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นางสาวปรียานุช เตชะเลิศมณี

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EVALUATION OF COSMETIC ACTIVITIES OF THE EXTRACTS FROM MUSHROOMS OF THE FAMILY BOLETACEAE IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Cosmetic Science
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เห็ดวงศ์โบลิทาซีอี (เห็ดโบลีท) เป็นเห็ดชนิดหนึ่งที่รับประทานได้และพบได้ในประเทศไทย มีงานวิจัยหลายงานที่รายงานว่าสารสกัดเห็ดชนิดนี้มีปริมาณสารกลุ่มฟีนอลิกสูงและมีฤทธิ์ต้านออกซิเดชันที่ดีเมื่อเปรียบเทียบกับสารสกัดเห็ดชนิดอื่น อย่างไรก็ตาม ยังมีการศึกษาน้อยเกี่ยวกับฤทธิ์ทางด้านเครื่องสำอางของเห็ดโบลีทโดยเฉพาะเห็ดโบลีทในประเทศไทย ในการศึกษานี้มีวัตถุประสงค์เพื่อประเมินหาปริมาณสารประกอบที่ออกฤทธิ์ทางชีวภาพของสารสกัดเห็ดโบลีทในประเทศไทยที่เตรียมได้จากส่วนดอกเห็ดและส่วนน้ำเพาะเลี้ยงเห็ด ได้แก่ สารกลุ่มฟีนอลิค สารกลุ่มฟลาโวนอยด์ สารกลุ่มคาร์โบไฮเดรต และสารกลุ่มโปรตีน นอกจากนี้ ยังมีการประเมินฤทธิ์ทางเครื่องสำอางแบบนอกกายของสารสกัดเห็ดวงศ์โบลิทาซีอี ได้แก่ ฤทธิ์การต้านออกซิเดชัน (การวิเคราะห์ดีพีพีเอช และเอบีทีเอส) ฤทธิ์การยับยั้งไทโรซิเนส ฤทธิ์การยับยั้งคอลลาจิเนส และฤทธิ์การยับยั้งอีลาสเตส และศึกษาหาความสัมพันธ์ระหว่างฤทธิ์ทางเครื่องสำอางกับปริมาณกลุ่มสารสำคัญที่มีอยู่ในสารสกัดเห็ดโดยทดสอบความสัมพันธ์จากค่าสถิติเพียร์สัน จากผลการทดลองพบว่า สารสกัดเห็ดโบลีทที่ได้จากดอกเห็ดมีปริมาณกลุ่มสารสำคัญมากกว่าสารสกัดที่ได้จากน้ำเพาะเลี้ยงเห็ด ยกเว้นสารกลุ่มคาร์โบไฮเดรต นอกจากนี้ สารสกัดจากดอกเห็ดยังแสดงฤทธิ์ทางเครื่องสำอางได้ดีกว่าสารสกัดที่ได้จากน้ำเพาะเลี้ยงเห็ด สารสกัด TISTR 55 FB แสดงปริมาณสารประกอบสูงที่สุดในกลุ่มฟีนอลิค (113.17 ± 1.17 มิลลิกรัมสมมูลของกรดแกลลิคต่อปริมาณสารสกัด 1 กรัม) และสารกลุ่มฟลาโวนอยด์ (12.52 ± 1.35 มิลลิกรัมสมมูลของควอเซตินต่อปริมาณสารสกัด 1 กรัม) และมีฤทธิ์ต้านออกซิเดชันดีที่สุดโดยการยับยั้งอนุมูลอิสระดีพีพีเอช (ความเข้มข้นในการยับยั้งอนุมูลอิสระ 50% เท่ากับ 13.62 ± 0.70 ไมโครกรัม/มิลลิลิตร) และเอบีทีเอส (ความเข้มข้นในการยับยั้งอนุมูลอิสระ 50% เท่ากับ 23.58 ± 0.30 ไมโครกรัม/มิลลิลิตร) และพบว่าสารสกัด TISTR 37 FB ที่ความเข้มข้น 500 ไมโครกรัม/มิลลิลิตร สามารถแสดงฤทธิ์ยับยั้งคอลลาจิเนส (63.81 ± 4.74%) และฤทธิ์ยับยั้งอีลาสเตส (44.44 ± 2.25%) ดีที่สุดเมื่อเทียบกับสารสกัดเห็ดอื่นที่ศึกษา นอกจากนี้ ไม่พบความสัมพันธ์หรือพบความสัมพันธ์ต่ำระหว่างกลุ่มสารสำคัญและฤทธิ์ทางเครื่องสำอางของสารสกัดเห็ด ยกเว้นฤทธิ์ต้านออกซิเดชันโดยการยับยั้งอนุมูลอิสระ ดีพีพีเอช ที่พบความสัมพันธ์ที่ดีกับสารกลุ่มฟีนอลิคในสารสกัดจากดอกเห็ด ผลแสดงให้เห็นว่าชนิดและส่วนของสารสกัดเห็ดมีผลต่อปริมาณกลุ่มสารสำคัญและฤทธิ์ทางเครื่องสำอางของสารสกัด การศึกษานี้แสดงให้เห็นว่าสารสกัดจากเห็ดวงศ์โบลิทาซีอีในประเทศไทยสามารถนำไปพัฒนาเป็นสารสำคัญใหม่จากธรรมชาติในผลิตภัณฑ์เครื่องสำอางต่อไปได้

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต	.....
สาขาวิชา	วิทยาศาสตร์เครื่องสำอาง	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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PREEYANUCH TACHALERDMANEE: EVALUATION OF COSMETIC ACTIVITIES OF THE EXTRACTS FROM MUSHROOMS OF THE FAMILY BOLETACEAE IN THAILAND. ADVISOR: DUSADEE CHARNVANICH, Ph.D., CO-ADVISOR: ASSOC. PROF. WARAPORN SUWAKUL, Ph.D., SOMPORN MOONMANGMEE, Ph.D., 94 pp.

Mushroom of the family Boletaceae (Boletes mushroom) is one of edible mushrooms and can be found in Thailand. There are many studies reporting that it had high content of polyphenols and showed good antioxidant activities when compared with other mushrooms. However, there are still few studies about cosmetic activities of Boletes mushroom extracts especially Boletes mushrooms in Thailand. The present study aimed to evaluate the content of bioactive compounds of fruiting body extracts and culture broth extracts from Boletes mushrooms locally grown in Thailand including phenolic compounds, flavonoid compounds, carbohydrate compounds and protein compounds. Moreover, *in vitro* cosmetic activities including free radical scavenging activities (DPPH and ABTS assays), anti-tyrosinase, anti-collagenase and anti-elastase activities of these extracts were evaluated. The relationship between their cosmetic activities and bioactive compound contents of mushroom extracts was examined with Pearson's correlation test. The results showed that Boletes mushroom extracts from fruiting body had higher content of bioactive compounds than culture broth extracts except carbohydrate compound. In addition, they also showed higher cosmetic activities than culture broth extracts. TISTR 55 FB extract showed the highest phenolic (113.17 ± 1.17 milligram gallic acid equivalent per gram extract) and flavonoid contents (12.52 ± 1.35 milligram quercetin equivalent per gram extract) and the greatest DPPH (IC_{50} 13.62 ± 0.70 microgram/millilter) and ABTS (IC_{50} 23.58 ± 0.30 microgram/milliliter) free radical scavenging activities. Moreover, TISTR 37 FB extract showed potent anti-collagenase (63.81 ± 4.74%) and anti-elastase activities (44.44 ± 2.25%) at the concentration of 500 microgram/milliliter when compared with other mushroom extracts studied. In addition, no or low correlation between their bioactive compounds and cosmetic activities was observed except DPPH scavenging activity which showed good correlation with phenolic content of fruiting body extracts. The results indicated that type and part of mushroom extracts affected their bioactive compounds and cosmetic activities. The present study exhibited that the mushroom extracts of the family Boletaceae in Thailand were promising for further development as new active natural ingredients in cosmetic products.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Cosmetic Science	Co-Advisor's Signature	.....
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CONTENTS

Page

Page

LIST OF TABLES

Page

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

%	=	percentage
ºC	=	celcius
µg	=	microgram
µg/ml	=	microgram per milliliter
µl	=	microliter
ABTS	=	2,2-azino-bisethylbenzthiazoline-6 sulfonic acid
$AlCl_3$	=	aluminium chloride
BSA	=	bovine serum albumin
BSAE	=	bovine serum albumin equivalent
CE	=	catechin equivalent
Cu^+	=	copper ion
DMSO	=	dimethyl sulfoxide
DPPH	=	2,2-diphenyl-1-picryl-hydrazyl
ECM	=	extracellular matrix
EGCG	=	(-)-epigallocatechin gallate
FC reagent	=	Folin-Ciocalteau reagent
g	=	gram
GAE	=	gallic acid equivalent
GC-MS	=	gas chromatography-mass spectometry
GE	=	glucose equivalent
HPLC-RI	=	high performance liquid chromatography-refractive Index

IC_{50}	=	50% inhibitory concentration
M	=	molar
mg	=	milligram
mg/ml	=	milligram per milliliter
ml	=	milliliter
mM	=	millimolar
mmol	=	millimole
MMPs	=	matrix metalloproteinases
MW	=	molecular weight
L-DOPA	=	3,4-dihydroxy-L-phenylalanine
nm	=	nanometer
PDA	=	potato dextose agar
PDB	=	potato dextose broth
QE	=	quercetin equivalent
SD	=	standard deviation
TEAC	=	trolox equivalent per gram extract
TPC	=	total phenolic content
U/ml	=	unit per milliliter
UV	=	ultraviolet
v/v	=	volume by volume
w/v	=	weight by volume
w/w	=	weight by weight
Zn	=	zinc

CHAPTER I

INTRODUCTION

In recent time, trends of natural healthcare products are more popular because consumers believe about safety of natural ingredients as compared with chemical ingredients. Therefore, natural ingredients are more interesting in using in many categories of industries including agricultural industry, food industry and especially cosmetic industry. During the economic slowdown, Thai natural ingredients in cosmetic products become a key element of marketing strategies for cosmetic industry (Rojanadilok, Nanagara, and Bunchapattanasakda, 2012). Marketing analysis for 10 years later of Thailand was found that the export volume (8.11%) and export value (4.22%) of herbals are continuously risen up. The market value of cosmetic products from natural extracts is 2000-3000 million baht per year. These data can be assumed that the popularity of natural ingredients in cosmetics is so high. Thus, the research in cosmetic activities of natural extracts in Thailand are needed for supporting the utilization of natural ingredients in Thailand (Chantuma and Khamanarong, 2013).

Natural ingredients in cosmetic products are produced from many sources including plants, animals, minerals and microorganisms. The previous study (Hyde, Bahkali, and Moslem, 2010) found that fungi extracts could be used in cosmetic products because of their cosmetic activities especially in anti-aging activity. Mostly active ingredient in fungi extracts is beta-glucan which is polysaccharide acting as moisturizer (Zulli et al., 1998) and stimulation of collagen synthesis (Pillai, Redmond, and Röding, 2005). Beta-glucan is extracted from various types of fungi such as yeast and mushroom (Du, Bian and Xu, 2014). The sources of beta-glucan affected the structure of beta-glucan showing various biological activities. Mostly used beta-glucan in cosmetic products is β-1,6-branched-β-1,3-glucan that could inhibit melanin synthesis and stimulate collagen synthesis. This beta-glucan structure is found not only in yeast extract (Zulli et al., 1998) but also in mushroom extract (Manzi and Pizzoferrato, 2000; Schmid et al., 2001; Fang et al., 2012). Beside beta-glucan, these

extracts composed of other bioactive compounds which may show cosmetic activities. Yeast extracts contained compounds of 2 groups including carbohydrate and protein (Tatjana, Marica, and Slavica, 2007), whereas mushroom extracts also had other bioactive compounds especially phenolic compounds that acted as a potent antioxidant (Carocho and Ferreira, 2013).

Mushrooms have been used and acclaimed as health products for a long time. Mushrooms can be divided into 2 groups including edible mushroom and medical mushroom (Badalyan, 2003). According to the literature, mushroom extracts are reported about many biological activities such as antimicrobial (Yamaç and Bilgili, 2006; Kitzberger et al., 2007), immunostimulatory (Y. O. Kim et al., 2005; Bhunia et al., 2011), antitumor (Zhang et al., 2007), antioxidant (Keleş, Koca, and Gençcelep, 2011) and cosmetic activities including moisturizing effect (Synytsya et al., 2009), anti-tyrosinase activity (Chien et al., 2008) and stimulation of collagen synthesis (Ruksirwanich et al., 2014). These studies showed that biological activities of mushroom extracts occurred from the effect of bioactive compounds such as polysaccharide (beta-glucan) (Synytsya et al., 2009), carotenoid (Robaszkiewicz et al., 2010), fatty acid (K. H. Kim, Choi, and Lee, 2012), protein (Hearst et al., 2010) , amino acid (Lee et al., 1999) and phenolic compounds (Reis et al., 2012).

In the present, mushroom extracts that are mostly studied and developed as new active ingredients for cosmetic products are came from foreign species. For example, *Ganoderma lucidum* (Lingzhi) and *Grifola frondosa* (Maitake) are very popular in cosmetic products because of their biological activities including anti-tyrosinase activity, fibroblast proliferation, stimulation of collagen synthesis and inhibition of melanogenesis (S. W. Kim et al., 2007; Chien et al., 2008). In addition, there are other mushroom species that are interesting for cosmetic application. For example, *Boletus edulis* mushroom which is in the family Boletaceae was reported as a good antioxidant (Kuka and Cakste, 2011) when compared with other mushroom extracts (S.-Y. Tsai, Tsai, and Mau, 2007; Ribeiro et al., 2008; Sarikurkcu, Tepe, and Yamac, 2008; Vamanu and Nita, 2013). Moreover, this mushroom extract also displayed high phenolic content (Sarikurkcu et al., 2008). In the present, *Boletus*

edulis extract is used in cosmetic products especially anti-aging product (Bros, 2013: online). Although there are many studies about biological activities of various mushroom extracts, the reports about mushroom extracts in Thailand have little information. For example, *Volvariella volvacea* (Hed fang) extract showed good antioxidant and stimulation of collagen synthesis when compared with other studied mushroom extracts (Ruksirwanich et al., 2014). Furthermore, there are a lot of Boletes mushrooms found in the forest of Thailand but no study about cosmetic activities of these Boletes mushroom extracts has been reported.

Mushroom extracts can be produced from 3 parts of mushroom including fruiting body, mycelium culture and culture broth. In the present, mostly studied mushroom extracts were obtained from fruiting body part which showed potential biological activity. However, limitations of fruiting body extracts were quality control and difficulty of scale up in industrial level because of limited seasonal harvesting (rare in nature) (Mikheil et al., 2010). To overcome these limitations, it is interesting to study and develop mushroom extracts from other parts such as mycelium culture and culture broth for using as active ingredients in cosmetic products. They can be controlled for the yield throughout the year and quality of the extract by controlling condition of cultivation (media component, temperature and pH). When studied the biological activities of mycelium culture extracts and culture broth extracts, the results showed that they had good antioxidant activity. However, culture broth extract from *Lignosus rhinoceratis* mushroom showed better antioxidant activity than mycelium culture extract (Lau et al., 2014). Many studies about antioxidant activity of mushroom extracts from culture broth were reported that they composed of good antioxidant compounds (H. Y. Kim et al., 2008; Lau et al., 2014; Ki et al., 2015; M. S. Lee et al., 2015).

In the present study, Boletes mushroom extracts in Thailand from fruiting body and culture broth were evaluated for content of bioactive compounds including phenolic compounds, flavonoid compounds, carbohydrate compounds and protein compounds. Moreover, their *in vitro* cosmetic activities including antioxidant, anti-tyrosinase, anti-collagenase, and anti-elastase activities were also determined. In

addition, correlation between their cosmetic activities and their bioactive compounds was also investigated. This research would provide beneficial information for finding new active ingredients in cosmetic products and promoting the use of natural products in Thailand.

The main objectives of this study were to investigate properties of Boletes mushroom extracts from fruiting body and culture broth as follows:

1. *in vitro* cosmetic activities including antioxidant activities (DPPH and ABTS assays), anti-tyrosinase activity, anti-collagenase activity and anti-elastase activity
2. content of bioactive compounds including phenolic, flavonoid, carbohydrate and protein compounds
3. correlation between their cosmetic activities and their bioactive compound contents

CHAPTER II

LITERATURE REVIEW

1. Trend in cosmetic application of the extract from microorganisms

In recent years, the cosmetic industry is more popular because of the attention about healthy life and beautiful personality. From this reason, they affected the market demand of personal care products such as skin lightening, anti-aging, anti-acne and moisturizer products. The current trends of cosmetic products are in going back to nature. Thus, the natural products for cosmetic benefits become favored and interesting to find new active ingredients from natural origin. Natural ingredients from Thailand are the key strategies in cosmetic industry.

There are many sources of natural ingredients including plants, animals, minerals and especially microorganisms (bacteria, fungi, yeast). According to various biological activities, the extracts from microorganisms become attractive to use as active ingredients in cosmetic products. For example, bacterial extract from the family Pseudomonadeceae has a capability to improve the appearance of skin not only by inhibiting elastase release but also maintaining moisture in the skin (Martin et al., 2004). The extract obtained from culture supernatant of *Lactobacillus rhamnosus* showed good free radical scavenging activity, reducing power, anti-tyrosinase activity and moisture retention (C. C. Tsai et al., 2013). Beta-glucan that is by product of yeast extracts (*Saccharomyces cerevisiae*) had been also used in cosmetic products for many years because of their attractive biological activities. Beta-glucan derived from microorganisms especially yeast and mushroom showed a good antioxidant activity and protects skin against oxidative stress (Donzis, 1993; Zulli et al., 1998; Du et al., 2014). Moreover, beta-glucan is mostly bioactive compound that is used as a cosmetic ingredient in various cosmetic products (B. C. Lee et al., 2003). In addition, there are other bioactive compounds that can be extracted from microorganisms. They are not only polysaccharide (beta-glucan) but also phenolic, flavonoid and protein compounds depending on the sources (bacteria, yeast and mushroom) and the extraction method.

2. Application of mushroom extract in cosmetics

For finding new active ingredients from nature, fungi especially mushrooms, were interesting sources for using as active ingredients in cosmetic products because of their various biological activities and high content of bioactive compounds (Hyde et al., 2010). Mushroom is divided into 2 groups including edible mushroom and medical mushroom. In Asia, a lot of mushrooms are widely used in traditional medicines due to their medical properties for a thousand years. Many mushrooms are good sources of various bioactive compounds including carbohydrate (beta-glucan; polysaccharide), phenolic compounds, vitamin, flavonoid and protein that affected their potent biological activities (Khatua, Paul, and Acharya, 2013). Recently, mushrooms have become more attractive not only as functional foods but also as active compounds in cosmetic products (S. W. Kim et al., 2007).

From the previous studies, there are many species of mushroom that showed interesting biological activities including antimicrobial (Yamaç and Bilgili, 2006; Kitzberger et al., 2007; Hearst et al., 2010), antitumor (Zhang et al., 2007; Fang et al., 2012; Liu, Huang, and Zhou, 2014), immunomodulotory (Y. O. Kim et al., 2005; Bhunia et al., 2011), anti-tyrosinase activity (Chien et al., 2008; N. Alam et al., 2011; Park, Kwon, and Lee, 2015) and free radical scavenging activity (Sengkhamparn and Phonkerd, 2014). Example of foreign mushroom extracts showing cosmetic activities were summarized in Table 1.

Table 1 Biological activities of foreign mushroom extracts

Mushroom	Parts	Biological activity	Bioactive compounds	Cosmetic Products	Reference
Ganoderma lucidum	fruiting body	antioxidant activity	triterpene (phenolic)	-	Smina et al. (2011)
	fruiting body mycelium culture	antioxidant activity	phenolic polysaccharide	-	Heleno et al. (2012)
	mycelium culture	anti-tyrosinase activity	-	whitening	Chien et al. (2008)
Aspergillus niger	mycelium culture	moisturizing effect	chitin-glucan	moisturizer	Gautier et al. (2008)
Schizophyllum commune	mycelium culture	moisturizing effect	shizophyllan (beta-glucan)	anti-aging moisturizer	Pirshahid et al. (2011); Du, Bian, and Xu (2014)
Pleurotus nebrodensis	fruiting body	antioxidant activity anti-tyrosinase activity	phenolic compounds	-	N. Alam, Yoon, and Lee (2011)
	fruiting body (fraction)	melanogenesis inhibition	-	whitening	Dangre et al. (2012)
Grifola frondosa	mycelium culture	antioxidant activity stimulation of collagen biosynthesis	polysaccharide protein	anti-aging	S. W. Kim et al. (2007)

In addition, there are many interesting mushrooms in Thailand that may be used as active ingredients but there are a few studied about biological activities for using as active ingredients in cosmetic products. For example, *Volvariella volvacea* (Hed Fang) aqueous extract showed good antioxidant activity (IC_{50} value of DPPH scavenging activity 2.17±0.22 mg/ml), lipid peroxidation inhibition and stimulation of collagen biosynthesis as compared with other mushrooms in this study (Ruksirwanich et al., 2014).

Boletes is the group of Boletaceae mushrooms that have unique appearance. The characteristics of Boletes mushrooms is the presence of many tubes underside of their cap. The tubes are so tightly packed that look like surface of sponge. Boletes is the mycorrhizal partners with trees. Boletes mushrooms can be found in the forest or urban ecosystem that have the ectomycorrhizal tree. (Kuo, 2013: online). Boletaceae mushrooms have many interesting biological activities (Table 2). The previous studies reported that mushroom extracts from the family of Boletaceae had greater antioxidant activities than other mushrooms studied. Mushroom in the family Boletaceae can be largely found in the forest of Thailand especially in the north-eastern parts. Recently, there is no studies about cosmetic activities of Boletaceae mushrooms in Thailand. Consequently, the investigation of extracts from Boletaceae mushrooms in Thailand is interesting as source of natural ingredients in cosmetic products which are necessarily.

Table 2 Biological activities of Boletaceae mushroom extracts

Mushroom extracts	Parts	Biological activity	Bioactive compounds	Cosmetic products	Reference
Boletus edulis (water extract)	fruiting body	DPPH scavenging activity (4.58±0.04 µmole trolox/g)	phenolic (5.17±0.09 mg GAE/g)	-	Y. Wang and Xu (2014)
Boletus edulis (ethanol extract)	fruiting body	DPPH scavenging activity (IC_{50}: 16±0.3 µg/ml)	phenolic (41.82±0.08 mg GAE/g) flavonoid (8.73±0.11mg catechin(CE)/g)	-	Vidović et al. (2010)
	fruiting body	increase skin barrier function	polysaccharide	Anti-aging	SOTHYS (2011)
Boletus luridus (water extract)	fruiting body	DPPH scavenging activity (4.82±0.23 µmole trolox/g)	phenolic (10.43±0.01 mg GAE/g)	-	Y. Wang and Xu (2014)
Boletus aereus (water extract)	fruiting body	DPPH scavenging activity (4.87±0.12 µmole trolox/g)	phenolic (10.18±0.13 mg GAE/g)	-	Y. Wang and Xu (2014)
Boletus aurantiacus (ethanol extract)	fruiting body	DPPH scavenging activity (IC_{50}: 24±0.4 µg/ml)	phenolic (36.43±0.09 mg GAE/g) flavonoid (17.62±0.05 mg CE/g)	-	Vidović et al. (2010)

3. Preparation of mushroom extracts

The biological or cosmetic activities of mushrooms occur from their cellular components and secondary metabolites. Secondary metabolites that are the products from growth, development and reproduction of mushroom cultivation have many bioactive compounds including alkaloids, amino acid, polysaccharide, phenolic acid, flavonoid and other chemical compounds. Interesting bioactive compounds of mushrooms can be prepared from different morphological stages (development stage) of mushroom cultivation including fruiting body and submerge culture (mycelia and culture broth or culture filtrate) (Rahi and Malik, 2016). Fruiting body is the spore-producing organ of fungus (mushroom). Submerge culture obtained 2 parts including mycelia and culture broth. Mycelia is the vegetative part (asexual reproduction) of mushroom that consist of many branches like hyphae. Mycelia can grow in many substrates such as soil, wood or culture media. Culture broth or culture filtrate is by product of mycelium culture (Stamets and Chilton, 1983). Mostly mushroom products are derived from fruiting bodies. Although mostly studied mushroom extracts are come from fruiting body due to various biological activities, they have some limitations about industrial production because of their seasonal harvesting. In addition, it is difficult to control the quality of batch. Therefore, other parts of mushrooms are interesting to develop for using in cosmetic industry (Vladimir, 2012). There are many advantages of using the mycelium culture and culture broth rather than the fruiting body. Mycelium extracts can be easily provided and is inexpensive when compared with fruiting body. In addition, preparation of extracts from mycelium culture and culture broth can be controllable to increase product yields for further using in cosmetic industry *(Park et al., 2015)*. Recently, there are many studies reporting about potent biological activity of mycelium mushroom extracts. For example, mycelium culture extract of *Agaricus brasilie*nsis showed greater free radical scavenging activity and metal chelating ability than fruiting body extract *(Carvajal et al., 2012)*. Mycelial polysaccharide extract from *Lepista sordida* (400 µg/ml) presented good DPPH radical scavenging activity (82 %), hydroxyl radical scavenging activity (76.9%) and superoxide radical scavenging activity

(70%) *(Zhong et al., 2013)*. In addition to mycelium extract, culture broth is one of the interesting sources that can be also used in cosmetic products in the future. When compared the biological activity between mycelium extracts and culture broth extracts, the studies exhibited the more potent biological activities of culture broth extracts than mycelium culture extracts. For example, the culture broth extracts from *Antrodia camphorate* showed greater antioxidant activity than the extracts from mycelium culture *(Song and Yen, 2002)*. Culture broth extract showed 0.74 ± 0.01 mM trolox equivalent antioxidant capacity (TEAC) but mycelium culture extracts was 0.21-0.63 mM trolox equivalent antioxidant capacity. This result is similar to the study of Lau et al. (2014) that reported the more potent antioxidant activity of culture broth extract (223.05 ± 8.26 mmol trolox equivalent per gram extract) of *Lignosus rhinocerotis* than mycelium extract (143 ± 13.42 mmol trolox equivalent per gram extract).

4. Evaluation of *in vitro* cosmetic activities

4.1 Antioxidant (free radical scavenging activity)

There are many methods for determination of antioxidant activity based on different principles. DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) methods are widely used for evaluation of antioxidant activity because of simple, accurate and inexpensive assay. Both assays are based on the ability of different compounds to act as free radical scavengers (Marinova and Batchvarov, 2011).

4.1.1 DPPH scavenging activity

The DPPH method is rapid, simple and convenient method that is used for screening free radical scavenging activity to act as hydrogen donors and free radical scavengers. DPPH assay is performed in an organic solvent system. Therefore, this method can be used for measurement antioxidant activity of lipophilic compounds or compounds with high lipid content (Damgaard et al., 2014). According to DPPH radical absorption at 517 nm, DPPH radical scavenging activity can be determined by monitoring the

decrease in this UV absorbance. From Figure 1, when DPPH radicals react with a substrate (AH) that can donate a hydrogen atom, this reaction displays as the loss of violet color (M. N. Alam, Bristi, and Rafiquzzaman, 2013).

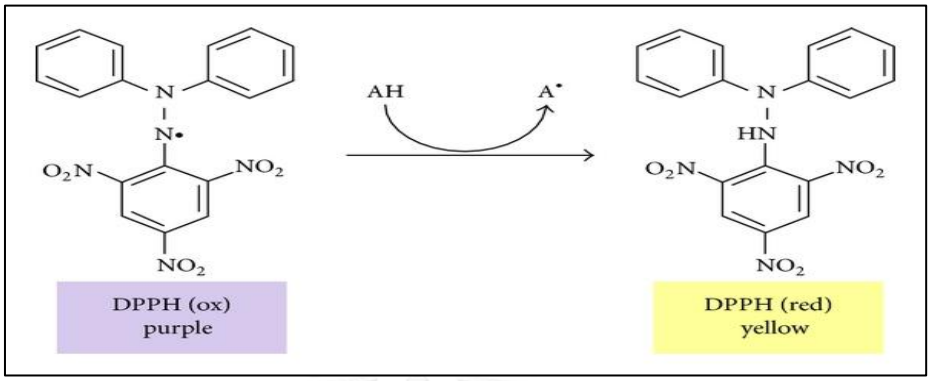

Figure 1 Mechanism of DPPH method (Teixeira et al., 2013)

4.1.2 ABTS scavenging activity

$ABTS^{\cdot+}$ is a stable cation that is not found in human body. ABTS has high water solubility and chemical stability. Therefore, it can be used for measurement hydrophilic antioxidant. The integration of $ABTS^{\cdot+}$ can be inhibited by the presence of antioxidant compounds in the reaction medium. The incubation time depends on the antioxidant ability of test sample (Antolovich et al., 2002). ABTS method is rapid and accurate to perform because it avoids interfere factor such as high temperature and pH value (Arnao, Cano, and Acosta, 2001). ABTS method can be used to evaluate the antioxidant activity by detection of the reaction between ABTS cation (blue-green chromophore) and antioxidant compounds at 734 nm as shown in Figure 2.

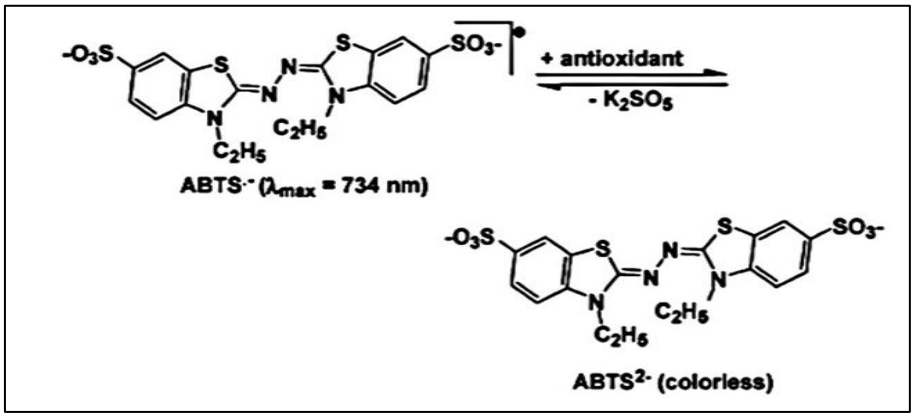

Figure 2 Mechanism of ABTS assay (Shalaby and Shanab, 2013)

DPPH and ABTS assayed were used to determine antioxidant activity of the extracts in this study because they can be used to measure antioxidant activity of lipophilic and hydrophilic compounds.

4.2 Anti-tyrosinase activity

For screening a new whitening agent, tyrosinase inhibitory activity have become interesting because whitening agents act specially to reduce the function of tyrosinase enzyme. Tyrosinase is a copper-containing enzyme that is the key enzyme in melanin biosynthesis. As shown in Figure 3, the biosynthetic pathway of melanin in melanocytes has three different reactions. The first reaction of this pathway is the hydroxylation of tyrosine to L-DOPA. Then, L-DOPA is oxidized to Dopaquinone. The last reaction is changing dopaquinone by a complex reaction involving cyclization and oxidative polymerization to form melanin (Lida et al., 1995; Parvez et al., 2007). Therefore, the potent tyrosinase inhibitor is strong against the oxidation of L-tyrosine and L-DOPA to dopaquinone.

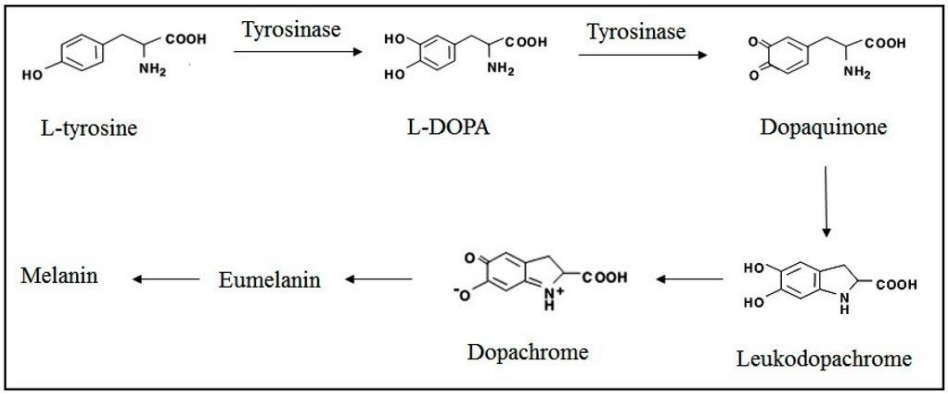

Figure 3 The biosynthesis pathway of melanin (Ribeiro et al., 2015)

DOPAchrome method using L-tyrosine or L-DOPA as a substrate is used to determine anti-tyrosinase activity by detection of the decrease of L-DOPA to DOPAchrome reaction. This reaction is evaluated by measurement the loss of color at 490 nm (Lida et al., 1995). The decrease of UV absorbance means the increase in anti-tyrosinase activity. For this method, mushroom tyrosinase is a critical role of tyrosinase inhibitor studies. This enzyme can be purified from the mushroom *Agaricus bisporus*. The limitation of this assay is the difference between mushroom tyrosinase and human tyrosinase that shows little difference effect especially lag time of the reaction between enzymes and substrate. Mushroom tyrosinase is a cytosol enzyme and tetramer type that highly glycosylated during its complex maturation process, whereas the human tyrosinase is membrane bonded and the monomer type. The study of human tyrosinase inhibitor is very limited because of the complicate method to purify human tyrosinase (T. S. Chang, 2009).

4.3 Anti-collagenase activity

In elderly people, the reduction of skin thickness occurs from the loss of collagen and elastin. The decrease of collagen content is 2% per year because of the production of matrix metalloproteinases (MMPs) (Farage, Miller, and Maibach, 2010). The extracellular matrix (ECM) function is to stabilize tissue structure and to influence the proliferation, development, metabolic function, migration of cells that contact this matrix. MMPs are enzymes which are divided

into subclasses, including collagenase, gelatinase and membrane type-MMPs which digest collagen and gelatin. MMPs are important for tissue remodeling under normal physiological conditions. Modifications of ECM have been recommended as a cause of wrinkle and the loss of elasticity. For limitation of degradation of ECM, tissue inhibitor of metalloproteinase (TIMPs) reacts with MMPs to maintain the integrity of healthy tissues (Azmi et al., 2014). There are many factors that can induce the synthesis of MMP in skin fibroblast (Sim et al., 2007) such as UV irradiation, ages and reactive oxygen species (ROS). For determination of anti-collagenase activity, Enzchek® gelatinase/collagenase assay kit (E-12055) has been used in many studies (Chaudhuri, 2004, 2005; Sim et al., 2007; Chanvorachote et al., 2009). Collagenase is zinc-containing metalloproteinase which is important to react with collagenase inhibitor (Abdul Wahab, 2014). The substrate in this assay (DQ™ gelatin) is labeled with quenched fluorescein. This substrate is digested by collagenase to produce highly fluorescent peptides. Therefore, the increase in fluorescence intensity is proportional to proteolytic activity that can be detected by a fluorescence microplate reader with absorption maxima at 485 ± 10 nm and emission detection at 530 ± 15 nm. A limitation of this assay kit is a substrate that is provided from this kit can be digested by other MMPs.

4.4 Anti-elastase activity

Elastin that is one of the major extracellular components of the dermis about 2-4% of the dermis matrix is responsible for the elasticity of the skin. So, the depletions of elastin can be caused of aging skin and wrinkle. Similar to collagen, elastin can be degraded by matrix metalloproteinases (elastase) (Farage et al., 2010). Elastase is a serine proteinase. This enzyme is responsible for the breakdown of elastin in ECM. The principle and limitation of anti-elastase activity is similarly to anti-collagenase activity. Elastin can be digested by other proteinase. EnzChek®elastase assay kit was performed for determination of anti-elastase activity of extract (Chattuwatthana and Okello, 2015). Therefore, the principle of this assay is based on the digestion of substrate (DQ™ elastin) by

elastase enzyme to yield high fluorescence fragments that can be detected with the excitation at 485 ± 10 nm and the emission at 530 ± 15 nm by fluorescent microplate reader. Elastase enzyme is disturbed by elastase inhibitor. Therefore, the decrease in fluorescence intensity means the increase in anti-elastase activity.

5. Correlation between *in vitro* cosmetic activities and bioactive compounds

5.1 Correlation between free radical scavenging activities and bioactive compounds

The previous studies reported good correlation between free radical scavenging activities (DPPH and ABTS) and phenolic or flavonoid compounds (X. Li, Wu, and Huang, 2009; Stanković, 2011; Oboh and Okhai, 2012). Phenolic compounds showed a good DPPH scavenging activity by the reaction between the phenols form (*o*-quinone intermediates) and hydrogen atom of DPPH radical. However, this reaction was dependent on the nature of the phenol. The hydroxyl groups of phenolic compounds acted as DPPH radical scavenger (Antolovich et al., 2002). The correlation between protein content and DPPH or ABTS scavenging activities showed low correlation (Vamanu, 2012). There was no study that reported about the correlation between carbohydrate content and free radical scavenging activities. Although phenolic or flavonoid compounds showed potent DPPH or ABTS scavengers, the previous study showed no correlation between DPPH scavenging activity and ABTS scavenging activity. This effect may be due to different types of polyphenols that showed the ability to scavenge DPPH or ABTS radicals. For example, ABTS radical scavengers depended on the molecular weight, the nature of hydroxyl groups and the number of aromatic rings of polyphenols. (Hagerman et al., 1998; Khan et al., 2012)

5.2 Correlation between anti-tyrosinase activity and bioactive compounds

The correlation between tyrosinase activity and phenolic compounds was moderate (Bravo, Alzate, and Osorio, 2016). Phenolic compounds that acted as

tyrosinase inhibitor should have hydroxyl group at the 6 position of phenolic structure (Dej-adisai, Parndaeng, and Wattanapiromsakul, 2016). However, there was no study that reported about the correlation between flavonoid, carbohydrate or protein compounds and anti-tyrosinase activity.

5.3 Correlation between anti-collagenase activity and bioactive compounds

The relationship between anti-collagenase activity and phenolic or flavonoid compounds showed intermediate correlation (Bravo et al., 2016). The anti-collagenase activity was occurred from several mechanisms. First, hydroxyl groups of phenolic or flavonoid compounds could interact with backbone or other functional groups of side chain of collagenase (Sin and Kim, 2005). Second, the benzene ring of phenolic compounds could react with collagenase for conformation change of enzyme (Madhan et al., 2007). In addition, collagenase contains a structural Zn ion active site. This active site plays a major role in facilitating interaction with an inhibitor (Bigg, Clark, and Cawston, 1994). Therefore, phenolic compounds that act as collagenase inhibitor should have hydroxyl groups or benzene ring in chemical structure such as epigallocatechin. However, there was no study that reported about the correlation between total protein content or total carbohydrate content and anti-collagenase activity.

5.4 Correlation between anti-elastase activity and bioactive compounds

The correlation between anti-elastase activity and phenolic compounds was intermediate (Bravo et al., 2016). Similar to anti-collagenase activity, the structure of polyphenol affected anti-elastase activity. In addition, there was the study that reported about the ability of anti-elastase activity of flavonoid compounds. The catechol group of flavonoid compounds showed high inhibitory of elastase (Kanashiro et al., 2007). However, there was no study about the correlation between flavonoid, carbohydrate or protein content and anti-elastase activity.

From the literature review, phenolic compounds may act as antioxidant, tyrosinase inhibitor, collagenase inhibitor and elastase inhibitor. These activities

can be occurred from partly phenolic compounds due to the suitable structure of phenolic compounds such as hydroxyl group in a side chain. From the correlation between DPPH scavenging activity and total phenolic content, the content of phenolic compounds may refer to antioxidant activity especially free radical scavenging activity. Therefore, determination of total phenolic content may be used as a parameter for screening new antioxidant compounds.

CHAPTER III

MATERIALS AND METHODS

Materials

1. 2,2-Azino-bisethylbenzthiazoline-6 sulfonic acid (ABTS) (Lot SLBM3808V, Sigma-Aldrich, USA)
2. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) (Lot STBD4146V, Sigma-Aldrich, USA)
3. 3,4-Dihydroxy-L-phenylalanine (L-DOPA) (Lot SLBF6724V, Sigma-Aldrich, USA)
4. 95% ethanol (The Liquor Distillery Organization, Thailand)
5. Absolute ethanol (Lot 150225-0119, B&J Scientific, Thailand)
6. Aluminium chloride (Lot 70838619, Ajax Finechem Pty Ltd, Australia)
7. Bovine serum albumin (BSA) (Lot 089K16291, Sigma-Aldrich, USA)
8. Copper (II) sulfate pentahydrate (Lot 014, Merck, Germany)
9. D-(+)-glucose anhydrous (Lot 0000156372, Himedia, India)
10. Dimethyl sulfoxide AR grade (DMSO) (Lot 15050344, RCI labscan, Thailand))
11. Di-sodium hydrogen phosphate anhydrous (Lot 434, Merck, Germany)
12. EnzCheck® elastase assay kit E12056 (Lot 1724825, Molecular probes, USA)
13. EnzCheck® gelatinase/ collagenase assay kit E 12055 (Lot 1717422, Molecular probes, USA)
14. (-)-Epigallocatechin gallate (EGCG) (Lot SLBL3516V, Sigma Aldrich, USA)
15. Folin-Ciocalteau reagent (Lot HC 304032, Merck, Germany)
16. Gallic acid (Lot SZE 61290, Sigma Aldrich, USA)
17. Kojic acid (Lot 097K2572, Sigma, USA)
18. L-(+)-ascorbic acid (Lot 402404, Carlo Erba, Italy)
19. Mushroom tyrosinase T3824 (Lot SLBJ5647V, Sigma-Aldrich, USA)

20. Phenol (Lot 908, Merck, Germany)
21. Potassium acetate (Lot 26385 May & Baker Ltd, UK)
22. Potassium persulfate (Lot BCBN 9478V, Sigma Aldrich, USA)
23. Potassium sodium (+) - tartrate (Lot 0310344, Ajax Finechem, Australia)
24. Quercetin (Lot 085K0720, Sigma Aldrich, USA)
25. Sodium carbonate (Lot 1006568 Ajax Finechem Pty Ltd, Australia)
26. Sodium dodecyl sulfate (SDS) (Lot 114372, usb, USA)
27. Sodium hydroxide (Lot 089K16291 Merck, Germany)
28. Sodium phosphate dibasic (Lot 50490, Sigma Aldrich, USA)
29. Sulfuric acid 98% AR grade (Lot K31905031314, Merck, Germany)

Apparatus

1. Analytical balance (GMPH, Sartorius, Germany)
2. High speed refrigerated centrifuge (Suprema 25, TOMY, Japan)
3. Freeze dryer (Super Modulyo, Thermo electron corporation, Canada)
4. Micropipette (Trefflab, Switzerland)
5. Microplate reader (FLUOstar OPTIMA multidetection microplate reader, BMG Babtech, Germany)
6. pH meter (S220K, Mettler Toledo, Switzerland)
7. Rotary evaporator (Hei-VAP evaporator, Heidolph, Germany)
8. UV-VIS spectrophotometer (UV-2450, Shimadzu, Japan)
9. Vortex mixer (Vortex-Genie 2, Scientific industry, USA)

Others

1. 96-well plates (Costar®, Corning®, USA)
2. Whatman filter paper No.1, 150 mm (Whatman International Ltd., UK)

Methods:

The experiments were divided into four parts:

1. Preparation of crude extracts from Boletes mushrooms locally grown in Thailand
2. Analysis of bioactive compounds in Boletes mushroom extracts
3. Evaluation of *in vitro* cosmetic activities of Boletes mushroom extracts
4. Analysis of data

Part 1. Preparation of crude extracts from Boletes mushrooms locally grown in Thailand

In this experiment, Boletes mushroom extracts were prepared from two parts of mushroom including fruiting body and culture broth. The details were as follows:

1.1 Boletes mushroom extracts from fruiting body (S.-Y. Tsai et al., 2007; Vidović et al., 2010)

Fresh fruiting bodies of Boletes mushrooms were collected from the central and the northeastern parts of Thailand (Table 3). After harvesting, fresh fruiting bodies were cleaned and extracted with 95% ethanol in the ratio of 1:5 for 7 days. Then, the residue was filtered out using Whatman No.1 filter paper. The resultant ethanol solution was evaporated in a rotary evaporator at 40°C. After ethanol was evaporated, the residue was dried by freeze-drying method. The percentage yield of the extract obtained was calculated from equation (1). All of samples were kept in an airtight container with silica gel at -20°C prior to further studies (Y. Wang and Xu, 2014). Stability of fruiting body extracts was determined by measuring the percentage of DPPH inhibition at the concentration of 100 µg/ml after storage at -20°C for 6 months.

$$\% \text{ yield} = \frac{\text{weight of freeze-dried extract}}{\text{weight of fresh fruiting body of mushroom}} \times 100 \qquad \text{Equation (1)}$$

1.2 Boletes mushroom extracts from culture broth

Boletes mushroom extracts from culture broth were obtained from Thailand Institute of Scientific and Technological Research (TISTR). Briefly, the strains of

Boletes mushroom were prepared from the collected fruiting body. This strain was cultured at 30ºC for 14 days in 5 liter flasks with 1000 ml of potato dextrose broth. To collect the yield, culture broths were centrifuged at 8000 rpm for 20 minutes and were filtered through Whatman No.1 filter paper. The supernatant was dried by freeze-drying method. The yield of the extract was expressed as gram of the dried extract per liter of culture media. All of samples were kept in an airtight container with silica gel at -20ºC prior to further evaluation (Lau et al., 2014).

Table 3 Details of Boletes mushroom extracts

Local name	**Source (Province)**	**Code name**	
		Fruiting body	**Culture broth**
Hed peaung nok yoong	Chaiyaphum	TISTR 14 FB	N/A
Hed tub tao	Phra Nakhon Si Ayutthaya	TISTR 31 FB	TISTR 31 CB
Hed peaung tub khwai	Roi Et	TISTR 36 FB	TISTR 36 CB
Hed peaung kom	Chaiyaphum	TISTR 37 FB	TISTR 37 CB
Hed peaung kao	Chaiyaphum	TISTR 50 FB	N/A
Hed peaung tub tao	Chaiyaphum	TISTR 55 FB	TISTR 55 CB
Hed peaung wan	Chaiyaphum	TISTR 56 FB	N/A

N/A: Not available

Part 2. Analysis of bioactive compounds in Boletes mushroom extracts

In this study, Boletes mushroom extracts were determined for the content of bioactive compounds including total phenolic content, total flavonoid content, total carbohydrate content and total protein content. Each determination was carried out in triplicate.

2.1 Total phenolic content

Total phenolic content was measured by using Folin-Ciocalteau method modified from Miliauskas, Venskutonis, and Beek (2004). Folin-Ciocalteau (FC) reagent is mixture of phosphomolybdate and phosphotungstate which can be reacted with phenolic compound to form blue complex. The chromogen can be detected by a UV-VIS microplate reader at 765 nm. Preparation method of reagent was showed in appendix A. Sample solution (20 µl) was mixed with 10% FC reagent (100 µl) in a 96-well plate. Then, 75% sodium carbonate solution (80 µl) was added into this mixture and incubated in darkness at room temperature for 60 minutes. Distilled water was used as a blank. After incubation, the mixture was measured at 765 nm by a UV-VIS microplate reader. For quantifying the phenolic content, the standard curve of gallic acid at the concentration range between 4-16 µg/ml was used. The results were expressed as milligrams (mg) of gallic acid equivalent (GAE) per gram (g) of the extract (mg GAE/g extract).

2.2 Total flavonoid content

Total flavonoid content of extracts was determined by aluminium chloride ($AlCl_3$) colorimetric assay with slightly modified protocol from C.-C. Chang et al. (2002) and Lin and Tang (2007). $AlCl_3$ can form an acid stable complex with flavonoid, flavonol and flavone structure (Bag, Devi, and Bhaigyabati, 2015). This complex can be measured by a UV-VIS microplate reader at 415 nm. The detail of reagent preparation was reported in appendix A. Twenty microliters of sample solution, 60 µl of absolute ethanol, 4 µl of 10% $AlCl_3$ solution, 4 µl of 1M potassium acetate solution and 112 µl of distilled water were added into a 96-well plate, respectively. The mixture solutions were incubated in darkness at room temperature for 45 minutes. Then, the absorbance of reaction mixture was measured by a UV-VIS microplate reader at 415 nm. Distilled water was used as a blank. Quercetin was used as a reference standard for determination of total flavonoid content. Various concentrations (3-15 µg/ml) of quercetin were used to make a standard curve. The results were expressed as milligrams of quercetin equivalent (QE) per gram of the extract (mg QE/g extract).

2.3 Total carbohydrate content

Phenol-sulfuric method was used to determine total carbohydrate content. In principle, carbohydrate compounds (simple sugars, oligosaccharide, polysaccharide) are broken down by the concentrated sulfuric acid to produce furan derivatives that can react with phenol to form yellow compounds (Nielsen, 2010). The colorimetric reaction can be measured by a UV-spectrophotometer at 490 nm. This assay was modified from the basic protocol of DuBois et al. (1956). Reagent preparation was mentioned in appendix A. Sample solution (0.5 ml) and 5% phenol solution were added into a test tube (16X160 mm) and mixed well by vortex mixer. Then, 1.5 ml of concentrated sulfuric acid was added directly to the liquid surface within 10 seconds. The mixture solution was mixed by vortex-mixer for 5 seconds and incubated at room temperature for 30 minutes. All test tubes were allowed to cool down to room temperature before measuring the absorbance by a spectrophotometer at 490 nm. Distilled water was used as a blank. The standard curve of glucose at the concentration range between 2-18 µg/ml was employed for quantifying the carbohydrate content. The results were expressed as milligrams of glucose equivalent (GE) per gram of the extract (mg GE/g extract).

2.4 Total protein content

Determination of total protein content in the extracts was detected by modification of Lowry method (Lowry et al., 1951).This method is based on the reaction of phenolic group of tyrosine and tryptophan residues (amino acid) in the protein extract with Folin-Ciocalteau (FC) reagent. The peptide bonds of protein react with copper under alkaline conditions to provide Cu^{+} that can react with FC reagent to produce blue complex (heteropolybdemun blue). The colorimetric assay can be measured by a UV-spectrophotometer at 760 nm. The preparation of reagents was obtained in appendix A. Sample solution (0.5 ml) and alkaline copper sulfate reagent (0.5 ml) were added into a test tube (13X100 mm). The mixture was incubated at 37° C for 10 minutes. After pre-incubation, 0.2 ml of FC solution was added to a test tube and incubated at 37°C for 20

minutes. Distilled water was used as a blank. Then, this solution was measured the absorbance at 760 nm by using a UV-spectrophotometer. The standard curve of bovine serum albumin (BSA) at the concentration range between 5-30 µg/ml was used for quantifying the protein content. The results were expressed as milligrams of BSA equivalent (BSAE) per gram of the extract (mg BSAE/ g extract).

Part 3. Evaluation of *in vitro* cosmetic activities of Boletes mushroom extracts

3.1 Free radical scavenging activities

Free radical scavenging activities can be determined by measuring the remaining concentration of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2-azino-bisethylbenzthiazoline-6 sulfonic acid (ABTS) radicals used as representative of free radical. Therefore, free radical scavenging activities of Boletes mushroom extracts were determined using 2 different methods as follows:

3.1.1 DPPH free radical scavenging activity

The DPPH assay is a simple colorimetric method of antioxidant activity based on the decrease in UV absorbance at 510 nm of DPPH radical after addition of an antioxidant compound in DPPH solution. The potential DPPH radical scavengers will change violet color of DPPH solution to yellow. This method was slightly modified from protocol of Miliauskas et al. (2004). The DPPH solution and sample solution were prepared as described in appendix A. Sample solution at the final concentration of 100 µg/ml was used for screening antioxidant activity. One hundred microliters of DPPH solution ($6x10^{-5}$ M) was mixed with other solutions to make final volume of 200 µl in a 96-well plate. In each well, the substance was added as follows below:

A (control)	:	100 µl DPPH solution + 100 µl absolute ethanol
B (blank of A)	:	200 µl absolute ethanol
C (sample)	:	100 µl sample solution + 100 µl DPPH solution

D (blank of C) : 100 µl sample solution + 100 µl absolute ethanol

The mixed solutions were incubated in darkness at room temperature for 60 minutes and then, the mixtures were measured at 510 nm using a UV-VIS microplate reader. Ascorbic acid and gallic acid were selected as positive controls. Absolute ethanol was used as a blank (B). DPPH free radical scavenging activity (% DPPH inhibition) of samples was calculated using the following equation (2):

DPPH inhibition (%) = $\left[\frac{(A-B)-(C-D)}{(\mathrm{A}-\mathrm{B})}\right]$ x 100 Equation (2)

Where A : the absorbance of DPPH solution and absolute ethanol

B : the absorbance of absolute ethanol

C : the absorbance of sample with DPPH solution

D : the absorbance of sample and absolute ethanol

3.1.2 ABTS free radical scavenging activity

ABTS assay is widely used to determine the antioxidant activity based on radical scavenging activity. This assay is based upon the ability of antioxidant compound to decolorize the ABTS radical cation ($ABTS^{\cdot+}$), which is blue solution at UV absorbance 734 nm. This method was slightly modified from protocol of K. J. Lee et al. (2014) that was performed in a 96-well plate. Preparation of reagents and samples was mentioned in appendix A. The substance was added in each well as follows:

A (control) : 20 µl distilled water + 180 µl ABTS solution

B (blank of A) : 200 µl distilled water

C (sample) : 20 µl sample solution + 180 µl ABTS solution

D (blank of C) : 20 µl sample solution + 180 µl distilled water

The mixed solutions were incubated in darkness at room temperature for 60 minutes. Then, they were measured at 734 nm by a UV-VIS microplate reader. Ascorbic acid was used as a positive control. Distilled water was employed as a blank. The ABTS radical scavenging activity (% ABTS inhibition) of extracts was calculated using the following equation (3):

ABTS inhibition (%) = $\left[\frac{(A-B)-(C-D)}{(A-B)}\right]$ x 100 Equation (3)

Where A : the absorbance of ABTS solution and distilled water

B : the absorbance of distilled water

C : the absorbance of samples with ABTS solution

D : the absorbance of samples and distilled water

3.2 Anti-tyrosinase activity

Tyrosinase is the key enzyme in melanogenesis that is the cause of skin hyperpigmentation. Anti-tyrosinase activity was determined by DOPAchrome method using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a substrate. After addition of L-DOPA, the enzyme reaction was detected by measuring the color changing in UV-absorbance at 490 nm due to DOPAchrome formation for 10 minutes. The potential anti-tyrosinase compound would decrease red color of DOPAchrome. In this study, Boletes mushroom extracts were screened for anti-tyrosinase activity using DOPAchrome method modified from the protocol of Lida et al. (1995) and Sritularak (2002). This method was performed in a 96-well plate. The reagent and preparation were shown in appendix A. The absorbance of the reaction mixture was measured in four wells (A, B, C and D). In each well, the substance was filled as shown below:

A (control) : 20 µl of mushroom tyrosinase solution (480 unit/ml) + 140 µl of phosphate buffer (pH 6.8) + 20 µl of distilled water or DMSO

B (blank of A) : 160 µl of phosphate buffer (pH 6.8) + 20 µl of distilled water or DMSO

C (sample) : 20 µl of mushroom tyrosinase solution (480 unit/ml) + 140 µl of phosphate buffer (pH 6.8) + 20 µl of sample solution

D (blank of C) : 160 µl of phosphate buffer solution (480 unit/ml) + 20 µl of sample solution

After pre-incubation in darkness at room temperature for 10 minutes, 20 µl of L-DOPA solution was added into the mixture solutions and was further incubated in darkness at room temperature for 20 minutes. Then, UV-absorbance of the reaction mixture was measured at 490 nm with a UV-VIS microplate reader. Kojic acid and distilled water were used as positive control and blank, respectively. Anti-tyrosinase activity (% tyrosinase inhibition) of Boletes mushrooms extracts was calculated using the following equation (4):

$$\text{Tyrosinase inhibition (\%)} = \left[\frac{(A-B)-(C-D)}{(A-B)}\right] \times 100 \qquad \text{Equation (4)}$$

Where A : The absorbance of blank solution with enzyme

B : The absorbance of blank solution without enzyme

C : The absorbance of sample solution with enzyme

D : The absorbance of sample solution without enzyme

3.3 Anti-collagenase activity

In this study, anti-collagenase activity was performed in a 96-well plate using a Enzchek® gelatinase/collagenase assay kit (E-12055; Molecular probe) (Chaudhuri, 2005). This assay kit contains substrate (DQ™ gelatin), type IV collagenase from *Clostridium histolyticum*, pH 7.4 Tris-HCl reaction buffer and 1,10-phenanthroline (general metalloproteinase inhibitor). DQ™ gelatin was used to determine the activity of matrix metalloproteinases 2 (MMP-2). This substrate is labeled by fluorescein that can be digested by gelatinase and collagenase to give highly fluorescent peptides. The increase or decrease in fluorescence intensity can be monitored with a fluorescence microplate reader set for excitation at 485 nm and emission at 535 nm. For measurement of potential gelatinase/collagenase inhibition activity, the decrease in

fluorescence intensity of the activity of samples solution compared with the enzyme activity alone was detected. This method was modified from the previous study (Pientaweeratch, 2014). The reagent and sample preparation were mentioned in appendix A. Sample solutions at the final concentration of 100 and 500 µg/ml in a 96-well plate were prepared for screening anti-collagenase activity. The absorbance of the reaction mixture was measured in four wells. The reagent and sample solution were added into each well as follows:

A (control) : 80 µl of 1X reaction buffer + 20 µl of DQ™ gelatin solution + 100 µl of 0.4U/ml collagenase solution

B (blank of A) : 180 µl of 1X reaction buffer + 20 µl of DQ™ gelatin solution

C (sample) : 80 µl of sample solution in 1X reaction buffer + 20 µl of DQ™ gelatin solution + 100 µl of 0.4 U/ml collagenase solution

D (blank of C) : 80 µl of sample solution in 1X reaction buffer + 20 µl of DQ™ gelation solution + 100 µl of 1X reaction buffer

The reaction mixtures were incubated in darkness at room temperature for 90 minutes. Then, the florescence intensity of the reaction mixtures was measured at excitation wavelength at 485 nm and emission wavelength at 535 nm with a fluorescent microplate reader. Solutions of 1,10-phenanthroline and (-)-epigallocatechin (EGCG) were used as positive controls. Then, anti-collagenase activity (% collageanse inhibition) of Boletes mushroom extracts was calculated by the equation (5) as follows:

$$\text{Collagenase inhibition (\%)} = \left[\frac{(A-B)-(C-D)}{(A-B)}\right] \times 100 \qquad \text{Equation (5)}$$

Where A : The fluorescence intensity of blank solution with enzyme

B : The fluorescence intensity of blank solution without enzyme

C : The fluorescence intensity of sample solution with

enzyme

D : The fluorescence intensity of sample solution without enzyme

3.4 Anti-elastase activity

Anti-elastase activity was performed in a 96-well plate by using EnzChek® E-12056 elastase kit (Molecular probe). This assay kit contains DQ™ elastin (substrate), elastase from pig pancreas, pH 8.0 Tris-HCI buffer and *N-methoxysuccinyl*-alanyl-alanyl-prolyl-valine-*chloromethyl ketone* (CMK). DQ™ elastin can be digested by elastase enzyme to provide high fluorescence fragment which is observed with the excitation wavelength at 485 nm and the emission wavelength at 535 nm using a fluorescence microplate reader. The potential elastase inhibitor displays a decrease in fluorescence intensity. This method was slightly modified from the protocol of Pientaweeratch (2014). Preparation method of reagent mixture was mentioned in appendix A. For screening study, the sample solutions were prepared to give at the final concentration of 100 and 500 µg/ml in each well in a 96-well plate. The absorbance of the reaction mixture (final volume 200 µl) was measured in four wells. In each well, the substance was added as shown below:

A (control) : 50 µl of 1X reaction buffer + 100 µl of elastase solution (0.4 U/ml)

B (blank of A) :150 µl of 1X reaction buffer

C (sample) : 50 µl of sample solution + 100 µl of elastase solution (0.4U/ml)

D (blank of C) : 50 µl of sample solution + 100 µl of 1X reaction buffer

After pre-incubation in darkness at room temperature for 15 minutes, 50 µl of DQ™ elastin solution was added into the mixture solution and was further incubated in darkness at room temperature for 30 minutes. Then, the florescence intensity of the reaction mixture was detected at excitation wavelength at 485 nm and emission wavelength at 535 nm using a fluorescent

microplate reader. CMK and EGCG were used as positive controls. The 1X reaction buffer was used as a blank. Anti-elastase activity (% elastase inhibition) of Boletes mushrooms extracts was calculated by the equation (6) as follows:

$$\text{Elastase inhibition (\%)} = \left[\frac{(A-B)-(C-D)}{(A-B)}\right] \times 100 \quad \text{Equation (6)}$$

Where A : The fluorescence intensity of blank solution with enzyme

B : The fluorescence intensity of blank solution without enzyme

C : The fluorescence intensity of sample solution with enzyme

D : The fluorescence intensity of sample solution without enzyme

4. Determination of IC_{50} value of cosmetic activities

Biological activities of Boletes mushroom extracts were expressed as % inhibition at the concentration of 100 or 500 μg/ml for screening study. The extracts that provided % inhibition more than 70% were selected for finding IC_{50} value (μg/ml) which was calculated from the equation of linear regression of the graph between concentration and % inhibition.

Part 4. Analysis of data

All of determinations were performed in triplicate. All data were reported as mean ± standard deviation (SD). Analysis of data was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using SPSS software (version 17.0 for window). The correlation between the biological activity and the content of bioactive compounds was examined by Pearson's correlation coefficient (r). Significance was determined at $p < 0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

Part 1. Preparation of crude extracts from Boletes mushrooms locally grown in Thailand

1.1 Boletes mushroom extracts from fruiting body

The extraction yield and bioactive compounds of natural extracts depend on the nature of extracting solvents because of the varied polarities and chemical characteristics of bioactive compounds (Sultana, Anwar, and Ashraf, 2009). In this study, Boletes mushrooms were collected from the central and the northeastern parts of Thailand (Figure 4). Fruiting bodies of Boletes mushrooms were extracted with 95% ethanol. The appearance and extraction yield of fruiting body extracts are shown in Figure 5 and Table 4. Ethanol is widely used for extraction of natural compounds because of its polarity and safety in using for consumers. Extraction of Boletes mushrooms (*Boletus edulis* and *Boletus auranticus*) was reported in many studies (S.-Y. Tsai et al., 2007; Vidović et al., 2010). The yield of Boletes mushroom extracts in this study was 2.56-3.40% by weight of fresh mushrooms. The extract yield from this study was lower than the yield of Boletes mushroom extracts (15.81-39.09%) from the previous studies (S.-Y. Tsai et al., 2007; Vidović et al., 2010) which used 50% and 95% ethanol, respectively. In addition, the previous studies used dried mushroom samples for extraction. Therefore, the different yields of Boletes mushroom extracts may be due to different types of mushrooms and characters of fresh or dried samples. The ethanol to water ratio affected the yield of extracts because of the various solubility of compounds in each sample (Cacace and Mazza, 2003; Yilmaz and Toledo, 2006; Spigno, Tramelli, and De Faveri, 2007). In this study, fresh fruiting body of Boletes mushrooms was immediately macerated with 95% ethanol because of limitation of mushroom life during collection. Freshly mushrooms are

highly perishable because they can deteriorate by their enzyme the loss of water. Therefore, 95% ethanol was used to preserve freshly harvested mushrooms.

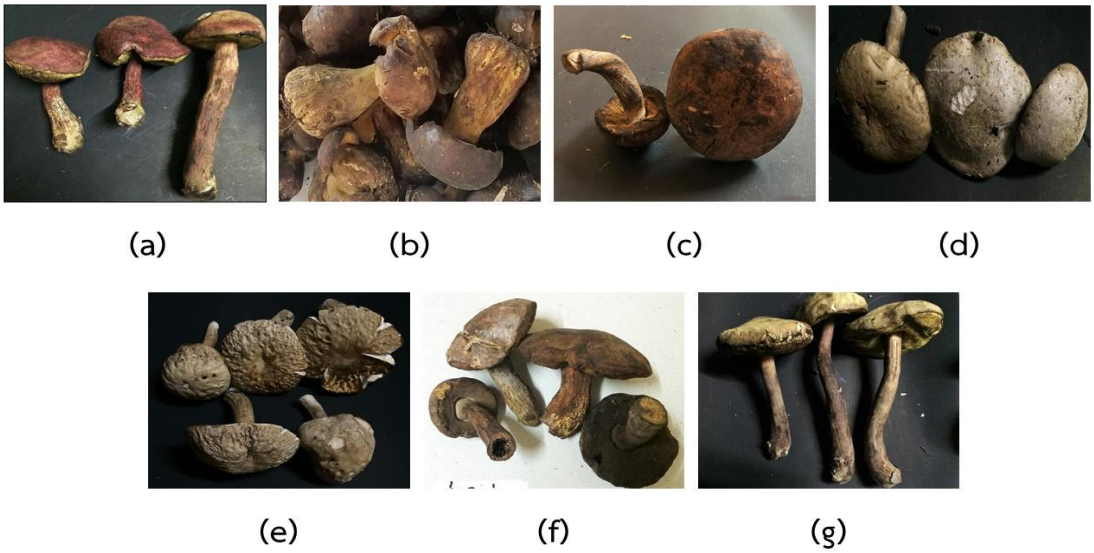

Figure 4 Physical appearance of fresh fruting body of Boletes mushrooms collected from the central and the northeastern parts of Thailand: TISTR 14 (a), TISTR 31 (b), TISTR 36 (c), TISTR 37 (d), TISTR 50 (e), TISTR 55 (f) and TISTR 56 (g)

Table 4 Appearance and yields of Boletes mushroom extracts from fruiting body (n=3, mean ± SD)

Mushroom extracts	Appearance	Yield (%w/w of fresh mushroom)
TISTR 14 FB	Dark brown paste	2.82 ± 0.12
TISTR 31 FB	Brown powder	3.16 ± 0.45
TISTR 36 FB	Dark brown powder	2.56 ± 0.16
TISTR 37 FB	Yellow brown powder	3.09 ± 0.36
TISTR 50 FB	Dark brown semisolid	3.40 ± 0.47
TISTR 55 FB	Black pellet	2.75 ± 0.15
TISTR 56 FB	Brown powder	2.72 ± 0.21

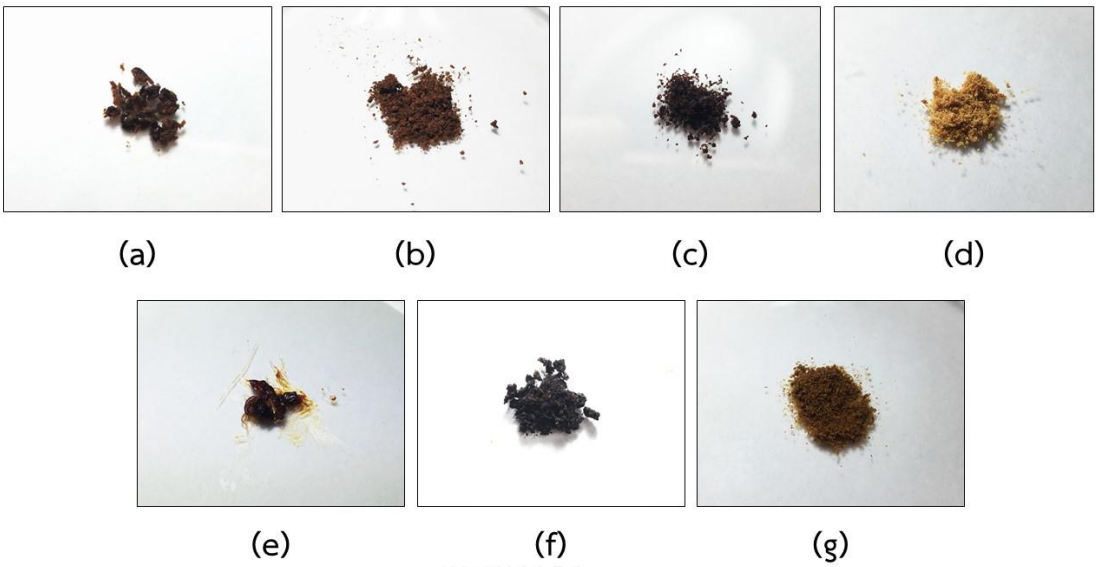

Figure 5 Appearance of Boleteaceae mushroom extracts from fruiting body: TISTR 14 FB (a), TISTR 31 FB (b), TISTR 36 FB (c), TISTR 37 FB (d), TISTR 50 FB (e), TISTR 55 FB (f) and TISTR 56 FB (g)

1.2 Boletes mushroom extracts from culture broth (culture filtrate)

Culture broth is a byproduct from mushroom cultivation which composed of many bioactive compounds. The previous study reported about antioxidant activity of culture broth of *Lignosus rhinocerotis* mushroom which showed better antioxidant activity than mycelium culture. This result may be due to different morphological stages of mushroom cultivation that gave different bioactive compounds (Lau et al., 2014). For Boletes mushrooms, they have few studies about biological activities and bioactive compounds especially culture broth extracts. In this study, culture broth extracts were prepared from submerge culture of Boletes mushrooms collected from the central and the north-eastern parts. In this study, potato dextrose broth (PDB) was used as a media. Potato dextrose broth or potato dextrose agar (PDA) was a simple media that was widely used for producing bioactive compounds in culture broth (Vladimir, 2012). Culture broth extracts in this study could be prepared from only 4 from 7 Boletes mushrooms because some Boletes mushroom samples were contaminated during mushroom cultivation.

The appearance and yield of culture broth extracts are showed in Table 5 and Figure 6. The yield of culture broth extracts of Boletes mushroom depended on type of mushroom. The yield of culture broth extracts (9.56-17.66 g dried media/liter of media) was lower than that of pure potato dextrose broth (20.40 g dried /liter of media) because of the nutritional requirement for development stage of each Boletes mushroom (S.-T. Chang and Miles, 1989). The culture broth extracts had different appearance from the fruiting body extracts of the same mushrooms. Culture broth extracts were sticky semisolid whereas mostly fruiting body extracts were powder. The difference of appearance may be due to various types and contents of bioactive compounds in the extracts. The culture broth extract had high sugar content which resulted in sticky mass of the extracts.

Table 5 Appearance and yield of Boletes mushroom extracts from culture broth

Mushroom extracts	**Appearance**	**Yield (g dried extracts/ L of media)**
TISTR 31 CB	Dark brown semisolid	10.32
TISTR 36 CB	Dark brown semisolid	9.56
TISTR 37 CB	Yellow brown semisolid	17.66
TISTR 55 CB	Dark brown semisolid	11.32

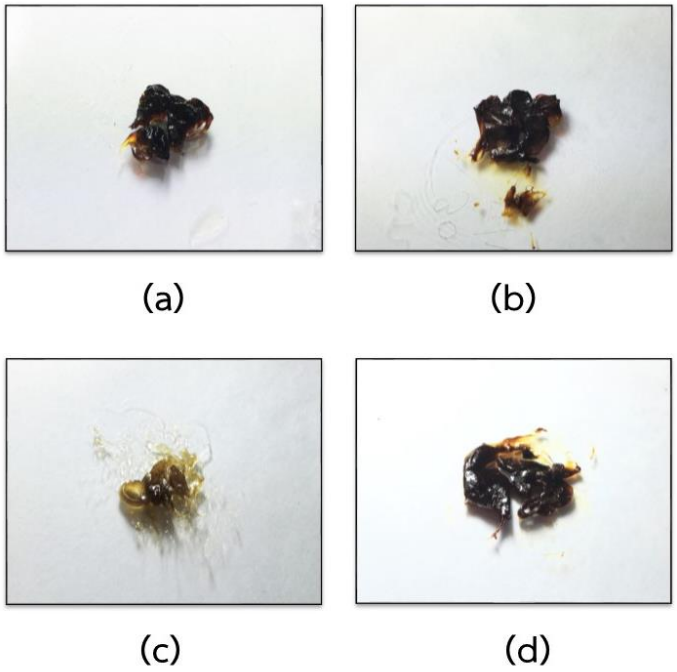

Figure 6 Appearance of Boletes mushroom extracts from culture broth: TISTR 31 CB (a), TISTR 36 CB (b), TISTR 37 CB (c) and TISTR 55 CB (d)

1.3 Biological stability of the Boletes mushroom extracts

The effect of 6-month storage at -20°C on DPPH radical scavenging activity of fruiting body extracts at the concentration of 100 µg/ml is present in Table 6. After 6-month storage, DPPH radical scavenging activity of mostly fruiting body extracts was not significantly different from the freshly prepared extracts (0-month storage) except TISTR 37 FB and TISTR 50 FB extracts. TISTR 37 FB and TISTR 50 FB extracts after 6-month storage showed significantly higher % DPPH inhibition than the freshly prepared extracts. This effect may be due to the good stability of phenolic compounds in each extract (Amoo et al., 2012). The culture broth extracts from Boletes mushrooms were not determined for the stability because they were obtained after fruiting body extracts (March 2016). They were studied in all tests within 1.5 month after the extraction.

Table 6 Effect of 6-month storage on DPPH scavenging activity of fruiting body extracts from Boletes mushrooms (n = 3, mean ± SD) (* Significantly different between 0 and 6 month of the same extracts at $p < 0.05$)

Mushroom extracts	% DPPH inhibition	
	0 month	6 months
TISTR 14 FB	83.34 ± 0.19	83.86 ± 0.51
TISTR 31 FB	56.30 ± 1.72	57.41 ± 1.23
TISTR 36 FB	80.66 ± 0.57	78.76 ± 0.30
TISTR 37 FB	74.47 ± 1.50*	78.68 ± 1.25*
TISTR 50 FB	43.72 ± 1.38*	56.93 ± 1.81*
TISTR 55 FB	93.89 ± 0.35	93.85 ± 0.12
TISTR 56 FB	77.99 ± 0.82	78.93 ± 1.44

Part 2. Analysis of bioactive compounds in Boletes mushroom extracts

In this study, bioactive compounds in Boletes mushroom extracts was determined in terms of total phenolic content, total flavonoid content, total carbohydrate content and total protein content.

2.1 Total phenolic content

Phenolic compounds are known as antioxidant compounds which contain one or more aromatic rings with one or more hydroxyl group (Dai and Mumper, 2010). Phenolic compounds including phenolic acid, flavonoid and tannin are secondary metabolites of fungal (mushroom) (Rahi and Malik, 2016). According to Figures 7 and 8, total phenolic contents (TPC) of fruiting body and culture broth extracts were ranged 26.70-113.17 mg GAE/g extract and 8.60-48.07 mg GAE/g extract, respectively. TISTR 55 FB extract had significantly the highest phenolic content and TISTR 31 CB had significantly the lowest phenolic content.

In this study, mostly Boletes mushroom extracts showed higher phenolic content than the previous studies which reported TPC of other Boletes mushroom extracts between 5.17-41.82 mg GAE/g extract (S.-Y. Tsai et al., 2007; Vidović et al., 2010; Guo et al., 2012). Different phenolic contents of Boletes mushroom extracts may be due to type of mushroom, age of fresh mushroom sample, extraction method and extraction solvent (Mattila et al., 2001; Dai and Mumper, 2010).

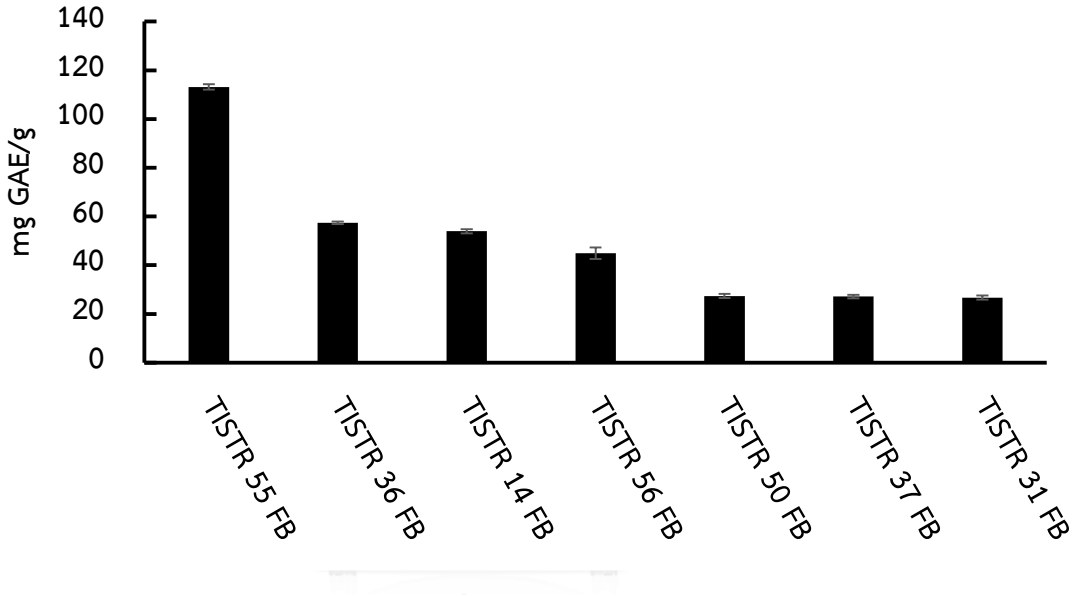

Figure 7 Total phenolic content of Boletes mushroom extracts from fruiting body (n = 3, mean ± SD)

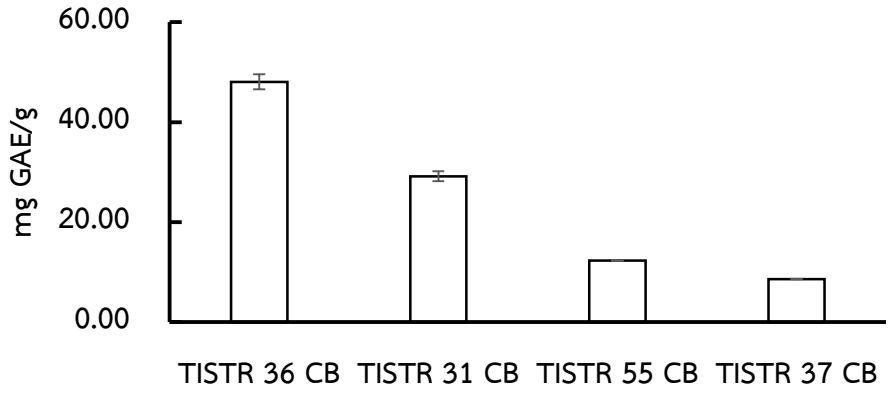

Figure 8 Total phenolic content of Boletes mushroom extracts from culture broth (n = 3, mean ± SD)

In addition, the fruiting body extracts expressed higher phenolic content than the culture broth extracts of the same mushrooms (Figure 9). For example, TISTR 55 FB extract showed the highest phenolic content (113.17 mg GAE/g) but that of its culture broth extract was low (12.31 mg GAE/g). This effect may be occurred from different parts of extraction and extraction method. In this study, fruiting body extracts were prepared with ethanol, whereas the preparation of culture broth extracts did not use organic solvent. For the culture broth extract in this study, potato dextrose broth was used as culture media because it showed faster radial growth of mycelia mushroom than other media. However, there was the previous study that reported about effect of different culture media on mycelia growth and biomass production (secondary metabolite). Other media such as Melin-Norkans medium (MMN) was reported about high bioactive compounds of culture broth extract from *Ganoderma lucidum* including phenolic acid and polysaccharide (Heleno et al., 2012). For Boletes mushroom cultivation in the further study, other culture media should be tested for preparation of culture broth extract.

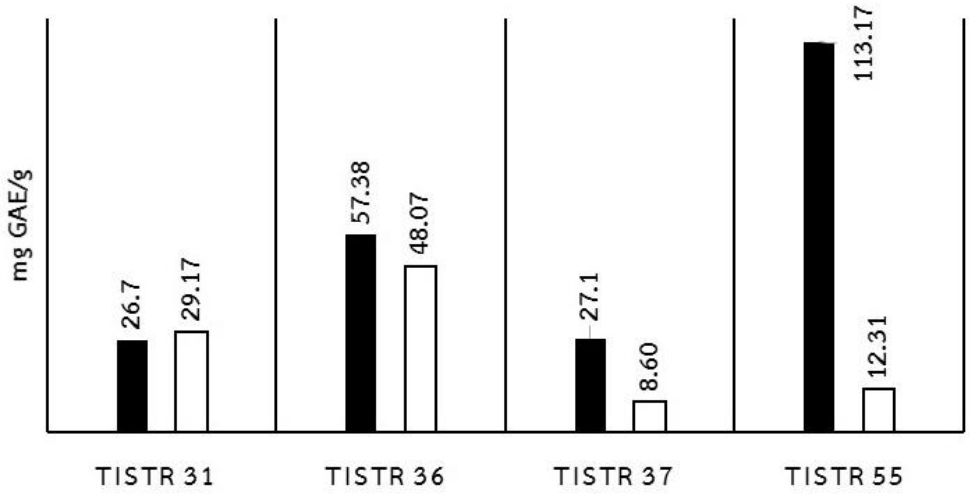

Figure 9 Comparison between total phenolic content of Boletes mushroom extracts from fruiting body (●) and culture broth (○)

2.2 Total flavonoid content

Flavonoids were multi-active components including antioxidant activity and soothing actions in cosmetic products (Arct and Pytkowska, 2008). They could be found in secondary metabolite of mushroom extracts. According to Figure 10, the results showed that Boletes mushroom extracts from fruiting body exhibited low total flavonoid content (2.65-12.52 mg QE/g extract) but extracts from culture broth were undetectable (less than 1 mg QE/g). This result agreed with the study of Vidović et al. (2010) and Kuka and Cakste (2011) that showed low flavonoid content in other Boletes mushroom extracts (*Boletus edulis* and *Boletus auranticus*). In this study, TISTR 55 FB extract showed significantly the highest flavonoid content (12.52 mg QE/g extract). This extract had higher flavonoid content than *Boletus edulis* extract (8.73 ± 0.11 mg CE/g) but lower than *Boletus auranticus* extract (17.62 ± 0.05 mg CE/g) (Vidović et al., 2010).

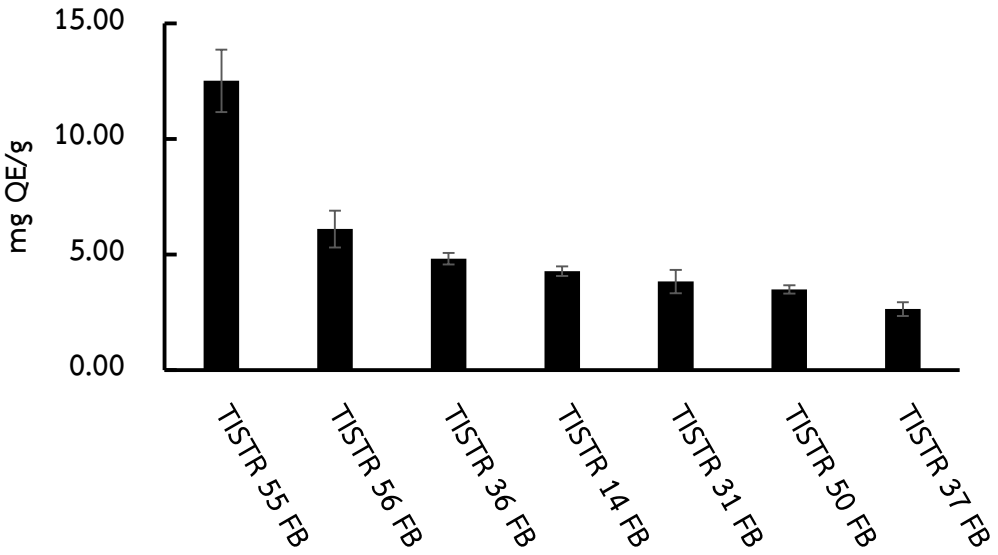

Figure 10 Total flavonoid content of Boletes mushroom extracts from fruiting body (n = 3, mean ± SD)

2.3 Total carbohydrate content

Carbohydrate including polysaccharides has been used as cosmetic ingredients for many years. There are many studies that reported mushrooms as the source of carbohydrate. Total carbohydrate content of Boletes mushroom extracts was examined using phenol sulfuric assay. Total carbohydrate content of fruiting body extracts showed in range between 50.28-317.82 mg GE/g extract (Figure 11). Fruiting body extracts from different Boletes mushrooms showed various carbohydrate content. In addition, solvent type used for extraction also affected their carbohydrate content. Barros et al. (2008) reported that the water extract of fruiting body from *Boletus edulis* mushroom had total carbohydrate content of 71.15 mg GE/g. In the present study, ethanolic extracts of Boletes mushrooms contained higher carbohydrate content than this water extract. This result may be due to different type of Boletes mushroom and extraction solvent.

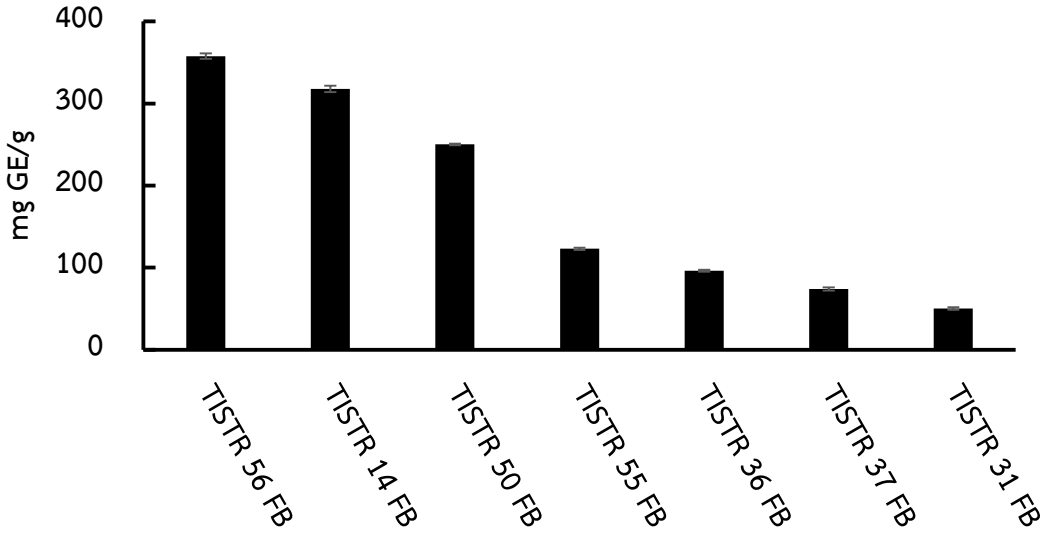

Figure 11 Total carbohydrate content of Boletes mushroom extracts from fruiting body (n = 3, mean ± SD)

Culture broth extracts of Boletes mushrooms showed very high total carbohydrate content (871.79-944.53 mg GE/g extract) (Figure 12). TISTR 36 CB and TISTR 37 CB extracts had significantly the highest carbohydrate content of all culture broth extracts. All culture broth extracts displayed largely higher total carbohydrate content than fruiting body extracts. The high carbohydrate content may be due to the components of culture media in which dextrose is a mainly compound. The other reason may be because mycelia of Boletes mushroom could secret exopolysaccharide into culture media. The exopolysaccharide in culture broth could be analyzed by HPLC-RI or GC-MS for characterization of free sugar content in extracts (Heleno et al., 2012; Zheng et al., 2014).

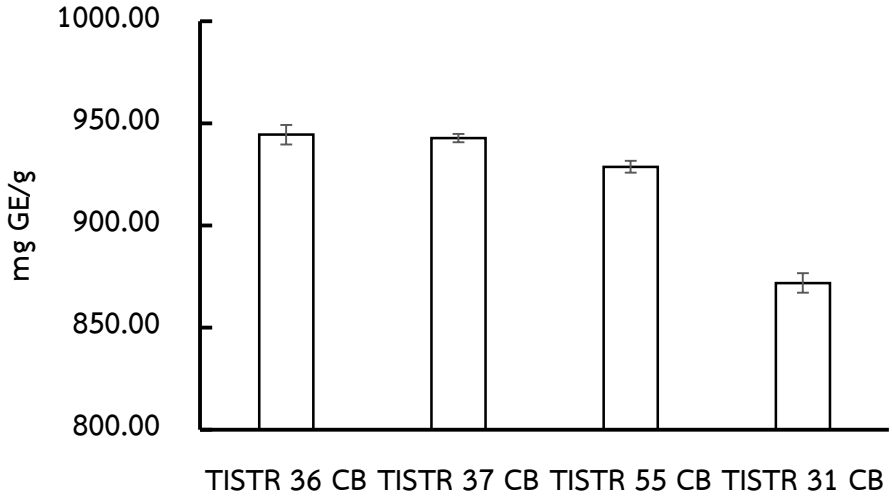

Figure 12 Total carbohydrate content of Boletes mushroom extracts from culture broth (n = 3, mean ± SD)

2.4 Total protein content

Protein or amino acid is usually used as active ingredients in cosmetic products showing the benefits for skin and hair by protection of their structure (Chvapil and Eckmayer, 1985). Mushroom was important source of protein (S.-T. Chang and Miles, 1989). Therefore, Boletes mushrooms may be used as source of protein for further using in food or cosmetic industry. Total protein content of Boletes mushroom extracts was examined using Lowry assay. Total protein contents of fruiting body extracts (102.61-542.50 mg BSAE/g) were significantly different (Figure 13). TISTR 55 FB extract had the highest protein content (542.50 ± 3.95 mg BSAE/g) whereas TISTR 50 FB extract had the lowest content (102.61 ± 2.66 mg BSAE/g). This result was consistent with the previous study that found high protein content of fruiting body extract from *Boletus edulis* (526 mg protein/g) (Kuka and Cakste, 2011). Therefore, total protein content of Boletes mushroom extracts depended on type of mushrooms. Moreover, it was influenced by different stages of fruiting body, time of harvest and collection techniques (Barros et al., 2007; Barros et al., 2008).

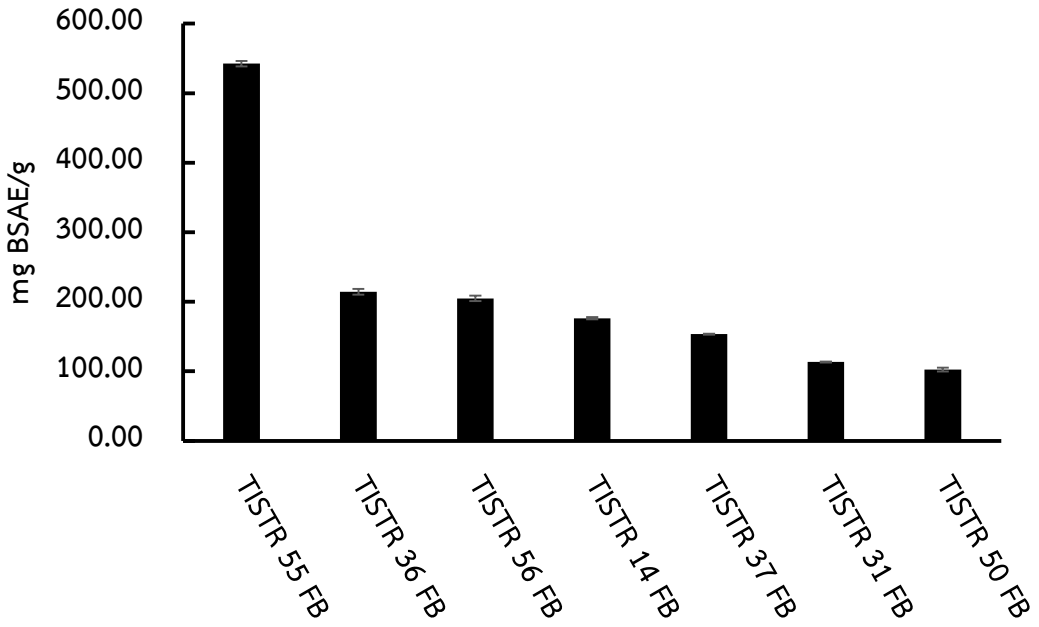

Figure 13 Total protein content of Boletes mushroom extracts from fruiting body (n = 3, mean ± SD)

Culture broth extracts of Boletes mushrooms showed total protein content between 7.34-112.06 mg BSAE/g (Figure 14). TISTR 36 CB extract showed significantly the highest protein content. TISTR 37 CB extract had the lowest protein content which was similar to the previous study showing low protein content of culture broth extract from *Lignosus rhinocerotis* (7.4 mg BSAE/g) (Lau et al., 2014). The culture broth extracts had lower total protein content than fruiting body extracts in the same mushrooms. This result could be assumed that different parts and types of Boletes mushrooms exhibited different total protein contents as discussed in results of their phenolic and carbohydrate content.

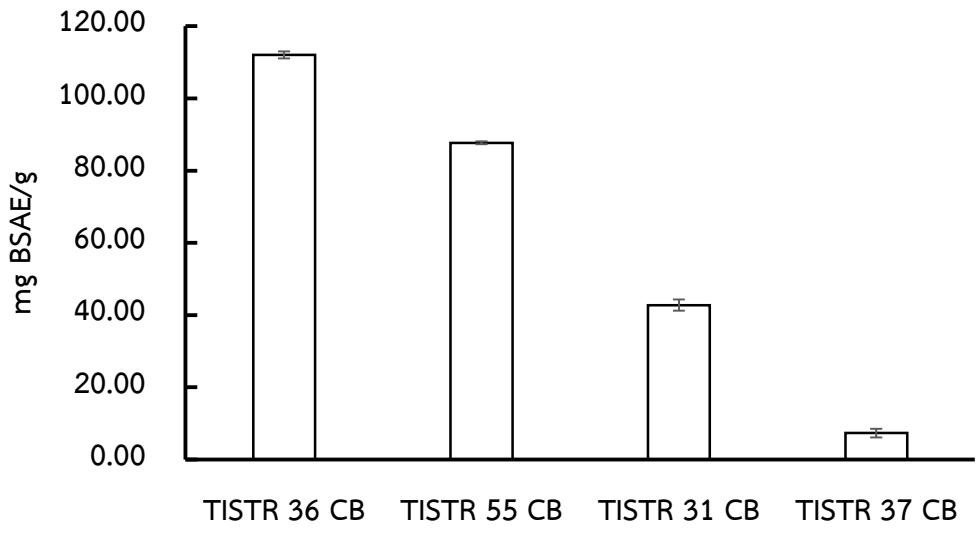

Figure 14 Total protein content of Boletes mushroom extracts from culutre broth (n = 3, mean ± SD)

All of total bioactive compounds studied in the Boletes mushroom extracts were concluded in Table 7. In summary, type and content of bioactive compounds in Boletes mushroom extracts depended on mushroom species, age of fruiting body or mycelium and nutritional substrate (Gursoy et al., 2009; Saraswaty et al., 2013). Type and content of bioactive compounds in the extracts leaded to the potential of their cosmetic activities. *In vitro* cosmetic activities of these extracts were evaluated to investigate correlation between their compound contents and activities.

Table 7 Content of bioactive compounds in Boletes mushroom extracts (n = 3, mean ± SD)

Mushroom extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	Total carbohydrate content (mg GE/g)	Total protein content (mg BSAE/g)
TISTR 14 FB	53.94 ± 0.84	4.29 ± 0.21	317.82 ± 3.95	176.29 ± 1.66
TISTR 31 FB	26.70 ± 0.89	3.84 ± 0.51	50.28 ± 1.44	113.35 ± 0.66
TISTR 36 FB	57.38 ± 0.56	4.83 ± 0.25	96.15 ± 1.26	214.37 ± 3.98
TISTR 37 FB	27.10 ± 0.75	2.65 ± 0.30	73.99 ± 2.02	153.48 ± 0.66
TISTR 50 FB	27.35 ± 0.84	3.49 ± 0.19	250.15 ± 1.06	102.61 ± 2.66
TISTR 55 FB	113.17 ± 1.17	12.52 ± 1.35	122.97 ± 1.41	542.50 ± 3.95
TISTR 56 FB	44.87 ± 2.35	6.11 ± 0.79	357.67 ± 3.32	204.80 ± 4.00
TISTR 31 CB	29.17 ± 0.98	UD	871.79 ± 4.78	42.75 ± 1.55
TISTR 36 CB	48.07 ± 1.53	UD	944.53 ± 4.78	112.06 ± 1.00
TISTR 37 CB	8.60 ± 0.04	UD	942.84 ± 2.03	7.34 ± 1.19
TISTR 55 CB	12.31 ± 0.09	UD	928.72 ± 2.90	87.71 ± 0.38

UD: undetectable (less than 1 mg QE/g)

Part 3. Evaluation of *in vitro* cosmetic activities of Boletes mushroom extracts

3.1 Free radical scavenging activity

Free radical can damage skin cell that is cause of skin aging. The role of antioxidant is to remove free radical. Antioxidants play an important part of cosmetic products especially personal care products. In this study, two methods (DPPH and ABTS assays) were used to evaluate free radical scavenging activity of Boletes mushroom extracts. The results were found that all of fruiting body extracts from Boletes mushrooms showed high antioxidant activities of both assays. DPPH (43.72-93.89%) and ABTS (36.58-99.76%) scavenging activities of each extract at the concentration of 100 µg/ml are shown in Figures 15 and 16, respectively. TISTR 55 FB extract displayed significantly the highest percentage of inhibition on both DPPH and ABTS methods. Moreover, free radical scavenging activities of TISTR 55 FB extract were higher than those of other Boletes mushrooms from the previous study. The ethanolic extract of *Boletus edulis* mushroom at the concentration of 1 mg/ml showed 72.20% and 83.75 % inhibition on DPPH and ABTS assays, respectively (Vamanu and Nita, 2013).

The extracts showing more than 70% inhibition were studied for finding IC_{50} value. IC_{50} values of DPPH (13.62-66.06 µg/ml) and ABTS (23.58-62.41 µg/ml) scavenging activities are presented in Figures 17 and 18. The sequence of their IC_{50} values of DPPH and ABTS assays corresponded to the result of their percentage of inhibition at the concentration of 100 µg/ml. TISTR 55 FB extract had the greatest free radical scavenging activity on both assays. This result agreed with the study of Gursoy et al. (2009) that different types of mushroom showed various free radical scavenging activities. In the present study, TISTR 37 FB and TISTR 36 FB extracts showed different effects on DPPH and ABTS scavenging activities. TISTR 37 FB extract showed greater activity on ABTS assay than DPPH assay. TISTR 36 FB extract showed higher effect on DPPH scavenging activity than ABTS scavenging activity. The different effect may be because of types of antioxidant compounds in each extract. DPPH assay was used to screen lipophilic antioxidant, whereas ABTS was suitable for hydrophilic antioxidant

compounds (Damgaard et al., 2014). However, Vidović et al. (2010) reported that DPPH IC_{50} values (μg/ml) of ethanolic extracts from *Boletus edulis* and *Boletus auranticus* were 16 and 24 μg/ml, respectively, which were similar to that of TISTR 55 FB extract in this study. Although TISTR 55 FB extract had lower antioxidant activity than positive controls (ascorbic acid and gallic acid), it showed potent DPPH and ABTS scavenging activities.

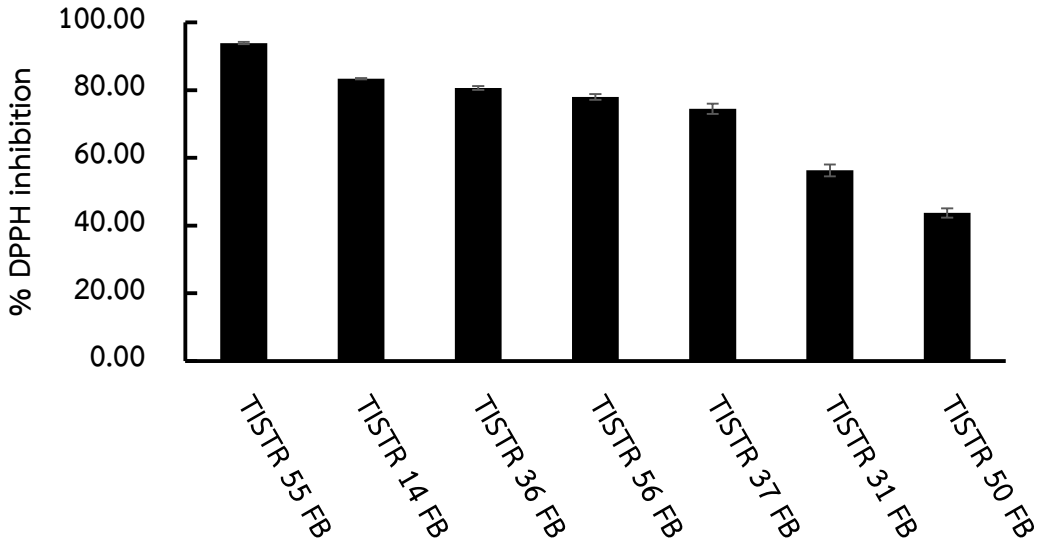

Figure 15 DPPH scavenging activity of Boletes mushroom extracts from fruiting body at the concentration of 100 μg/ml (n = 3, mean ± SD)

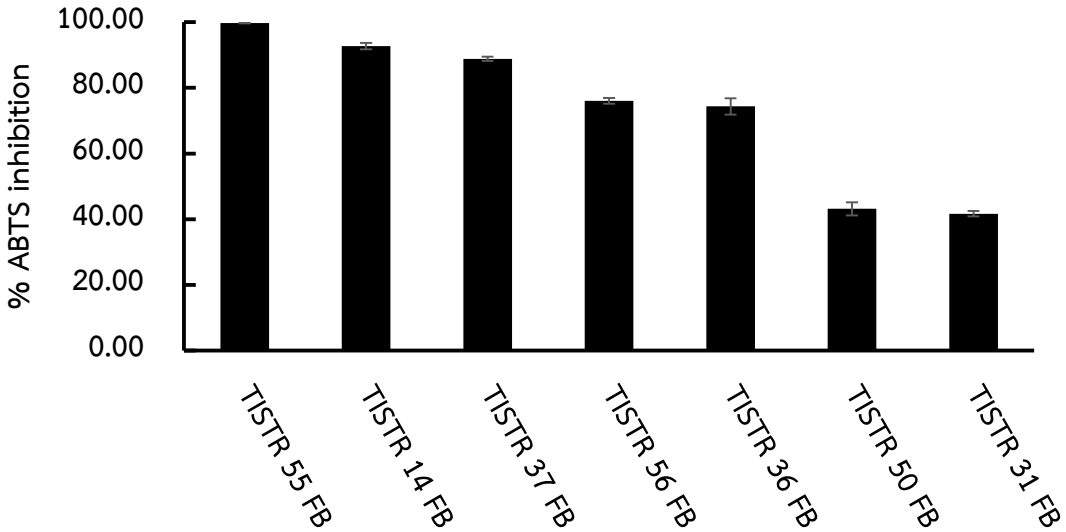

Figure 17 ABTS scavenging activity of Boletes mushroom extracts from fruiting body at the concentration of 100 µg/ml (n = 3, mean ± SD)

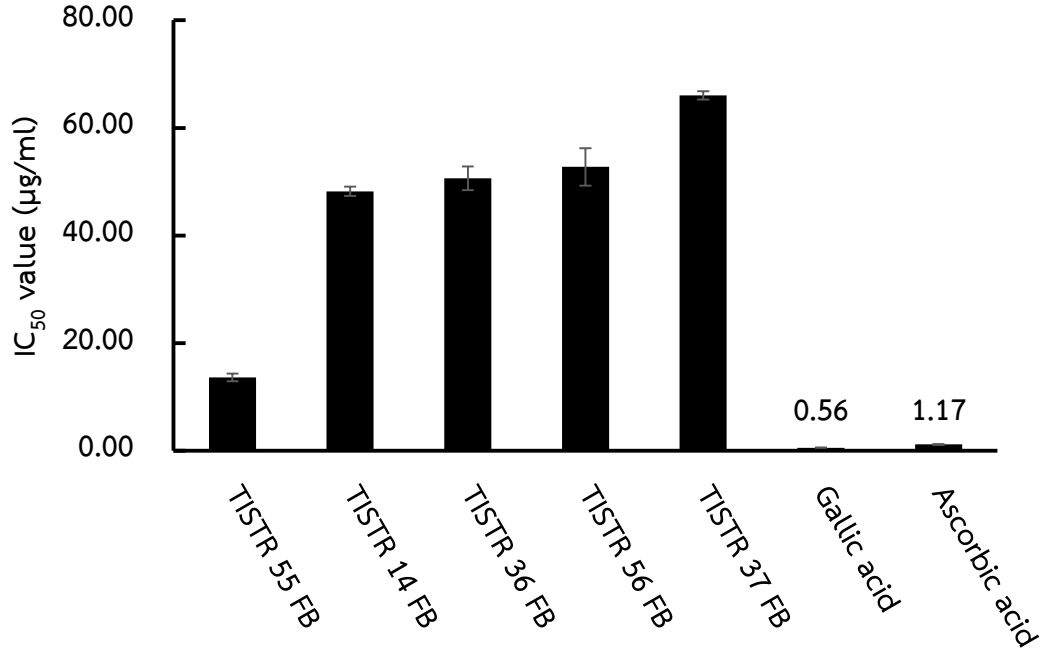

Figure 16 DPPH IC_{50} value of Boletes mushroom extracts from fruiting body that showed % inhibition more than 70% (n = 3, mean ± SD)

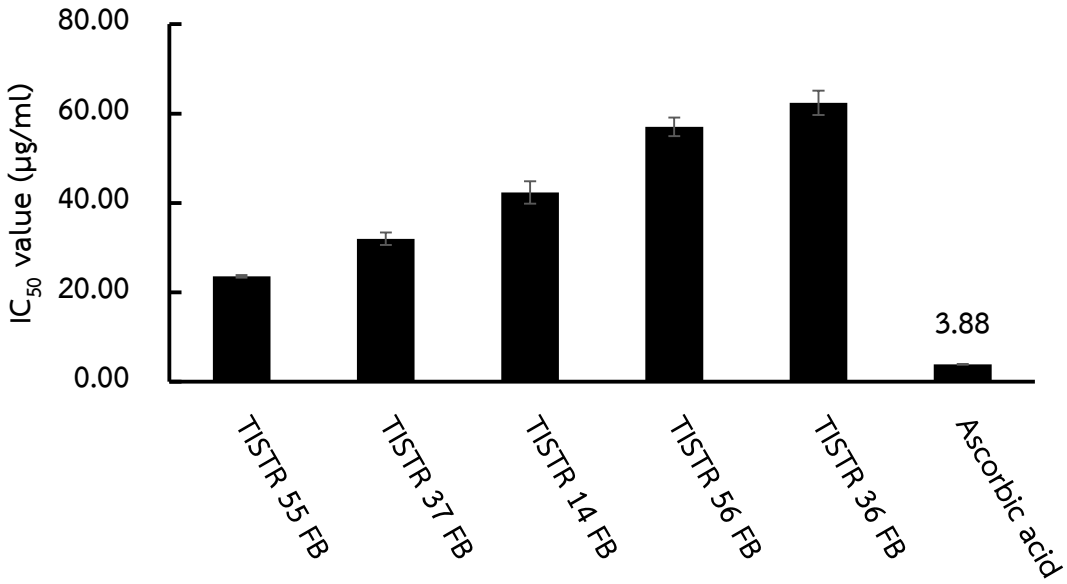

Figure 18 ABTS IC_{50} value of Boletes mushroom extracts from fruiting body that showed % inhibition more than 70% (n = 3, mean ± SD)

Boletes mushrooms extracts from culture broth at the concentration of 100 µg/ml showed DPPH (9.85-38.62%) and ABTS (13.88-41.85%) scavenging activities (Figures 19 and 20). Each culture broth extract presented different effects on both assays. For example, TISTR 55 CB extract showed the highest % DPPH inhibition but not show the highest % ABTS inhibition. This effect may be occurred from different mechanisms of free radical scavenging between DPPH and ABTS radicals as discussed in the result of free radical scavenging activity of fruiting body extracts. The study about cosmetic activities especially antioxidant activity of culture broth extracts had little information. Ming Yeou Lung, Tsai, and Huang (2010) reported low DPPH scavenging activity of methanolic extract from culture broth of *Phellinus igniarus* mushroom (30% DPPH inhibition at the concentration of 10 mg/ml). This extract showed lower scavenging effect on DPPH radicals than all of Boletes mushroom extracts in this study. The different antioxidant activities of the culture broth extracts from this study and from the previous study may be due to different type of mushroom and condition of mycelium culture. For example, Saraswaty et al. (2013) reported that age of

mycelium culture of *Fennelia nivea* fungus affected its antioxidant activity. When increased the cultivation period, the antioxidant activity of the culture broth extract was enhanced.

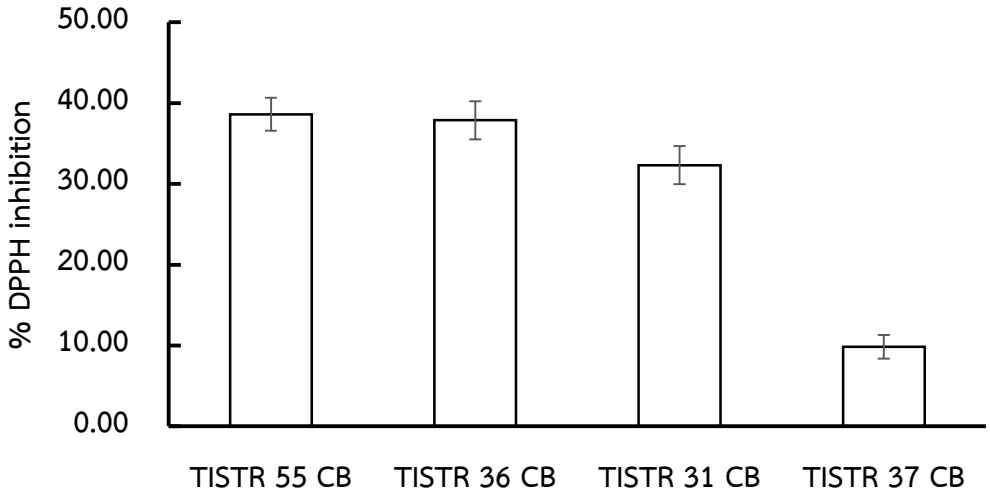

Figure 19 DPPH scavenging activity of Boletes mushroom extracts from culture broth at the concentration of 100 µg/ml (n = 3, mean ± SD)

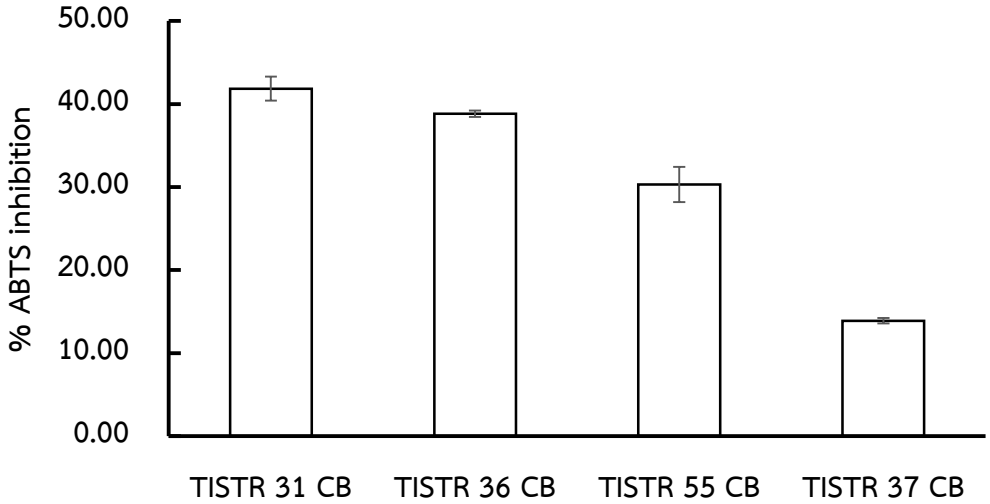

Figure 20 ABTS scavenging activity of Boletes mushroom extracts from culture broth at the concentration of 100 µg/ml (n = 3, mean ± SD)

DPPH and ABTS scavenging activities of fruiting body and culture broth extracts were compared. It was found that all fruiting body extracts showed higher percent DPPH and ABTS inhibition than culture broth extracts of the corresponding mushroom. TISTR 37 showed considerably different DPPH and ABTS scavenging activity between fruiting body extract and culture broth extract. Fruiting body extract of TISTR 37 showed high DPPH scavenging activity but culture broth extract displayed low activity. This result may be due to difference of bioactive compounds in each part of mushroom extracts that can affect their antioxidant activity (Saraswaty et al., 2013; Liu et al., 2014). In addition, Culture condition (time or culture media) of Boletes mushrooms may also influence to free radical scavenging activity of culture broth extracts. The effect of bioactive compounds in Boletes mushroom extracts was discussed on their cosmetic activities in Part 4.

Free radicals can affect cellular functions and cellular constituents. Therefore, the active ingredient having free radical scavenging activity is a primary effective antioxidant (M. Y. Lung and Chang, 2011). Recently, many studies showed that mushroom extracts are acted as free radical scavengers (Lo and Cheung, 2005; Smina et al., 2011; Dangre et al., 2012). In this study, Boletes mushroom extracts could be used as antioxidant compounds due to good free radical scavenging activity.

2.2. Anti-tyrosinase activity

Tyrosinase is a multifunctional copper-containing enzyme that can be extracted from different sources including plants, mammalian melanoma tumor and fungi especially mushroom. It is responsible for melanin synthesis. For finding new whitening agent, tyrosinase inhibitor is a part of mechanism to against melanin production that cause skin hyperpigmentation *(Parvez et al., 2007)*. The hydroxyl groups of phenolic compounds in mushroom extracts may affect anti-tyrosinase activity because hydroxyl groups can form a hydrogen

bond with active site of tyrosinase enzyme (N. Alam et al., 2011). In this study, DOPAchrome method was used to determine the anti-tyrosinase activity of Boletes mushroom extracts. The results were reported in term of the percentage of tyrosinase inhibition.

According to Table 8, the percentage of tyrosinase inhibition of Boletes mushroom extracts at the concentration of 100 and 500 µg/ml was reported. The result was found that both fruiting body and culture broth extracts showed no anti-tyrosinase activity in both concentrations except TISTR 36 FB extract. However, mostly Boletes mushroom extracts at the concentration of 500 µg/ml presented lower % tyrosinase inhibition than the extracts at the concentration of 100 µg/ml. This may be due to effect of the mushroom extracts on tyrosinase activity which should be further studied. The previous studies reported that tyrosinase enzyme could be extracted from fruiting body of *Agaricus bisporus* in which enzyme was similar to human tyrosinase (Friedman and Daron, 1977; Zaidi, Ali, and Ali, 2014). These results in the present study were different from the previous studies. N. Alam et al. (2011) reported that acetonic extract from fruiting body of *Pleurotus nebrodensis* showed 55.76% tyrosinase inhibition at the concentration of 1 mg/ml. Chien et al. (2008) reported that the ethanolic extract from *Ganoderma lucidum* showed the highest anti-tyrosinase activity when compared with other mushrooms. At the concentration of 100 µg/ml, mycelia extract of this mushroom showed 40% tyrosinase inhibition. When increased its concentration to 1000 µg/ml, the percentage of tyrosinase inhibition was 80%.

Table 8 Anti-tyrosinase activity of Boletes mushroom extracts at the concentration of 100 and 500 µg/ml (n = 3, mean ± SD)

Mushroom extracts	% Tyrosinase inhibition	
	100 µg/ml	500 µg/ml
TISTR 14 FB	-13.01 ± 0.78	-32.43 ± 1.67
TISTR 31 FB	-3.92 ± 1.52	-1.93 ± 0.43
TISTR 36 FB	31.18 ± 1.49	15.47 ± 3.14
TISTR 37 FB	8.17 ± 0.46	-18.32 ± 1.24
TISTR 50 FB	4.28 ± 1.11	-5.80 ± 1.63
TISTR 55 FB	-11.33 ± 2.33	-24.17 ± 1.43
TISTR 56 FB	5.26 ± 0.70	-17.37 ± 1.92
TISTR 31 CB	-3.67 ± 1.07	-5.72 ± 2.02
TISTR 36 CB	7.00 ± 3.52	-14.00 ± 1.68
TISTR 37 CB	-0.77 ± 0.88	-2.00 ± 1.74
TISTR 55 CB	-5.89 ± 3.94	-19.64 ± 1.73
Kojic acid *(60 µg/ml)*	74.58 ± 2.93	

2.3 Anti-collagenase activity

The collagenase assay kit was used for anti-aging activity of the extracts. MMP-2 could degrade collagen, gelatin and elastin in skin which were cause of the lack of skin elasticity contributing to wrinkle formation. DQ™ gelatin was used as a substrate to exhibit the MMP-2 activity. There were a few studied on anti-collagenase activity of mushroom extracts especially Boletes mushroom extracts. As shown in Table 9, the anti-collagenase activity of Boletes mushroom extract at the concentration of 100 and 500 µg/ml is reported. The extracts at 100 µg/ml showed no anti-collagenase activity. When their concentrations were increased to 500 µg/ml, their activity was improved. However, more concentration of the extracts could not be studied because of their solubility limitation in the used solvent. The highest % collagenase inhibition was found in TISTR 37 FB extract that showed 63.81 ± 4.74% at the concentration of 500

μg/ml (IC_{50} value of 390.74 ± 2.75 μg/ml). However, its activity was still low when compared with 15 μg/ml EGCG (84.53 ± 3.14%) and 0.1 mM 1,10-phenanthroline (71.91 ± 1.85%).

Anti-collagenase activity of mushroom extracts obtained from different parts showed different effects. In this study, this activity of culture broth extracts was lower than that of fruiting body extracts from the same mushrooms at the concentration of 500 μg/ml. For example, TISTR 37 FB extract had the highest anti-collagenase activity, whereas TISTR 37 CB extract had the lowest activity. This effect may occur from difference of extraction method and bioactive compounds in each extract that could react with collagenase enzyme. The correlation between their activity and bioactive compounds was discussed in Part 4. The anti-collagenase activity of different parts of mushroom extracts showed different effects. TISTR 37 FB extract had the highest anti-collagenase activity whereas TISTR 37 CB extract had the lowest activity. This effect may occur from difference of extraction methods and bioactive compounds in each extract that can react with collagenase enzyme as discussion in Part 4.

2.4 Anti-elastase activity

Anti-elastase activity was also determined for screening anti-aging effect of Boletes mushroom extracts. According to Table 10, the anti-elastase activity of Boletes mushroom extracts at the concentration of 100 and 500 μg/ml is reported. The fruiting body extracts at 100 μg/ml showed no activity. When increased the concentration to 500 μg/ml, the activity of fruiting body extracts was increased. As discussed in 2.3, more concentration of the extracts could not be studied. The extracts at 500 μg/ml exhibited low or medium effects for elastase inhibition when compared with 0.01 mM CMK (82.07 ± 1.37%) and EGCG at 15 μg/ml (69.04 ± 2.47%). TISTR 37 FB extract showed the highest anti-elastase activity. Culture broth extracts of both concentrations showed no or low anti-elastase activity. The extracts at the concentration of 500 μg/ml from

fruiting body displayed higher % elastase inhibition than culture broth extracts. The results of their anti-elastase activity were similar to anti-collagenase activity. For example, TISTR 37 FB extract showed the highest anti-elastase activity but TISTR 37 CB extract gave the lowest activity. As discussed in anti-collagenase (2.3) activity, extraction method and bioactive compounds in each extract also affected anti-elastase activity.

Table 9 Anti-collagenase activity of Boletes mushroom extracts at the concentration of 100 and 500 µg/ml (n = 3, mean ± SD)

Mushroom extracts	**% Collagenase inhibition**	
	100 µg/ml	**500 µg/ml**
TISTR 55 FB	-14.19 ± 4.29	22.07 ± 2.44
TISTR 50 FB	-25.96 ± 3.32	-12.29 ± 1.27
TISTR 36 FB	-19.83 ± 4.69	19.66 ± 1.46
TISTR 56 FB	-25.29 ± 3.42	-4.91 ± 1.40
TISTR 37 FB	-3.76 ± 1.53	63.81 ± 4.74
TISTR 31 FB	-19.73 ± 2.42	10.92 ± 0.62
TISTR 14 FB	-16.73 ± 3.99	-6.66 ± 3.32
TISTR 55 CB	0.84 ± 3.42	3.95 ± 2.23
TISTR 36 CB	-2.81 ± 1.61	12.48 ± 2.36
TISTR 37 CB	2.31 ± 2.16	3.57 ± 4.11
TISTR 31 CB	-2.52 ± 4.17	8.24 ± 3.40
0.1 mM 1,10-phenanthroline	71.91 ± 1.85	
EGCG (15 µg/ml)	84.53 ± 3.14	

Table 10 Anti-elastase activity of Boletes mushroom extracts at the concentration of 100 and 500 μg/ml (n = 3, mean ± SD)

Mushroom extracts	**% Elastase inhibition**	
	100 μg/ml	**500 μg/ml**
TISTR 14 FB	6.57 ± 1.76	30.21 ± 1.54
TISTR 31 FB	12.12 ± 2.94	35.29 ± 3.75
TISTR 36 FB	-11.86 ± 3.24	29.22 ± 1.61
TISTR 37 FB	-9.08 ± 2.68	44.44 ± 2.25
TISTR 50 FB	-3.80 ± 0.86	26.46 ± 1.76
TISTR 55 FB	-2.25 ± 4.26	33.79 ±2.65
TISTR 56 FB	4.74 ± 3.56	26.46 ± 3.96
TISTR 31 CB	3.45 ± 1.05	6.72 ± 1.87
TISTR 36 CB	-3.33 ± 1.52	2.64 ± 3.91
TISTR 37 CB	-15.15 ± 1.96	-10.04 ± 3.93
TISTR 55 CB	4.78 ± 2.19	-0.28 ± 2.02
0.01 mM CMK	82.07 ± 1.37	
EGCG (15 μg/ml)	69.04 ± 2.47	

Part 4. Correlation between *in vitro* cosmetic activities and bioactive compounds

4.1 Correlation between free radical scavenging activities and bioactive compounds

4.1.1 Fruiting body extracts

From the results in Part 2, bioactive compounds of the Boletes mushroom extracts could be divided into 3 groups (high, medium and low contents). Total phenolic content of fruiting body extracts (26.7-113.17 mg GAE/g) was divided into high (more than 80 mg GAE/g), medium (40-80 mg GAE/g) and low (less than 40 mg GAE/g) phenolic content groups. Their total protein content (102.61-542.50) mg BSAE/g) was also divided into 3 groups such as high (more than 300 mg BSAE/g), medium (150-300 mg BSAE/g) and low (less than 150 mg BSAE/g) protein content groups. Moreover, their total carbohydrate content (50.28-357.67 mg GE/g) was divided into 3 groups including high (more than 800 mg GE/g), medium (200-800 mg GE/g) and low (less than 200 mg GE/g). Group of their total flavonoid content was not divided because of very low content.

For antioxidant activities as reported in Part 3, DPPH (43.72-93.89%) and ABTS (36.58-99.76%) scavenging activities could be divided into 3 groups such as high (more than 80%), medium (60-80%) and low (less than 60%) activities groups.

According to Figure 21(a) and Table 11, fruiting body extracts showed significantly good correlation (r=0.760) between total phenolic content and DPPH radical scavenging activity. This result agreed with the previous study that phenolic compounds affected to DPPH radical scavenging activity by their chemical structure (Dai and Mumper, 2010). Phenolic compounds have hydroxyl groups that can donate a hydrogen atom or an electron to free radicals. Moreover, they have extended conjugated aromatic system to delocalize an unpaired electron (Dai and Mumper, 2010). Total flavonoid content of the extracts showed insignificantly medium correlation with DPPH scavenging activity

(r=0.622) (Figure 22(b)). This result was inconsistent with the previous study that showed high correlation between DPPH radical scavenging activity and total flavonoid content (Andrea Bunea et al., 2011). The result may be because of low flavonoid content of fruiting body extracts or different types of flavonoid compounds in each extract resulting in different DPPH scavenging activity. For carbohydrate content, no correlation between this content and DPPH scavenging activity was observed as shown in Figure 21(c). Protein contents in fruiting body extracts insignificantly correlated (r=0.726) with DPPH radical scavenging activity (Figure 21 (d)). This correlation may be because of carboxyl or hydroxyl group of amino acid that could act as good scavenger (Okada and Okada, 1998).

From Figure 21, the outliers of each plot were observed and they may be a cause of low correlation between mostly bioactive compounds and % DPPH inhibition. The extracts being the outliers for the plots of total phenolic, flavonoid and protein compounds showed low content of bioactive compounds and low DPPH scavenging activity (TISTR 31 FB and TISTR 50 FB), whereas those for the plot of carbohydrate content showed medium content but low % DPPH inhibition (TISTR 14 FB, TISTR 50 FB and TISTR 56 FB). For example, when the graphs were conducted for only high and medium % DPPH inhibition groups (more than 60% DPPH inhibition), the correlation between total phenolic content (r=0.975) or total flavonoid content (r=0.892) or total protein content (r=0.901) and % DPPH inhibition were significantly higher. This result may be due to variety of DPPH scavenging activity of low phenolic content group (TISTR 31 FB, TISTR 37 FB and TISTR 50 FB extracts). TISTR 37 FB extract showed high DPPH scavenging activity but TISTR 31 FB and TISTR 50 FB extracts did not. This result may be occurred from the different presence of potent phenolic or flavonoid or protein compounds in fruiting body extracts that could inhibit DPPH radical (Okada and Okada, 1998; Khan et al., 2012). For carbohydrate content, the correlation was higher (r= 0.756) when it was conducted for only low carbohydrate content group (less than 200 mg GE/g). This result may be due to variety of DPPH scavenging activity of medium carbohydrate content group. For example, TISTR 14 FB and TISTR 56 FB extracts showed good activity but TISTR

50 FB did not. In addition, it may result from different types of carbohydrate compounds in each extract. Polysaccharides showed more antioxidant activity than monosaccharide due to the structure of polysaccharide that can donate hydrogen atom from one of the internal monosaccharide units (B. J. Wang et al., 2005).

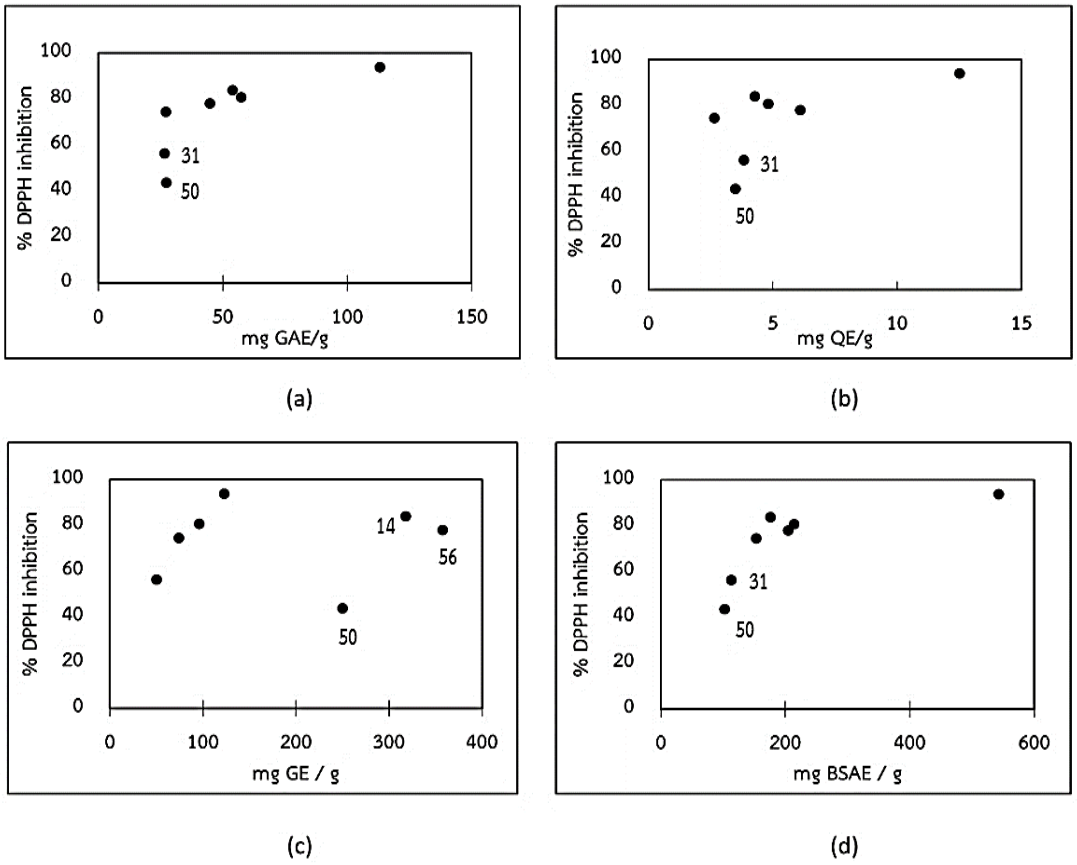

Figure 21 Plot between % DPPH inhibition and content of bioactive compounds; total phenolic content (a), total flavonoid content (b), total carbohydrate content (c) and total protein content (d) of fruiting body extracts from Boletes mushrooms

For ABTS radical scavenging activity, its correlation with bioactive compounds of fruiting body extracts is presented in Figure 22 and Table 11. It showed low correlation between this activity and all of bioactive compounds except total carbohydrate content which showed no correlation. The result was similar to the previous study that showed low correlation between ABTS scavenging activity and total phenolic content of plant extracts (Pukumpuang, 2012). For flavonoid content in fruiting body extracts, low correlation disagreed with the previous study that reported high correlation between flavonoid content and ABTS radical scavenging activity of wheat (*Triticum aestivum L.*) (Y. Li et al., 2015). Low flavonoid content in Boletes mushroom extracts may result in no clear effect of this content on ABTS scavenging activity.

The plots of correlation between % ABTS inhibition and bioactive compounds were observed the same outliers as the result of % DPPH inhibition. However, the correlations between % ABTS inhibition and the content of bioactive compounds did not higher when they were conducted for only high and medium % ABTS inhibition groups. So, it could be assumed that the content of bioactive compounds (phenolic, flavonoid and protein compounds) of Boletes mushroom extracts tested did not affect their ABTS scavenging activity. Fruiting body extracts may have other compounds such as organic acid that could react with ABTS radical (Carvajal et al., 2012). For carbohydrate compound, the correlation was higher (r=0.823) when it was conducted for only low carbohydrate content group (less than 200 mg GE/g). The effect of total carbohydrate content of the Boletes mushroom extracts on ABTS scavenging activity was similar to the result of DPPH scavenging activity as discussed above. The medium carbohydrate content groups including TISTR 14 FB, TISTR 50 FB and TISTR 56 FB extracts showed different ABTS scavenging activity. TISTR 56 FB extract had higher carbohydrate content (357.67 mg GE/g) than TISTR 14 FB (317.82 mg GE/g) but it showed lower ABTS scavenging activity (77.63%) than TISTR 14 FB extract (92.70%). TISTR 50 FB also had medium carbohydrate content (250.15 mg GE/g) but it showed low % ABTS inhibition (43.14%).

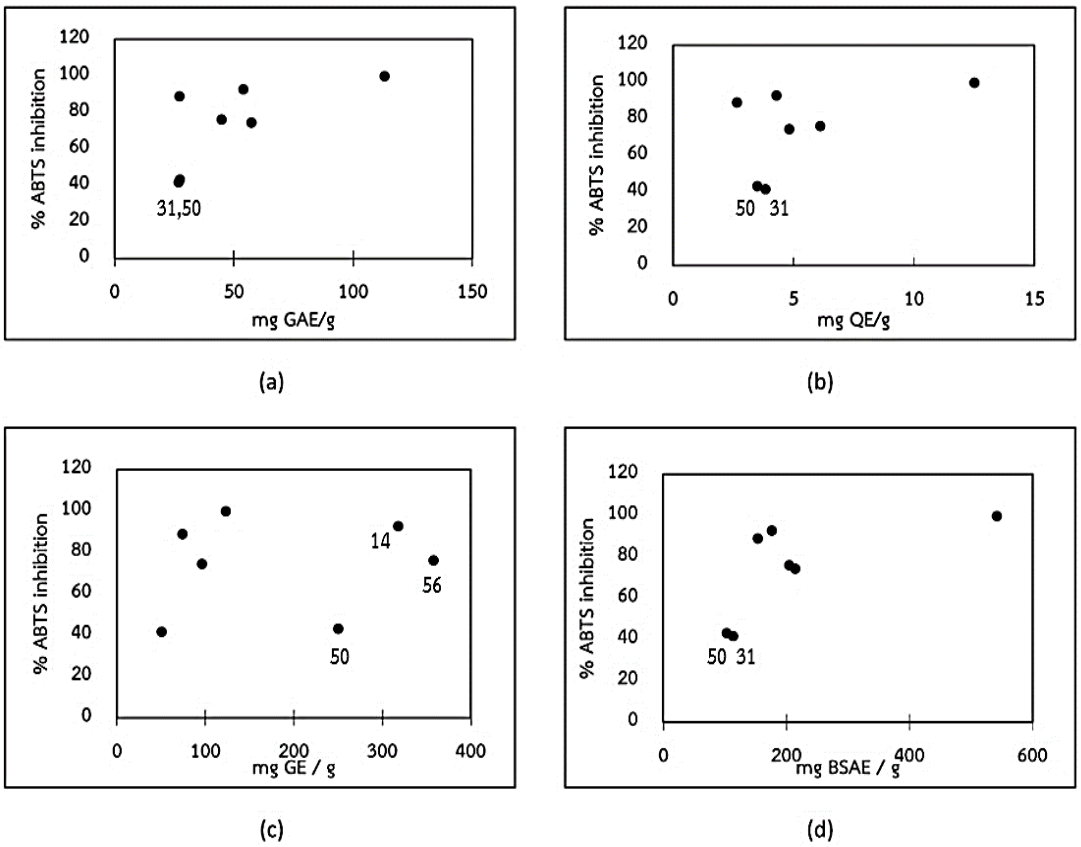

Figure 22 Plot between % ABTS inhibition and content of bioactive compounds; total phenolic content (a), total flavonoid content (b), total carbohydrate content (c) and total protein content (d) of fruiting body extracts from Boletes mushrooms

Table 11 Pearson's coefficient for the correlation between bioactive compounds and free radical scavenging activities of fruiting body extracts from Boletes mushrooms (*Significant at $p < 0.05$)

Bioactive compounds	Pearson's correlation coefficient (r)	
	DPPH	ABTS
Total phenolic content	0.760*	0.655
Total flavonoid content	0.622	0.501
Total carbohydrate content	0.039	0.109
Total protein content	0.726	0.646

4.1.2 Culture broth extracts

The correlation between free radical scavenging activities (DPPH and ABTS assays) and bioactive compounds including total phenolic, total carbohydrate and total protein contents of culture broth extracts from Boletes mushrooms is presented in Figure 23 and Table 12. The culture broth extracts showed insignificant correlation between TPC and free radical scavenging activities in both assays showed. For DPPH scavenging activity, the correlation was insignificantly increased (r=0.952) when it was plotted for only TISTR 31 CB, TISTR 37 CB and TISTR 36 CB extracts. TISTR 55 CB extract had very low TPC (12.31 mg GAE/g) but it showed DPPH scavenging activity similar to TISTR 36 CB extract that had TPC 48.07 mg GAE/g extract. The correlation between ABTS scavenging activity and TPC was also insignificantly increased (r=0.899) when it was conducted only TISTR 31 CB, TISTR 37 CB and TISTR 55 CB extracts. TISTR 36 CB extract had the highest TPC (48.07 mg GAE/g), whereas it did not show the highest ABTS scavenging activity. Although the correlation was increased when the plot was cut the outlier from the graph, it showed insignificant correlation between free radical scavenging activities on

both assays and TPC of culture broth extracts as discussed in correlation between DPPH scavenging activity and TPC of fruiting body extracts. Number of the culture broth extracts may be too less to observe the correlation in the present study.

From Figure 23(c-d) and Table 12, no correlation between free radical scavenging activities and total carbohydrate content of culture broth extracts was observed. For DPPH scavenging activity, the correlation with their total phenolic content was higher (r=0.951) when it was conducted for only TISTR 31 CB, TSITR 36 CB and TISTR 55 CB extracts. TISTR 37 CB extract was the outlier of this plot. It had high carbohydrate content but low DPPH scavenging activity. This result may be due to content of dextrose from culture media that affected the content of carbohydrate compound in this extracts. In addition, it may be influenced from type and content of carbohydrate compounds that could react with DPPH or ABTS radicals as discussed in section 4.1.1.

As reported in Figure 23(e-f) and Table 12, DPPH scavenging activity of culture broth extracts showed insignificantly good correlation (r=0.886) with total protein content, whereas ABTS scavenging activity showed lower correlation (r=0.627). In addition, the correlation between DPPH scavenging activity and total protein content was insignificantly increased (r=0.928) when it was conducted for only TISTR 31 CB, TISTR 37 CB and TISTR 55 CB extracts. TISTR 36 CB extract had the highest total protein content (112.06 mg BSAE/g) but showed DPPH scavenging activity similar to TISTR 55 CB extract that had total protein content of 87.71 mg BSAE/g. For ABST scavenging activity, the correlation with total protein content was insignificantly increased (r=0.993) when it was plotted for only TISTR 36 CB, TISTR 37 CB and TISTR 55 CB extracts. TISTR 31 CB extract had low total protein content (42.75 mg BSAE/g) but showed the highest ABTS scavenging activity. This result may be due to the influence of other bioactive compounds in the extracts or the presence potent protein compounds showing free radical scavenging activity by hydroxyl groups or carboxyl groups of amino acid (Okada and Okada, 1998).

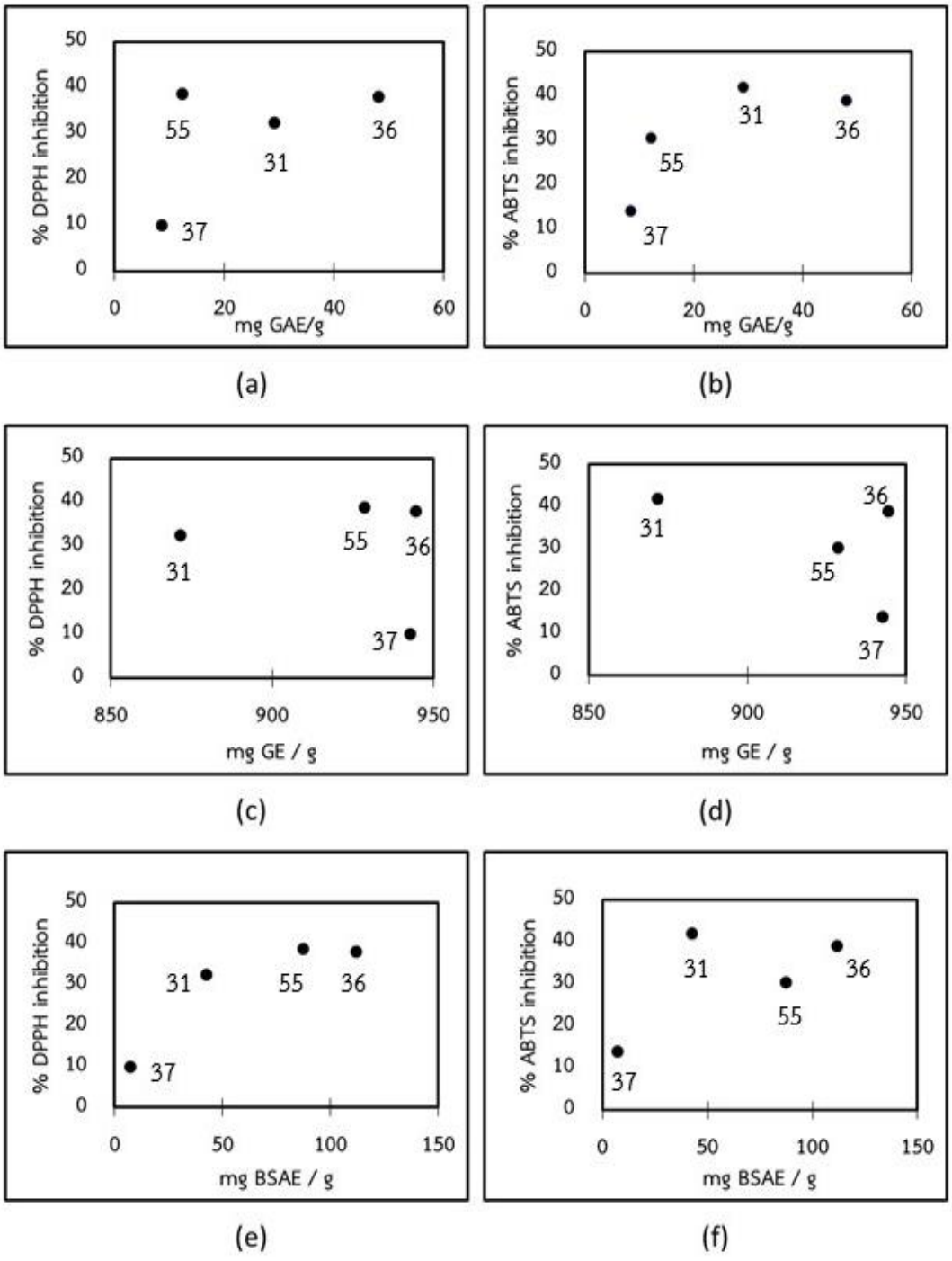

Figure 23 Plots between free radical scavenging activity including DPPH (left) and ABTS (right) and bioactive compounds; total phenolic content (a,b), total carbohydrate content (c,d) and total protein content (e,f) of culture broth extracts from Boletes mushrooms

Table 12 Pearson's coefficient for the correlation between bioactive compounds and free radical scavenging activities of culture broth extracts from Boletes mushrooms

Bioactive compounds	Pearson's correlation coefficient (r)	
	DPPH	ABTS
Total phenolic content	0.563	0.759
Total carbohydrate content	-0.217	-0.567
Total protein content	0.886	0.627

4.2 Correlation between anti-collagenase activity and bioactive compounds

From the result in Part 3., the anti-collagenase activity of fruiting body extracts from Boletes mushroom (10.92-63.81%) could be divided into 3 groups: high (more than 60%), medium (40-60%) and low (less than 40%) activity groups. Positive values of % collagenase inhibition were plotted against different bioactive compounds of the fruiting body extracts at the concentration of 500 µg/ml as shown in Figure 24. Table 13 shows no correlation between anti-collagenase activity and all of bioactive compounds. This result disagreed with the previous study that reported moderate correlation between total phenolic content and anti-collagenase activity of Andean fruits (Bravo et al., 2016). From Figure 24, it was observed that values of TISTR 37 FB extract was the outlier of every plot. When the plots were conducted for only low anti-collagenase activity group (TISTR 55 FB, TISTR 36 FB and TSITR 31 FB extracts), the correlations were insignificantly increased in total phenolic content (r=0.886), total flavonoid content (r=0.741), total carbohydrate content (r=0.986) and total protein content (r=0.818). TISTR 37 FB extract had low values of all bioactive compounds investigated but showed the highest anti-collagenase activity. This effect may be due to the presence of potent phenolic compounds that could react with collagenase enzyme (Madhan et al., 2007). Phenolic compounds have hydroxyl group that can act as hydrogen bond donors or acceptors with backbone functional

group including hydroxyl, amino and carboxyl groups of collagenase enzyme (Madhan et al., 2007). In addition, TISTR 37 FB may contain other bioactive compounds that can act as a collagenase inhibitor by other mechanisms.

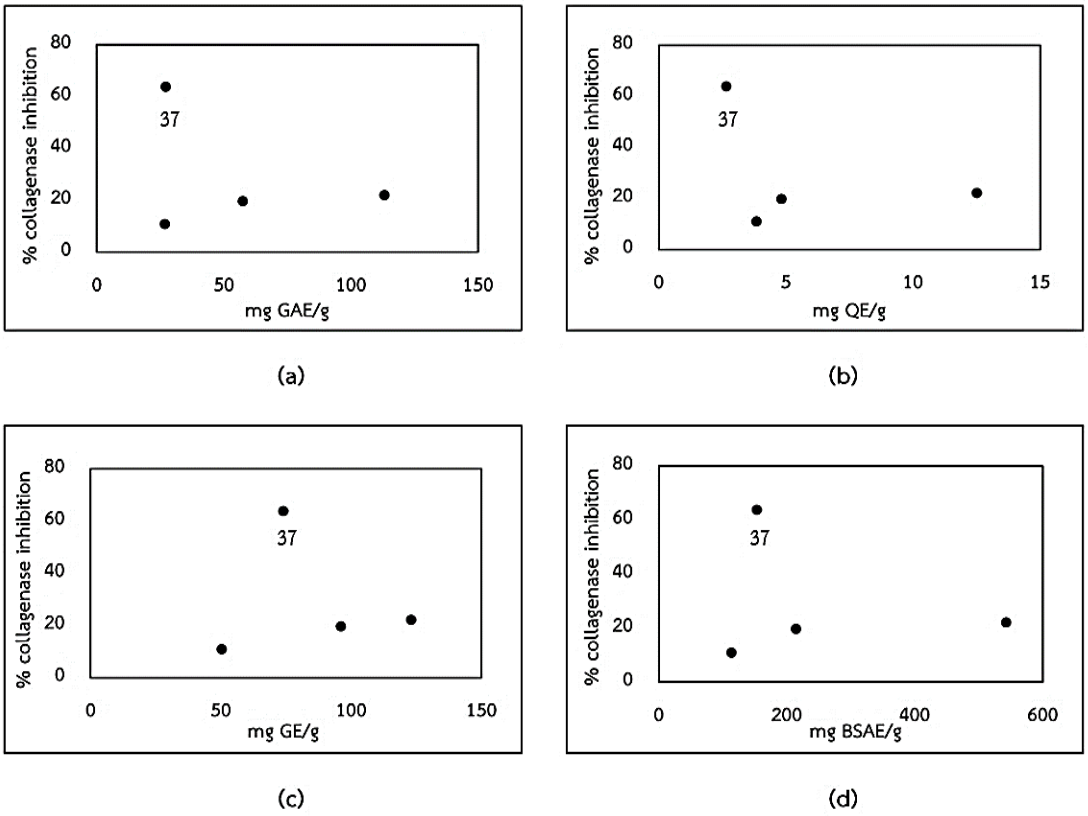

Figure 24 Plots between anti-collagenase activity and bioactive compounds; total phenolic content (a), total flavonoid content (b), total carbohydrate content (c) and total protein content (d) of fruiting body extracts from Boletes mushrooms

Table 13 Pearson's coefficient for the correlation between anti-collagenase activity and bioactive compounds of fruiting body extracts from Boletes mushrooms

Bioactive compounds	Total phenolic content	Total flavonoid content	Total carbohydrate content	Total protein content
Anti-collagenase activity	-0.307	-0.353	-0.056	-0.187

4.3 Correlation between anti-elastase activity and bioactive compounds

From the result of anti-elastase activity (26.46-44.44%) of fruiting body extracts from Boletes mushrooms in Part 3, it could be divided into 3 groups including, high (more than 60%), medium (40-60%) and low (less than 40%) activity groups. Percentage of elastase inhibition was plotted against all of bioactive compounds of the extracts at the concentration of 500 µg/ml as shown in Figure 25. From Table 14, there was no correlation between anti-elastase activity and all of bioactive compounds. However, low correlations were observed in total phenolic content (r=0.349), total flavonoid content (r=0.361) and total protein content (r=0.374) when the plots were performed on only low anti-elastase activity group (TISTR 31 FB, TISTR 36 FB and TISTR 55 FB extracts). TISTR 37 FB extract was the outlier in every plot as discussed in section 4.2. The result of phenolic content disagreed with the previous study that reported moderate correlation between anti-elastase activity and phenolic content of fruit of Andean plants (Bravo et al., 2016). Similar to the correlation with anti-collagenase activity, the result may be due to difference in content of potent phenolic compounds that could react with elastase enzyme in each extract (J. H. Kim et al., 2009; Popoola et al., 2015). Flavonoid compounds could be act as elastase inhibitors because the structure of

flavonoid compounds including quercetin and myricetin had catechol group that significantly inhibited elastase enzyme (Kanashiro et al., 2007).

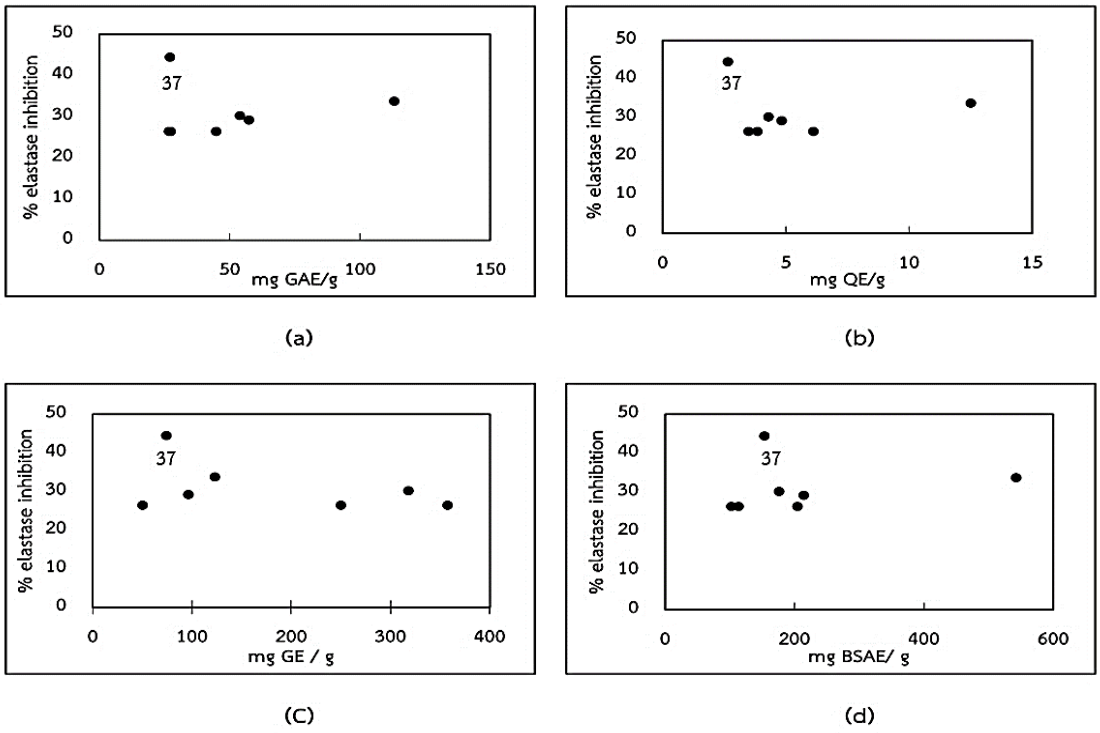

Figure 25 Plots between anti-elastase activity and bioactive compounds; total phenolic content (a), total flavonoid content (b), total carbohydrate content (c) and total protein content (d) of fruiting body extracts from Boletes mushrooms

Table 14 Pearson's coefficient for the correlation between anti-elastase activity and bioactive compounds of fruiting body extracts from Boletes mushrooms

Bioactive compounds	Total phenolic content	Total flavonoid content	Total carbohydrate content	Total protein content
Anti-elastase activity	-0.105	-0.129	-0.681	0.041

From the results of correlation study in the present study, they could be assumed that total phenolic content may be used as a parameter for finding new antioxidant compounds from Boletes mushroom extracts. For other bioactive compounds including flavonoid, carbohydrate and protein, they were not suitable for using as a parameter to screen new antioxidants. In addition, there were no correlation between bioactive compounds and activities of anti-collagenase and anti-elastase. Therefore, total phenolic content, total flavonoid content, total carbohydrate content and total protein content could not be used as parameters for screening new collagenase inhibitors and elastase inhibitors.

In the present study, the Boletes mushroom extracts obtained from different parts including fruiting body and culture broth showed different contents of bioactive compounds and the cosmetic activities. Fruiting body extracts from Boletes mushrooms investigated had interesting cosmetic activities especially TISTR 37 FB and TISTR 55 FB extracts. TISTR 37 FB extract presented good free radical scavenging activity, anti-collagenase activity and anti-elastase activity. TISTR 55 FB extract exhibited potent DPPH and ABTS scavenging activities. These cosmetic activities of Boletes mushroom extracts may be result from the potent bioactive compounds (phenolic, flavonoid or protein compounds), combination of many bioactive compounds or other bioactive compounds in the extracts.

CHAPTER V
CONCLUSION

In this study, fruiting body extracts and culture broth extracts from Boletes mushroom locally grown in Thailand were prepared. They were determined for the content of bioactive compounds including phenolic, flavonoid, carbohydrate and protein compounds. In addition, their *in vitro* cosmetic activities including DPPH and ABTS free radical scavenging activities, anti-tyrosinase activity, anti-collagenase activity and anti-elastase activity were also evaluated and compared between fruiting body extracts and culture broth extracts. The correlation between their *in vitro* cosmetic activities and bioactive compounds was investigated.

The results obtained in this study can be summarized as follows:

1. Yield of ethanolic extracts from fruiting body of Boletes mushrooms was varied from 2.56-3.40% by weight of the fresh mushrooms, whereas yield of culture broth extracts was ranged from 9.56-17.66 gram of dried extract per liter of media.
2. Boletes mushroom extracts obtained from fruiting body had higher contents of phenolic compounds (8.60-113.17 mg GAE/g), flavonoid compounds (2.65-12.52 mg QE/g) and protein compounds (7.34-542.50 mg BSAE/g) than the culture broth extracts. For carbohydrate compounds, the culture broth extracts (871.79-944.53 mg GE/g) presented more content than the fruiting body extracts (50.28-357.67 mg GE/g).
3. The DPPH (9.85-93.89%) and ABTS (13.88-99.76%) scavenging activities of fruiting body extracts at concentration of 100 µg/ml were higher than those of the culture broth extracts. TISTR 55 FB extract showed the highest DPPH and ABTS inhibition. Moreover, this extract showed the lowest IC_{50} values of DPPH (13.62 µg/ml) and ABTS (23.58 µg/ml) scavenging activities.
4. All of Boletes mushroom extracts investigated showed no anti-tyrosinase activity except TISTR 36 FB extract.

5. Anti-collagenase activity of Boletes mushroom extracts at concentration of 500 µg/ml showed no effect or low to medium activity. TISTR 37 FB extract exhibited the highest percentage of collagenase inhibition.

6. Anti-elastase activity of Boletes mushroom extracts at concentration of 500 µg/ml showed no effect or low to medium activity. TISTR 37 FB extract showed the highest anti-elastase activity.

7. The good correlation between total phenolic content and DPPH scavenging activity of fruiting body extracts was significantly found. However, there was no or low correlation between other biological activities and other bioactive compounds of Boletes mushroom extracts.

The extracts from Boletes mushrooms locally grown in Thailand presented interesting cosmetic activities and bioactive compounds. The results exhibited that the fruiting body extracts of TISTR 37 FB and TISITR 55 FB showed promising cosmetic activities that can be developed as new active ingredients in cosmetic products. TISTR 55 FB extract had very potent antioxidant activity, whereas TISTR 37 FB extract showed multifunctional effect including antioxidant, anti-collagenase and anti-elastase activities. The extracts of TISTR 37 FB and TISTR 55 FB should be evaluated for physicochemical properties to further develop as cosmetic products. For culture broth extracts, however, properties of these extracts may be resulted from unsuitability of culture condition affecting secondary metabolites secreted from mycelia into broth. Therefore, the effect of culture condition on properties of the Boletes mushroom extracts from culture broth should be further investigated.

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APPENDIX

PREPARATION OF REAGENTS

1. **Reagents preparation for Folin-Ciocalteau method (Determination of total phenolic content)**

   a) Preparation of 75 g/L of sodium carbonate solution

   Sodium carbonate (3.75 g) was dissolved in 50 ml of distilled water.

   b) Preparation of 10% v/v Folin-Ciocalteau (FC) reagent

   FC reagent (1ml) was mixed with 9 ml of distilled water.

   c) Preparation of a standard compound (gallic acid)

   Stock solution (2 mg/ml) was prepared by dissolving gallic acid (20 mg) with absolute ethanol (10 ml). Then, 1000 µl of this stock solution was adding into 10 ml of absolute ethanol to prepare standard curve at the concentration range between 4-6 µg/ml.

   d) Preparation of a test sample solution

   The sample solutions were prepared by sonicating the mushroom extracts with 70 % v/v ethanol for fruiting body extracts and distilled water for culture broth extracts in water for 3-5 minutes to make initial concentration of 10 mg/ml. Stock solution was diluted with 70% v/v ethanol (fruiting body extracts) or distilled water (culture broth extracts) for preparing various concentration before adding into each well.

2. **Reagents preparation for aluminium chloride complex (Determination of total flavonoid content)**

   a) Preparation of 10% w/v aluminium chloride solution

   Aluminium chloride (1 g) was dissolved in 10 ml of distilled water.

   b) Preparation of 1 M potassium acetate (MW 98.15)

   Potassium acetate (4.0975 g) was dissolved in 50 ml of distilled water.

   c) Preparation of a standard solution (quercetin)

To prepare stock solution (2 mg/ml), quercetin (20 mg) was dissolved in 10 ml of absolute ethanol. Then, working solution (500 µg/ml) was prepared by adding 2.5 ml of stock solution into 10 ml of absolute ethanol for preparation of standard curve at the concentration range between 3-15 µg/ml.

d) Preparation of test samples

The sample solutions were prepared by sonicating the mushroom extracts with 70% v/v ethanol for fruiting body extracts and distilled water for culture broth extracts in water for 3-5 minutes to make initial concentration of 15 mg/ml before adding into 96-well plate.

3. Reagents preparation for Phenol-sulfuric assay (Determination of total carbohydrate content)

a) Preparation of 5% w/v phenol solution

Phenol (5 g) was dissolved in distilled water 100 ml

b) Preparation of a standard compound (glucose)

Glucose (5 mg) was dissolved in 10 ml of distilled water. Then, this stock solution (500 µg/ml) was diluted with water to prepare standard curve at the concentration range between 2-18 µg/ml.

c) Preparation of test samples

The sample solutions were prepared by sonicating the mushroom extracts with 70 % v/v ethanol for fruiting body extracts and distilled water for culture broth extracts for 3-5 minutes to make initial concentration of 2 mg/ml before adding into a test tube.

4. Reagents preparation for Lowry assay (total protein content)

a) Preparation of 50% v/v Folin-Ciocalteau solution

Folin-Ciocalteau reagent (5 ml) was mixed with 5 ml of distilled water.

b) Preparation of alkaline copper sulfate reagent

The reagent mixture was prepared as follow:

Solution A :2% w/v sodium carbonate in 0.1 sodium hydroxide with 0.5% w/v sodium dodecyl sulfate

Solution B: :0.5% w/v copper (II) sulfate pentahydrate in 0.1% w/v potassium tartrate

Analytical reagent was prepared by mixing 50 ml of solution A and 1 ml of solution B.

c) Preparation of a standard compound (Bovine Serum Albumin; BSA)

Stock solution (500 µg/ml) was prepared by dissolving BSA (5 mg) in 10 ml of distilled water. For preparation of standard curve at the concentration range between 5-30 µg/ml, this stock solution was diluted with water to give various concentration.

d) Preparation of a test sample solution

The sample solutions were prepared by sonicating the mushroom extracts with 70 % v/v ethanol for fruiting body extracts and distilled water for culture broth extracts for 3-5 minutes to make initial concentration of 2 mg/ml before adding into a test tube.

5. Reagents preparation for DPPH assay

a) Preparation of $6x10^{-5}$M DPPH (2,2-diphenyl-1-picryl-hydrazyl) solution (MW 394.3)

DPPH (1.18 mg) was dissolved in 50 ml of absolute ethanol.

b) Preparation of positive control (ascorbic acid, gallic acid)

Stock solution (1 mg/ml) was prepared by dissolving substance (10 mg) with 10 ml of absolute ethanol. Then, this stock solution was diluted with absolute ethanol to give the final concentration range between 0.5-2 µg/ml.

c) Preparation of test sample solution

The test samples were prepared with initial concentration of 500 µg/ml in 70% v/v ethanol for the fruiting body extracts and in distilled water

for the culture broth extracts. For screening DPPH scavenging activity, the extract solutions were diluted with 70% v/v ethanol or distilled water to give the final concentration of 100 µg/ml in each well.

6. Reagents preparation for ABTS assay

a) Preparation of ABTS solution

The ABTS reagent was prepared by mixing 5 ml of 7 mM ABTS (2,2-azino-biethylbenzthiazoline-6 sulfonic acid) solution (MW 548.7) with 88 µl of 140 mM potassium persulfate solution (MW 270.22). Then, the mixed solution was kept in darkness at room temperature for 16 hours to all free radical generation and was diluted with distilled water (1:49, v/v) to obtain the absorbance 0.700 ± 0.05 at 734 nm.

b) Preparation of a positive control (ascorbic acid)

Stock solution (1 mg/ml) was prepared by dissolving ascorbic acid (10 mg) in 10 ml of distilled water. Then, stock solution was diluted with distilled water for preparing various concentration (0.5-2 µg/ml) before adding into each well.

c) Preparation of the test samples

The test samples were prepared with initial concentration of 1000 µg/ml in 70% v/v ethanol for the fruiting body extracts and in distilled water for the culture broth extracts. For screening ABTS scavenging activity, the extracts solutions were added in 70% v/v ethanol or distilled water to give the final concentration of 100 µg/ml in each well.

7. Reagents preparation for anti-tyrosinase activity assay

a) Preparation of a phosphate buffer solution (pH 6.8)

Solution A : 20 mM of sodium phosphate dibasic (312 mg) was dissolved in 100 ml of distilled water.

Solution B : 20 mM of di-sodium hydrogen phosphate anhydrous (284 mg) was dissolved in 100 ml of distilled water.

Then, solution A and B were mixed to obtain phosphate buffer solution pH 6.8

b) Preparation of 0.85 mM L-DOPA (3,4-dihydroxy-L-phenylalanine) solution (MW 197.19)

L-DOPA (0.8 g) was dissolved in 5 ml of 20 mM phosphate buffer (pH 6.8).

c) Preparation of 480 unit/ml mushroom tyrosinase solution (labeled activity 5771 unit/mg)

Mushroom tyrosinase (0.83 mg) was dissolved in 10 ml of 20 mM phosphate buffer (pH 6.8).

d) Preparation of a positive control (kojic acid)

Stock solution (1 mg/ml) was prepared by dissolving kojic acid (10 mg) in 10 ml of distilled water. This stock solution was diluted with 20 mM phosphate buffer to prepare various concentration (15-60 µg/ml) before adding into 96-well plate.

e) Preparation of a test sample solution

The sample solution was prepared by sonicating the extracts with dimethyl sulfoxide (DMSO) for dissolving the fruiting body extracts and with distilled water for the culture broth extracts for 3-5 minutes to make initial concentration of 10 mg/ml. For screening tyrosinase inhibitory of Boletes mushroom extracts, the sample solutions were diluted with 20 mM phosphate buffer to give final concentration of 100 and 500 µg/ml in each well.

8. Reagents preparation for anti-collagenase activity assay (Enzchek® gelatinase/collagenase assay kit E-12055)

a) Preparation of 1X reaction buffer (200ml)

10X reaction buffer (20 ml) was diluted in 180 ml distilled water.

b) Preparation of DQ™ gelatin solution

A DQ™ gelation stock solution (1.0 mg/ml) was prepared by adding 1 ml of distilled water directly to the vials containing the lyophilized substrate. Then, 125 µg/ml stock solution was prepared by adding 1 ml of this stock solution to 7 ml of 1X reaction buffer. When 20 µl of this solution was used, the final concentration of DQ™ gelatin solution in each well was 12.5 µg/ml.

c) Preparation of *Clostridium* collagenase solution

A 1000 U/ml stock solution of the *Clostridium* collagenase was prepared by adding 0.5 ml of distilled water directly to the vial containing lyophilized collagenase enzyme. Then, 0.4 U/ml working stock solution was prepared by adding 20 µl of the stock solution (1000U/ml) into 50 ml of 1X reaction buffer. When 100 µl of this solution was used, the final concentration of collagenase solution in each well was 0.2 U/ml.

d) Preparation of 1,10-phenanthroline (collagenase inhibitor)

1,10-phenanthroline (9.9 mg) was dissolved with 25 µl of ethanol. A 10 mM stock solution was prepared by adding 10 µl of this stock solution to 2 ml of 1X reaction buffer. Then, 0.05 mg/ml working solution was prepared by adding 16 µl of stock solution into 624 µl of 1X reaction buffer.

e) Preparation of positive control ((-)-epigallocatechin; EGCG)

Stock solution was prepared by dissolving 1 mg of EGCG in 10 ml of distilled water. Then, this stock solution was diluted with 1X reaction buffer for using as positive control.

f) Preparation of test sample solution

For preparation of sample solution, the extracts were sonicated with 70%v/v ethanol (fruiting body extract) or distilled water (culture broth extract) for 3-5 minutes to make initial concentration of 10 mg/ml. The sample solutions were diluted with 1X reaction buffer to give final concentration of 100 and 500 µg/ml in each well for screening anti-collagenase activity of extracts.

9. Reagents preparation for anti-elastase activity assay (Enzchek® elastase assay kit E-12056)

a) Preparation of 1X reaction buffer (60ml)

10X reaction buffer (6 ml) was diluted with 54 ml of distilled water.

b) Preparation of DQ™ elastin solution

A DQ™ elastin stock solution (1000 µg/ml) was obtained by adding 1 ml of distilled water directly to the vial containing lyophilized substrate. Then, 100 µg/ml DQ™ elastin solution was prepared by adding the stock solution (1000 µg/ml) into 9 ml of 1X reaction buffer. When 50 µl of this working solution was used in each well, the final concentration of DQ™ elastin solution was 25 µg/ml.

c) Preparation of an elastase solution

A 100 U/ml stock solution was prepared by adding distilled water directly to vial containing lyophilized elastase enzyme. Then, 0.4 U/ml of working solution was obtained by adding 100 µl of this stock solution (100 U/ml) into 25 ml of 1X reaction buffer. When 100 µl of this working solution was used in each well, the final concentration was 0.2 U/ml

d) Preparation of 1 mM *N-methoxysuccinyl*-alanyl-alanyl-prolyl-valine-*chloromethyl ketone* (CMK) solution as positive control (elastase inhibitor)

The 50 µl of DMSO was directly added into the vial of CMK 1 mM working solution was prepared by adding 20 µl of this stock solution to 380 µl of 1X reaction buffer. Then, this stock solution was diluted with 1X reaction buffer to make 0.04 mM for using as positive control in this assay.

e) Preparation of a positive control (EGCG)

For preparation of stock solution (1 mg/ml), EGCG (1 mg) was dissolved in 10 ml of distilled water. Before adding into 96-well plate, working solution was prepared by diluting this stock solution by 1X reaction buffer.

f) Preparation of a test sample solution

The samples were prepared by sonicating the solid extracts with 70% v/v ethanol for the extracts from fruiting body and with distilled water for the extracts from culture broth in water for 3-5 minutes to make initial concentration of 10 mg/ml. To screen anti-elastase activity of Boletes mushroom extracts, the sample solutions were added in 1X reaction buffer to give final concentration of 100 and 500 µg/ml in each well.

VITA

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