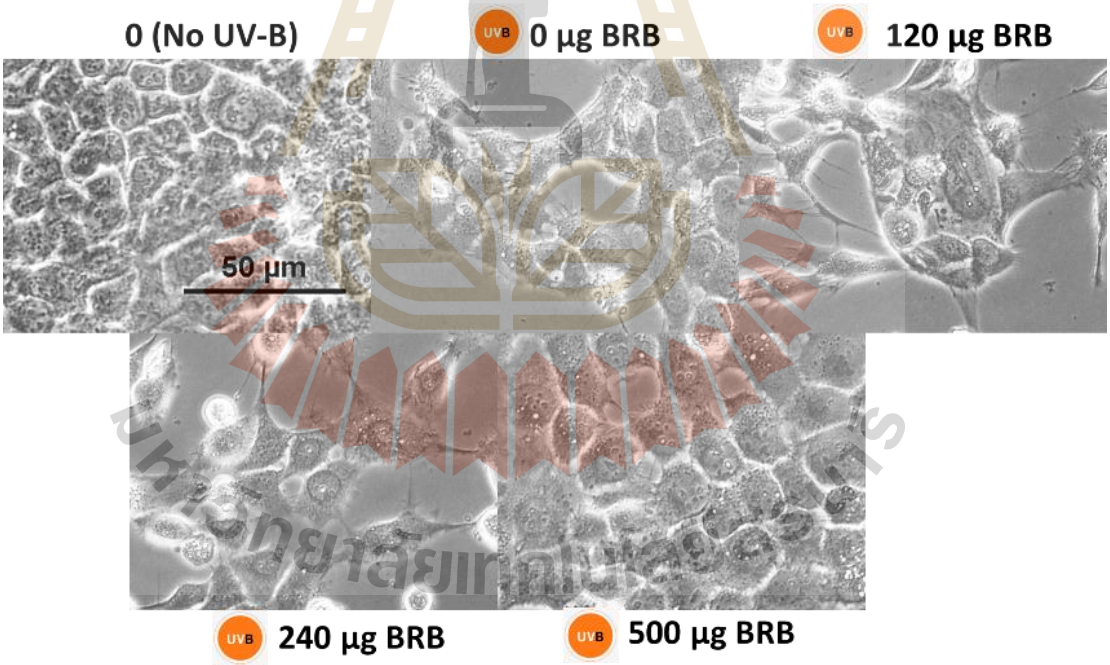

Figure 4.4 Effect of photoprotective properties on UVB stress in HaCaT cells

4.4.3 UVB induced intracellular ROS generation

UVB is a well-known environmental stressor that can generate ROS within skin cells. Moreover, oxidative stress can affect damage to intracellular

components, including DNA, lipids, and proteins and is involved in changes in expression of several genes. Bioactive phenolic compounds are secondary metabolites of plants and exert in molecular function of anti-aging, antioxidant, and anti-inflammatory (Sangkitikomol et al., 2010; Zhang et al., 2006). Therefore, the intracellular antioxidant activity of BRB extract was evaluated. UVB induced intracellular ROS in HaCaT keratinocytes cells and the scavenging activity of BRB extract on these was investigated via a DCFHDA assay. The ROS level increased after UVB irradiated when compared to the untreated cells (Figure 4.5). Treatment with either BRB extract or PA (protocatechuic acid as positive control) significantly attenuated the ROS generation in the cells in a concentration-dependent way.

In addition, fluorescence image results indicated a substantial decreased level of fluorescence in treated cells, compared to untreated cells (Figure 4.6 and 4.7). Treatment with BRB extract resulted in a significant reduction of intracellular ROS levels similar to those of non-irradiated cells in both quantitative and qualitative results. Several studies have searched and reported that natural phenolic compounds obtained from plant extracts can efficiently decrease the level of ROS generation against UVB stress (Cha et al., 2014; Murapa et al., 2012; Singh & Agarwal, 2005). Furthermore, our previous results indicated that BRB has antioxidant activity in $ABTS^{+}$ and DPPH. Moreover, quantification results of BRB extract revealed the presence of six phenolic acids in the extract and syringic acid was the most dominant.

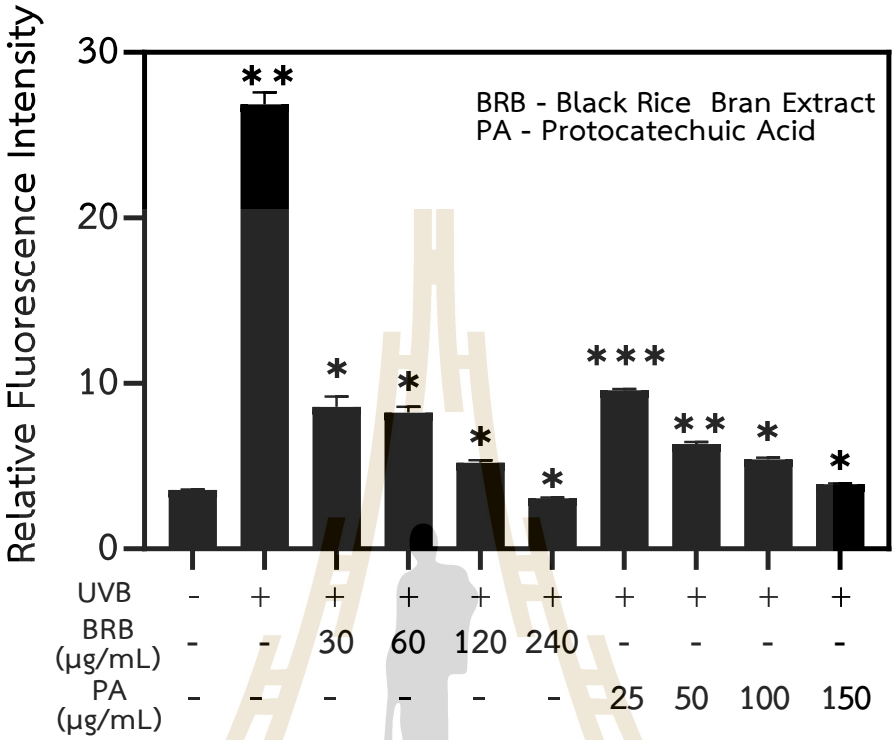

Figure 4.5 Reactive Oxygen Scavenging Activity of BRB Extract. The results are given as the mean ± standard error of the mean (%), $n = 5$; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

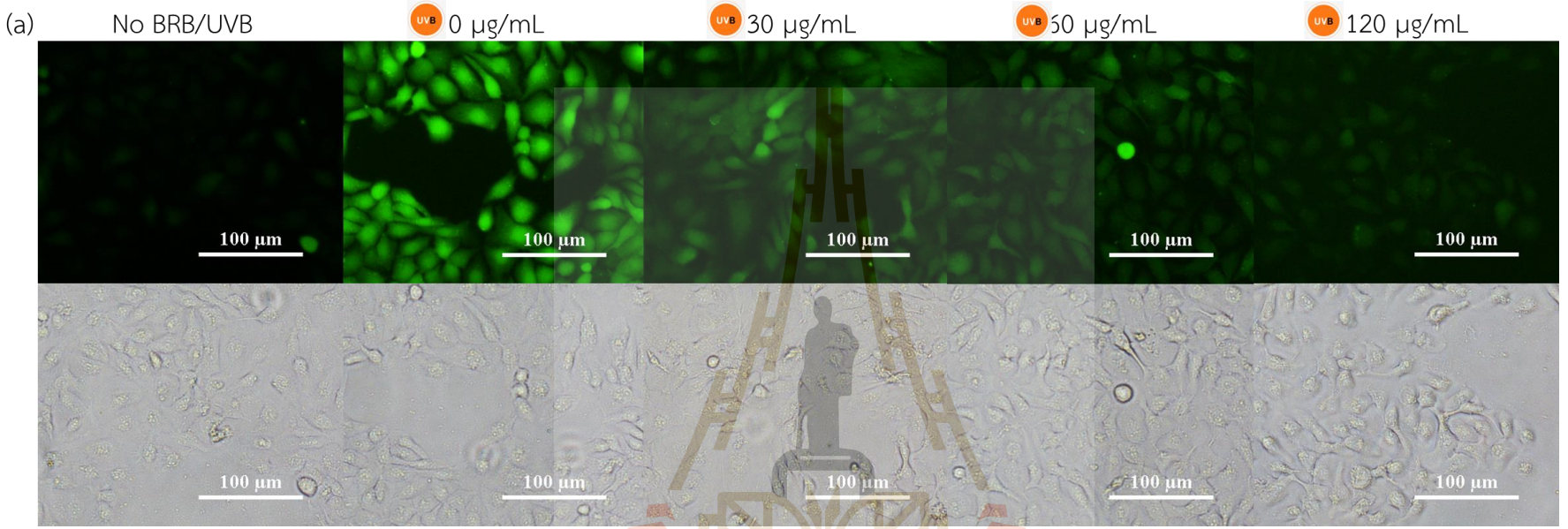

Figure 4.6 Representative fluorescence-microscopy images (a) BRB treated on UVB-irradiated HaCaT cells.

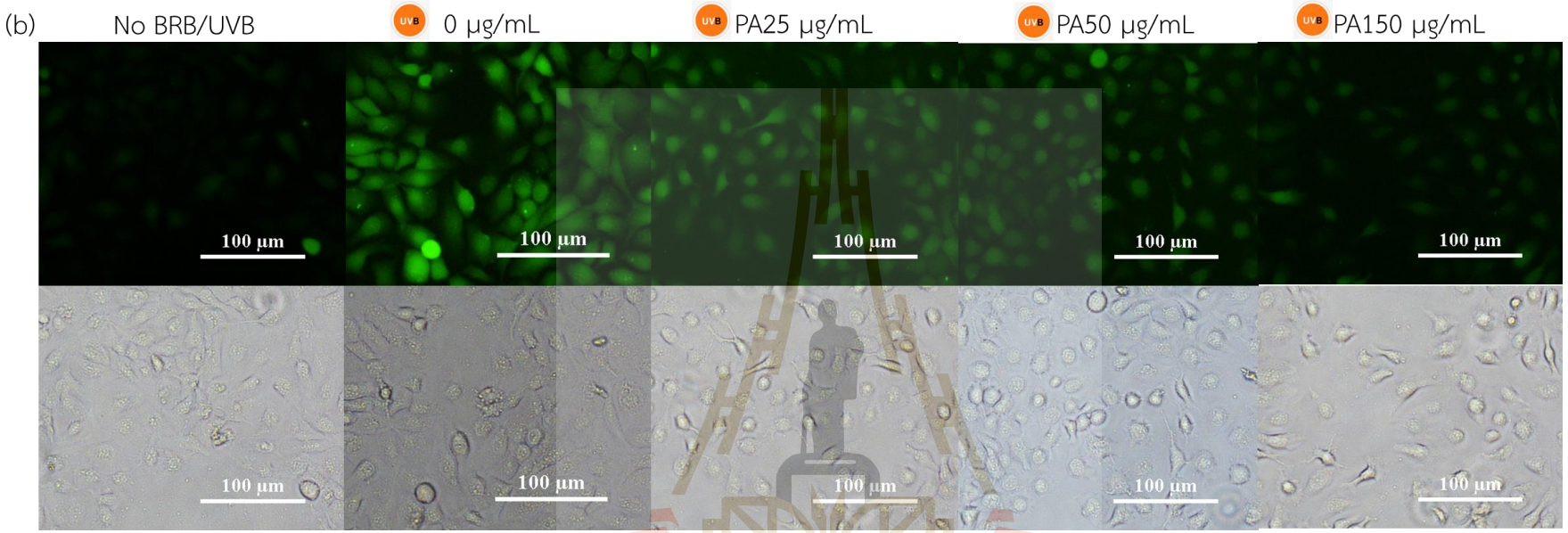

Figure 4.7 Representative fluorescence-microscopy images Protocatechuic acid (PA) treated on UVB-irradiated HaCaT cells.

4.4.4 Metalloproteinase (MMP-1 and MMP-2), COL1A and Elastin gene expression in HaCaT cells

The aging of the skin is regulated by the enhancement of MMPs by UVB irradiation. MMP-1 and MMP-2 are known to be involved in damage to the ECM matrix. In addition, their gene regulation is shown to be upregulated during UVB stress and consistent with our results (Inomata et al., 2003). As shown in Figure 4.8, the level of MMP-1 and MMP-2 expression were significantly upregulated in UVB irradiated cells but downregulated in BRB extract-treated cells in a concentration-dependent for both genes. In contrast, MMP-1 mRNA level of expression was 1.2±0.1 fold higher while MMP-2 level of expression was 1.8±0.2 fold in UVB stress cells than in no UVB stress cells. We have convincingly demonstrated that BRB extract effectively inhibits the expression of UVB-induced MMP-1 and MMP-2 in HaCaT cells.

Moreover, COL1A gene expression was examined followed UVB stress and BRB treatment. COL1A gene expression was significantly suppressed after UVB was exposed to the cells. Recovery of COL1A gene expression was explored in all treatments (BRB at 30-240 µg/mL and protocatechuic acid at 150 µg/mL). The result of gene expression level of COL1A revealed that BRB extract has ability to increase the collagen synthesis. In addition, mRNA level of expression of elastin exerted similar effect as COL1A gene (Figure 4.9). As previously reported, UVB induced MMP-1 expression through pathways involving activator protein-1 (AP-1) and the mitogen-activated protein kinase (MAPK) signaling pathway (Dunaway et al., 2018; Matrisian, 1992). In addition, other studies stated that black rice extract inhibited MMP-1 and MMP-3 expression and exerted the possible antioxidative effects through AP-1 activity and MAPK signaling in human dermal fibroblasts (Han et al., 2018). Our findings agree with these previous studies, underlining the potential of syringic acid to downregulate MMP expression and its anti-aging properties through the AP1 pathway. They suggested that syringic acid exerted anti-aging effects in human keratinocytes,

affirming the critical transcriptional activity of the AP-1 promotes cell proliferation, and collagen degradation. Hence, BRB extract emerges as a compelling and efficient natural extract for rejuvenating human keratinocytes as it component syringic acid.

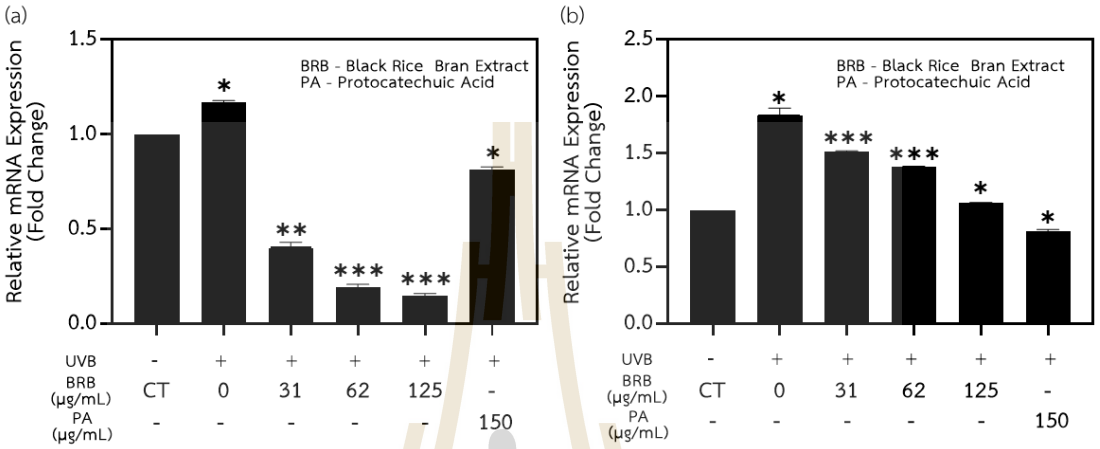

Figure 4.8 The inhibition effect of BRB on (a) MMP-1 and (b) MMP-2 expression in HaCaT cells. (PA - Protocatechuic Acid). The results are given as the mean ± standard error of the mean (%), n = 5; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

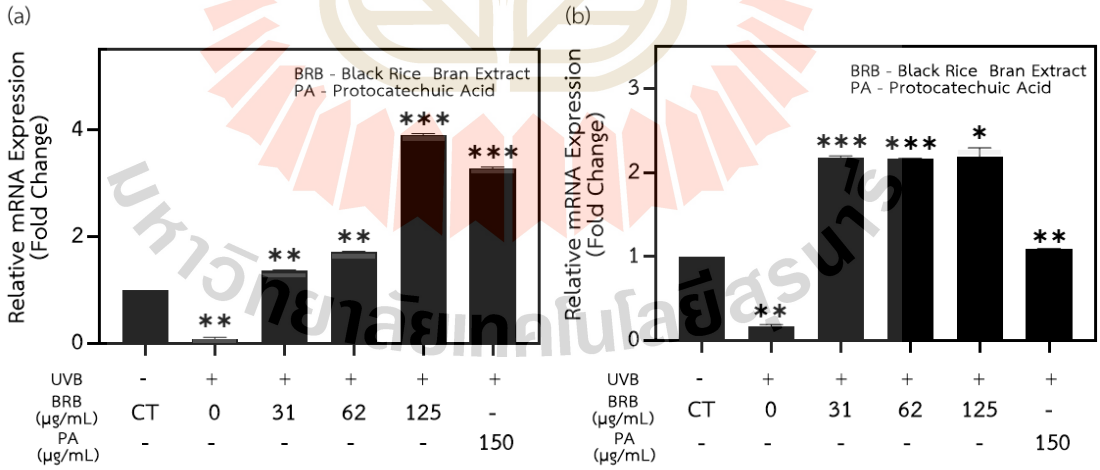

Figure 4.9 The effect of BRB on (a) COL1A and (b) Elastin expression in HaCaT cells. (PA - Protocatechuic Acid). The results are given as the mean ± standard error of the mean (%), n = 5; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

4.5 Conclusions

Notably, in the context of skin health, collagen plays a critical role in maintaining its elasticity and strength. Reduced collagen expression is often observed in photoaged skin. Our findings align with this knowledge, as UVB-exposed HaCaT cells exhibited a decrease in type I collagen gene expression levels, which, significantly, was rescued by the application of BRB extract. Our results point to the potential of BRB extract in preventing or counteracting UVB-induced alterations in the extracellular matrix (ECM) through a dual mechanism, involving the suppression of MMP expression and the promotion of collagen expression. Moreover, our study also considered the influence of oxidative stress triggered by UVB irradiation. It is well-established that oxidative stress leads to the overexpression of MMP-1 and MMP-2 via pathways involving AP-1 and MAPK signaling. Our research indicates that BRB extract is efficacious in reducing intracellular ROS generation in HaCaT cells. These outcomes can be attributed to the antioxidative properties of BRB extract, underscoring its potential as a protective strategy against photodamage.

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CHAPTER V

SUMMARY AND RECOMMENDATION

5.1 Future Research Directions and Recommendations

This study looked at the combination of enzymatic pretreatment, Lactic acid bacteria fermentation, and ultrasonic-assisted extraction. The research discovered cases in which the synergistic combination of enzymatic pretreatment and fermentation did not correspond to the increased extraction efficiency normally associated with UAE. These results highlighted the significance of a thorough knowledge of the unique characteristics and needs of the sources, enzyme selection, and microorganisms involved in this process. It is possible that not all source materials are appropriate for this combination approach, and differences in content, structure, and biological elements may limit the practicality of such a technique. Overall, future research should concentrate on adapting the enzymatic and fermentation processes for particular source materials, enzymes, and microorganisms, as well as improving the UAE parameters to optimize phenolic component extraction.

Because the integration strategy did not correspond with our main goal, we concentrated primarily on enhancing the ultrasonic-assisted extraction conditions. The effectiveness and potential of Ultrasonic-Assisted Extraction (UAE) as a beneficial approach for the extraction of phenolic compounds from Black Rice Bran were proven via optimization. Furthermore, these findings demonstrated improved efficiency in extraction time, personnel, and energy utilization as compared to traditional approaches. Furthermore, the BRB extract demonstrated antioxidant activity as proven by its ability to scavenge free radicals in tests such as ABTS and DPPH, indicating potential in vivo uses. Furthermore, when UVB irradiated HaCaT cells were treated with BRB extract, intracellular reactive oxygen species (ROS) levels were reduced, highlighting its potential as an anti-oxidative stress agent. Furthermore, the findings provide light on the potential advantages of BRB extract in terms of skin

health and cellular function. Our findings show that BRB extract causes a statistically significant increase in collagen gene expression in human keratinocytes, confirming its significance in collagen production. We found that BRB extract significantly decreased MMP gene expression, indicating a probable mechanism by which BRB extract contributes to reduced matrix breakdown in the skin.

These results not only corroborate prior study in the area, but also provide new insights into the use of BRB extract in cosmetics and dermatological investigations. Future research should look at the role of major signaling pathways, such as the MAPK and NF-KB pathways, in mediating the effects of BRB extract on cellular responses to produce a better understanding of the molecular processes at work. Furthermore, studying the regulation of cytokines, such as IL-6, TNF-α, and TGF-β in response to BRB extract administration might give insights into the extract's immunomodulatory capabilities, which has implications for inflammation-related skin diseases. Further study should concentrate on clarifying the crosstalk between these pathways and cytokines, revealing possible synergistic or antagonistic interactions that may be exploited for therapeutic applications in dermatology.

APPENDIX

APPENDIX

Black Rice Bran Fermentation

1.1 Abstract

The research explores the integration of enzymatic pretreatment, lactic acid bacteria fermentation, and extraction of phenolic compounds from fermented biomass. The research problem is that there have been no studies on the integration of these processes. The study aimed to investigate the effects of the integration technique on the total phenolic content of fermented black rice bran biomass. The total phenolic content was not improved in fermented biomass extract when compared to non-fermented.

1.2 Introduction

Lactic acid bacteria can significantly decrease the toxicity level of primary compounds by metabolizing the products. For example, metabolized products of caffeic, p-muramic and protocatechuic acids have lower antimicrobial activity than the original form (Curiel et al., 2010). Structural modifications of polyphenols are the individual characteristics of lactic acid bacteria. Due to this fact, their effects are changeable on the accessibility of certain compounds (Gross et al., 2010). Fermentation using lactic acid bacteria *L. paracasei*, *L. plantarum* and *L. acidophilus* are able to increase the total content of phenolic compounds up to 1,500 mg/ml. The possible mechanisms of hydrolytic enzymes synthesized by LAB which hydrolyses the complex phytochemical compounds to simpler structure (Free Form). As other bacterial strains, hydrolytic enzymes synthesis can also be significant differences within the species. Moreover, increasement of lactic acid produced by lactic acid bacteria may help phenolic compounds as stabilizing agent.

In addition, lactic acid bacteria can hydrolyze the insoluble bound phenolic acids to soluble phenolic acids though the activities of various enzymes such as cellulase, esterase, and decarboxylase to hydrate the insoluble bound phenolic acids. During fermentation processes, the levels of nutrients, soluble dietary fiber, and total phenolic content can be increased which have been shown that fermentation using probiotics can increase the content of active compounds and antioxidant compounds included in rice bran (Le et al., 2019). There are not many articles in black rice bran fermentation with lactobacillus fermentation after pretreatment with α-amylase while most of research focusing on coculture fermentation of lactobacillus with fugus *Rhizopus* with the aim of increasing protein contents and free phenolic content (Fritsch et al., 2016).

Although phenolic compounds have large potential of applicability in industries, there has still remaining challenge which need to develop the strategies to improve their bioavailability. Most of the research have focused on the extraction of phenolic compounds from fermented rice bran which was obtained by providing synthetic nutrients for microorganisms instead of using the help of enzymes to improve the fermentation process. To improve the fermentation without supplying additional nutrients, pretreated by steaming with thermostable α-amylase can provide nutrient source for lactic acid bacteria growth. Liu, Zhang, et al., (2017)(Liu et al., 2017) reported this process can increase the total phenolic contents and antioxidant activity was higher than single fermentation due to the production of phenolic esterase and carbohydrase, which hydrolyze the ester bond between phenolics and cell wall components. However, no study on the extraction of phenolic compounds from α-amylase pretreated and fermented rice bran with the ultrasonic assisted technique have been reported.

Hence, this study focuses on integration of enzymatic pretreatment, lactic acid bacteria fermentation without nutrient supplement and ultrasonic assisted extraction on black rice bran to extract the phenolic compounds from fermented biomass.

1.3. Materials and methods

1.3.1 Materials

Five strains of freeze-dried *Lactobacillus* were given from Assistant Professor Dr. Sureelak Rodtong, School of Microbiology, Suranaree University of Technology. *L. fermentum* M19 strain was a gift from Associate Professor Dr. Kaemwich Jantama, School of Biotechnology, Suranaree University of Technology. Lactobacillus MRS agar and broth were purchased from HiMedia Laboratories, India.

1.3.2 Inoculum preparation and growth rate determination

Lactobacillus MRS agar and broth were prepared as described by manufacturer protocol. Each strain was streaked onto MRS agar and incubated at 37 ℃ for 48 h. A working culture solution was prepared with a single colony of each bacterial species and incubated for an overnight. For the growth rate determination, 200 µl of inoculum was added to 20 ml of autoclaved MRS nutrient broth in a sterile 50 ml conical tubes. 1 ml of culture suspension was collected at every 6 h interval and measured optical density (OD) at the wavelength of 600 nm by spectrophotometer in relation to a MRS broth blank.

1.3.3 Colony forming unit per milliliter (CFU/ml) versus OD_{600} standard curve

Overnight bacterial broth was centrifuged at 4,000 rpm and cell pellets were collected by washing with 0.9% (w/v) NaCl. Cell pellets were resuspended in 0.9% NaCl and proceeded 1:10 serial dilution from previous dilution into the subsequent tube containing 9 ml of 0.9% NaCl. The optical density of culture was checked at OD_{600}. 100 µl of culture suspension was spread onto MRS agar and enumerated at 37 ℃ for until colonies grown on plates. 30-300 colonies on plates were used to calculate the CFU/mL. Standard curve was plotted colony forming units against optical density.

1.3.4 Black rice bran fermentation

Black rice bran was fermented using lactic acid bacteria after enzymatic treatment. For the enzymatic pretreatment, 0.5%, 1% and 3% (v/v) of glucoamylase

were applied. In brief, 40 g of black rice bran, distilled water and glucoamylase were mixed thoroughly and adjusted to the final volume 150 mL. Enzymatic activity was activated at 57 °C in the water bath. Then, inactivation was proceeded by autoclaving at 121 °C for 15 min. Autoclaved samples were cooled down to room temperature and 10% (v/v) of fresh inoculum was added. After 48 h fermentation, fermented rice bran biomass was obtained by manual press method. Fermented biomass was dried in a hot air oven at 50 °C for overnight (Khan et al., 2020).

1.3.5 Preliminary extraction parameters optimization

Extraction parameters such as ethanol concentration, temperature, and pH were optimized. 20g of black rice bran was mixed with 100 ml of different concentrations of ethanol. Extraction was conducted using ultrasonic water bath for 30 min at set extraction parameters. Ethanol concentration (0%, 20%, 40%, 60%, 80%, and 100%), temperature (30, 50, 70 and 80 °C) and pH (1-5) were selected as extraction parameters for the extraction.

1.3.6 Phenolic compounds extraction from fermented biomass

20g of fermented biomass were mixed with 100 ml of ethanol and extracted in an ultrasonic water bath for 30 min at 50 °C. Fermented biomass extract was collected after centrifuged at 4,000 rpm for 30 min and filtrated with the Whatman No.1 filter.

1.3.7 Total phenolic and antioxidant activity analysis

Total phenolic content, total anthocyanin content, total flavonoid content, $ABTS^{+}$ and DPPH antioxidant assays were followed as stated in Chapter 3 (3.3.4 – 3.3.7).

1.4 Results and discussion

1.4.1 Black rice bran fermentation

The growth curve of five strains were observed by measuring optical density at 600 nm. The list of five lactobacillus strains was shown in Table 1. The bacterial growth curve demonstrated the lag phase, log phase and stationary phase

(Figure 1a). Log phase of the growth was selected for the utilization of lactic acid bacteria for black rice bran fermentation experiments. pH is an important indicator of the fermentation process and pH changes indicate the microbial metabolic activity. The pH of the black rice bran culture decreased after fermentation and was found below pH 4 for lactic acid bacterial fermentation while pH 6 for nonfermented (Figure 1b).

Table 1 *Lactobacillus* Strains

No	Strains	
1	*Lactobacillus brevis*	TISTR 860
2	*Lactobacillus fermentum*	TISTR 876
3	*Lactobacillus plantarum*	TISTR 877
4	*Lactobacillus acidophillus*	TISTR 1388
5	*Lactobacillus acidophillus*	M19

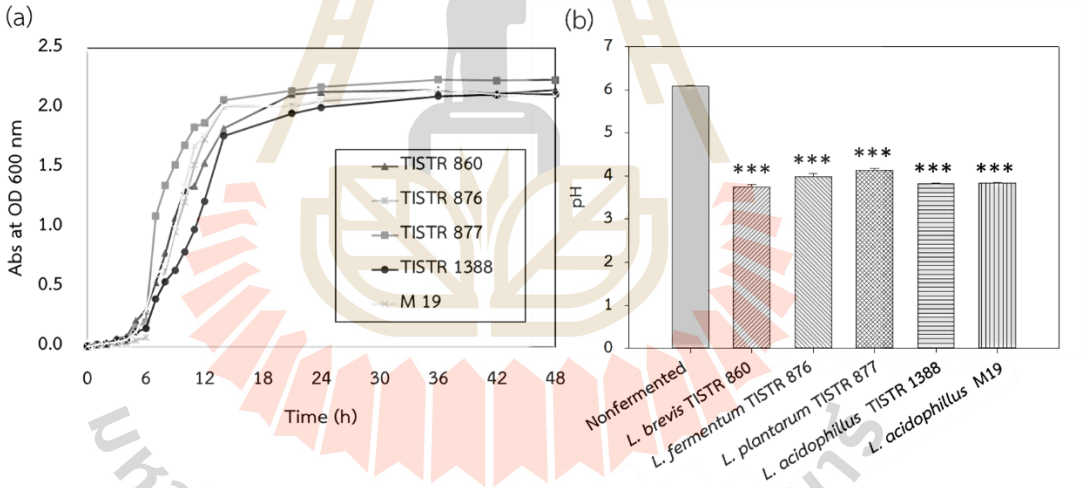

Figure 1 (a) Bacterial growth curve and (b) changes in pH

1.4.2 Antioxidant activity of fermented black rice bran biomass extract

Antioxidant activity of fermented biomass extract (FBE) was expressed as percent scavenging activity. The $ABTS^{+}$ scavenging activity of the FBE which was fermented with *L. fermentum* TISTR 876, *L. plantarum* TISTR 877, *L. acidophillus* TISTR 1388 and M19 were higher than non-fermented extract (NFE) (Figure 2). However, DPPH scavenging activity of FBE was lower than NFE (Figure 3). Among

these strains, *L. fermentum* TISTR 876 and *L. acidophillus* M19 were selected for the integrated fermentation with enzymatic pretreatment based on both antioxidant assays.

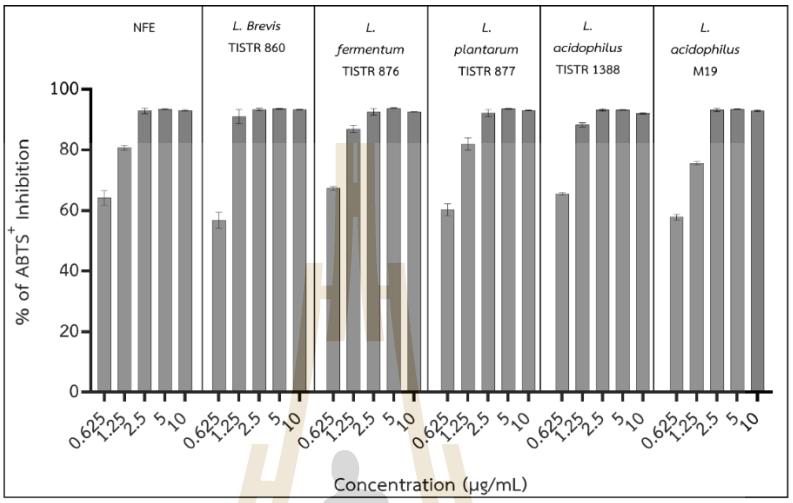

Figure 2 ABTS^{+} and DPPH scavenging activity

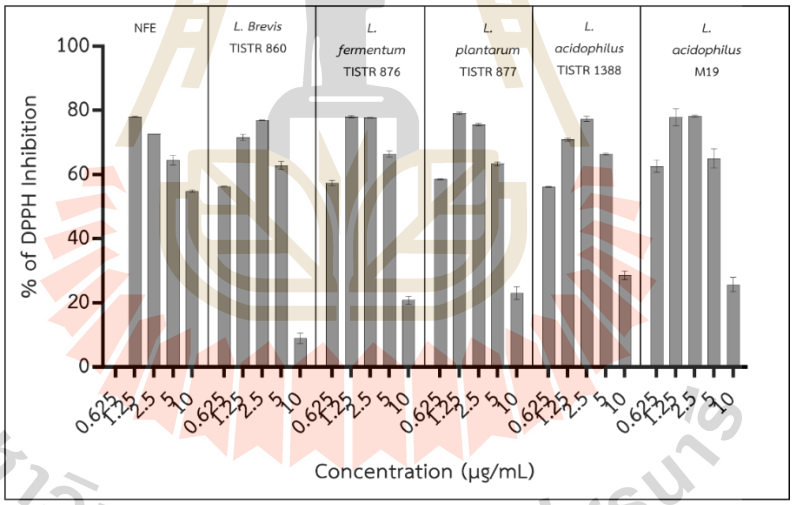

Figure 3 ABTS^{+} and DPPH scavenging activity

1.4.3 Preliminary optimization for the black rice bran extraction

For the preliminary optimization, the extraction condition for the phenolic compounds from black rice bran such as ethanol concentration, temperature and pH were optimized. The total phenolic content of the black rice bran extract (BRBE) was higher at 40% and 60% ethanol, 50-80 °C and pH 3 (Figure 4).

Hence, 50 % ethanol concentration, 50 °C and pH 3 were selected as optimal extraction condition for fermented biomass.

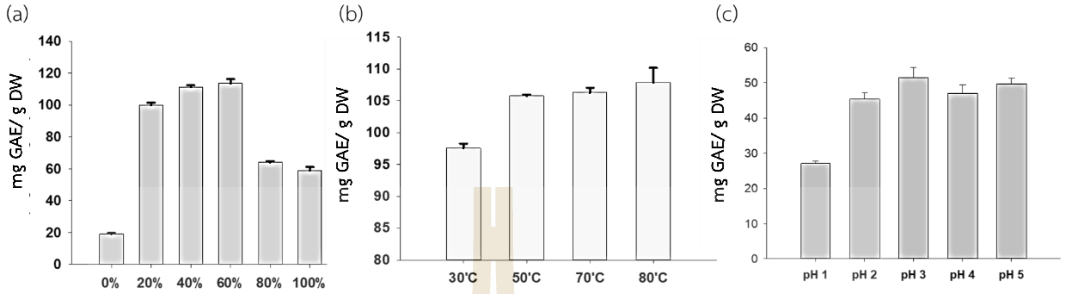

Figure 4 TPC in preliminary optimization (a) ethanol concentration, (b) temperature and (c) pH

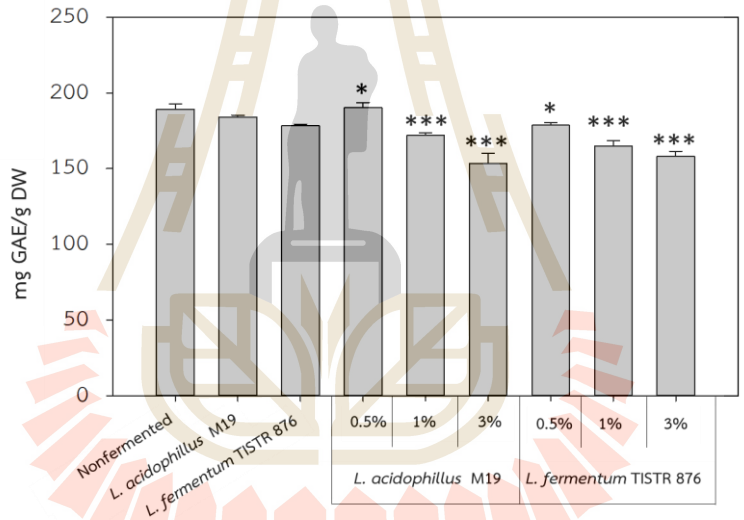

Figure 5 Total phenolic content in fermented biomass extract

Total phenolic contents from the fermented biomass extract, which was pretreated with α-amylase, indicated that the integration of enzymatic pretreatment and lactic acid bacteria fermentation did not significantly improve the total phenolic content compared to non-fermented and single lactic acid bacteria fermentation (Figure 5). This observation might be attributed to the fact that a considerable portion of total phenolics could have accumulated in the fermented supernatant. The studies by Liu, Wen, et al. (2017) highlighted that fermentation increased the total phenolic content in the aqueous solution from rice bran after pretreatment

with gelatinization and liquefaction. Additionally, the effects of fermentation are closely related to the enzymes secreted by the microbes, which may influence the phenolic profiles in the substrate and potentially lead to the formation of new compounds (Fritsch, Heinrich, Vogel, & Toelstede, et al., 2016). The presence of these enzymes and the alteration of phenolic profiles could potentially account for the apparent absence of a higher total phenolic content in the fermented biomass extract despite the fermentation process (Kwaw et al., 2018).

1.5. Conclusion

In summary, fermentation led to significant pH reductions and acid secretion indicated the fermentation process had occurred. The antioxidant activity of fermented biomass extract (FBE) varied with higher ABTS+ scavenging activity compared to non-fermented extract (NFE), while DPPH scavenging activity was comparatively lower. Optimal extraction conditions yielded higher total phenolic content in black rice bran extract (BRBE). The integration of enzymatic pretreatment with lactic acid bacteria fermentation did not substantially enhance the total phenolic content in the fermented biomass. This observation suggests the possible accumulation of total phenolics in the fermented supernatant. Enzymes secreted during fermentation might have influenced phenolic profiles, potentially leading to unwanted compound formations. These findings pointed out the complexity of enzymatic activities and phenolic transformations in fermentation, prompting careful selection of bacterial strains and enzymes for black rice bran fermentation.

1.6 References

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BIOGRAPHY

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