Songklanakarin J. Sci. Technol.
42 (4), 865-872, Jul. - Aug. 2020

http://www.sjst.psu.ac.th

Original Article

Anti-oxidative, anti-inflammatory, and anti-estrogenic effects of mushroom *Mycoamaranthus cambodgensis* (Pat.) Trappe, S. Lumyong, P. Lumyong, Sanmee & Zhu L. Yang extract

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Received: 7 March 2019; Revised: 29 April 2019; Accepted: 15 May 2019

Abstract

Mycoamaranthus cambodgensis (Pat.) Trappe, S. Lumyong, P. Lumyong, Sanmee & Zhu L. Yang (Hymenogasteraceae) is a wild edible mushroom found in the deciduous forests of Thailand. It is used as a folk medicine for postpartum recovery. This study aimed to investigate the anti-oxidative, anti-estrogenic, and anti-inflammatory activities of *M. cambodgensis* extract (MCE). The MCE showed high anti-oxidative activity. From the DPPH and FRAP methods, the 50% effective concentrations were 55.51±3.62 µg/mL and 166.64±11.43 µg/mg, respectively. The extract contained high total phenolic (113.80±3.13 mg tannic acid equivalent/g) and flavonoid contents (2.61±0.12 mg quercetin equivalent/g). The MCE decreased pro-inflammatory mediator gene expressions including COX-2, IL-1β, IL-6, iNOS, and TNF-α. In 17β-estradiol (E2)-induced MCF-7 cells, the MCE at 12.5 µg/mL synergistically augmented the proliferative effect of E2, whereas MCE at 50–100 µg/mL significantly suppressed this E2 effect. The potent anti-oxidant content and anti-estrogenic activity of MCE may correlate to its anti-inflammatory activity and traditional uses. This mushroom is a good candidate for further natural product development.

Keywords: *Mycoamaranthus cambodgensis*, anti-oxidative activity, anti-estrogenic activity, anti-inflammatory activity

1. Introduction

Estrogen is a major risk factor for breast cancer development via both the estrogen receptor (ER)-dependent and independent mechanisms. In the ER-dependent pathway, estrogen activates estrogen-response gene expression, stimulates cell proliferation and expansion of estrogen-responsive neoplastic cells (Cavalieri *et al.*, 2006; Yager & Davidson, 2006). For the ER-independent pathway, estrogen induces genotoxic estrogen metabolites and the formation of free radicals and reactive oxygen species, leading to DNA damage (Clemons & Goss, 2001, Patel & Bhat, 2004, Yager, 2000,

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Yager & Davidson, 2006). Oxidative stress is associated with estrogen-induced carcinogenesis (Bhat, Calaf, Hei, Loya, & Vadgama, 2003; Mense *et al.*, 2008). Mense and colleagues (Mense *et al.*, 2008) reported an important role of estrogen in oxidative stress induction that leads to breast cancer development in *in vivo*. During estrogen exposure in female ACI rats, estrogen induced breast morphological changes and increased breast cell proliferation. In addition, lipid peroxidation increased and anti-oxidant enzymes were upregulated which suggested elevated hydrogen peroxide production and oxidative stress in the cells (Mense *et al.*, 2008).

Inflammation, especially chronic and unregulated inflammation, plays an important role in cancer development. Immune and stromal cells surrounding the tumor microenvironment secrete several pro-inflammatory mediators which promote cell transformation and malignancy such as tumor necrosis factor (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β), cyclooxygenase-2 (COX-2) (Culig, 2011; Landskron, De la Fuente, Thuwajit, Thuwajit, & Hermoso1, 2014). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced from inflammatory processes are also involved in carcinogenesis (Landskron *et al.*, 2014). Suppressive effects of anti-inflammatory agents on pro-inflammatory mediator production can help prevent and treat cancers (Rayburn, Ezell, & Zhang, 2009; Todoric *et al.* 2016).

Mycoamaranthus cambodgensis (Pat.) Trappe, S. Lumyong, P. Lumyong, Sanmee & Zhu L. Yang. is an edible mushroom and naturally found in the forests of Thailand and several countries in southeastern Asia (Lumyong, Sanmee, Lumyong, Yang, & Trappe, 2003). It is in the family Hymenogasteraceae (Chandrasrikul *et al.*, 2011). The thin outer membranous layer of the mushroom is yellowish (Figure 1). Its white gleba changes to dark brown gleba upon maturation (Lumyong *et al.*, 2003). In Thailand, the mushroom is used in traditional medicine to help maintain regular menstrual cycles, treat tumors, and nourish health. The nutritional, phenolic, and flavonoid content of *M. cambodgensis* was recently investigated (Srikram & Supapvanich, 2016); however, there has been no scientific study of the pharmacological effects on anti-estrogenic and anti-inflammatory activities which correlate with its traditional uses. Therefore, the objectives of this study were to investigate the anti-oxidative, anti-estrogenic, and anti-inflammatory activities of *M. Cambodgensis* aqueous ethanolic extract *in vitro*.

2. Materials and Methods

2.1 Extract preparation

Fresh *M. cambodgensis* fruiting bodies were collected from a community forest in Wattana Nakorn District, Sa Kaeo Province, Thailand (13º74′49″ N, 102º28′70″ E) in October 2014. A specimen (No. N. Fangkrathok M001) was deposited in the herbarium at the Faculty of Agricultural Technology, Burapha University Sakaeo Campus, Sa Kaeo, Thailand. Powder of the dried mushroom was macerated in 50% ethanol for 7 d at room temperature. The filtered extract was concentrated under rotary evaporator and the yield of crude *M. cambodgensis* extract (MCE) was 15.1% w/w dry weight.

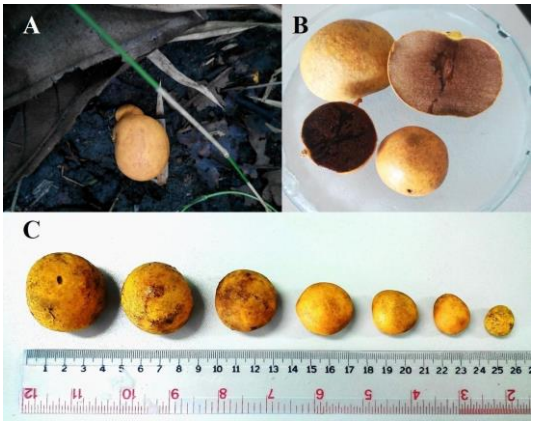

Figure 1. *M. cambodgensis* fruiting body morphology. A) a fruiting body in natural habitat, B) light and dark brown gleba with yellowish thin outer membranous layer, C) various sizes of fruiting bodies.

2.2 Determination of anti-oxidative activity

2.2.1 DPPH radical scavenging assay

Anti-oxidative activity was determined using DPPH radical scavenging assay (Oh, Kim, Choi, Chung, & Ham, 2008). Briefly, MCE was diluted with methanol. A quantity of 200 µL of 1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was added with diluted MCE (2800 µL). After 15 min, absorbance of the bleaching solution was measured at 515 nm. Radical scavenging inhibition was calculated and expressed as 50% effective concentration (EC_{50}). Ascorbic acid and α-tocopherol were used as positive compounds.

2.2.2 Ferric reducing antioxidant power assay

Anti-oxidative activity was also determined using the ferric reducing antioxidant power (FRAP) assay (Lado, Then, Varga, Szoke, & Szentmihályi, 2004). Briefly, FRAP reagent (50 mL of 300 mM acetate buffer pH 3.6, 5 mL of 10 mM 2,4,6-tripyridyl-s-triazine solution and 5 mL of 20 mM $FeCl_3$ solution) was freshly prepared. Ferrous sulfate ($FeSO_4$) was used as a standard and diluted in 40 mM HCl, whereas the MCE sample was diluted in ethanol. The diluted MCE or $FeSO_4$ solution (6 µL) was mixed with distilled water (18 µL) and FRAP reagent (180 µL). After 4 min, the solution was measured at the absorbance of 600 nm. The relative anti-oxidative activity was calculated by comparison with a standard curve.

2.3 Determination of total phenolic content

The Folin-Ciocalteu method was used to determine the total phenolic content (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, tannic acid or MCE was diluted with ethanol and then 0.5 mL of the tannic acid solution or MCE was mixed with the Folin reagent (0.25 mL) and 20% sodium carbonate solution (0.25 mL). After 40 min, the solution absorbance was measured at 725 nm. Total phenolic content was calculated by comparison with a tannic acid standard curve and expressed as mg tannic acid equivalent/g (mg TAE/g).

2.4 Determination of total flavonoid content

Total flavonoid content was determined by using aluminum chloride colorimetric method as modified from Chandra and colleagues (Chandra *et al.*, 2014). Briefly, quercetin or MCE was diluted in ethanol and then 0.5 mL of quercetin or MCE was mixed with 2% aluminum chloride (0.5 mL). After 1 h, the solution absorbance was measured at 420 nm. By comparison with quercetin standard curve, the sample absorbance was calculated and expressed as mg quercetin equivalent/g (mg QCE/g).

2.5 Determination of anti-inflammatory activity

RAW264.7 cells, murine macrophages were cultured in a 96-well plate (1×10^4 cells/well) containing culture medium (10% fetal bovine serum and 1% penicillin-streptomycin supplemented with Dulbecco's modified Eagle's medium [DMEM]) and incubated in a CO_2 incubator at 37 ºC for 24 h. The MCE was diluted in culture medium and then added to the cells in the presence or absence of 1 µg/mL *Escherichia coli* lipopolysaccharide (LPS). After 24-h incubation, the cells were analyzed for cell viability using Alamar blue assay (Page, Page, & Noel, 1993). Cell viability was calculated and expressed as 50% inhibitory concentration (IC_{50}).

For anti-inflammatory activity, RAW264.7 cells were cultured overnight in a 12-well plate (1×10^5 cells/well) and then treated with MCE and positive control compounds including indomethacin (inhibitors for IL-1β, IL-6, TNF-α, and COX-2 gene expression) and aminoguanidine (inducible nitric oxide synthase or iNOS gene expression inhibitor). After incubation for 22 h, LPS (final concentration of 1 µg/mL) was added and then further incubated for 2 h. Harvested cells were extracted for total RNA using a GE Healthcare extraction kit. Total RNA (40 ng) was used to synthesize first-strand cDNA using the Omniscript Reverse Transcriptase Kit. Each cDNA sample was mixed with Taq polymerase and deoxynucleotide mix, and each primer included β-actin (Won *et al.*, 2006), IL-1β (Sugawara, Yamada, & Mizuno, 2003), IL-6 (Sugawara *et al.*, 2003), TNF-α (Won *et al.*, 2006), COX-2 (Won *et al.*, 2006), and iNOS (Won *et al.*, 2006). Amplification was completed using 30 cycles and the polymerase chain reaction (PCR) amplification conditions followed Won and colleagues (Won *et al.*, 2006) and Sripanidkulchai and colleagues (Sripanidkulchai, Junlatat, Wara-aswapati, & Hormdee, 2009). PCR products were then analyzed on 1.5% agarose gel, visualized using Novel Joice staining. The product densities were measured by a Gel Documentation and System Analysis machine. The relative mRNA expression level was analyzed by comparison with β-actin and expressed as IC_{50}.

2.6 Determination of anti-estrogenic activity

The breast cancer cell line MCF-7 was cultured in an estrogen limited cell culture medium (phenol red-free minimum essential medium [MEM] supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 µg/mL insulin, and 1% of an antibiotic-antimycotic mixture in the absence of fetal bovine serum) and incubated in CO_2 incubator at 37 ºC for 3 d. The cells were then harvested and re-cultured (1×10^4 cells/well, 100 µL/well) with a working culture medium (phenol red-free MEM supplemented with all those supplements and 5% dextran-coated charcoal-treated fetal bovine serum) in a 96-well plate. MCE was diluted to various concentrations with the working culture medium and then 100 µL was added into the cells in the presence and absence of 17*β*-estradiol (E2) at the final concentration of 200 pM. In order to confirm cell proliferating activity of E2 against the tested MCF-7, the cells were treated with various concentrations of E2 (final concentrations of 2, 20, and 200 pM). After incubation for 4 d, cell viability was analyzed using Alamar blue assay (Page *et al.*, 1993). Cell proliferation was calculated from fluorescent intensity value and compared with untreated cells. The results were expressed as % cell proliferation.

2.7 Statistical analysis

All experiments were performed in triplicate. The results are expressed in mean±SD. One-way ANOVA and multiple comparison least significant difference (LSD) were analyzed using SPSS version 16.0.

3. Results

3.1 Anti-oxidative activity, total phenolic and total flavonoid contents of MCE

MCE showed high anti-oxidative activity (EC_{50} of 55.51±3.62 µg/mL) in the DPPH assay and a high FRAP value (166.64±11.43 µg/mg) (Table 1). The extract contained both phenolics and flavonoids in concentrations of 113.80±3.13 mg TAE/g and 2.61±0.12 mg QCE/g, respectively. These chemical contents might play roles in the anti-oxidative activity.

3.2 Anti-inflammatory activity of MCE

The cell viability of RAW264.7 cells was analyzed to obtain a safe concentration of MCE. In the presence or absence of LPS, MCE at a concentration lower than 200 µg/mL showed very low cytotoxicity (data not shown). MCE showed strong anti-inflammatory activity against LPS-induced RAW264.7 cells (Figure 2). Without LPS, MCE did not increase any gene expressions compared with untreated cells. The levels of genetic expression of all pro-inflammatory mediators were elevated when treated with LPS alone. MCE significantly decreased the expression of the elevated IL-1β and IL-6 genes in dose-dependent manners. In particular, MCE at concentrations of 100–200 µg/mL could decrease these gene expression levels to a level similar to indomethacin at 50 µg/mL. MCE significantly suppressed the LPS-induced COX-2 expression in a dose-dependent manner and MCE at the concentration of 200 µg/mL could suppress COX-2 expression to nearly the same level as indomethacin. MCE could significantly reduce LPS-induced TNF-α expression with strong activity at the concentration of 200 µg/mL. Interestingly, MCE strongly suppressed LPS-induced expression of the iNOS gene, as observed by an extract concentration of 200 µg/mL that reduced the iNOS gene expression to the baseline level of untreated cells. In addition, MCE at the concentration of 100 µg/mL could decrease iNOS

Table 1. Anti-oxidative activity, total phenolic and flavonoid contents of *M. cambodgensis* extract (MCE).

Extract/Standard compounds	Anti-oxidant activity		Total phenolic content (mg TAE/g)	Total flavonoid content (mg QCE/g)
	DPPH (EC_{50}, µg/mL)	FRAP (µg/mg)		
MCE	55.51±3.62	166.64±11.43	113.80±3.13	2.61±0.12
Ascorbic acid	3.84±0.09	ND	ND	ND
α-Tocopherol	7.75±0.03	ND	ND	ND

DPPH=2,2-diphenyl-1-picrylhydrazyl, EC_{50}=50% effective concentration, FRAP=ferric reducing antioxidant power, TAE=tannic acid equivalent, QCE=quercetin equivalent, ND=no determination

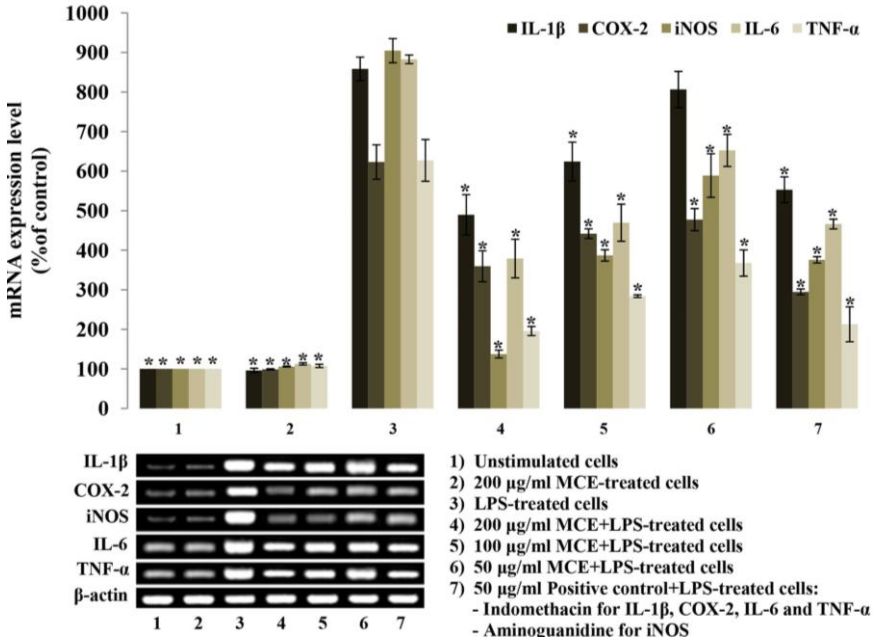

Figure 2. Effects of *M. cambodgensis* extract (MCE) on pro-inflammatory mediator gene expressions of LPS-induced RAW264.7 cells.

gene expression to a level similar to 50 µg/mL of amino-guanidine which is an iNOS gene expression suppressor. These results indicated a potent anti-inflammatory property of MCE.

3.3 Anti-estrogenic activity of MCE

The ER positive breast cancer cell line MCF-7 was evaluated for cell proliferation in responsiveness to E2 induction (Figure 3). In the presence of E2 (2–200 pM), MCF-7 was induced to proliferate in a dose-dependent manner. Without E2, MCE at concentrations of 12.5–100 µg/mL did not increase cell proliferation except for MCE at a concentration of 50 µg/mL that slightly increased the cell proliferation. With 200 pM E2 at a concentration of 12.5 µg/mL, MCE significantly increased cell proliferation higher than the effect of E2 alone which indicated that MCE at a lower concentration might synergistically augment the inductive effect of E2 on cell proliferation. However, MCE at concentrations of 50–100 µg/mL strongly suppressed the inductive effect of E2 on cell proliferation. Interestingly, MCE at a concentration of 50 µg/mL completely inhibited the effect

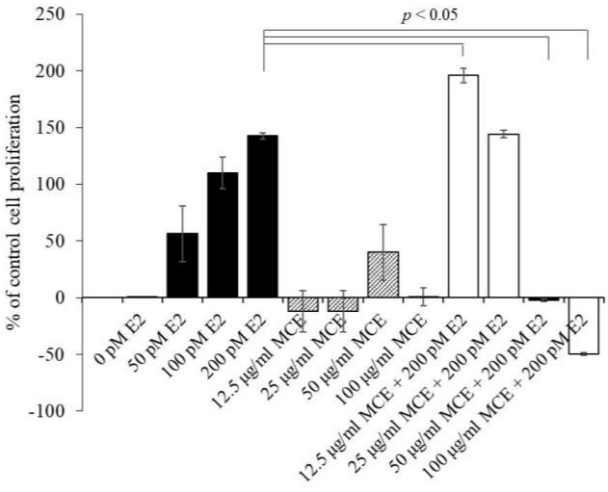

Figure 3. Effects of *M. cambodgensis* extract (MCE) on MCF-7 cell proliferation in the presence and absence of E2.

of E2 to a level similar to untreated cells in the absence of E2. MCE at a concentration of 100 µg/mL not only completely inhibited the effect of E2 but also decreased cell proliferation to a lower level than the untreated cells in the absence of E2 (Figure 3). These results indicated that MCE alone did not have estrogenic properties, but the combination of E2 with MCE showed strong anti-estrogenic activity against MCF-7 cells.

4. Discussion

With nutritional and medicinal properties, mushrooms have been reported to have several pharmaceutical effects such as anti-tumor, anti-inflammatory, anti-viral, and immunomodulatory activities (Elsayed, Enshasy, Wadaan, & Aziz, 2014; Wasser, 2002). Abundant bioactive substances were found in mushrooms such as polysaccharides, phenolics, polyphenolics, flavonoids, terpenoids, ergosterols, and peptides. Many compounds from mushrooms were reported to show significant anti-inflammatory activity such as ergosterol from *Inonotus obliquus* (Ma, Chen, Dong, & Lu, 2013), ganoderic acid from *Ganoderma lucidum* (Akihisa *et al.*, 2007), cordymin from *Ophiocordyceps sinensis* (formerly known as *Cordyceps sinensis*) (Qian, Pan, & Guo, 2012), and fucomannogalactan from *Lentinula edodes* (Carbonero *et al.*, 2008). *M. cambodgensis* is a wild mushroom that is used as a folk medicine by Thai folk healers in the treatment of postpartum health problems and tumors. Recently, we reported that *M. cambodgensis* fruiting bodies contained D-mannitol. This bioactive compound showed anti-inflammatory activity against RAW264.7 cells via suppression of COX-2, IL-6, iNOS, and TNF-α genes (Fangkrathok, Yodsaoue, Junlatat, Sripanidkulchai, & Jaisamut, 2017). In the present study, ethanolic extract of *M. cambodgensis* or MCE showed anti-oxidative properties. Moreover, its activity was relatively lower than α-tocopherol and ascorbic acid by approximately 7 and 14 times, respectively. The high total phenolics and flavonoids contents might be associated with the anti-oxidative activity of the extract. Mannitol has free radical scavenging properties (Andre & Villain, 2017); therefore, it might also play a role in the anti-oxidative activity of MCE.

In this study, MCE showed strong anti-inflammatory activity by suppression of the LPS-induced pro-inflammatory mediator gene expressions including IL-1β, IL-6, COX-2, iNOS, and TNF-α. MCE could reduce these gene expressions at a similar or a better degree than the positive control suppressors, indomethacin and aminoguanidine. The suppressive effect on pro-inflammatory mediator genes, especially iNOS, was possibly due to its anti-oxidative activity and chemical contents including phenolics, flavonoids, and mannitol (Fangkrathok *et al.*, 2017). In LPS-exposed macrophages, reactive molecules such as hydroxyl free radicals, hydrogen peroxide, and nitrogen monoxide are produced and involved in cellular signaling for pro-inflammatory gene expressions. These pro-inflammatory mediators including COX-2, iNOS, TNF-α, IL-1β, and IL-6 also play roles in oxidative stress-induced inflammation (Federico, Morgillo, Tuccillo, Ciardiello, & Loguercio, 2007; Hussain & Harris, 2007). This oxidative stress and inflammatory environment can harm surrounding cells, and if this harm occurs over the long term, it may lead to carcinogenesis (Hussain & Harris, 2007; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). In addition, an imbalance between reactive free radicals and endogenous anti-oxidative defense mechanisms can cause several diseases such as chronic inflammation, diabetes, cardiovascular diseases, ischemia, anemia, and cancer (Arulselvan *et al.*, 2016; Ravipati *et al.*, 2012). Consumption of natural anti-oxidant containing diets or dietary supplements can be an alternative way to prevent illness. Both mycelia and fruiting bodies of many edible mushrooms are well-known as natural anti-inflammatory sources that exert a health beneficial potency. Such mushrooms include *Agaricus bisporus* (Moro *et al.*, 2012), *O. militaris* (formerly known as *Cordyceps militaris*) (Won & Park, 2005), *Grifola frondosa* (Zhang, Mills, & Nair, 2002), *Ganoderma lucidum* (Dudhgaonkar, Thyagarajan, & Sliva, 2009), *Inonotus obliquus* (Ma *et al.*, 2013), *Lentinula edodes* (Carbonero *et al.*, 2008), and *Lentinus polychrous* (Fang krathok, Junlatat, & Sripanidkulchai, 2013). The data from other studies suggest that *M. combodgensis* can also be a good anti-oxidative and anti-inflammatory source for the treatment and prevention of illnesses.

Moreover, MCE at concentrations of 50–100 µg/mL also showed strong anti-estrogenic activity against E2-induced MCF-7 cell proliferation. However, MCE at a concentration of 12.5 µg/mL showed synergistic activity when co-treated with 200 pM E2 which resulted in increased breast cancer cell proliferation. MCE showed biphasic effect of both stimulation and suppression on E2-induced MCF-7 cell proliferation that might depend on its concentration or ER binding interference with a dual effect of estrogen on breast cancer cell proliferation. Estrogen signaling in breast cancer is very complex and involved with subsequent signaling to modulate many different targets. The dual role of E2 on target cells depends on the E2 concentration and the type of ER. Estrogen at low concentrations in the range of picograms can promote cell proliferation. These low concentrations are sufficient to increase breast cancer risk in postmenopausal woman (Chatrite, Cortes-Prieto, Philippe, Wright, & Pasqualini, 2007). Dose-dependent interaction of phytoestrogens with E2 was previously reported. Low concentrations (0.01–10 µM) of genistein and coumestrol significantly enhanced E2-induced MCF-7 cell proliferation by increasing DNA synthesis, whereas at high concentrations (>10 µM) the compounds caused inhibition (Wang & Kurzer, 1998). MCE at a low concentration in this study may also exert a synergistic activity on cell proliferation.

In contrast, estradiol at high concentrations (5–10 µM) can inhibit ER positive breast cancer cell proliferation by changing cell cycle kinetic parameters (Reddel & Sutherland, 1987). Le Bail and colleagues (Le Bail, Varnat, Nicolas, & Habrioux, 1998) reported anti-proliferative activity of flavonoids on MCF-7 cells. At a high concentration of 50 µM, 7-methoxyflavanone and 7,8-dihydroxyflavone possessed anti-estrogenic and anti-proliferative activities through ER-independent mechanisms. So and colleagues (So, Guthrie, Chambers, & Carroll, 1997) reported anti-proliferating activity on MCF-7 cells of several flavonoids including gelangin, genistein, quercetin, baicalein, hesperetin, and neringenin. Only genistein showed competitive ER binding activity with estrogen. The others reported that flavonoids possibly exerted anti-proliferating activity by other mechanisms such as binding to type II estrogen binding sites (Markaverich, Roberts, Alejandro, & Clark, 1984; So *et al.*, 1997), direct or

indirect impairing the binding of ER to the estrogen response element (ERE), and affecting estrogen-induced transcription (Demirpence *et al.*, 1994; So *et al.*, 1997). Demirpence and colleagues (Demirpence *et al.*, 1994) and Swami and colleagues (Swami, Krishnan, & Feldman, 2000) reported the anti-estrogenic effect of 1,25-dihydroxyvitamin D3 or vitamin D3, active form of vitamin D, and its possible mechanism against MCF-7 cells by interfering with the estrogenic action at the ERE level and suppressing estrogen actions. Edible mushrooms contain an abundance of ergocalciferol or vitamin D2 that can be hydroxylated to 25-hydroxyvitamin D in the liver and then further hydroxylated to 1,25-dihydroxyvitamin D3 in the kidney (Ozzard, Hear, Morrison, & Hoskin, 2008). Studies in human and rats showed that ingestion of edible mushrooms can increase the level of serum vitamin D (Outila, Mattila, Piirornen, & Lamberg-Allardt, 1999, Jasinghe & Perera, 2005; Jasinghe, Perera, & Barlow, 2005). Therefore, mushroom consumption, including *M. cambodgensis*, may provide flavonoids and vitamin D that can help prevent and suppress breast tumor. This effective ability of MCE supports the traditional use of *M. cambodgensis* fruiting bodies. However, in order to better understand the mechanism of anti-estrogenic activity of MCE, further studies in the ER competitive binding and signaling cascade are needed.

Moreover, vitamin D3 is important for balancing menstrual cycle and ovary function. A peak rising serum level of 1,25-dihydroyvitamin D3 was observed at a regular ovulation period (Gray, McAdoo, Hatley, Lester, & Thierry, 1982), whereas a low level of this vitamin D3 was associated with irregular cycles (Jukic, Steiner, & Baird, 2015), which indicated that vitamin D3 may play an important role in regulating the menstrual cycle and ovulatory functions. Vitamin D2 from mushroom consumption may help to increase vitamin D3 in serum resulting in regulatory cycle of female reproductive system. This biological effect of vitamin D in mushrooms may support the traditional use of *M. combodgensis* for postpartum nourishment.

5. Conclusions

M. combodgensis extract showed anti-oxidative, anti-inflammatory, and anti-estrogenic activities which may be associated in part with its chemical composition including phenolics, flavonoids, and D-mannitol. These results support the use of *M. combodgensis* as a traditional medicine and can be a good candidate for natural product development. However, an *in vivo* study is needed and its mechanism of action needs to be explored.

Acknowledgements

This work was financially supported by the Research Grant of Burapha University through National Research Council of Thailand (Grant no. 129/2560). We thank the Center for Research and Development of Herbal Health Products, Khon Kaen University, Khon Kaen, Thailand for use of the facilities.

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