ANTI-MELANOGENESIS AND ANTI-INFLAMMATORY ACTIVITIES OF SELECTED MEDICINAL AND CULINARY MUSHROOMS

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FACULTY OF SCIENCE
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ANTI-MELANOGENESIS AND ANTI-INFLAMMATORY ACTIVITIES OF SELECTED MEDICINAL AND CULINARY MUSHROOMS

ABSTRACT

Ten medicinal and culinary mushrooms which commonly available in Malaysia market, namely *Agaricus bisporus* (white and brown varieties), *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frondosa*, *Hypsizygus marmoreus*, *Lentinula edodes*, *Pleurotus eryngii*, *Pleurotus floridanus* and *Pleurotus pulmonarius* were investigated for their anti-melanogenesis and anti-inflammatory activity in attempt to study their potentials to be used in cosmeceuticals. The mushrooms were extracted with hot water and freeze-dried prior for testing. The anti-melanogenesis activity of mushroom extracts were determined by cell-free mushroom tyrosinase assay, followed by cell viability assay, measurement of intracellular melanin content and cellular tyrosinase assay using B16F10 murine melanoma cells. Whilst, the anti-inflammatory activity of the mushroom extracts was tested by measuring the level of nitric oxide (NO), tumor necrosis factor alpha (TNF-α) and interleukin (IL)-10 excreted by RAW 264.7 murine macrophage cells. Out of the ten extracts, *A. bisporus* (brown), *P. floridanus* and *P. pulmonarius* effectively reduced the intracellular melanin content and cellular tyrosinase activity in B16F10 cells. *A. bisporus* (brown) was the best extract in reducing intracellular melanin content to $57.05 \pm 3.90\%$ at concentration of log 3.0 with no toxicity effects on B16F10 cells. This extract also reduced cellular tyrosinase activity to $17.93 \pm 2.65\%$, which performed better than positive control, kojic acid ($33.81 \pm 7.41\%$). In parallel, *A. bisporus* (brown) extract has appreciable anti-inflammatory activity by reducing the NO and TNF-α level ($66.82 \pm 2.81\%$ and $73.67 \pm 2.97\%$, respectively) at highest concentration tested. A reduction of NO level was also being observed for *P. floridanus* and *P. pulmonarius* extracts at similar

concentrations. Based on the effectiveness of single extract to inhibit melanogenesis and inflammatory response, three combination mushroom extracts comprise of *G. lucidum* with *P. floridanus* or *P. pulmonarius* at 1:1 ratio was formulated and the synergistic effect of these combined extracts was evaluated. The results revealed that combined mushroom extracts did not have synergistic effect in these biological activities. In summary, single extract was more active than the combine extracts. Our findings also showed that *A. bisporus* (brown) extract has potential to be used as natural ingredients in cosmeceutical products.

Keywords: mushrooms, melanogenesis, tyrosinase, inflammatory, cosmeceuticals

AKTIVITI ANTI-MELANOGENESIS DAN ANTI-RADANG BAGI CENDAWAN PERUBATAN DAN MASAKAN YANG TERPILIH

ABSTRAK

Sepuluh cendawan perubatan dan masakan yang biasa di pasaran Malaysia, iaitu *Agaricus bisporus* (jenis putih dan coklat), *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frondosa*, *Hypsizygus marmoreus*, *Lentinula edodes*, *Pleurotus eryngii*, *Pleurotus floridanus* and *Pleurotus pulmonarius* telah dikaji untuk aktiviti anti-melanogenesis dan anti-radang dalam usaha mengenal pasti potensi mereka untuk digunakan dalam kosmeseutikal. Cendawan-cendawan telah diekstrak dengan air panas dan dikering-bekukan sebelum kajian dijalankan. Aktiviti anti-melanogenesis ekstrak cendawan telah ditentukan melalui esei tirosinasa cendawan sel-bebas, diikuti oleh esei viabiliti sel, pengukuran kandungan melanin intrasel dan esei tirosinasa selular menggunakan sel-sel melanoma tikus B16F10. Manakala, aktiviti-aktiviti anti-radang ekstrak-ekstrak cendawan telah diuji dengan mengukur tahap nitrik oksida (NO), faktor nekrosis tumor alfa (TNF-α) dan interleukin (IL)-10 yang dirembeskan oleh sel-sel makrofag tikus RAW 264.7. Daripada sepuluh ekstrak, *A. bisporus* (coklat), *P. floridanus* dan *P. pulmonarius* telah mengurangkan kandungan melanin intrasel dan aktiviti tirosinasa selular pada sel B16F10 secara berkesan. *A. bisporus* (coklat) adalah ekstrak yang terbaik dalam pengurangan kandungan melanin intrasel ke 57.05 ± 3.90% pada kepekatan log 3.0 tanpa kesan toksiksiti pada sel-sel B16F10. Ekstrak ini juga mengurangkan aktiviti tirosinasa selular ke 17.93 ± 2.65%, menunjukkan prestasi yang lebih baik daripada kawalan positif, asid kojik (33.81 ± 7.41%). Pada masa yang sama, ekstrak *A. bisporus* (coklat) mempunyai aktiviti anti-radang yang agak ketara dengan mengurangkan tahap NO dan TNF-α (66.82 ± 2.81% dan 73.67 ± 2.97%, masing-masing) pada kepekatan tertinggi yang diuji. Pengurangan tahap NO juga diperhatikan

dalam ekstrak *P. floridanus* dan *P. pulmonarius* pada kepekatan yang serupa. Berdasarkan keberkesanan ekstrak tunggal untuk menghalang melanogenesis dan tindak balas keradangan, tiga ekstrak cendawan gabungan yang terdiri daripada *G. lucidum* dengan *P. floridanus* atau *P. pulmonarius* pada nisbah 1:1 telah diformulasikan dan kesan sinergi ekstrak gabungan ini telah dinilai. Hasil kajian menunjukkan bahawa ekstrak cendawan gabungan tidak mempunyai kesan sinergi dalam aktiviti-aktiviti biologi ini. Secara ringkas, ekstrak tunggal adalah lebih aktif berbanding dengan ekstrak gabungan. Penemuan kami juga menunjukkan bahawa ekstrak *A. bisporus* (coklat) mempunyai potensi untuk digunakan sebagai bahan semula jadi dalam produk kosmeseutikal.

Kata kunci: cendawan, melanogenesis, tirosinasa, keradangan, kosmeseutikal

ACKNOWLEDGMENTS

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MTT	3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
CO_2	Carbon dioxide
cm	Centimeter
JNK	c-Jun N-terminal kinase
cAMP	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase-2
°C	Degree celcius
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's medium
eNOS	Endothelial nitric oxide synthase
ELISA	Enzyme-linked immunosorbent assay
e.g.	Example
FBS	Fetal bovine serum
g	Gram
g	Gravity
iNOS	Inducible nitric oxide
IFN-γ	Interferon-γ
IL	Interleukin
kDa	Kilo dalton
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	Lipopolysaccharide
μg	Microgram
μg/ml	Microgram per milliliter

μl	Microliter
MITF	Micropthalmia-associated transcription factor
mm	Millimeter
mM	Millimolar
MAP kinase	Mitogen-activated protein kinase
M	Molar
NED	N-(1-napthyl)ethylenediamide dihydrochloride
nm	Nanometer
nM	Nanomolar
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NF-κB	Nuclear factor-kappa B
L-NAME	N_ω-Nitro-L-arginine methyl ester hydrochloride
OD	Optical density
pg	Page
%	Percentage
ONOO−	Peroxynitrite
PMSF	Phenylmethylsulfonyl fluoride
PBS	Phosphate buffered saline
H_3PO_4	Phosphoric acid
pH	Potential of hydrogen
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
AKT	Protein kinase B
rpm	Revolutions per minute

STAT3	Signal transducer and activator of transcription 3
NaOH	Sodium hydroxide
Na$_2$HPO$_4$	Sodium phosphate dibasic
NaH$_2$PO$_4$	Sodium phosphate monobasic
SEM	Standard error of mean
SAPKs	Stress-activated protein kinases
O2 •-	Superoxide radicals
TLR-4	Toll-like receptor-4
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor alpha receptor
TRPs	Tyrosinase related proteins
UV	Ultraviolet light
UVR	Ultraviolet rays
FDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
α-MSH	α-melanocyte stimulating hormone

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Mushroom has been consumed as food since ancient times due to its nutritional value, taste and flavor (Wani *et al.*, 2010). Aside from its ability to combat various diseases, mushrooms also have high potential to be used as natural ingredients for cosmeceutical products (Hyde *et al.*, 2010). In a review by Taofiq *et al.* (2016), numerous extracts and bioactive compounds from mushrooms have high potential to be used in cosmetics, cosmeceuticals and nutricosmetics. Mushrooms have numerous biological activities which beneficial to be developed as cosmeceutical product such as anti-inflammatory (Choi *et al.*, 2014), anti-tyrosinase (Huang *et al.*, 2014), antioxidant (Liu *et al.*, 2014), antimicrobial (Kaur *et al.*, 2016) and anti-collagenase (Bae *et al.*, 2005) activities.

Increased production and accumulation of melanin in the skin could cause acquired hyperpigmentation such as melasma, age spots and freckles (Briganti *et al.*, 2003). As a crucial enzyme for melanogenesis, tyrosinase enzyme is widely used as the target molecules in anti-melanogenesis study (Chaiprasongsuk *et al.*, 2016; Manse *et al.*, 2016). Previously, several medicinal and culinary mushrooms such as *Pleurotus, Flammulina* and *Ganoderma* genus were reported to have good tyrosinase inhibitory activity (Rout & Banerjee, 2007; Chien *et al.*, 2008; Alam *et al.*, 2010; Alam *et al.*, 2012; Nagasaka *et al.*, 2015) and skin whitening potential.

The cosmetic industry generally assumes that inflammation has a negative effect on the condition and appearance of skin (Zhang & Falla, 2009). One of the factor that trigger inflammatory response occur in the skin is by ultraviolet rays (UVR) exposure from the sun (Nicolaou *et al.*, 2011). Nuclear factor-kappa B (NF-κB) is a transcription factor which regulates the expression of several cytokines during inflammation. NF-κB signaling pathway has been widely studied and it is a common target molecule for anti-

inflammatory response (Zhai *et al*., 2016). The inhibition of pro-inflammatory mediators such as nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) were other target molecules to prevent prolong inflammation (Dong *et al*., 2017). Interleukin (IL)-10 has important regulatory effect on inflammatory response due to its capacity to inhibit production of pro-inflammatory cytokines (Malefyt *et al.*, 1991). As an anti-inflammatory cytokines, IL-10 was commonly used as a marker for anti-inflammatory response of the compounds or extracts (Gasparrini *et al*., 2017).

With the increasing worldwide demand for cosmeceutical products, there is a large market of cosmeceutical products awaiting us. The general procedures in the present study are outlined in Figure 1.1.

Objectives of study

The main objectives of the present study were as follows:

i.   to determine the anti-melanogenesis effect of selected mushroom extracts through inhibition of tyrosinase activity and melanin synthesis.

ii. to evaluate the anti-inflammatory activity of selected mushroom extracts and expression of anti-inflammatory-related agents.
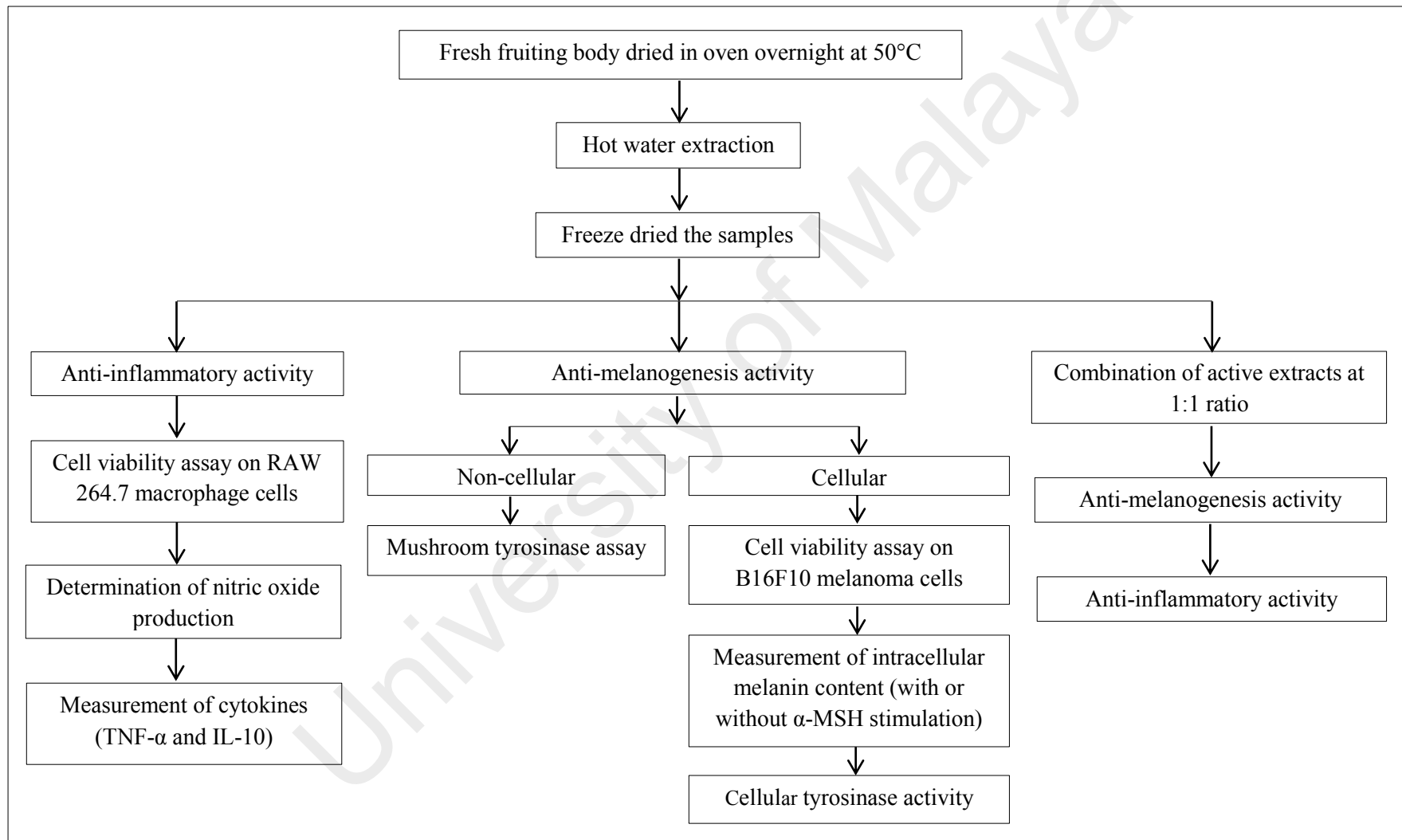
Figure 1.1: Outline of general procedure

CHAPTER 2: LITERATURE REVIEW

2.1 Skin

Skin is a highly organized and differentiated structure which consists of various cell types. The main cells in skin tissue are keratinocytes, fibroblasts and melanocytes (Reemann *et al*., 2014). Skin coats the whole external surface of body and it joins with mucous membranes of digestive system, respiratory system, urogenital system and conjunctiva of eyelids. Skin lining the external auditory meutus of the ear and the external surface of the eardrum (Arda *et al.*, 2014).

The skin has four functional layers, namely stratum corneum, epidermis and dermis and subcutaneous fat (Nash *et al.*, 2007). The stratum corneum is the outermost surface of skin which is critically important in controlling water loss (Ikekawa, 1995). Epidermis is made up of four to five layers of cells which mostly consist of keratinocytes. The average of epidermis thickness is 100 μm, but this varies considerably with body area considered, for instance, the thickness of epidermis on eyelids is 50 μm and 1 mm thickness on the palms and soles (Kanitakis, 2001). The dermis which located beneath the epidermis, is a supportive, compressible and elastic connective tissue protecting the epidermis, its appendages and the vascular and nervous plexuses running through it (Kanitakis, 2001). Subcutaneous fatty layers provide the structural framework of skin in the form of collagen and elastin and it is where the vascular system, which supplies the nutrients to the skin and many appendages like hair follicles, sweat and sebaceous gland reside (Nash *et al.*, 2007). The structure of human skin is shown in Figure 2.1.

Skin is a complex organ due to the diversity of cell types, their interaction and their function (Nash *et al*., 2007). The foremost role of facial skin is certainly to reflect our identity and mood. Besides that, our facial skin becomes the barrier to maintain safe environment that enables an organism to protect the DNA and reproduce it. Facial skin helps to maintain the body content, fluids, proteins and electrolytes which are crucial to maintain the integrity of the body. Facial skin becomes the first line of defense for external environmental hazard and plays vital role in wound healing and repair of injury (Arda *et al*., 2014).
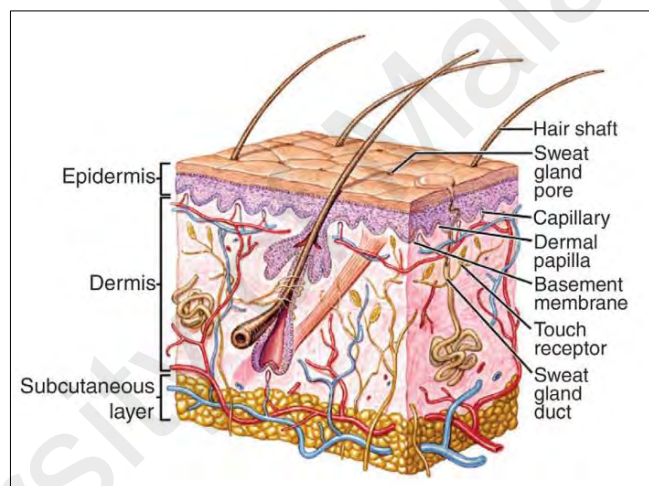
Figure 2.1: Structure of human skin (MacNeil, 2008)

2.2 Skin care and cosmeceuticals

Cosmetic and skin care products are the part of everyday grooming. Applying of cosmetics or beauty products on skin will not cause the skin to change or heal, the products are just meant to cover and beautify (Vaishali *et al.*, 2013). The term 'cosmeceutical' was coined in 1961 by Raymond Reed, founding members of the United State society of Cosmetic Chemist. He originally thought of the word to describe 'active' and science-based cosmetics. Albert Kligman further popularized the word and

concept with the development of prescription-strength tretinoin for the enhance appearance of ultraviolet (UV) damaged and wrinkled skin in 1970s.

A 'drug' is a compound used in the treatment and prevention of disease, or are intended to affect physiologic function or structure of the body. A 'cosmetic' is a substance that cleanses or enhances the appearance of the skin without therapeutic benefit. Cosmeceuticals is a hybrid between drugs and cosmetic product which intended to enhance both health and beauty of the skin through external application (Pieroni *et al.*, 2004). Consumers generally believe cosmeceuticals are regulated and tested as drugs due to their biological activities offer to the consumers (Newburger, 2009).

According to the United States Food and Drug Administration (FDA), the Food, Drugs and Cosmetic Act; a product can be a drug, a cosmetic, or a combination of both, but the term 'cosmeceutical' has no meaning under the law (Mukul *et al.*, 2011). Thus, cosmeceuticals are not legal terms acknowledged by the FDA (Newburger, 2009). Cosmeceutical products are often tested through *in vitro* studies using silicone replicas of skin and, at best, clinical trials are small and open-label studies which usually supported by cosmetic company (Mullaicharam *et al.*, 2013).

Azelaic acid, licorice acid, kojic acid, aloesin and arbutin are the active compounds frequently used in cosmeceutical formulation (Draelos, 2008). Azelaic acid is well known for its antibacterial properties and commonly used in acne cream (Charnock *et al.*, 2004). Licorice extract is commonly used in the cosmeceutical product to reduce inflammation (Aly *et al.*, 2005) while kojic acid is well known for whitening cosmeceuticals and it works through the inhibition of crucial enzyme in melanogenesis (tyrosinase) (Cabanes *et al.*, 1994). Arbutin, vitamin C, alpha tocopherol (vitamin E), niacimide, orchid extract, aloe vera extract, pycnogenol, marine algae extract, cinnamic acid, flavonoids, green tea extract and aloesin are reported to have anti-melanogensis

effect and a number of above compounds have been used as whitening cosmeceuticals (Sarkar *et al.*, 2013).

2.3 Mushrooms

Mushrooms are very large and diversified group of macrofungi belonging to Basidiomycetes and Ascomycetes; with a cell cycle including the formation of sexual spores (Elsayed *et al.*, 2014). According to dictionary of the fungi, there are a total of 97, 330 discovered species of fungi which include slime molds, lichen forming fungi, chromistan fungi, chytridiaceous fungi, yeast, molds and mushroom producing filamentous fungi (Patel *et al.*, 2012). Mushroom is characterized by distinctive fruiting body which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Lindequist *et al.*, 2005). Mushrooms have high nutritional value as they are rich source of carbohydrates, lipids, proteins, amino acids, vitamins, minerals and trace elements (Kalač, 2009; Wang *et al*., 2014).

Traditionally, a wide variety of mushrooms have been used in many different cultures for the maintenance of health as well as the prevention and treatment of diseases through immunomodulatory and anti-cancer properties (Valverde *et al.*, 2015). For thousands of years, mushrooms have been used in cuisine due to their unique flavor which will enhance the taste of food (Badole *et al.*, 2008). Culinary mushrooms not only provide mouth-watering dishes, they are also rich in bioactive compounds which lead to various biological activities such as antioxidant, anti-tyrosinase and anti-inflammatory activities (Barros *et al.*, 2007; Jedinak *et al.*, 2011; Nagasaka *et al*., 2015).

2.3.1 Polysaccharides of mushroom

Polysaccharides from mushroom composed of biopolymers which function in structural support, serve as energy, or excreted extracellularly for cell protection and attachment to other surface (Giavasis, 2014). Polysaccharides can be extracted through several methods such as hot water extraction, dilute alkaline extraction and enzymolysis methods (Shi, 2016). Polysaccharide is made up of long chain monosaccharide units linked through glycosidic bond. Polysaccharide can interconnect through several points to form wide variety of branched or linear structures (Wasser, 2002). Polysaccharide is a major component in mushroom which accounts 30-70% of mushroom dry weight (Cheung, 2013). Mushrooms contain different types of polysaccharide such as chitin, hemicellulose, α- and β-glucans, mannans, xylans and galactans (Singdevsachan *et al.*, 2016). Alpha and beta glucans are among well characterized polysaccharides in fungi (Rodrigues *et al.*, 2011).

Degree of branching, molecular weight and conformation (triple helix, single helix or random coil) determine the biological activity of polysaccharide (Meng *et al.*, 2016). Fungal polysaccharides are well known for their immnomodulatory and antitumor activity (Ma *et al.*, 2014; Li *et al.*, 2016; Minato *et al.*, 2016; Castro-Alves *et al.*, 2017). Immunomodulatory activity of heteroglycan from *Lentinus fusipes* worked through NO stimulation in macrophage cells (Manna *et al.*, 2017). An 899 kDa polysaccharide from *Grifola frondosa* stimulated immune response in macrophage by enhancing proliferation and phagocytosis activity (Ma *et al.*, 2015). Polysaccharide from *Pleurotus eryngii* inhibited the growth of implanted tumor in mice and improved the immune function (Liu *et al.*, 2015). Other biological activities reported for mushroom polysaccharide were anti-diabetic (Zhang *et al.*, 2016), anti-coagulant (Román *et al.*, 2016), antitumor (Zhao *et al.*, 2016), antioxidant (Thetsrimuang *et al.*, 2011), anti-inflammatory (Ruthes *et al.*, 2013) and prebiotic (Synytsya *et al.*, 2009).

For cosmeceutical applications, polysaccharide extracts are known for their hydrating properties on the skin due to hydroxyl groups which generally interact strongly with water (Semenzato *et al.*, 2014). Mushroom polysaccharides exhibited good anti-aging, anti-inflammatory, antimicrobial and antioxidant activities which is suitable for cosmeceutical application (Chen *et al.*, 2012; Zhong *et al.*, 2013; Li & Shah, 2016; Dong *et al.*, 2017).

2.3.2 Mushroom samples used in this study

2.3.2.1 *Agaricus bisporus*

The appearances of *A. bisporus* (brown and white variety) are shown in Figure 2.2 and 2.3, respectively. *A. bisporus* which is known as table mushroom, cultivated mushroom or button mushroom, is a culinary basidiomycete fungus. It is one of the most widely cultivated mushrooms in the world. The original wild form of *A. bisporus* bears a brownish cap and dark brown gills (brown button mushroom), but more familiar is the current variant with a white form, having white cap, stalk and flesh and brown gills (white button mushroom) (Jagadish *et al.*, 2009).

A. bisporus is considered as a valuable health food with high contents of polyphenols, ergothioneine, vitamins and minerals (Dubost *et al.*, 2007). Moreover, Thangthaeng, *et al.* (2015) reported that consumption of *A. bisporus* mushroom improved memory function in rats. Semi-purified polysaccharide extracts from *A. bisporus* contain (1→6),(1→4)-linked α-glucan, (1→6)-linked β-glucan, and mannogalactan which possessed immunomodulatory effects on human monocytic cells (Smiderle *et al.*, 2011). Compounds such as catechin, caffeic acid, ferulic acid and myricetin from *A. bisporus* may contribute to the potent antioxidant activity of this mushroom (Liu *et al.*, 2013). White button mushrooms exhibited anti-proliferative, pro-apoptotic properties and inhibits prostate tumor growth in mice (Adams *et al.*,

2008). White button mushrooms also able to lower blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats (Jeong *et al*., 2010).

Figure 2.2: The appearance of *A. bisporus* (brown)

Figure 2.3: The appearance of *A. bisporus* (white)

2.3.2.2 *Flammulina velutipes*

F. velutipes is known as golden needle mushroom or enokitate (Jing *et al*., 2014). It is one of the most popular culinary fungi and widely cultivated in the world due to its attractive taste and high nutritional values (Johansen *et al*., 2005). *F. velutipes* has low calorie with high content of polysaccharide, amino acids, fibre and vitamin (Leifa *et al*., 2001).

A number of bioactive compounds have been isolated and identified in *F. velutipes* such as flavonoids, glycosides, proteins, polysaccharide and phenol (Yang *et al*., 2012; Kang *et al*., 2014). Polysaccahrides from *F. velutipes* has antioxidative and renoprotective effect in mice (Lin *et al*., 2016). Due to its antioxidant activity, flavonoids from *F. velutipes* has neuroprotective effect against damaged brain cells (Hu *et al*., 2016). *F. velutipes* also contain immunomodulatory protein which is feasible for medical applications (Lin *et al*., 2013). Sterols from *F. velutipes* showed toxicity effect against several cancer cell, thus has high potential to be developed as potent antitumor agents (Yi *et al*., 2013). The appearance of *F. velutipes* is shown in Figure 2.4.

Figure 2.4: The appearance of *F. velutipes*

2.3.2.3 *Ganoderma lucidum*

G. lucidum is Basidiomycetes belonging to the *Ganodermataceae* family and the order *Polyparales* (Yan *et al.*, 2010; Hasnat *et al.*, 2015). *G. lucidum* has several common names such as Lingzhi (China); Youngzhi (Korea); Munnertake, Sachitake, and Reishi (Japan). *G. lucidum* is used widely for prevention and treatment of variety illness (Paterson, 2006). It is a popular folk medicine for various ailments including allergy, insomnia, bronchitis, chronic hepatitis, hyperglycemia, hypertension, hepatopathy, nephritis, cancer, inflammation and gastric ulcer (Sliva, 2003).

Different compounds have been extracted from *G. lucidum* and scientific studies revealed various biological activities of *G. lucidum* extract such as anti-virus (El-Mekkawy *et al.*, 1998), anti-hypertensive (Morigiwa *et al.*, 1986), anti-inflammatory (Liu *et al.*, 2015; Cai *et al.*, 2016) and antitumor (Joseph *et al.*, 2011). The appearance of *G. lucidum* is shown in Figure 2.5.

Figure 2.5: The appearance of *G. lucidum*

2.3.2.4 *Grifola frondosa*

G. frondosa is a Basidiomycetes fungus from order *Aphyllopherales* and family *Polyporaceae.* It is known as hen-of-wood or maitake mushroom (Shih *et al.*, 2008). *G. frondosa* has been valued for traditional medicine and being used as health food for long time in China, Japan and other Asian countries (Meng *et al.*, 2017). The fruiting body of *G. frondosa* is composed of multiple, overlapping caps with diameter of 2-10 cm and sharing a common base (Mau *et al.*, 2001).

It contains approximately 86% of moisture, 59% carbohydrates, 21% crude proteins, 10% crude fiber and 3% crude fat (Mau *et al.*, 2001). Intra- and extracellular β-polysaccharide from *G. frondosa* exerted immunostimulatory activity by inducing tumor necrosis TNF-α production in human peripheral blood mononuclear cells (Švagelj *et al.*, 2008). Heteropolysaccahride from *G. frondosa* is reported to possess anti-viral activity against a causative pathogen for hand-foot-and-mouth disease (Zhao *et al.*, 2016). The water soluble polysaccharides from *G. frondosa* exerted antitumor activity through its immunomodulatory activity (Mao *et al.*, 2015). Besides that, polysaccharide from *G. frondosa* mushroom has antioxidant activity and hepatoprotctive effect on liver cells (Chen *et al.*, 2012; Ma *et al.*, 2015). Fruiting body of *G. frondosa* mushroom also showed anti-diabetic activity in mouse (Keiko *et al.*, 1994). The appearance of *G. frondosa* is shown in Figure 2.6.

Figure 2.6: The appearance of *G. frondosa*

2.3.2.5 *Hypsizygus marmoreus*

H. marmoreus is known as bunashimeji or hon-shimeji (Zhang *et al*., 2015). *H. marmoreus* is one of the important culinary mushrooms in East Asia, such as in China, Japan and Korea (Lee *et al*., 2012). The fruiting body of *H. marmoreus* comprises long stipes and closed caps, which are spotted or marbled. When the cap of fruiting body matured, the dark tan cap turns to gray-brown and then creamy brown. *H. marmoreus* has mildly sweet nutty flavour and crunchy texture (Lee *et al*., 2007). The unique flavor of *H. marmoreus* is contributed by various free amino acids and carbohydrates in the mushroom (Harada *et al*., 2003).

Polysaccharide from *H. marmoreus* is reported to have good immunomodulatory property and the glycoprotein has anti-luekemic activity (Lee *et al*., 2011; Tsai & Ma, 2013). As reported by Lee *et al*. (2007), *H. marmoreus* is able to protect human body against oxidative damage due to its antioxidant activity which is contributed by its phenolic compounds. The appearance of *H. marmoreus* is shown in Figure 2.7.

Figure 2.7: The appearance of *H. marmoreus*

2.3.2.6 *Lentinula edodes*

L. edodes is from the family of *Agaricaceae* (Boer *et al.*, 2004). *L. edodes* is regarded as functional food since it is traditionally being used to treat various ailments such as tumors, flu, heart diseases, high blood pressure, obesity, problems related to sexual dysfunction and ageing, diabetes, liver ailments, respiratory diseases, exhaustion and weakness (Breene, 1990). *L. edodes* is a culinary medicinal mushroom with antimicrobial and anti-inflammatory properties (Carbonero *et al.*, 2008; Kaur *et al.*, 2016).

Lentinan which is a glucan from *L. edodes*, is one of a well-studied medicinal fungal metabolites responsible for antibacterial activity. The average of molecular weight of lentinan is 500, 000 Da and the main chain of lentinan composed of β-(1,3)-D-glucose residues with β-(1,6)-D-glucose side groups attached to every third of main chain (Bisen *et al.*, 2010). Eritadenine and L-ergothioneine are the other well studied bioactive compounds from *L. edodes* (Baba *et al.*, 2015). Low molecular weight lignin from *L. edodes* is reported to be responsible for its anti-viral activity against hepatitis-C

virus (Matsuhisa *et al*., 2015). Aside from its *in vitro* antitumor activity, *L. edodes* also have great antioxidant activity through its capability to scavenge radical formation (Finimundy *et al*., 2013). The appearance of *L. edodes* is shown in Figure 2.8.

Figure 2.8: The appearance of *L. edodes*

2.3.2.7 *Pleurotus eryngii*

P. eryngii which is from *Pleurotaceae* family is a culinary mushroom which has medicinal properties (Miyazawa *et al.*, 2008; Lv *et al.*, 2009). *P. eryngii* is locally known as king oyster mushroom (Ryu *et al.*, 2015). In English, *P. eryngii* is called king trumpet mushroom and known as eringi in Japan (Kikuchi *et al*., 2016). As a popular edible mushroom, *P. eryngii* may have potential bioctive fuction as described in Traditional Chinese Medicine (Chen *et al*., 2014). Fruiting bodies of *P. eryngii* are used for food and food-flavouring materials due to their nutritional and medicinal values. *P. eryngii* has also been inoculated into cooked rice and fermented to produce nutraceutical rice product (Liu *et al*., 2013).

Biologically active compounds have been isolated from *P. eryngii* such as polysaccharide, lipid, sterol, peptide and dietary fibre (Chen *et al*., 2012). Polysaccharide from mycelia of *P. eryngii* showed hepatoprotective effect by reducing hepatic lipid level in mice (Xu *et al*., 2017). Intracelllular polysaccharide from *P. eryngii* has been proved to exert antioxidant activity (Zhang *et al*., 2016). A water soluble polysaccharide with (1→6)-linked galactose residues from *P. eryngii* possessed good immunoregulatory activity by inducing macrophage to release pro-inflammatory factor such as NO, TNF-α, IL-1 and IL-6 (Xu *et al*., 2016). Polysaccharide from *P. eryngii* comprised mainly glucose residue and is important nutritional ingredients for anti-cancer health benefit (Ren *et al*., 2016). Besides that, polysaccharide from *P. eryngii* have antibacterial properties by inhibiting the growth of *Escherichia coli* (Li & Shah, 2014). Chen *et al.* (2016) reported that polysaccharide from *P. eryngii* could be therapeutic agent for hyperlipidemia and hyperglycemia. Ethanolic extract of *P. eryngii* also showed good antioxidant and anti-inflammatory activity, contributed by its phytochemical components including phenolic acids, flavonoids, tocopherols and carotenoids (Lin *et al*., 2014). The appearance of *P. eryngii* is shown in Figure 2.9.

Figure 2.9: The appearance of *P. eryngii*

2.3.2.8 *Pleurotus floridanus*

P. floridanus is a common mushroom species in tropical West African and Southern part of Asia. It grows in large numbers as a group on fallen trees, log of woods and wooden poles. The measurement of cap may range from 5.0 to 7.5 cm diameter while the stipe is 0.5 to 2.5 cm length and the spore is in cream-white color. *P. floridanus* is a culinary and highly nutritous mushroom (Adenipekun & Gbolagade, 2006).

P. floridanus species also known as *P. florida* (Rout *et al.*, 2005) and contains valuable nutritional values such as polysaccahride, proteins, fibre, minerals and low lipid cotent (Alam *et al.*, 2008; Ahmed *et al.*, 2009). A water soluble $(1\rightarrow 6)$-β-D-glucan stimulated macrophage by producing high amount of NO, thus providing immune-enhancing activity of *P. floridanus* mushroom (Das *et al.*, 2010). *P. floridanus* also have good antioxidant and antitumor activities (Jose & Janardhanan, 2001). The appearance of *P. floridanus* is shown in Figure 2.10.

Figure 2.10: The appearance of *P. floridanus*

2.3.2.9 *Pleurotus pulmonarius*

P. pulmonarius is a warm weather oyster mushroom because its mycelium and fruiting body can grow under wide range of temperatures ranging from 10-31°C. This signifies that its fruiting body is capable to withstand high ambient temperature with promising fresh market potentials (Shen *et al.*, 2014). Phytochemical studies revealed that *P. pulmonarius* contains several polysaccharide, high amount of protein, essential amino acids and vitamins (Baggio *et al.*, 2012). A dose-dependent inhibition of nociceptive response in mice was detected from mannoglactan of *P. pulmonarius* (Smiderle *et al.*, 2008).

P. pulmonarius was reported to exhibit good antioxidant and anti-inflammatory activity (Komura *et al.*, 2010; Khatun *et al.*, 2015). *P. pulmonarius* has potential to be used in the treatment of human liver cancer as it suppresses liver cancer development and progression through inhibition of VEGF-induced PI3K/AKT signaling pathway (Xu *et al.*, 2012). These biological activities of *P. pulmonarius* proved the medicinal values of the mushroom which has been used in traditional medicine. The appearance of *P. pulmonarius* is shown in Figure 2.11.

Figure 2.11: The appearance of *P. pulmonarius*

2.4 Melanogenesis

2.4.1 Overview of melanogenesis

Melanogenesis is a biosynthetic pathway for the formation of melanin in human skin (Tsatmali *et al.*, 2002). Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) signaling pathway is the primary cascade in melanogenesis (Jung *et al.*, 2009). Tyrosinase is a multifunctional, glycosylated and copper containing oxidase enzyme which play key role in the first and only rate limiting-steps in melanogenesis process (Uiterkamp & Mason, 1973). Tyrosinase catalyzes the conversion of L-tyrosine to dopaquinone (Tsatmali *et al.*, 2002). The first two steps in melanogenesis are rate limiting steps in melanin synthesis (Chang, 2009). The downstream pathway in melanogenesis can proceed spontaneously at physiological pH value (Halaban *et al.*, 2002).

Melanin plays a pivotal role in the absorption of free radicals generated within the cytoplasm and protects the host from various type of ionizing radiation, including ultraviolet radiation (UVR) (Parvez *et al.*, 2006). Human melanocytes produce two types of melanin, the brown-black eumelanin and reddish-yellow phaeomelanin (Thody *et al.*, 1991; Tsatmali *et al.*, 2002). Large amounts of phaeomelanin are found in epidermis of skin type I and II and in red hair. Eumelanin predominates in the individual with dark skin and hair. The photoprotective effect of eumelanin is more effective than phaeomelanin (Thody *et al.*, 1991; Tsatmali *et al.*, 2002).

Upon stimulation by ultraviolet light (UV), cAMP-dependent pathway is a notable pathway in melanogenesis (Busca & Ballotti, 2000). Based on the study carried out by Hunt *et al.* (1995), eumelanin was the major pigment in the epidermis while phaeomelanin was the major pigment in the cultured melanocytes. Excessive melanin production or abnormal distribution of melanin can cause hyperpigmentation of the skin (Chang, 2012).

UVR is the main stimulus for melanogenesis which in turn causes tanning of the skin (Park *et al.*, 2009). Two distinct phases of tanning response that have been reported are immediate pigmentation and delayed pigmentation. Both types of pigmentation have strong genetic determinant and are generally more evident in individuals with dark skin and hairs (Costin & Hearing, 2007). Immediate pigmentation appears 5-10 minutes after exposure to UVR, and it will disappear minutes or days later. Immediate pigmentation does not depend on increased melanin synthesis but on the oxidation of pre-existing melanin and redistribution of melanosomes to the epidermal upper layers. Delayed pigmentation occurs 3-4 days after exposure to ultraviolet rays and will disappear within weeks. Delayed pigmentation occurs due to increased level of epidermal melanin, especially eumelanin which provides photoprotection (Videira *et al.*, 2013). Excessive melanin production leads to hyperpigmentation (Ahn *et al*., 2006) and can be recognized by darkening or increasing natural color of the skin due to increased deposition of melanin pigment in the epidermis and/or dermis (Konrad & Wolff, 1973).

2.4.2 Target molecules for melanogenesis inhibition

Natural melanogenesis inhibitors act through the down regulation of tyrosinase activity. The mechanism might directly inhibit tyrosinase catalytic activity, accelerate tyrosinase degradation, inhibit tyrosinase gene expression or directly down-regulate the tyrosinase protein (Chang, 2012). Tyrosinase inhibitors are the most popular and widely-used hypopigmenting agents as only melanocytic cells produce tyrosinase enzyme (Han *et al.*, 2015). Clinical hypopigmenting agents rarely used melanogenesis inhibitors targeting to the gene expression or protein degradations because of non-specific binding and global effects via intracellular signaling pathways (Huang *et al*., 2012). Most of the researches had been conducted on using mushroom tyrosinase enzyme to study the anti-melanogenesis effect of the compounds or extracts (Seo *et al.*,

2003; Xie *et al.*, 2003). Mushroom tyrosinase is well-studied enzyme and easily be purified from mushroom *A. bisporus* (Espin *et al.*, 1998).

Micropthalmia-associated transcription factor (MITF) is a melanocyte-specific transcription factor which is crucial for melanocyte development and differentiation (Bertolotto *et al.*, 1996). MITF belongs to the basic helix-loop-zip family of transcription factors. It regulates both melanocyte proliferation as well as melanogenesis. MITF plays crucial role to regulate tyrosinase and related proteins (TRPs) and many melanocytes structural proteins (Solano *et al.*, 2006). The ability of MITF to regulate transcription is controlled by two opposing signaling pathways, namely the pro-proliferative MAP kinase pathway and the anti-proliferative stress-activated p38 pathway (Vance & Goding, 2004). The sample that possessed inhibitory effect on MITF expression will be an inhibitor for the whole melanogenesis process (No *et al.*, 2006). Melanogensis synthesis can also be inhibited by disrupting the intracellular trafficking of tyrosinase-related proteins and lysosome-associated membrane protein (Ni-Komatsu *et al.*, 2008).

2.5 Inflammation

2.5.1 Overview of inflammation

Loss of function and pain, heat, redness and swelling are common sign of inflammation (Taofiq *et al.*, 2016). Macrophages derived from monocytes play a key role in initiating the first line of defense in host immunity against foreign pathogens (Liu *et al.*, 2014). The complex process of inflammation started by the binding of pro-inflammatory mediators such as lipopolysaccharide (LPS), IL-1β and interferon-γ (IFN-γ) to toll-like receptor-4 (TLR-4), which in turn stimulate macrophage through inflammatory NF-κB signaling pathway (Lee *et al.*, 2012).

NF-κB is a transcription factor that plays a prominent role in immune and inflammatory response *via* regulation of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α), anti-inflammatory cytokine (e.g. IL-10), chemokines (e.g. IL-8), growth factor and inducible enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Lawrence *et al.*, 2002; Chan *et al.*, 2015; Choi *et al.*, 2015). Activation of NF-κB occurs through phosphorylation of IκB-α subunits and resulting p65-p50 heterodimer to migrate into nucleus and up-regulating the expression of pro-inflammatory genes (Lawrence *et al.*, 2002; Li *et al.*, 2017). Subsequently, pro-inflammatory mediators such as NO, TNF-α, IL-6 and prostaglandin E_2 (PGE_2) will be up-regulated (Song *et al.*, 2016). Therefore, reduction of inflammatory mediator may become useful marker for assessing anti-inflammatory activity of extract or isolated compounds (Kim *et al.*, 2013).

2.5.2 Nitric oxide (NO) gas

NO is a colorless gas at room temperature involved in the regulation of physiological mechanism in cardiovascular, nervous and immunological system (Zamora *et al.*, 2000). NO is a short-lived free radical and has very small size which enables to diffuse freely within cells from its site of formation to its site of action (Aktan, 2004).

Biochemical pathway for NO synthesis is through L-arginine:NO pathway. Nitric oxide synthase (NOS) enzyme is responsible for the conversion of L-arginine to L-citrulline and the co-product, NO. The three distinct isoform of NOS available are endothelial NO synthase (eNOS), neuronal NO synthase (nNOS) and iNOS. The iNOS is not expressed in resting cells, but produced massively after the cells being induced by LPS or cytokines (Moncada, 1999). Excessive production of NO by iNOS will cause toxicity to the cells. The toxic effect of NO was predominantly due to the formation of

peroxynitrite (ONOO−) in the reaction with superoxide radicals (O2 •-). The peroxynitrite interacts with lipids, DNA and proteins *via* direct oxidative reactions or *via* indirect and radical-mediated mechanisms. The production of peroxynitrite causes a crucial pathogenic mechanism such as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer and neurodegenerative disorders (Pacher *et al.*, 2007). Several methods are available for measurement of NO, however, diazotization method using Griess reagent is the most widely used method (Archer, 1993; Zhou *et al*., 2008).

2.5.3 Pro-inflammatory cytokines

Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) are produced by activated macrophages and involved in the up-regulation of inflammatory response (Zhang & An, 2007). Pro-inflammatory cytokines exert a wide range of effects that produce inflammation, including induction of vascular endothelial receptors required for migration of immune cells out of the circulation and into the area of inflammation, and attracting and activating additional macrophages and neutrophils to assist in the destruction of foreign particles (Watkins *et al.*, 1995).

TNF-α acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 to regulate apoptotic pathways, NF-κB activation of inflammation, and activate stress-activated protein kinases (SAPKs) (Boka *et al*., 1994). Monocytes and macrophages are primary cells to release IL-1β during cell injury, infection, invasion, and inflammation. Other non-immune cells such as fibroblasts and endothelial cells also secrete IL-1β (Copray *et al*., 2001). IL-6 plays a key role in the acute phase inflammatory response as defined by a variety of clinical and biological features such as the production of acute phase proteins. IL-6 also exerts stimulatory effects on T- and B-cells, thus favoring chronic inflammatory responses (Gabay, 2006).

2.5.4 Anti-inflammatory cytokines

IL-10 is a potent anti-inflammatory cytokines among other family members from cytokine type II (IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29) (Mosser & Zhang, 2008). Homodimers of IL-10 interact with its heterodimeric receptor complex to regulate the biological activity in immune cells, keratinocyte and endothelial cells (Groux & Cottrez, 2003). IL-10 exerts its anti-inflammatory activity through the inhibition of iκB kinase and by inhibiting NF-κB in the nucleus, subsequently reduced the expression of pro-inflammatory mediators (Driessler *et al.*, 2004). Besides that, the reductions of pro-inflammatory mediators by IL-10 also work through other pathway such as STAT-3 pathway (Takeda *et al*., 1999).

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and reagents

Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium hydroxide (NaOH), phosphate buffered saline (PBS), Dulbecco's Modified Eagle's medium (DMEM), penicillin/streptomycin (100X), amphotericin B, fetal bovine serum (FBS), accutase, 3-(4,5-dimetyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), α-melanocyte stimulating hormone (α-MSH), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), LPS, N-(1-napthyl)ethylenediamide dihydrochloride (NED), phosphoric acid (H_3PO_4) N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and Kojic acid were purchased from Sigma-Aldrich Company. The L-3,4-dihydroxyphenylalanine (L-DOPA) and sulfanilamide were purchased from MP Biomedicals company.

3.2 Mushroom samples

Nine medicinal and culinary fresh mushrooms samples were purchased from the market on 1st of July 2015, namely *Agaricus bisporus* (brown variety)*, A. bisporus* (white variety)*, Flammulina velutipes, Grifola frondosa, Hypsizygus marmoreus, Lentinula edodes, Pleurotus eryngii, P. floridanus* and *P. pulmonarius. Ganoderma lucidum* was bought in dried form.

3.3 Preparation of hot water extract of mushrooms

Any debris was cleaned up from the fruiting bodies of mushroom samples and they were cut into small pieces to speed up the drying process. The hot water extraction of mushroom was carried out according to the method described by Cheng *et al.* (2013). Briefly, the samples were dried in oven (Memmert, Germany) at 50°C for overnight. Dried mushroom samples were ground into fine powder, kept in sealed plastic bags and stored in -20°C prior the extraction process. The powdered fruiting bodies were then subjected to hot water extraction at ratio (1:10 w/v) at 100°C for one hour. The extract which was cooled at room temperature was then centrifuged at 10, 000 rpm at 4°C for 20 minutes. The supernatant was filtered using Whatman filter paper Grade one (GE healthcare, UK) to remove the any debris from the extract. The extraction was repeated for three times. The resulting aqueous extract was freeze-dried and kept at 4°C prior use.

3.4 Cell lines and culture medium

The B16F10 melanoma and RAW 264.7 macrophage cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM medium, supplemented with 10% FBS, penicillin/streptomycin and amphotericin B. The cells were incubated in a CO_2 incubator with humidified atmosphere containing 5% CO_2 at 37°C and were sub-cultured every three days.

3.5 Cell viability assay (MTT assay)

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay was conducted according to Mosmann (1983) with modifications. Briefly, B16F10 melanoma cells (3×10^3 cells/well) or RAW 264.7 macrophage cells (4×10^3 cells/well) were seeded in 96-well plate and left to adhere overnight. Two hundreds microliter (200 µl) of mushroom extracts at concentrations of log 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 was added to each well and incubated for 48 hours (B16F10 melanoma cells) or 24 hours (RAW 264.7 macrophage cells). Wells containing untreated cells (without addition of any extract) were regarded as an untreated control (negative control). The MTT solution (5 mg/ml) was then added to all the wells for three hours following which the purple formazan precipitates were dissolved in DMSO. The plates were then read on a microplate reader using a test wavelength of 570 nm with reference of 650 nm. Kojic acid was used as positive control for anti-melanogenesis assay (B16F10 melanoma cells) while L-NAME was used for anti-inflammatory assay (RAW 264.7 macrophage cells). Cell viability was calculated using the following formula: (%) = (A_{sample} / $A_{control}$) $\times$ 100, where A_{sample} and $A_{control}$ are the absorbance from the mixture with, or without the addition of mushroom extract, respectively.

3.6 Anti-melanogenesis activity of mushroom extract
3.6.1 Mushroom tyrosinase assay

The effect of mushroom extracts on cell-free mushroom tyrosinase activity was determined spectrophotometrically as described previously by Alam *et al*. (2010). The tyrosinase activity was determined using L-DOPA as a substrate. Briefly, each well in 96-well plate contained 40 µl of mushroom extract or kojic acid at concentration of log 2.0, 2.2, 2.4, 2.6, 2.8 or 3.0, 80 µl of sodium phosphate buffer (0.1 M, pH 6.8), 40 µl of 300 unit/ml tyrosinase in 0.1 M sodium phosphate buffer (pH 6.8) and 40 µl 2.5 mM

L-DOPA in 0.1 M sodium phosphate buffer (pH 6.8). The mixture was incubated for 10 minutes at 37°C. The absorbance was measured at 475 nm using a microplate reader. Each sample was accompanied by a blank containing all components except L-DOPA. Kojic acid was used as positive control. The results were compared with a control consisting of deionized water in place of sample. The percentage of tyrosinase activity was calculated as follow: $(A_{sample} / A_{control}) \times 100$ %, where A_{sample} and $A_{control}$ are the absorbance from the mixture with, or without the addition of mushroom extract, respectively.

3.6.2 Measurement of intracellular melanin content

The intracellular melanin content was measured as described previously by Huang *et al*. (2012). The B16F10 melanoma cells were seeded with density 1×10^5 cells/dish in 60 mm dish and incubated overnight in order for cells to adhere. The cells were treated with mushroom extracts or kojic acid (positive control) at concentrations of log 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 in the presence or absence of α-MSH for 48 hours. After treatment, the cells were detached by incubation with accutase for four minutes and subsequently centrifuged at 4,000 rpm for 15 minutes and the cell pallets were solubilized in 1 N NaOH at 80°C for one hour. The melanin content was measured by measuring the absorbance at 405 nm using a microplate reader. The percentage of melanin content was calculated relative to the untreated control.

3.6.3 Cellular tyrosinase assay

Cellular tyrosinase activity was measured using a previously described method by Han *et al*. (2015). Wells of 60 mm dish were seeded with B16F10 melanoma cells at a density of 1×10^5 cells/dish and incubated overnight to allow them to adhere. The cells were treated with selected concentration of mushroom extracts (*A. bisporus*

(brown), *P. eryngii, P. floridanus, P.* pulmonarius) or kojic acid (positive control; log 2.8) together with 100 nM α-MSH for 48 hours. *A. bisporus* (brown), *P. eryngii, P. floridanus, P. pulmonarius* at concentration of log 2.8 or 3.0 or both were selected for cellular tyrosinase activity assay based on their ability to reduce intracellular melanin content in B16F10 melanoma cells below 80% (Table 4.3) and recorded cell viability above 65% (Table 4.2). The cells were lysed with 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM PMSF in ice for 30 minutes. The lysates were then clarified by centrifugation at 13, 000 rpm for 20 minutes at 4°C. Enzyme activity was normalized to protein concentration as determined by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, US). The reaction mixture consisting of 50 µg protein (adjusted with 0.1 M sodium phosphate buffer, pH 6.8) and 100 µl of 5 mM L-DOPA was added to each well of 96-well plate. The reaction mixture was incubated at 37°C for one hour. The absorbance was measured at 475 nm using a microplate reader. Tyrosinase activity was calculated by the following formula: Tyrosinase activity (%) = (A_{sample} / $A_{control}$) × 100, where A_{sample} and $A_{control}$ are the absorbance from the mixture with, or without the addition of test mushroom extract, respectively.

3.7 Anti-inflammatory activity of mushroom extract

3.7.1 Determination of nitric oxide (NO) production

The nitric oxide (NO) assay was performed as previously described by Chan *et al*. (2015). Briefly, RAW 264.7 macrophage cells were seeded with 2×10^5 cells/well and incubated overnight. The attached cells were co-incubated with 1 µg/ml LPS in the presence of various mushroom extracts at concentration of log 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 at 37°C for 24 hours. The quantity nitrite in the culture medium was measured as an indicator of NO production. An amount of 100 µl of cell culture medium was mixed

with 100 µl of Griess Reagent (1% sulfanilamide and 0.1% NED in 5% H_3PO_4) and incubated at room temperature for 10 minutes. The absorbance at 540 nm was then measured with a microplate reader. L-NAME was used as positive control. The percentage of NO production was calculated by the following formula: NO production (%) = (A_{sample} / $A_{control}$) × 100, where A_{sample} and $A_{control}$ are the absorbance from the mixture with, or without the addition of mushroom sample, respectively.

3.7.2 Measurement of TNF-α and IL-10 production

In this assay, the cells were prepared as in the NO assay (Section 3.7.1). Six mushroom extracts namely *A. bisporus bisporus* (brown), *A. bisporus* (white), *G. lucidum, H. marmoreus, P. floridanus* and *P. pulmonarius* were selected for the measurement of TNF-α and IL-10 production based on their ability to reduce NO production in RAW 264.7 macrophage cells below 90% at selected concentration(s) (Table 4.5). After 24 hours incubation with six selected concentration(s) of mushroom extracts, the culture media in each well was collected and the presence of TNF-α and IL-10 were assayed using enzyme-linked immunosorbent assay (ELISA) kit (Abcam, UK). All reagents and solutions required for this assay were provided in the ELISA kit.

Briefly, 100 µl of cell culture media was pipetted into a 96-well microplate coated with either anti–mouse TNF-α or anti-mouse IL-10 and was incubated overnight at 4°C. The medium was discarded, and the well was washed four times with 300 µl washing buffer. Biotinylated anti–mouse TNF-α or IL-10 antibody (100 µl) was added into each well, and the plate was incubated for one hour at room temperature with gentle shaking. The solution was discarded, and the wells were washed before 100 µl of horseradish peroxidasestreptavidin solution was added into each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. After the final wash, 100 µl TMB One Step Substrate Reagent was added to each well, and the plate was

incubated for another 30 minutes in the dark with gentle shaking. Fifty microliter (50 μl) of stop solution was added into each well to stop the color development, and the absorbance at 450 nm was immediately measured in microplate reader. The percentage of TNf-α and IL-10 was calculated relative to the untreated control.

3.8 Preparation of combination mushroom extracts

In cosmeceutical industry, several active ingredients are always combined in a specific ratio to optimize their biological activities, as the active ingredients may work synergistically and enhance the desired effect. The mushroom extract which has anti-melanogenesis activity on B16F10 melanoma cells (*A. bisporus* (brown), *G. lucidum, P. floridanus* and *P. pulmonarius*; section 4.3.2 and 4.3.3) could probably has better anti-melanogenesis and anti-inflammatory activities when combined with other mushroom extract at specific ratio. In this study, several mushroom extracts (*A. bisporus* (brown), *G. lucidum, P. floridanus* and *P. pulmonarius*) were combined at ratio 1:1 at different concentrations as it will be much clearer to evaluate the synergistic effect when they were in the same ratio.

In intracellular melanin content assay, *G. lucidum* extract reduced intracellular melanin content in B16F10 melanoma cells to $83.72 \pm 3.25\%$ at concentration of log 2.4 (Table 4.3). This result showed the lowest intracellular melanin content compared to other nine mushroom extracts at similar concentration (log 2.4). Thus, *G. lucidum* extract at concentration of log 2.4 was selected to be used in all combination mushroom extracts. *A. bisporus* (brown), *P. floridanus* and *P. pulmonarius* extracts at concentration of log 3.0 showed anti-tyrosinase activity (Table 4.4). Due to their good anti-melanogenesis activity, these three mushroom extracts were selected to combine with *G. lucidum* extract to form three different combination mushroom extracts (1:1 ratio) at specific concentrations. The three combination prepared was GL2.4ABB3.0,

GL2.4PF3.0 and GL2.4PP3.0. The description of each combination extract is summarized in Table 3.1.

Table 3.1: The list and description of combination mushroom extracts

Combination	Description
GL2.4ABB3.0	The combination comprise of *G. lucidum* extract at concentration of log 2.4 and *A. bisporus* (brown) at concentration of log 3.0.
GL2.4PF3.0	The combination comprise of *G. lucidum* extract at concentration of log 2.4 and *P. floridanus* at concentration of log 3.0.
Gl2.4PP3.0	The combination comprise of *G. lucidum* extract at concentration of log 2.4 and *P. pulmonarius* at concentration of log 3.0.

Anti-melanogenesis activity of the combination mushroom extracts was studied using the same procedure as single extract. Mushroom tyrosinase assay was excluded because all single mushroom extracts have a poor inhibitory effect on mushroom tyrosinase enzyme activity. The three combined mushroom extracts (GL2.4ABB3.0, GL2.4PF3.0 and GL2.4PP3.0) were screened for their cell viability in B16F10 melanoma cells. The combination mushroom extracts were then tested for their anti-melanogenesis activity through melanin content assay and cellular tyrosinase activity. The combination mushroom extract which has toxicity effect on B16f10 melanoma cells (cell viability below 65%) was not included in the subsequent anti-melanogenesis and anti-inflammatory assays.

The combination mushroom extract which did not have toxicity effect on B16F10 melanoma cells were then tested for their anti-inflammatory activity in RAW 264.7 macrophage cells. The effect of combination mushroom extract on cell viability was tested by MTT assay. Anti-inflammatory activity of combination mushroom extracts were studied by measuring NO, TNF-α and IL-10 production in RAW 264.7 macrophage cells.

3.9 Statistical analysis

All data are presented as the mean $\pm$ standard error of mean (SEM) of three independent experiments and statistically significant differences from the untreated control was analyzed using student's t-test ($p < 0.05$).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Extraction yield of mushroom extracts

Hot water extraction yielded mainly polysaccharides as most of the polysaccharides are soluble in hot water (Dore *et al*., 2007; Shi, 2016). The other compounds like proteins or lipids probably will not contain in this extract due to the different solubility and molecular properties. Normally, proteins are extracted through cold water extraction (Dan *et al.*, 2016). Hot water extract might not contain free proteins as high temperature may cause denaturation of protein structure (Moriyama *et al*., 2008). The hot water extraction yield of ten medicinal and culinary mushrooms is listed in Table 4.1. The final weight of mushroom greatly reduced compared to the initial weight. The weights of all mushroom extracts were lower than the final weight of mushroom. This is due to the loss of water content as 90% of the mushroom weight are water (Kao *et al.*, 2013).

F. velutipes and *P. eryngii* gave the highest percentage of yield (0.05%). The results obtained was similar to Chen *et al*. (2013) where these mushrooms were among the species that yielded high polysaccharide content. In *F. velutipes*, the polysaccharide mainly linked though β-linkage with triple helical structure (Smiderle *et al*., 2006; Yang *et al*., 2012). L-arabinose was the highest monosaccharide unit followed by D-galactose, D-glucose and D-mannose (He *et al.*, 2012). β-Glucan was the main polysaccharide in *P. eryngii* with the main chain of (1→3)-linked glucose (Ikekawa, 1995; Carbonero *et al*., 2006). *G. lucidum* and *G. frondosa* extracts yield with only 0.01%. Based on the previous study conducted by Kozarski *et al*. (2011), *G. lucidum* contained mostly α-glucan (94%) and low polysaccharides. This result is in agreement to the current yield for *G. lucidum* extract where this species yield the least.

Table 4.1: Yield of hot water extraction of medicinal and culinary mushrooms

Extract	Initial weight (g)	Final weight (g)	Weight of extract (g)	Percentage of yield (%)[a]
A. bisporus (brown)	400.00	29.80	15.10	0.04
A. bisporus (white)	400.00	30.60	14.50	0.03
F. velutipes	500.00	57.00	23.20	0.05
*G. lucidum**	101.00	101.00	1.01	0.01
G. frondosa	500.00	46.00	7.10	0.01
H. marmoreus	500.00	47.80	19.30	0.04
L. edodes	500.00	45.80	11.10	0.02
P. eryngii	500.00	44.60	23.00	0.05
P. floridanus	500.00	55.96	13.32	0.03
P. pulmonarius	500.00	48.70	16.40	0.03

[a] The percentage of yield was calculated relative to the initial weight of mushroom samples. * Sample was purchased in dried form.

4.2 Effect of mushroom extract on B16F10 melanoma and RAW 264.7 macrophage cells

In the current study, the anti-melanogenesis and anti-inflammatory activity of ten selected medicinal and culinary mushroom extracts was investigated using cell-based assay. Firstly, the cell viability was observed with MTT assay after treatment with mushroom extracts at different concentrations. The percentage of viable cells was determined in order to exclude the possibility that anti-melanogenesis and anti-inflammatory response of mushroom extracts were caused by cytotoxicity effect. The viability of B16F10 melanoma and RAW 264.7 macrophage cells after exposure to mushroom extracts are shown in Table 4.2. In this study, 65% of viable cell at any concentration of mushroom extract was set as non-toxic to B16F10 melanoma cells whereas 50% was set for RAW 264.7 macrophage cells (Chan *et al.*, 2011; Razali *et al.*, 2014).

For *G. lucidum* extract, the B16F10 cell viability was 45.32 ± 5.38% at concentration of log 2.6 and the viable cells were reduced to 11.00 ± 3.19% at concentration of log 2.8 in RAW 264.7 macrophage cells. *H. marmoreus* extract reduced cell viability to 63.50 ± 1.73% at concentration of log 2.6 in B16F10 melanoma cells but did not show appreciable toxic effect on RAW 264.7 macrophage cells. A reduction of cell viability (43.24 ± 4.48%) was observed for *P. pulmonarius* extracts on RAW 264.7 macrophage cells at the highest concentration. *A. bisporus* (white) extract promoted the cell growth of B16F10 melanoma cells when the concentration was increased. As a positive control for anti-melanogenesis assay, kojic acid reduced the cell viability of B6F10 melanoma cells below 65% at concentration of log 3.0. L-NAME, the positive control for anti-inflammatory assay recorded cell viability above 50% at all tested concentrations. The mushroom extract, which has exerted cellular toxicity below threshold level was excluded from subsequent anti-melanogenesis (intracellular melanin content assay and cellular tyrosinase assay) and anti-inflammatory assays (NO assay and determination of TNF-α and IL-10 using ELISA kit).

Table 4.2: The effect of mushroom extracts on B16F10 melanoma and RAW 264.7 macrophage cells

Extract	Cell viability on B16F10 melanoma cells (%)[a]						Cell viability on RAW 264.7 macrophage cells (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
Untreated control	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
A. bisporus (brown)	103.79	102.06	103.84	106.53	94.72	77.09	66.89	64.22	60.45	57.64	55.38	54.10
	± 3.64	± 2.00	± 2.90	± 2.30*	± 0.86*	± 2.21*	± 0.61*	± 0.78*	± 0.70*	± 0.29*	± 0.20*	± 0.32*
A. bisporus (white)	82.96	83.64	84.22	83.34	88.15	92.36	106.55	91.76	83.63	77.07	78.56	75.45
	± 2.27*	± 2.31*	± 3.49*	± 1.99*	± 2.56*	± 2.09*	± 4.11*	± 0.86*	± 0.69*	± 1.67*	± 0.75*	± 1.16*
F. velutipes	88.62	92.23	90.46	89.38	86.77	87.35	90.80	86.17	78.82	74.10	71.81	72.33
	± 4.15*	± 5.33	± 4.88	± 4.19*	± 3.37*	± 4.14*	± 3.23*	± 3.42*	± 2.12*	± 2.17*	± 2.83*	± 6.26*
G. lucidum	100.56	95.75	87.66	45.32	14.51	5.33	95.45	91.94	88.51	65.69	11.00	6.70
	± 1.49	± 3.55	± 3.50*	± 5.38*	± 4.59*	± 1.10*	± 3.68	± 5.66	± 2.72*	± 2.68*	± 3.19*	± 1.13*
G. frondosa	94.99	95.46	96.22	95.67	97.16	89.47	104.93	92.88	92.13	92.12	83.99	82.74
	± 3.89	± 4.65	± 4.14	± 5.05	± 11.09	± 3.78*	± 5.17	± 4.58	± 2.72*	± 1.81*	± 1.20*	± 2.07*
H. marmoreus	91.95	78.23	73.22	63.50	52.88	40.04	104.42	102.57	99.33	90.88	80.36	67.49
	± 3.17*	± 3.92*	± 3.04*	± 1.73*	± 1.06*	± 0.60*	± 1.12*	± 1.04*	± 1.89	± 0.88*	± 0.85*	± 1.41*
L. edodes	82.67	85.62	79.97	80.47	79.85	70.90	86.99	86.43	78.59	74.13	69.90	61.57
	± 1.79*	± 2.18*	± 2.26*	± 1.82*	± 0.60*	± 1.38*	± 4.45*	± 2.33*	± 0.72*	± 1.20*	± 1.02*	± 0.95*
P. eryngii	99.24	96.93	94.50	98.85	91.66	86.94	95.05	90.96	82.54	80.02	77.25	73.42
	± 1.40	± 3.54	± 5.35	± 3.96	± 3.95	± 1.78*	± 2.24	± 1.37*	± 1.02*	± 0.99*	± 2.18*	± 0.55*
P. floridanus	105.69	106.09	105.72	107.06	101.08	93.71	74.52	76.73	75.61	71.41	68.30	61.16
	± 2.61	± 4.58*	± 5.48*	± 2.37*	± 2.94	± 1.58*	± 2.45*	± 1.85*	± 2.36*	± 2.11*	± 1.57*	± 0.74*

Table 4.2, continued

Extract	Cell viability on B16F10 melanoma cells (%)[a]						Cell viability on RAW 264.7 macrophage cells (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
P. pulmonarius	116.41 ± 3.77*	113.32 ± 3.92*	110.97 ± 2.18*	115.95 ± 3.79*	109.83 ± 3.12*	98.65 ± 3.37*	65.41 ± 4.58*	61.58 ± 2.08*	57.16 ± 2.02*	55.75 ± 2.70*	50.00 ± 3.73*	43.24 ± 4.48*
Kojic acid	104.36 ± 2.99	99.93 ± 1.72	103.46 ± 3.42	97.40 ± 2.78	77.65 ± 4.22*	55.76 ± 2.41*	-	-	-	-	-	-
L-NAME	-	-	-	-	-	-	84.00 ± 5.66*	83.49 ± 5.51*	81.36 ± 2.58*	64.77 ± 2.48*	73.47 ± 1.08*	67.29 ± 1.17*

Untreated control readings (from well with cells but without any extract) were set as 100% and readings of experimental well were expressed as percentage of untreated controls. *$p < 0.05$ significantly different from untreated control (negative control). [a] Relative activity (%) = Percentage of sample versus untreated control.

4.3 Anti-melanogenesis activity of mushroom extract

4.3.1 Inhibitory effect of extracts on mushroom tyrosinase activity

To determine whether the ten selected mushroom extracts affect tyrosinase activity directly, mushroom tyrosinase assay was performed by using L-DOPA as substrate and mushroom tyrosinase as enzyme source. Mushroom tyrosinase is widely used as target enzyme in screening potential inhibitors of melanogenesis due to specificity and availability of the enzyme (Souza *et al*., 2012; Chang *et al*., 2015).

The percentage of mushroom tyrosinase activity was presented in Figure 4.1. Based on the bar chart shown in Figure 4.1, the percentages of mushroom tyrosinase activity for all mushroom extracts were more than 80% compared to the positive control. Mushroom tyrosinase activity for all the mushroom extracts were not concentration dependent except for *G. lucidum* extract. However, the inhibitory activity for *G. lucidum* extract was weak. At concentration of log 3.0, mushroom tyrosinase activity for *G. lucidum* extract was 82.28 ± 0.54%. As positive control, kojic acid reduced mushroom tyrosinase activity to 17.83 ± 0.66% at concentration of log 2.0. Kojic acid showed excellent inhibition on mushroom tyrosinase activity and was used as positive control for anti-melanogenesis study because it has known inhibitory effect on tyrosinase activity and melanin synthesis (Garcia & Fulton, 1996; Arung *et al*., 2006;).

Chan *et al.* (2011) reported that ethanolic extract of brown seaweed (*Sargassum polycystum*) was not active in mushroom tyrosinase assay but showed good results in cell-based assay using B16F10 melanoma cells, might be due to the difference in tyrosinase enzyme structure where mushroom tyrosinase is tetramer proteins structure while mammalian tyrosinase is a monomer (Parvez *et al*., 2007). Other than that, mushroom tyrosinase is located in the cytososol while tyrosinase in mammalian cells cell is membrane bonded and highly glycosylated (Nishioka, 1978). These factors are

possible reason for poor inhibition of the extracts on mushroom tyrosinase activity in this study. Therefore, anti-melanogensis activity of ten mushroom extract was then studied in cells-based assay using B16F10 melanoma cells.
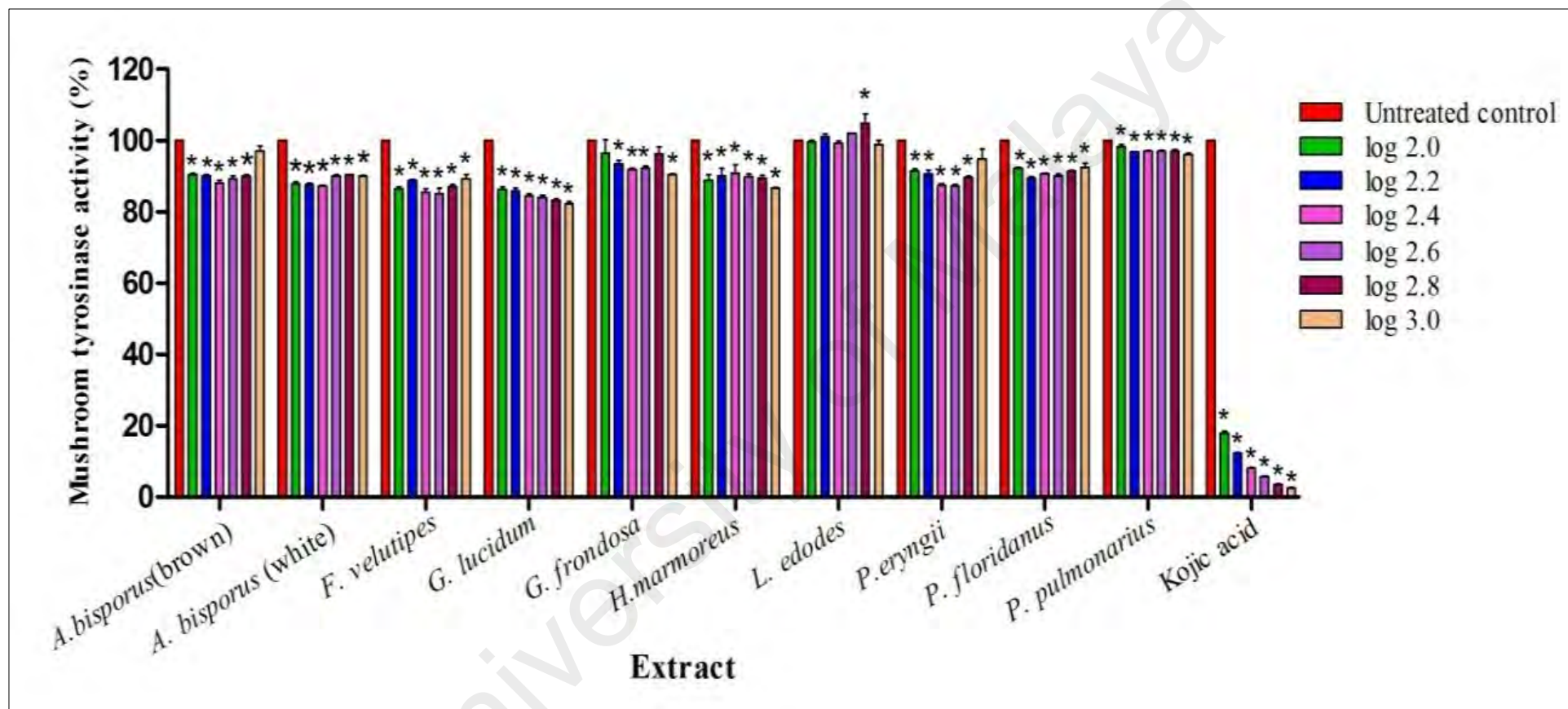
Figure 4.1: The effects of ten mushroom extracts and kojic acid on mushroom tyrosinase activity
The readings of experimental wells were expressed as percentage of untreated control which was set as 100%. *$p < 0.05$ was statistically significant compared with the value of untreated control

4.3.2 Inhibitory effect of mushroom extracts on intracellular melanin content in B16F10 melanoma cells

In cellular assay, the murine B16F10 melanoma cell line was used to study the anti-melanogenic activity of the ten selected mushroom extracts because those cells contain tyrosinase enzyme which is critical for melanin biosynthesis (Busca & Ballotti, 2000). Besides, murine B16F10 melanoma cell line can also be stimulated with α-MSH and cultured *in vitro* (An *et al*., 2008).

The inhibitory effect of ten mushroom extracts on melanin content in B16F10 melanoma cells is shown in Table 4.3. The *A. bisporus* (brown) extract showed the lowest intracellular melanin content (57.05 ± 3.90%) at the highest concentration without any toxicity effect (cell viability above 65%). Intracellular melanin content for cells treated with *P. eryngii, P. floridanus* and *P. pulmonarius* were below 80% at concentration of log 3.0, showing moderate ability to inhibit melanin production in B16F10 melanoma cells. Significant reduction of intracellular melanin content was observed for cells treated with *A. bisporus* (white), *F. velutipes, L. edodes,* (log 3.0), *G. lucidum* and *H. marmoreus* extracts (log 2.4) at the highest concentration, but the percentage was above 80%. *G. frondosa* extract was the least effective to reduce melanin content in B16F10 melanoma cells at the highest concentration (91.17 ± 6.49%). The percentage of intracellular melanin for kojic acid treated cells was 47.40 ± 1.87% at concentration of log 2.8. Significant reduction of melanin content in B16F10 melanoma cells at non-lethal concentration might indicate anti-melanogenesis activity of the extracts (Jeong *et al*., 2013). Based on the results obtained in the intracellular melanin content assay, four mushrooms extracts (*A. bisporus* (brown), *P. eryngii, P. floridanus,* and *P. pulmonarius*) which recorded a reduction of intracellular melanin content below 80% were selected for intracellular melanin content assays in α-MSH stimulated B16F10 melanoma cells and cellular tyrosinase assay.

Upon exposure to α-MSH, the expression of tyrosinase, TRP-1 and TRP-2 protein in B16F10 melanoma cells were increased. Therefore, the concentration of melanin in α-MSH stimulated B16F10 melanoma cell was higher than non-stimulated cells (Han *et al*., 2014). Table 4.3 also presented the level of intracellular melanin content in B16F10 melanoma cells after co-incubation with 100 nM α-MSH and selected mushroom extracts for 48 hours. At concentrations of log 2.8 and 3.0, the melanin productions for *A. bisporus* (brown) extract treated cells was suppressed to 23.96 ± 1.56% and 15.24 ± 0.59% respectively, showing the lowest intracellular melanin content. Intracellular melanin content of cells treated with α-MSH stimulation for *P. floridanus* extract was reduced to 80.53 ± 2.64% whereas *P. pulmonarius* extract was 71.36 ± 2.06% at concentration of log 3.0. The α-MSH stimulated B16F10 melanoma cells for with *P. eryngii* extract recorded the highest intracellular melanin content (90.80 ± 2.55%). Lastly, kojic acid recorded intracellular melanin of 17.36 ± 0.21% at the highest concentration tested. The reduction of intracellular melanin content in α-MSH stimulated B16F10 melanoma cells by the mushroom extracts further proved their anti-melanogenesis activity.

Table 4.3: Effect of mushroom extracts on intracellular melanin content in B16F10 melanoma cells

Extract	Melanin content (%)[a]						Melanin content with α-MSH stimulation (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
Untreated control	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
A. bisporus (brown)	106.07	101.30	99.63	88.42	71.88	57.05	-	-	-	-	23.96	15.24
	± 4.43	± 1.66	± 3.67	± 3.15*	± 2.54*	± 3.90*					± 1.56*	± 0.59*
A. bisporus (white)	111.93	101.37	108.74	111.59	102.31	82.88	-	-	-	-	-	-
	± 5.45	± 4.71	± 1.38*	± 0.82*	± 5.90	± 3.68*						
F. velutipes	102. 91	111.17	101.99	100.25	98.78	87.26	-	-	-	-	-	-
	± 6.50	± 5.19	± 2.78	± 3.30	± 3.19	± 3.14*						
G. lucidum	92.90	98.97	83.72	-	-	-	-	-	-	-	-	-
	± 3.01	± 1.80	± 3.25*									
G. frondosa	100.01	102.17	103.81	103.73	101.17	91.17	-	-	-	-	-	-
	± 0.90	± 1.45	± 3.10	± 2.09	± 4.26	± 6.49						
H. marmoreus	99.28	96.48	91.35	-	-	-	-	-	-	-	-	-
	± 1.97	± 3.16	± 2.68*									
L. edodes	102.90	98.18	97.82	98.04	99.08	85.62	-	-	-	-	-	-
	± 3.49	± 4.56	± 4.82	± 2.69	± 3.02	± 3.07*						
P. eryngii	100.88	104.47	96.92	98.70	93.11	73.05	-	-	-	-	-	90.80
	± 6.62	± 3.06	± 4.01	± 1.61	± 3.58	± 1.02*						± 2.55*

Table 4.3, continued

Extract	Melanin content (%)[a]						Melanin content with α-MSH stimulation (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
P. floridanus	106.40	105.96	103.10	109.77	94.72	77.31	-	-	-	-	-	80.53
	± 4.19	± 1.90*	± 1.89	± 3.88	± 2.00	± 3.44*						± 2.64*
P. pulmonarius	109.74	106.18	101.10	99.84	81.90	76.38	-	-	-	-	-	71.36
	± 3.66	± 4.73	± 2.22	± 1.87	± 3.58*	± 0.99*						± 2.06*
Kojic acid	83.61	80.25	70.36	58.29	47.40	-	-	58.61	48.91	38.13	27.60	17.36
	± 2.60*	± 1.66*	± 1.11*	± 3.81*	± 1.87*			± 3.14*	± 1.97*	± 0.60*	± 0.61*	± 0.21*

Untreated control was set as 100% and the data from experimental were expressed as percentage of untreated controls. *$p < 0.05$ significantly different from untreated control. [a] Relative activity (%) = Percentage of sample versus untreated control. The percentage of melanin content in α-MSH stimulated cells for kojic acid at concentration of log 3.0 was extrapolated from the graph.

4.3.3 Inhibitory effect of mushroom extracts on cellular tyrosinase activity

Tyrosinase inhibitors are widely-used hypopigmenting agents because they have a high specific in targeting tyrosinase enzyme without any side effects (Nagasaka *et al.*, 2015). Intracellular tyrosinase activity for the four selected mushroom extracts (*A. bisporus* (brown), *P.eryngii, P. floridanus* and *P. pulmonarius*) were assessed using L-DOPA as the substrates and the results were presented in Table 4.4. The *A. bisporus* (brown) extract treated cells showed the lowest percentage, indicating the best tyrosinase inhibitory activity. At concentration of log 2.8, the cellular tyrosinase activity was 32.65 ± 2.40% and further reduced to 17.93 ± 2.65% at concentration of log 3.0. These results showed that the inhibitory activity of *A. bisporus* (brown) was better than kojic acid (positive control) as it recorded cellular tyrosinase acitivity by 33.81 ± 7.41% at concentration of log 3.0. Both *P. floridanus* and *P. pulmonarius* extracts significantly reduced tyrosinase activity of treated cells to 93.08 ± 1.63% and 84.41 ± 2.26% at concentration of log 3.0, respectively.

Polysaccharides in *A. bisporus* and *P. pulmonarius* were mainly β-glucans whereas *P. florida* comprised of branched αβ-glucan with (1→3)-linked-α-D-Glucose as the main chain (Santos-Neves *et al.*, 2008; Baggio *et al.*, 2010; Kozarski *et al.*, 2011). These polysaccharides might contribute to the anti-tyrosinase activity of these extract in B16F10 melanoma cells. The hydroxyl group in the active compounds in mushroom extracts (*A. bisporus* (brown), *P. floridanus* and *P. pulmonarius*) could bind to the active site of tyrosinase enzyme, resulting in steric hindrance or changed conformation (Baek *et al.*, 2008). Besides that, hydroxyl group from active compounds in the mushroom extracts could form hydrogen bond to the active site of tyrosinase enzyme and therefore lower the enzymatic activity (Alam *et al.*, 2011). The inhibitory effect shown by the three selected mushroom extracts [*A. bisporus* (brown), *P. floridanus* and

P. pulmonarius] indicate that these extracts reduced melanogenesis in B16F10 melanoma cells by inhibiting the tyrosinase.

   Poor inhibitory effect on cellular tyrosinase activity was observed for *P. eryngii* extract at concentration of log 3.0 (96.49 ± 3.26%). Anti melanogenesis activity of this extract (*P. eryngii*) might work through other mechanism such as interference with melanosome maturation and transfer (Solano *et al*., 2006). Intracellular tyrosinase activity for kojic acid treated cells was 33.81 ± 7.41% at the same concentration.

Table 4.4: Effect of four mushroom extracts on cellular tyrosinase activity

Extract	Cellular tyrosinase activity (%)[a]	
	log 2.8	log 3.0
Untreated control	100.00	100.00
A. bisporus (brown)	32.65 ± 2.40*	17.93 ± 2.65*
P. eryngii	-	96.49 ± 3.26
P. floridanus	-	93.08 ± 1.63*
P. pulmonarius	-	84.41 ± 2.26*
Kojic acid	46.64 ± 5.74*	33.81 ± 7.41*

Untreated control readings (from dish containing media and α-MSH only) were set as 100% and readings of experimental dish were expressed as percentage of untreated controls. *$p < 0.05$ significantly different from untreated control. [a] Relative activity (%) = Percentage of sample versus untreated control. The percentage of cellular tyrosinase activity for kojic acid at concentration log 3.0 was extrapolated from the graph.

4.4 Anti-inflammatory activity of mushroom extracts

4.4.1 Inhibitory effect of mushroom extracts on NO level in LPS-stimulated RAW 264.7 macrophage cells

Exposure of the skin to ultraviolet B (UVB) rays resulting in pro-inflammatory mediator production such as prostaglandin, which is expected to act on the melanocytes to evoke sun tanning (Prunieras, 1986). Hyperpigmentation and inflammation have strong correlation as melanocyte activity is strongly influenced by inflammatory mediators and cytokines (Morelli & Norris, 1993). Anti-inflammatory assessment of the ten mushroom extracts were examined using RAW 264.7 macrophage cells as it is able to mimic the inflammatory response taking place in the biological system when stimulated with an endotoxin, LPS (Dong *et al.*, 2017). Upon stimulation with LPS, iNOS is highly expressed in macrophage and subsequently increase the concentration of NO (Gupta *et al.*, 2010). The level of NO was used as the inflammatory marker as overproduction of NO by iNOS is cytotoxic in inflammation (Kröncke *et al.*, 1997).

As illustrated in Table 4.5, *A. bisporus* (brown) extract significantly inhibited NO production at the six tested concentrations without a toxic effect on RAW 264.7 macrophage cells (Table 4.2). At concentration of log 3.0, *A. bisporus* (brown) extract was able to suppress the NO level in LPS-stimulated RAW 264.7 macrophage cells to $66.82 \pm 2.81\%$. A study by Moro *et al.* (2012) reported that *A. bisporus* extract significantly reduced NO level in LPS-stimulated RAW 264.7 cells. Fucogalactans, which is a type of polysaccharide from *A. bisporus* mushroom showed anti-inflammatory effect in mice (Komura *et al.*, 2010). Thus, the anti-inflammatory activity of *A. bisporus* (brown) extract in this present study could be due to the fucogalactans contained in the extract.

H. marmoreus extract significantly reduced the level of NO production from LPS-stimulated cells to 60.64 ± 0.36% at concentration of log 3.0. The *G. lucidum* extract reduced NO level to 88.29 ± 5.66% at concentration of log 2.0 and gradually reduced to 78.99 ± 4.18% at concentration of log 2.6. The *A. bisporus* (white), *P. floridanus* and *P. pulmonarius* extracts were able to reduce the NO level below 90% at the highest concentration (log 2.8 or log 3.0). Im *et al.* (2014) showed the hot water extract of *P. floridanus* mushroom exert inhibitory activities on NO production, iNOS protein expression and thus reduced inflammatory response in RAW 264.7 macrophage cells. The reduction of NO level in RAW 264.7 macrophage cells by *P. floridanus* in the current study could be due to the inhibition of iNOS expression.

The reduction of NO level for *F. velutipes* treated RAW 264.7 macrophage cells were less than 10% at concentration of log 3.0, indicate weak anti-inflammatory activity. *G. frondosa, L. edodes* and *P. eryngii* extracts were unable to reduce the level of NO in RAW 264.7 cells even at the highest concentration. The induction of NO production in RAW 264.7 macrophage cells for these three extracts (*G. frondosa, L. edodes* and *P. eryngii*) might indicate that they have good immunostimulatory activity instead of anti-inflammatory activity. L-NAME, a standard iNOS inhibitor, significantly reduced NO level to 64.71 ± 0.96% at concentration of log 2.0. Mushroom extracts at selected concentration(s) which recorded NO production below 90% was selected for TNF-α and IL-10 levels determination using ELISA kit in order to study their effect on cytokine productions.

Table 4.5: Effect of mushroom extracts on NO production in RAW 264.7 macrophage cells

Extract	NO production (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
Untreated control	100.00	100.00	100.00	100.00	100.00	100.00
A. bisporus (brown)	81.46 ± 7.38*	79.77 ± 6.97*	76.41 ± 5.41*	76.45 ± 5.96*	72.44 ± 3.94*	66.82 ± 2.81*
A. bisporus (white)	101.37 ± 1.25	102.42 ± 2.20	100.67 ± 1.24	98.79 ± 1.46	94.38 ± 1.02*	85.97 ± 0.73*
F. velutipes	101.84 ± 1.26	100.99 ± 1.13	101.88 ± 1.11	101.72 ± 1.04	99.51 ± 1.39	93.44 ± 2.18*
G. lucidum	88.29 ± 5.66*	87.23 ± 4.88*	81.24 ± 4.00*	78.99 ± 4.18*	-	-
G. frondosa	107.10 ± 1.76*	103.98 ± 2.80	107.84 ± 2.56*	111.86 ± 2.37*	111.05 ± 1.97*	103.73 ± 2.68
H. marmoreus	102.28 ± 0.98*	100.51 ± 0.46	96.76 ± 0.99*	90.23 ± 1.38*	80.46 ± 1.52*	60.64 ± 0.36*
L. edodes	107.90 ± 2.08*	109.06 ± 2.36*	110.81 ± 1.89*	112.03 ± 1.51*	109.19 ± 0.86*	101.21 ± 0.54
P. eryngii	103.68 ± 1.14*	102.68 ± 0.93*	104. 46 ± 0.86*	105.01 ± 0.99*	105.25 ± 1.34*	104.00 ± 2.26
P. floridanus	99.28 ± 0.70	99.74 ± 0.18	96.33 ± 0.37*	93.47 ± 0.91*	88.55 ± 2.05*	82.69 ± 3.31*

Table 4.5, continued

Extract	Melanin content (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
P. pulmonarius	96.01 ± 2.63	95.71 ± 2.85	93.56 ± 1.95*	93.35 ± 2.85*	89.17 ± 3.53*	-
L-NAME	64.71 ± 0.96*	56. 78 ± 1.32*	46.28 ± 1.88*	37.01 ± 1.65*	28.67 ± 1.21*	21.92 ± 0.70*

Untreated control readings (from well containing medium only) were set as 100% and readings of experimental well were expressed as percentage of untreated controls. *$p < 0.05$ significantly different from untreated control. [a] Relative activity (%) = Percentage of sample versus untreated control.

4.4.2 Inhibitory effect of selected mushroom extracts on TNF-α and IL-10 production in LPS-stimulated RAW 264.7 macrophage cells

Based on the result presented in Table 4.6, *A. bisporus* (brown) extract suppressed the level of TNF-α to $73.67 \pm 2.97\%$ at concentration of log 3.0 and increased IL-10 level at all the tested concentrations, except log 2.2 and log 2.4. The results showed that the level of IL-10 secretion for cells treated with *A. bisporus* (brown) extract was not concentration-dependent. The anti-inflammatory activity of *A. bisporus* (brown) was not effective in regulating the expression of anti-inflammatory cytokines (IL-10), but more effective to reduce the level of pro-inflammatory cytokines (TNF-α) at the highest concentration (log 3.0). At concentration log 3.0, *P. floridanus* extract recorded TNF-α level at $80.21 \pm 19.91\%$ and IL-10 at $272.16 \pm 41.41\%$. The *P. pulmonarius* extract at concentration log 2.8 reduced TNF-α level to $60.17 \pm 9.90\%$ and increased the level of IL-10 to $186.17 \pm 59.54\%$. According to Jedinak *et al*. (2011), anti-inflammatory activity of *Pleurotus* species works through the inhibition of NF-κB signaling pathway by inhibiting NO, TNF-α, IL-6 and other inflammatory mediators. Based on the results obtained in the current study, the mechanism of anti-inflammatory activity of *P. floridanus* and *P. pulmonarius* extracts might involve the NF-κB signaling pathway.

The *G. lucidum* extract increased both TNF-α and IL-10 level above 100% at most of the tested concentrations. Feng *et al.* (2010) obtained similar result for lycopene, a natural antioxidant compound that possessed anti-inflammatory activity without any inhibitory effect on TNF-α level. Anti-inflammatory activity of lycopene involved mitogen activated protein kinase (MAPK) and NF-κB signaling pathways. In their observation, lycopene significantly inhibited ERK1/2 and p38 MAPK activation but had no effect on activation of c-Jun N-terminal kinase (JNK) signaling pathway.

Another study conducted by Moro *et al*. (2012), showed that the expression of TNF-α was regulated by c-Jun N-terminal kinase (JNK) signaling pathway.

The increment of TNF-α and IL-10 for *H. marmoreus* extract was concentration-dependent. Among the six selected mushroom extracts, only *A. bisporus* (white) extract increased the level of TNF-α and reduced IL-10 at concentration of log 3.0. NF-κB signaling pathway is a complex pathway which involves various signaling molecules (Hoesel & Schmid, 2013). Anti-inflammatory effect of *H. marmoreus* and *A. bisporus* (white) extracts might not target the molecules which regulate the expression of TNF-α in RAW 264.7 macrophage cells, thus increasing this pro-inflammatory cytokine when stimulated with LPS. This study demonstrated that all the six selected mushroom extracts inhibited inflammatory response mainly through the attenuation of NO productions and less effective to regulate the expression of cytokines in RAW 264.7 macrophage cells.

Table 4.6: The effect of mushroom extract on TNF-α and IL-10 production in RAW 264.7 macrophage cells

Extract	TNF-α production (%)[a]						IL-10 production (%)[a]					
	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0	log 2.0	log 2.2	log 2.4	log 2.6	log 2.8	log 3.0
Untreated control	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
A. bisporus (brown)	113.42 ± 2.17*	110.83 ± 3.46*	108.64 ± 4.49	113.82 ± 2.63*	115.52 ± 8.21	73.67 ± 2.97*	115.80 ± 8.47	82.33 ± 10.49	85.49 ± 7.35	111.76 ± 12.42	126.53 ± 24.41	107.49 ± 8.69
A. bisporus (white)	-	-	-	-	-	107.17 ± 19.15	-	-	-	-	-	93.64 ± 28.91
G. lucidum	111.76 ± 2.74*	111.78 ± 0.35*	110.53 ± 2.64*	122.08 ± 2.31*	-	-	124.33 ± 20.55	93.04 ± 13.24	104.86 ± 24.28	158.13 ± 19.84*	-	-
H. marmoreus	-	-	-	-	74.30 ± 6.00*	95.12 ± 8.25	-	-	-	-	66.36 ± 15.43	95.48 ± 23.40
P. floridanus	-	-	-	-	78. 84 ± 7.02*	80.21 ± 19.91	-	-	-	-	139.02 ± 39.47	272.16 ± 41.41*
P. pulmonarius	-	-	-	-	60.17 ± 9.90*	-	-	-	-	-	186.17 ± 59.54	-

Untreated control (from well containing media and LPS only) was set as 100% and the experimental were expressed as percentage of untreated control. *$p < 0.05$ indicate significant difference from the untreated control. [a] Relative activity (%) = Percentage of sample versus untreated control.

4.5 Anti-melanogensis and anti-inflammatory activity of combination mushroom extracts

4.5.1 Effect of single and combination mushroom extracts on B16F10 melanoma and RAW 264.7 macrophage cells

The viability of B16F10 melanoma and RAW 264.7 macrophage cells after exposure to single and combination mushroom extracts are presented in Table 4.7. The viability of B16F10 melanoma cells for combination GL2.4PF3.0 and GL2.4PP3.0 was slightly lower than their single extract where both of the combination recorded cell viability around 74%. Combination GL2.4ABB3.0 showed the lowest cell viability in B16F10 melanoma cells ($46.11 \pm 4.11\%$). Due to the greater cytotoxicity effect in B16F10 melanoma cells (cell viability below 65%), combination GL2.4ABB3.0 was excluded from MTT assay in RAW 264.7 macrophage cells and subsequent anti-melanogenesis (intracellular melanin content assay and cellular tyrosinase assay) and anti-inflammatory assays (NO assay and determination of TNF-α and IL-10 using ELISA kit).

For RAW 264.7 macrophage cells, combination GL2.4PF3.0 and GL2.4PP3.0 have cell viability higher than their single extracts where both of the combinations recorded cell viability above 100%. Therefore these combinations (GL2.4PF3.0 and GL2.4PP3.0) were further tested for their anti-melanogenesis (intracellular melanin content assay and cellular tyrosinase assay) and anti-inflammatory activity (NO assay and determination of TNF-α and IL-10 using ELISA kit).

Table 4.7: The effect of single and combination mushroom extracts on B16F10 melanoma and RAW 264.7 macrophage cells

Extract	Cell viability (%)[a]	
	B16F10 melanoma cells	**RAW 264.7 macrophage cells**
Untreated control	100.00	100.00
GL2.4	87.66 ± 3.50*	88.51 ± 2.72*
ABB3.0	77.09 ± 2.21*	54.10 ± 0.32*
PF3.0	93.71 ± 1.58*	61.16 ± 0.74*
PP3.0	98.65 ± 3.37	43.24 ± 4.48*
GL2.4ABB3.0	46.11 ± 4.11*	-
GL2.4PF3.0	74.73 ± 0.73*[#^]	105.74 ± 2.44*[#^]
GL2.4PP3.0	74.80 ± 2.06*[#+]	103.63 ± 2.84[#+]

Untreated control readings (from well containing medium only) were set as 100% and the readings of experimental were expressed as percentage of untreated control. GL2.4: *G. lucidum* at concentration of log 2.4; ABB3.0: *A. bisporus* (brown) at concentration of log 3.0; PF3.0: *P. floridanus* at concentration of log 3.0; PP3.0: *P. pulmonarius* at concentration of log 3.0; GL2.4ABB3.0: Combination of *G. lucidum* at concentration of log 2.4 with *A. bisporus* (brown) at concentration of log 3.0; GL2.4PF3.0: Combination of *G. lucidum* at concentration of log 2.4 with *P. floridanus* at concentration of log 3.0; GL2.4PP3.0: Combination of *G. lucidum* at concentration of log 2.4 with *P. pulmonarius* at concentration of log 3.0. [a] Relative activity (%) = Percentage of sample versus untreated control. *$p < 0.05$ was statistically significant compared with the value of untreated control. [#]$p < 0.05$ was statistically significant compared with the value of single extract, GL2.4. [^]$p < 0.05$ was statistically significant compared with the value of single extract, PF3.0. [+]$p < 0.05$ was statistically significant compared with the value of single extract, PP3.0.

4.5.2 Inhibitory effect of single and combination mushroom extracts on intracellular melanin content in B16F10 melanoma cells and cellular tyrosinase activity

Table 4.8 showed the percentage intracellular melanin content in B16F10 melanoma cells. Both of the selected combination mushroom extracts, GL2.4PF3.0 and GL2.4PP3.0 significantly reduced intracellular melanin content to $69.37 \pm 1.22\%$ and $64.39 \pm 1.62\%$, respectively. To further prove their synergistic effect on anti-melanogenesis activity in B16F10 melanoma cells, the intracellular melanin content for cells treated with combination of GL2.4PF3.0 and GL2.4PP3.0 extracts with α-MSH stimulation were then assessed.

The intracellular melanin content for the combination GL2.4PF3.0 and GL2.4PP3.0 in α-MSH stimulated B16F10 melanoma cells were almost similar to their single extracts. The percentage of intracellular melanin content for cells treated with combination GL2.4PF3.0 was $87.28 \pm 3.30\%$ and did not show significant different to its single extract (pg 46, Table 4.3). The intracellular melanin content for cells treated with combination GL2.4PP3.0 was $83.48 \pm 2.52\%$ and the value was slightly lower than its single extract, GL.2.4 ($93.99 \pm 2.53\%$).

Based on the result presented in Table 4.8, both combinations of GL2.4PF3.0 and GL2.4PP3.0 recorded cellular tyrosinase activity $91.63 \pm 0.74\%$ and $86.61 \pm 1.82\%$, respectively. The cellular tyrosinase activity for these combination mushroom extracts also did not show significant different compared to their single extracts.

The results obtained in anti-melanogenesis activity indicate that combined mushroom extracts were unable to work optimally to reduce melanogenesis process in B16F10 melanoma cells as the percentage of intracellular melanin content in α-MSH stimulated cells and cellular tyrosninase activity were almost similar to their single extracts. The reduction of intracellular melanin content in non-stimulated cells could be

due to the reduction of cell number as the percentage of cell viability for combination mushroom extracts was lower than their single extracts.

Molecular weight, degree of branching, conformation and purity may influence the biological activity of polysaccharide (Smiderle $et\ al.$, 2010). Complex structure of polysaccharides for combination mushroom extracts may cause poor anti-melanogenesis activity in B16F10 melanoma cells.

Table 4.8: The effect of single and combination mushroom extract on intracellular melanin content in B16F10 melanoma cells and cellular tyrosinase activity

Extract	Melanin content (%)[a]	Melanin content with α-MSH stimulation (%)[a]	Cellular tyrosinase activity (%)[a]
Untreated control	100.00	100.00	100.00
GL2.4	83.72 ± 3.25*	93.99 ± 2.53	89.30 ± 0.92*
PF3.0	77.31 ± 3.44*	80.53 ± 2.64*	93.08 ± 1.63*
PP3.0	76.38 ± 0.99*	71.36 ± 2.06*	84.41 ± 2.26*
GL2.4PF3.0	69.37 ± 1.22 *[#]	87.28 ± 3.30*	91.63 ± 0.74*
GL2.4PP3.0	64.39 ± 1.62*[#+]	83.48 ± 2.52*[#+]	86.61 ± 1.82*

Untreated control was set as 100% and the data from experimental were expressed as percentage of untreated control. GL2.4: $G.\ lucidum$ at concentration of log 2.4; PF3.0: $P.\ floridanus$ at concentration of log 3.0; PP3.0: $P.\ pulmonarius$ at concentration of log 3.0; GL2.4PF3.0: Combination of $G.\ lucidum$ at concentration of log 2.4 with $P.\ floridanus$ at concentration of log 3.0; GL2.4PP3.0: Combination of $G.\ lucidum$ at concentration of log 2.4 with $P.\ pulmonarius$ at concentration of log 3.0. [a] Relative activity (%) = Percentage of sample versus untreated control. *$p < 0.05$ was statistically significant compared with the value of untreated control. [#]$p < 0.05$ was statistically significant compared with the value of single extract, GL2.4. [+]$p < 0.05$ was statistically significant compared with the value of single extract, PP3.0.

4.5.3 Inhibitory effect of single and combination mushroom extracts on NO, TNF-α and IL-10 level in LPS-stimulated RAW 264.7 macrophage cells

As shown in Table 4.9, the level of NO for combination GL2.4PF3.0 and GL2.4PP3.0 did not have significant difference from untreated controls. The PP3.0 extract was not tested in NO assay as it has cell viability below 50%. Both combination GL2.4PF3.0 and GL2.4PP3.0 did not have direct effect iNOS expression or iNOS activity as the levels of NO excreted by LPS-stimulated RAW 264.7 macrophage cells was almost similar with untreated control. Overproduction of NO in macrophage is toxic and may lead to various pathology of inflammatory diseases Including rheumatoid arthritis, diabetes, septic shock, transplant rejection, and multiple sclerosis (Aktan, 2004).

The combination mushroom extracts (GL2.4PF3.0 and GL2.4PP3.0) only affect the expression cytokines in RAW 264.7 macrophage cells by reducing the level of TNF-α and increased IL-10 production. The level of TNF-α for combination GL2.4PF3.0 was 74.50 ± 10.18% while combination GL2.4PP3.0 was 83.55 ± 5.73%. Besides, both combinations GL2.4PF3.0 and GL2.4PP3.0 increased the IL-10 level above 100%.

The complex structure of polysaccharide in combination GL2.4PF3.0 and GL2.4PP3.0 may enhance the ability of polysaccharides to regulate the expression of cytokines in RAW 264.7 macrophage cells. The ability of the combined mushroom extracts to regulate cytokines expression without regulating NO production will bring adverse effect as overproduction of NO by iNOS will cause pathogenesis of various diseases. Therefore, the combination mushroom extracts were not suitable to be used in cosmeceutical application as these extracts were unable to reduce the production of NO in RAW 264.7 macrophage cells. Queiros *et al.* (2009) reported the poor synergistic effect of combined extract for culinary mushroom in antioxidant activity. This finding

further support the current results where culinary mushroom might have a poor synergistic effect on biological activities.

Table 4.9: The effect of single and combination mushroom extracts on NO, TNF-α and IL-10 production in RAW 264.7 macrophage cells

Extract	NO production (%)[a]	TNF-α production (%)[a]	IL-10 production (%)[a]
Untreated control	100.00	100.00	100.00
GL2.4	81.24 ± 4.18*	110.53 ± 2.64*	104.86 ± 24.28
PF3.0	82.69 ± 3.31*	80.21 ± 19.91	272.16 ± 41.41*
GL2.4PF3.0	101.45 ± 3.34[#^]	74.50 ± 10.18*[#]	162.36 ± 22.75*[^]
GL2.4PP3.0	105.81 ± 2.93[#]	83.55 ± 5.73*[#]	237.03 ± 17.02*[#]

Untreated control (from well containing media and LPS only) was set as 100% and the data from experimental were expressed as percentage of untreated control. GL2.4: *G. lucidum* at concentration of log 2.4; PF3.0: *P. floridanus* at concentration of log 3.0; PP3.0: *P. pulmonarius* at concentration of log 3.0; GL2.4PF3.0: Combination of *G. lucidum* at concentration of log 2.4 with *P. floridanus* at concentration of log 3.0; GL2.4PP3.0: Combination of *G. lucidum* at concentration of log 2.4 with *P. pulmonarius* at concentration of log 3.0. [a] Relative activity (%) = Percentage of sample versus untreated control. *$p < 0.05$ was statistically significant compared with the value of untreated control. [#]$p < 0.05$ was statistically significant compared with the value of single extract, GL2.4. [^]$p < 0.05$ was statistically significant compared with the value of single extract, PF3.0.

4.6 Overall discussion

The realm of cosmeceuticals is rapidly expanding mainly due to the accessibility of new ingredients, financial bonus for developing new products, consumer demand and better knowledge of skin physiology (Draelos, 2008). Nowadays, there is a growing consumer demand for personal care products containing natural ingredients without any adverse effects. There has been dramatic growth in the sales of natural and organic personal care products in supermarkets, drug stores, salons and pharmacies across the world (Antignac *et al.*, 2011). The consumer demand for products that are perceived as healthier, organic and ecological is the major driver of natural and/or organic ingredient market (Antignac *et al.*, 2011). The finding of new natural products

which could be used as one of the ingredients in cosmeceutical products will give a great advantage to the cosmeceutical industry.

As an Asian country, we are exposed to sunlight daily and this situation may lead to several skin disorders like sun tanning or hyperpigmentation. Hyperpigmentation is not harmful to the skin, but it can cause significant cosmetic disfigurement and become a persistent psychological burden for the patient due to the limited efficacy of available treatment. Besides that, disfiguring facial lesion can significantly affect a person's overall emotional well-being and can contribute to decrease in social function, productivity at work, school and self-esteem (Ali *et al.*, 2015).

In vitro anti-melanogenesis and anti-inflammatory activity of selected medicinal and culinary mushroom have been studied due to the strong correlation of these two biological activities. These parameters have been used to measure the potential of single mushroom extracts and their combinations in whitening cosmeceutical application. The *A. bisporus* (brown) extract exhibited the best extract to inhibit melanogenesis process through direct inhibition on tyrosinase activity and has appreciable anti-inflammatory activity by reducing pro-inflammatory mediator level. *P. floridanus* and *P. pulmonarius* extracts showed moderate inhibitory activity in melanogensis and inflammatory response with similar mechanism as *A. bisporus* (brown) extract. The *P. eryngii* extract also showed moderate inhibitory activity in melanogenesis without direct effect on tyrosinase enzyme activity. However, the *P. eryngii* extract did not have an inhibitory effect on the inflammatory response in RAW 264.7 macrophage cells. *F. velutipes* extract showed weak inhibitory activity for both biological activities tested (anti-melanogenesis and anti-inflammatory activities). *A. bisporus* (white) and *L. edodes* extracts also showed weak melanogenesis inhibitory response in B16F10 melanoma cells while *H. marmoreus* extract was only good to inhibit inflammatory response in RAW 264.7 macrophage cells. Anti-inflammatory activity of *G. lucidum* extract was

better than its anti-melanogenesis activity while *G. frondosa* extract has poor inhibitory activities for both anti-melanogenesis and anti-inflammatory activities. In anti-inflammatory assay, the mushroom extracts were only effective to reduce NO production in RAW 264.7 macrophage cells without consistent inhibitory effect on cytokine production. In the current study, the combined mushroom extracts did not show synergistic effect on anti-melanogenesis and anti-inflammatory activities. Based on the results obtained in the current study, single extract was more effective in reducing melanogenesis (B16F10 melanoma cells) and inflammatory response (RAW 264.7 macrophage cells).

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

This study demonstrated that *A. bisporus* (brown) extract possessed the strongest inhibitory activity on melanogensis process and inflammation. *A. bisporus* (brown) extract inhibited melanogensis process in B16F10 melanoma cells by reducing the cellular tyrosinase activity, and subsequently reduced the production of intracellular melanin content. It also reduced inflammation by inhibiting NO and TNF-α production in RAW 264.7 macrophage cells. In the current study, the combined extracts did not show synergistic effect on anti-melanogenesis and anti-inflammatory activities. Therefore, the single extract will be more preferable to be used for further studies.

In conclusion, *A. bisporus* (brown) extract has high potential to be developed as natural ingredients as brightening agent in cosmeceutical products, and sooth the inflammatory response on the skin. However, further study in molecular level need to be conducted in order to clearly understand the mechanisms of inhibition for both biological activities. *In vivo* study should also be conducted to understand the mechanism of the mushroom extract in complex biological system of organism. Other than that, the penetration efficacy of the extract through the skin also need to be evaluated as penetration of cosmeceutical products through epidermis of skin is one of important parameter to measure the effectiveness of a product.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of presentations:

Mat Saad, H., Tan, Y. S., & Sim, K. S. (2016, Dec). *Synergistic effect of selected edible mushrooms on melanogenesis inhibition in B16F10 melanoma cells.* Paper presented at 21st Biological Science Graduate Congress, Kuala Lumpur, Malaysia.

Mat Saad, H., Tan, Y. S., & Sim, K. S. (2016, Oct). *Anti-melanogenesis effect of edible mushrooms on B16F10 melanoma cells.* Paper presented at 41st Annual Conference of Malaysian Society for Biochemistry and Molecular Biology, Kuala Lumpur, Malaysia.