Current Applied Science and Technology Vol. 17 No. 2 Jul.-Dec. 2017

Inhibitions of Key Enzymes Relevant to Obesity and Diabetes of Thai Local Mushroom Extracts

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Abstract

The aim of this experiment was to determine inhibitory activities against lipase (anti-obesity property) as well as α-amylase and α-glucosidase (anti-diabetic property) of fifteen Thai local mushroom extracts including Kai-khao (*Russula* sp.), Kai-kiew (*Russula* sp.), Nha-lae (*Russula* sp.), Sa-med (*Boletus* sp.), E-tun (*Russula* aff. sp.), Ra-ngok-khao (*Amanita* aff. sp.), Kor-kar-dang (*Russula* sp.), Por-nung (*Astraeus* sp.), Por-fai (*Astraeus* sp.), Ra-ngok-luang (*Amanita* sp.), Kor-kar-khao (*Russula* sp.), Nam-mak (*Macowanites* sp.), Koh-noi (*Russula* aff. sp.), Din-khao (*Russula* sp.) and Khao-pang (*Russula* sp.) in Amnat-Charoen province, Thailand. All mushrooms were extracted using ddH_2O (20 mg/mL) in a 50°C water bath shaker for 8 hrs. The enzyme inhibitory activities were performed using a colorimetric microplate assay. The results showed that anti-lipase activities of mushroom extracts (4 mg/mL) were in the range of 12-77% inhibition. Din-khao mushroom exhibited the highest inhibition, while Por-fai and Por-nung mushrooms exhibited the lowest. The anti-α-glucosidase activities of mushroom extracts (5 mg/mL) were in the range of 10-79% inhibition. Sa-med mushroom exhibited the highest inhibition, while Kor-kar-dang mushroom exhibited the lowest. However, the inhibition of α-amylase could not be detected in this study. These findings are useful for potential health promotion for Thai local mushroom consumption in controlling obesity and diabetes through key enzymes inhibition.

Keywords: enzyme inhibitory activity, obesity, diabetes mellitus, Thai local mushroom

1. Introduction

Obesity and diabetes arecurrently major health problems in Thailand. Obesity is defined by an abnormal body fat mass accumulation [1], while diabetes is defined as the body's inability to produce enough insulin or respond to insulin [2]. The common underlying cause of both diabetes and obesity is insulin resistance. Medicinal approach for treatment/prevention of these diseases has currently been focussed on key enzyme inhibition. For obesity, inhibition of lipase, the key enzyme that hydrolyzes triacylglycerols to 2-monoacylglycerols and fatty acids, can lead to a slower rate of dietary fat absorption [3]. Likewise, inhibition of α-amylase and α-glucosidase, the key enzymes that control diabetes through hydrolysis of polysaccharide into smaller saccharide subunits, can delay carbohydrate degradation and glucose absorption [4].

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Currently, synthetic drugs for treatment of obesity and diabetes such as orlistat (anti-lipase agent) and acarbose (anti-α-amylase and anti-α-glucosidase agent) have been reported regarding their severe side effects [5]. Therefore, green medicine from natural products is presently of interest. Mushroom, a popular food ingredient around the world, is a group of macrofungi with low calorie content and high fiber [6]. It also contains essential proteins [7], polysaccharides, and significant quantities of micronutrients [8]. Besides, many scientific researches have reported that biochemical properties of mushrooms have positive effects on the treatment of some non-communicable diseases. Previous studies had suggested that some mushrooms possessed inhibitory activities against lipase [9, 10], α-amylase and α-glucosidase [11]. However, no information on the lipase, α-amylase, and α-glucosidase inhibitory activities of edible mushrooms in Thailand has been previously reported.

In Amnat-Charoen province, many edible wild mushrooms can be found in deciduous forests due to damp weather that is suitable for mushroom growth. Most of these mushrooms cannot be farm-grown, thus the mushrooms can only be harvested from nature, causing mushroom production to be different depending on the weather of each particular year. Thus, extinction of these wild edible mushrooms is highly possible. To promote wild edible mushrooms conservation, providing knowledge, especially biological property on diseases prevention of these mushrooms, to the locals is very important. Controlling of obesity and diabetes through key enzyme inhibition is of interest, since these diseases aremajor public health problems in Thailand. Therefore, the aims of this research was to investigate lipase,α-amylase and α-g

2. Materials and Methods

2.1 Sample collection and preparation

Fifteen wild edible mushrooms including Kai-khao (*Russula* sp.), Kai-kiew (*Russula* sp.), Nha-lae (*Russula* sp.), Sa-med (*Boletus* sp.), E-tun (*Russula* aff. sp.), Ra-ngok-khao (*Amanita* aff. sp.), Kor-kar-dang (*Russula* sp.), Por-nung (*Astraeus* sp.), Por-fai (*Astraeus* sp.), Ra-ngok-luang (*Amanita* sp.), Kor-kar-khao (*Russula* sp.), Nam-mak (*Macowanites* sp.), Koh-noi (*Russula* aff. sp.), Din-khao (*Russula* sp.) and Khao-pang (*Russula* sp.) mushrooms were harvested from Dong Yai community forest, Hua Taphan district, Amnat-Charoen province, Thailand. All fresh mushrooms were cleaned with ddH_2O and freeze-dried using a Lyo GT2-S-type freeze dryer (lyophilizer from GEA freeze-drying equipment, Köln, NW, Germany). The freeze-dry samples were grinded by a grinder (Philips 600W from Philips Electronic Co., Ltd., Jakarta, Indonesia) before storing in a vacuum aluminum foil bag. Mushroom powders were then kept in a -20°C freezer within 2 months for further analysis.

2.2 Sample extraction

All mushrooms were extracted under the optimized extraction conditions as previously reported [12]. Mushroom powder was briefly extracted in ddH_2O (20 mg/mL) by vortexing the mixture for 1 minute before shaking in water bath shaker at 50°C for 8 hours. The mixture was then centrifuged at 1190*xg* for 15 minutes using a Rotina 38R centrifuge (Hettich Lab Technology, Tuttlingen, Germany). The supernatant was collected and stored at -40°C for further analysis on the enzyme inhibitory assay.

2.3 Lipase inhibitory assay

The lipase inhibitory activity was modified from the method of Choi *et al*. [13]. The assay consisted of 50 μL mushroom extract (4 mg/mL well^{-1}), 100 μL *Candida rugosa* lipase (2 μg well^{-1}) in enzyme buffer (50 mM Tris, 10 mM KCl, 1 mM EDTA containing 0.1% (w/v) BSA, pH 8.0), 40 μL 2,3-dimercapto-1-propanol tributyrate (DMPTB, 0.05 mM well^{-1}) in assay buffer (50

mM Tris containing 10 mM KCl and 1 mM EDTA, pH 7.2) and 10 µL 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.8 mM well^{-1}) in 50 mM KPB (pH 7.4). The lipase reaction was monitored at a wavelength of 412 nm. The enzyme inhibitory activities were determined by a colorimetric assay using a microplate reader (Synergy HT multi–detection microplate reader, BioTek Instruments, Inc., Winooski, VT) with a Gen5 data analysis software. The results were expressed as a percentage of inhibitory activity using the following the equation;

$$\%\text{inhibition}=100\times(1-((B-b)/(A-a)),$$

where, A is the initial velocity of the control reaction with the enzyme (control), a is the initial velocity of the control reaction without the enzyme (control blank), B is the initial velocity of the enzyme reaction with mushroom extract (sample) and b is the initial velocity of the reaction with mushroom extract but without the enzyme (sample blank). Orlistat, a commercially available lipase inhibitor, was used as a positive control.

2.4 α-Glucosidase inhibitory assay

The α-glucosidase inhibitory activity was performed according to the protocol of You *et al*. [14]. The assay consisted of 50 µL mushroom extract (5 mg/mL well^{-1}), 100 µL *Saccharomyces cerevisiae* α-glucosidase (0.5 µg well^{-1}), and 50 µL *p*-nitrophenyl-α-D-glucopyranoside (pNPG, 0.5 mM well^{-1}) in 50 mM KPB (pH 7.0). The reaction was monitored at a wavelength of 405 nm. The percentage of inhibition was calculated as above. Acarbose, a competitive inhibitor, was used as a positive control.

2.5 α-Amylase inhibitory assay

The α-amylase inhibitory activity was investigated according to the method by Funke and Melzig [4]. The assay was comprised of mushroom extract (5 mg/mL well^{-1}), porcine pancreatic α-amylase (4 mg well^{-1}), and *p*-nitrophenyl-α-D-maltopentaoside (PNPG-5, 1.25 mM well^{-1}) in 50 mM KPB (pH 7.0) and 200 mM KCl. The reaction was monitored at a wavelength of 405 nm. The percentage of inhibition was calculated as above. Acarbose was used as a positive control.

2.6 Statistical analysis

The experiments were performed in triplicate. The data were expressed as mean ± standard deviation (SD). All statistical analyses were evaluated using a GraphPad Prism software version 6.01 for Windows (GraphPad Software, Inc., San Diego, CA, www.graphpad.com). The significance of differences was defined at $p < 0.05$ using one way analysis of variance (ANOVA), followed by Duncan multiple range test for differences between means values.

3. Results and Discussion

3.1 Lipase inhibitory activity

The lipase inhibitory activities of fifteen Thai local mushroom extracts (4 mg/mL) were in the range of 12-77% inhibitions (Table 1). Din-khao mushroom exhibited the highest lipase inhibitory activity, while Por-fai and Por-nung mushrooms exhibited the lowest. Anti-lipase activities were also observed in Japanese mushrooms, in which 8 edible mushrooms (0.1 mg/mL) from Nagano including *Agaricus blazei*, *Grifola frondosa, Hericium erinaceus, Hypsizygus marmoreus, Lyophyllum shimeji, Sparassis crispa* and *Pleurotus eryngii* exhibited lipase inhibitory activities of 20.8-113.8% inhibitions [1]. In addition, 13 species of edible Korea mushroom (0.5 mg/mL) from Chungnam provinces including *Cordyceps militaris*, *Hericium erinaceum*, *Ganoderma lucidum*, *Inonotus obliquus*, *Phellinus linteus*, *Ganoderma lucidum*, *Agaricus blazei*, *Pleurotus osteratus*,

Lentinus edodes, *Collybia velutipes*, *Auricularia auricular*, *Agaricus bisporus*, and *Fomitopsis pinicola* exhibited 10.8-72.5% lipase inhibitions [9]. Additionally, the anti-lipase activities in 8 edible Spain mushrooms (0.17 mg/mL) from Madrid including *Boletus edulis, Amanita ponderosa, Lentinula edodes, Marasmius oreades, Morchella conica, Lyophyllum shimeji, Pleurotus eryngii* and *Grifola frondosa,* exhibited 10-20% inhibition [10]. Comparing to orlistat (the IC_{50} of 1.7 μg/mL, Table 1 and Figure 1), these mushrooms exhibited much lower anti-lipase inhibitions, suggesting that mushrooms may not contain effective anti-lipase agents.

Table 1. The percentage of lipase and α-glucosidase inhibitory activity compared with concentration of commercial drugs (orlistat and acarbose) in 15 wild edible mushroom extracts from Amnat-Charoen province, Thailand

Mushrooms	Percentage of enzyme inhibition	
	#Lipase	*α-Glucosidase
Din-khao	76.74±2.54[a]	23.96±1.33[e,f]
E-tun	62.71±3.38[b]	24.01±2.09[e,f]
Koh-noi	51.98±2.77[c]	37.76±3.51[c]
Kai-kiew	41.02±2.74[d]	24.50±1.68[e,f]
Kor-kar-dang	34.93±1.01[e]	9.72±0.85[i]
Nam-mak	34.86±3.42[e]	12.59±1.10[h]
Kai-khao	33.23±2.32[e]	41.96±3.94[b]
Ra-ngok-khao	30.25±2.41[f]	25.54±2.23[e]
Khao-pang	25.92±1.95[g]	28.89±1.92[d]
Nha-lae	25.30±1.76[g]	36.57±2.27[c]
Sa-med	21.70±1.99[h]	78.75±4.53[a]
Kor-kar-khao	19.43±1.71[h]	15.08±1.46[h]
Ra-ngok-luang	19.42±1.79[h]	22.66±1.89[f,g]
Por-fai	12.73±1.14[i]	9.85±0.70[i]
Por-nung	11.53±0.99[i]	20.34±1.78[g]
Orlistat	IC_{50} = 1.7 μg/mL	-
Acarbose	-	IC_{50} = 116.2 μg/mL

The percentage of inhibitory activities were expressed by mean±SD (n=3). The different superscript letters showed the significantly difference of inhibitory activities among various mushrooms at $p<0.05$ using one-way ANOVA followed by Duncan's multiple rang test.
#The concentration of mushroom extracts = 4 mg/mLwell^{-1}.
*The concentration of mushroom extracts = 5 mg/mLwell^{-1}

It was previously suggested that lipase inhibitory activities of mushrooms were varied, depending on extraction conditions such as extraction solvent, incubation time and shaking temperature. For extraction solvent, it was previously reported that mushrooms extracted with water most likely exhibited higher lipase inhibitory activities than the ones extracted with absolute methanol [1]. Besides, a study comparing mushrooms extracted with water, absolute methanol and 50% (v/v) aqueous methanol suggested that 50% (v/v) aqueous methanolic extract exhibited the highest lipase inhibitory activity [10, 15]. These results suggested that anti-lipase agents from mushroom likely composed of functional group(s) with polarity index between 5.1 (methanol) to 10.2 (water). However, our research focused on key enzymes inhibitory activity with potential

application in human consumption, thus water extract was an appropriate solvent for extraction in this study. For extraction time, it was suggested that methanolic extract of *Phellinus linteus* exhibited optimal lipase inhibitory activity at extraction time of 24-72 hours. The thermal stability of mushrooms being extracted in boiling waterfrom 5 minutes to 2 hours also showed significant effect on lipase inhibitory activity [1]. It was found that mushrooms exhibited lipase inhibitory activity up to 1 hour; however, continual heating resulted in increased inhibitory activity [1]. Furthermore, the lipase inhibitory activity of mushroom was found to be stable under the period of incubation time (24-72 hours) at the temperature of 40-60°C and a wide range of pH (4-8) [9].

Generally, polyphenols from plants are expected to function as anti-lipase agents [16-18]. For example, polyphenol-rich extracts from berries (blueberries, cloudberries, arctic bramble, lingonberries, strawberries and raspberries; 50 μg/mL GAE phenols) were found to exhibit effective lipase inhibitions *in vitro* (10-80% inhibition), while cloudberry showed a saturation effective activity with the IC_{50} of 5 μg/mL phenols [18]. However, it was previously reported that mushrooms contain very few phenolic acids and no detected flavonoids [19]. Thus, anti-lipase agents from mushrooms may be polysaccharides and protein, since it has been previously reported that these macro-compounds could effectively retard lipase reaction; for examples, β-glucans (homopolysaccharides) and polysaccharides (heteropolysaccharides) from *Ganoderma lucidum* [20], crude protein contents and free amino acids from *Phellinus linteus* extract [21], and β-glucan from *Auricularia auricular-judae* and *Tremella fuciformis* [22].

Din-khao mushroom with 77% lipase inhibition, which is equivalent to mushroom at 8.4 mg fresh weight, was compared to orlistat at the same percentage of inhibition (equivalent to 1.35 μg). The recommended dose of orlistat in adult under obesity is 120 mg, three times a day. Thus, obese patients are needed to consume orlistat at the concentration of 360 mg/day. These quantities were equivalent to consumption of mushroom at 2.4 kg/day to receive the same effect as olistat uptake under the hypothesis that all bioactive compounds in mushroom are absorbed into body and are completely effective toward lipase. However, consumption of high amount of mushroom is unrealistic in daily life, since one serving of mushroom only contains 100-200 g fresh weight of mushroom. Thus, mushroom consumption should be promoted as alternative pathway for obesity prevention rather than treatment.

3.2 α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activities of mushroom extracts (5 mg/mL) were in the range of 10-79% inhibition (Table 1). Sa-med mushroom exhibited the highest α-glucosidase inhibitory activity, while Kor-kar-dang mushroom exhibited the lowest. Comparing to acarbose (the IC_{50} of 116.2 μg/mL, Table 1 and Figure 1.), these mushrooms exhibited much lower anti-α-glucosidase inhibitions. Likewise, an *in vitro* research on Thai mushrooms, *Lentinula edodes* (Hed-hom) and *Pleurotus pulmonarius* (Hed-nang-fa-pu-tan), suggested that their IC_{50} values were 29-804 mg/mL, which are also higher than acarbose (the IC_{50} of 0.06 mg/mL) [23]. Besides, the research on 7 edible mushrooms from Japan including *Agaricus bisporus, Hypsizigus marmoreus, Pleurotus ostreatus, Lentinus edodes, Pleurotus eringii, Grifola frondosa* and *Flammulina velutipes* exhibited α-glucosidase inhibitory activities in the range of 20-87% inhibition at 25 mg equivalent fresh weight (FD) of water extracts [24]. However, anti-α-glucosidase activities of 6 medicinal mushrooms in Nantou, Taiwan including *Grifola frondosa*, *Hericium erinaceum*, *Agaricus blazei*, *Ganoderma lucidum*, *Coriolus versicolor*, and *Phellinus linteus* reported that their inhibitory activities (IC_{50} of 0.04-0.17 mg/mL) were stronger than acarbose (IC_{50} of 4.69 mg/mL) [11]. Different IC_{50} values reported in each literature might be a result of different methodologies of the enzyme assay. In the study of Su *et al*. [11], which reported high IC_{50} value of acarbose, it was indicated that the enzyme assay was performed using starch as a substrate and dinitrosalicylic acid (DNS) as an indicator. The DNS can react with reducing sugar, forming 3-amino-5-nitro salicylic acid, which can be detected at 540 nm. It was also suggested that method of DNS

normally overestimated the results of the assay [25]. Other experiments were performed using pNPG, the substrate with attached indicator, thus providing easier, less complicated, more reliable enzyme assay.

Figure 1. Size, shape and color of fresh edible Thai local mushrooms used in this study, which included **(a)** Kai-khao (*Russula* sp.), **(b)** Kai-kiew (*Russula* sp.), **(c)** Nha-lae (*Russula* sp.),**(d)** Sa-med (*Boletus* sp.),**(e)** E-tun (*Russula* aff. sp.), **(f)** Ra-ngok-khao (*Amanita* aff. sp.), **(g)** Kor-kar-dang (*Russula* sp.), **(h)** Por-nung (*Astraeus* sp.), **(i)** Por-fai (*Astraeus* sp.)**, (j)** Ra-ngok-luang (*Amanita* sp.), **(k)** Kor-kar-khao (*Russula* sp.), **(l)** Nam-mak (*Macowanites* sp.), **(m)** Koh-noi (*Russula* aff. sp.), **(n)** Din-khao (*Russula* sp.) and **(o)** Khao-pang (*Russula* sp.) mushrooms.

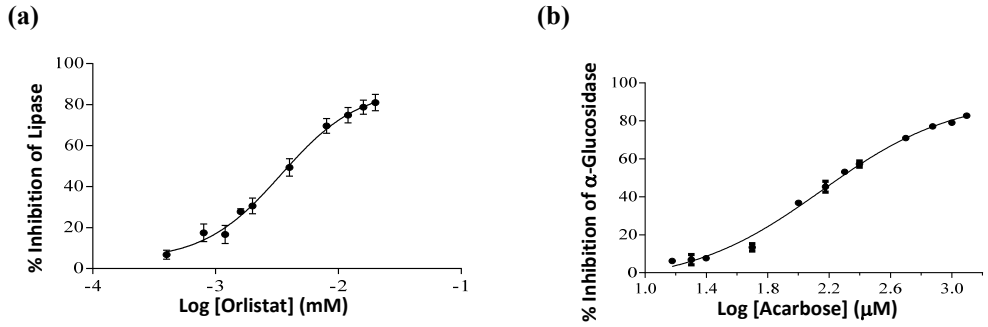

Figure 2. The plots of enzyme inhibitory activities of (**a**) lipase and orlistat and (**b**) α-glucosidase and acarbose for determination of the IC_{50} values.

It was previously suggested that the concentration of mushroom extract and extraction solvents significantly affected anti-α-glucosidase activity [24]. The ratio between solvent and solute (mushroom) as well as polarity of solvent should be optimized in order to achieve maximum efficiency in α-glucosidase inhibitions. For example, 25 and 100 mg equivalent FD of water extracted mushroom exhibited greater α-glucosidase inhibitory activities than 50 and 200 mg equivalent FD mushroom extracted with ethyl acetate [24]. Besides, it was previously reported that mushroom extracted with hot water (IC_{50} of 50 mg/mL) exhibited weaker anti-α-glucosidase activity than the one extracted with 80% (v/v) aqueous ethanol (IC_{50} of 29 mg/mL) [23]. These results suggested that anti-α-glucosidase agents from mushroom likely compose of functional group(s) with polarity index between 5.2 (absolute ethanol) to 10.2 (water).

Furthermore, qualities of carbohydrate such as fatty acid, oligosaccharides, polysaccharides and sugars that were found in various mushrooms are expected to be able to function as anti-α-glucosidase inhibitor. Previous researches suggested that oleic acid and linoleic acid from *Grifola frondosa* extract [11], water-soluble polysaccharide from *Auricularia auricula-judae* Quel. (Hed-hoo-noo) extract [26], polysaccharides from *Ganoderma lucidum* (Hed-lin-jue) extract [27], β-glucan and oligosaccharides from *Agaricus blazei* (Hed-kra-dum) extract [28], *exo*-polymer from *Ganoderma applanatum* (Hed-lin-jue-dang) and *Collybia confluens* [29], trehalose from*Grifola frondosa* (Hed-mai-ta-ke) [24], and salacinol and kotalanol from *Salacia* sp. [30] showed anti-α-glucosidase activities. Thus, it is possible that consumption of these mushrooms with bioactive components might decrease occurrence of diabetic through key enzyme inhibitory pathway.

Sa-med mushroom with 79% α-glucosidase inhibition, which is equivalent to mushroom at 15 mg fresh weight, was compared to acarbose at the same percentage of inhibition (equivalent to 118 μg). The recommended dose of acarbose inadult under diabetes is 25 mg, three times a day. Thus, diabetes patientsare needed to consume acarbose at the concentration of 75 mg/day. These quantities were equivalent to consumption of mushroom at 9.53 g/day to receive the same effect as acarbose uptake under the hypothesis that all bioactive compounds in mushroom are absorbed into body and are completely effective toward α-glucosidase. Due to low quantity of mushroom, mushroom consumption could possibly be promoted as alternative pathway for diabetic prevention and treatment. However, further information on *in vivo* bioavailability and absorption of bioactive components of mushrooms is required to be confirmed before passing judgement on its applications.

3.3 α-Amylase inhibitory activity

On the other hand, inhibition of α-amylase, another key enzyme that controls diabetes, of mushrooms (5 mg/mL) could not be detected under this study. However, previous study suggested that 6 medicinal mushrooms (*G. frondosa, H. erinaceum, A. blazei, G. lucidum, C. versicolor* and *P. linteus*) from Nantou, Taiwan showed anti-α-amylase activities (IC_{50} of 1.20-6.90 mg/mL), which are weaker than acarbose (IC_{50} of 0.04 mg/mL) [11]. Likewise, *L. edodes* and *P. pulmonarius* mushrooms from Thailand exhibited the IC_{50} values of 230-590 mg/mL, which are much higher than the IC_{50} value of acarbose (0.01 mg/mL) [23]. Thus, it is possible that the mushrooms in our experiment might possess α-amylase inhibitory activities if higher concentration of mushrooms (>5 mg/mL) was used in our experiment. However, in order to achieve anti-α-amylase activities from high concentration of mushroom extracts, it may indicate that these mushrooms may not contain effective anti-α-amylase agents. As stated previously that mushrooms contain very few phenolic acids and no detected flavonoids [19] and most effective anti-α-amylase agents are phenolics [31], it is possible that no detected anti-α-amylase activities as being observed in mushroom extracts in this present study might be due to the lack of sufficient amount of these bioactive compounds.

Therefore, the quantity and quality of enzyme inhibitory activities in different mushroom extracts might vary according to extraction conditions (type of solvent, extraction temperature and incubating time) and mushroom species (strains, development stage, cultivation and fruiting condition). Thus, consumers should consume a variety of mushroom types for better health as well as a decreased rate of morbidity and mortality.

4. Conclusions

All fifteen edible Thai local mushrooms from Dong Yai community forest, Amnat-Charoen province, Thailand exhibited various ranges of lipase and α-glucosidase inhibitory activities. Din-khao mushroom exhibited the highest lipase inhibitory activity, while Por-fai and Por-nung mushrooms exhibited the lowest. In addition, Sa-med mushroom exhibited the highest α-glucosidase inhibitory activity, while Kor-kar-dang mushroom exhibited the lowest. However, α-amylase inhibitory activity was not be detected under this extraction concentration. Since mushrooms were previously reported to contain low phenolics, other bioactive components such as polysaccharides, fatty acids and proteins/peptides are proposed to be responsible for these inhibitory activities. However, further studies are required to confirmthis hypothesis. The types and quantity for these bioactive components are needed to clarify their functions and mechanismson enzyme inhibitory reactions.

5. Acknowledgements

The authors would like to express our sincere appreciation to Dr. Thitiya Boonpratuang (The National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani, Thailand), Ms. Tuksaporn Thummarukcharoen (The National Science and Technology Development Agency (NATDA), Pathum Thani, Thailand) and Dr. Prapawan Sawhasan (Agriculture Science program, Agriculture Science division, Mahidol University, Kanchanaburi, Thailand) for mushroom identification. This research work was supported by the grant from Biodiversity-Based Economy Development Office (BEDO), Thailand. Mushroom samples were obtained from Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG).

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