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α-Glucosidase and Aldose Reductase Inhibitory Activities from the Fruiting Body of *Phellinus merrillii*

Guan-Jhong Huang,[*,†] Wen-Tsong Hsieh,[‡] Heng-Yuan Chang,[†,§] Shyh-Shyun Huang,[†] Ying-Chih Lin,[∥] and Yueh-Hsiung Kuo[*,†,⊥,#,▽]

[†]School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 404, Taiwan

[‡]Department of Pharmacology, China Medical University, Taichung 404, Taiwan

[§]Department of Pharmacy, Yongkang Veterans Hospital, Tainan 710, Taiwan

[∥]Department of Optometry, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli 356, Taiwan

[⊥]Department of Chemistry, National Taiwan University, Taipei 106, Taiwan

[#]Agricultural Biotechnology Research Center, Academic Sinica, Taipei 115, Taiwan

[▽]Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung 404, Taiwan

ABSTRACT: The inhibitory activity from the isolated component of the fruiting body *Phellinus merrillii* (PM) was evaluated against α-glucosidase and lens aldose reductase from Sprague−Dawley male rats and compared to the quercetin as an aldose reductase inhibitor and acarbose as an α-glucosidase inhibitor. The ethanol extracts of PM (EPM) showed the strong α-glucosidase and aldose reductase activities. α-Glucosidase and aldose reductase inhibitors were identified as hispidin (**A**), hispolon (**B**), and inotilone (**C**), which were isolated from EtOAc-soluble fractions of EPM. The above structures were elucidated by their spectra and comparison with the literatures. Among them, hispidin, hispolon, and inotilone exhibited potent against α-glucosidase inhibitor activity with IC_{50} values of 297.06 ± 2.06, 12.38 ± 0.13, and 18.62 ± 0.23 μg/mL, respectively, and aldose reductase inhibitor activity with IC_{50} values of 48.26 ± 2.48, 9.47 ± 0.52, and 15.37 ± 0.32 μg/mL, respectively. These findings demonstrated that PM may be a good source for lead compounds as alternatives for antidiabetic agents currently used. The importance of finding effective antidiabetic therapeutics led us to further investigate natural compounds.

KEYWORDS: Chinese herb, *Phellinus merrillii*, α-glucosidase, aldose reductase, hispidin derivatives, flavonoid

INTRODUCTION

Diabetes mellitus is a common disease with many complications such as atherosclerosis, cardiac dysfunction, retinopathy, neuropathy, and nephropathy.[1] α-Glucosidase (EC 3.2.1.20) catalyzes the final step in the digestive process of carbohydrates. Its inhibitors can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia and could be useful for treating diabetic and/or obese patients.[2] α-Glucosidase inhibitors such as acarbose, miglitol, and voglibose are known to reduce postprandial hyperglycemia primarily by interfering with the carbohydrate digestive enzymes and by delaying glucose absorption. Aldose reductase (AR) (E.C.1.1.1.21) is the first enzyme in the polyol pathway; it catalyzes the reduction of the aldehyde functionality of D-glucose to form D-sorbitol with concomitant conversion of NADPH to $NADP^+$.[3] It is generally accepted that this polyol pathway plays an important role in the development of some degenerative complications of diabetes. The elevated blood glucose levels, characteristic of diabetes mellitus, cause a significant flux of glucose through the polyol pathway in tissues such as nerves, retina, lens, and kidney, where glucose uptake is independent of insulin.[4] Thus, AR inhibitors have attracted attention in the research of diabetic complication treatments.

The inhibitory activities of plant phytochemicals, including polyphenols, against carbohydrate hydrolyzing enzymes contribute to the lowering of postprandial hyperglycemia in diabetic management as observed in vivo.[5] In vivo studies have shown the phenolic compounds in plant materials are capable of reducing oxidative stress by scavenging reactive oxygen species and preventing cell damage in diabetic rats.[6] The phenolic compounds in edible plants are currently regarded as natural antioxidants, and their antioxidant activities are important for human health.[7]

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial medicines. They produce various classes of secondary metabolites with interesting biological activities and thus have the potential to be used as valuable chemical resources for drug discovery.[8] Several mushrooms belonging to the genera *Inonotus* and *Phellinus* have been used as traditional medicines for the treatment of gastrointestinal cancer, cardiovascular disease, heart diseases, stomach ailments, and diabetes.[9] Interestingly, these mushrooms commonly produce a number of yellow antioxidant pigments that comprise hispidin derivatives and phenols.

Phellinus merrillii (PM) is a mushroom that belongs to the genus *Phellinus* and is commonly called "Sangwhang" in Taiwan. It is popular in oriental countries and has been traditionally used

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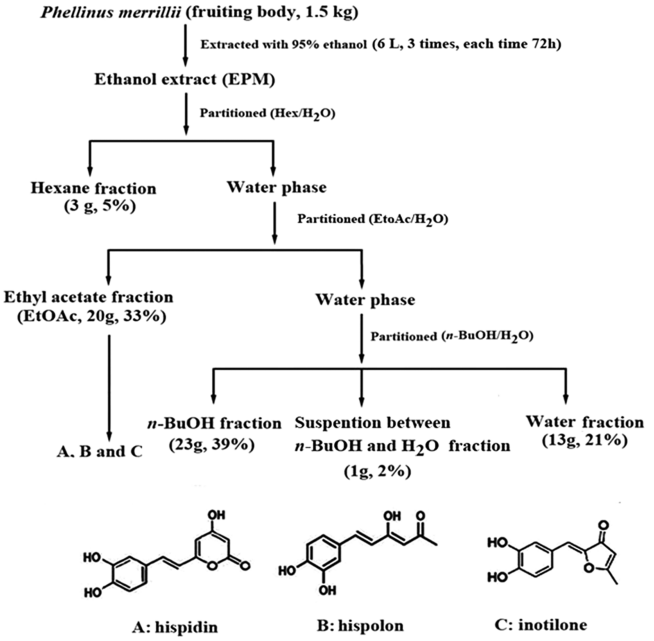

Figure 1. Top: A flowchart of the separated method procedure for isolation of active components from PM. Bottom: The structures of hispolon (**A**), hispidin (**B**), and inotilone (**C**).

as a food and medicine. Sangwhang contains many bioactive compounds and is known for improving health and preventing and remedying various diseases, such as gastroenteric disorders, lymphatic diseases, and cancer.[10−12] However, this is the first time that phenolic compounds including compounds **A**, **B**, and **C** have been identified from the fruiting body of PM. Moreover, there have been few studies on the effect of the mushroom's constituents on α-glucosidase and AR inhibitory activity. Therefore, we investigated the inhibitory effect of the fruiting body of PM on α-glucosidase and AR to evaluate its potential in treating diabetic complications. Active compounds isolated from the fruiting body of PM may be a good source for lead compounds as alternatives for antidiabetic agents currently used. Antidiabetic therapeutics are important events; we are encouraged to study the active principles for researching the lead compounds.

MATERIALS AND METHODS

Materials. DL-Glyceraldehyde, PNP-glycoside, PIPES, NADPH, *N*-(1-naphthyl) ethylenediamine, and quercetin were purchased from Sigma Chemical (St. Louis, MO). PM was supplied by the Po-Zone Enterprises Co. Ltd. (Taiwan), and identified by Drs. Yu-Cheng Dai (Institute of Applied Ecology, Chinese Academy of Science, China), and Sheng-Hua Wu (Department of Botany, National Museum of Natural Science, Taiwan).

Isolation and Determination of the Active Compounds. The fruiting body of PM (about 1.5 kg, air dry weight) was powdered and extracted with 6 L of 95% EtOH at room temperature (three times, 72 h each). Extracts were filtered and combined together and then evaporated at 40 °C (N-11, Eyela, Japan) to dryness under reduced pressure to give a dark brown residue (60 g). The yield obtained for PM is about 4%. The crude extract was suspended in H_2O (1 L) and then partitioned with 1 L of *n*-hexane (×2), 1 L of EtOAc (×2), and 1 L of *n*-butanol (×2), successively. It yielded five fractions, a *n*-hexane-soluble fraction, EtOAc-soluble fraction, *n*-butanol-soluble fraction, suspended fraction, and water-soluble fraction. The yield of every fraction is shown in Figure 1. The weights of every fraction are 3, 20, 23, and 13 g for *n*-hexane, ethyl acetate, *n*-butanol, and suspension between *n*-BuOH and H_2O fractions, respectively.

The active components were purified from the EtOAc-soluble portion (10 g) by a bioassay-guided separation. A portion of the active EtOAc fraction (10 g) was subjected to silica gel chromatography using stepwise $CHCl_3$−MeOH (9:1, 8:2, and 1:1 v/v) as the eluent. Final purification was achieved by preparative HPLC [Spherisorb ODS-2 RP18, 5 μm (Promochem), 250 mm × 25 mm, acetonitrile−H_2O (83:17 v/v), at a flow rate of 10 mL/min and UV detection at 375 nm]; yields were as follows: 200 mg of hispidin (**A**), 150 mg of hispolon (**B**), and 100 mg of inotilone (**C**). The identification of three compounds **A**−**C** was performed by comparing their physical spectral data with literature values.[12−14]

Inhibition Assay for α-Glucosidase Activity. The α-glucosidase inhibitory effect of the fractions of PM was assayed according to the procedure described previously by Matsui et al.[15] with minor modifications. Briefly, the enzyme reaction was performed using PNP-glycoside as a substrate in 0.1 M PIPES buffer, pH 6.8. The PNP-glycoside (2.0 mM) was premixed with samples at various concentrations, and the mixture was added to an enzyme solution (0.01 units) to make 0.5 mL of final volume. The reaction was terminated by adding 1 mL of 0.64% *N*-(1-naphthyl)-ethylenediamine solution (pH 10.7). The enzymatic activity was quantified by measuring the *p*-nitrophenol released from PNP-glycoside at 400 nm wavelength. All reactions were carried out at 37 °C for 30 min with three replications. Acarbose was used as a positive control. PIPES buffer was used in blank experiments and expressed as ΔA_{blank}/min. The concentration of the extracts required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

Measurement of AR Activity in Vitro. Crude AR was prepared as in the following steps: Lenses were removed from Sprague−Dawley (SD) rats weighing 250−280 g and were kept frozen until use. A rat lens homogenate was prepared in accordance with the method described by Hayman and Kinoshita.[16] A partially purified enzyme, with a specific activity of 6.5 U/mg, was routinely used in the evaluations of enzyme inhibition. The partially purified material was separated into 1.0 mL aliquots and stored at −40 °C. The AR activity was spectrophotometrically assayed by measuring the decrease in NADPH absorption at 340 nm over a 4 min period, using DL-glyceraldehyde as a substrate. Each 1.0 mL cuvette contained equal units of enzyme, 0.10 M sodium phosphate buffer (pH 6.2) and 0.3 mM NADPH, both with and without a 10 mM concentration of the substrate and an inhibitor.[17] Sodium phosphate buffer was used in blank experiments and expressed as ΔA_{blank}/min. The concentration of the extracts required to inhibit 50% of AR activity under the assay conditions was defined as the IC_{50} value.

Statistical Analysis. Experimental results were presented as the means ± standard errors (SEs) of three parallel measurements. The statistical analyses were performed by one-way analysis of variance, followed by Dunnett's *t* test. The difference was considered to be statistically significant when the *p* value was less than 0.05.

RESULTS AND DISCUSSION

Many of bioactive chemicals from plants are largely free from adverse effects and have excellent pharmacological actions; they could lead to the development of new classes of possibly antidiabetic agents. Therefore, we investigated the inhibitory effect of the fruiting body of PM on α-glucosidase and AR to evaluate its potential in treating diabetic complications. We evaluated that the EtOAc fraction showed the strongest potential of inhibiting α-glucosidase and AR activities (Table 1).

Inhibition Assay for α-Glucosidase Activity. The percentages of α-glucosidase inhibitory activity of the fraction of the EPM are shown in Table 1. The qualities of enzymatic inhibition in the fraction of the EPM were determined by calculating IC_{50}, with lower

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Table 1. α-Glucosidase and AR Inhibitory Activity from the Fruiting Body of PM[a]

extract and fractions	IC_{50} (μg/mL) α-glucosidase inhibitor	IC_{50} (μg/mL) AR inhibitor
ethanol extract	13.73 ± 0.43	12.55 ± 1.37
hexane soluble fraction	332.55 ± 16.35	240.36 ± 1.37
EtOAc soluble fraction	9.34 ± 0.23	7.57 ± 0.36
BuOH soluble fraction	9.25 ± 0.41	14.12 ± 1.53
suspension between *n*-BuOH and H_2O fraction	14.35 ± 0.42	33.90 ± 5.57
H_2O soluble fraction	231.59 ± 10.14	104.53 ± 9.01

[a] Values represent means ± SEs of three parallel measurements ($P < 0.05$).

Table 2. α-Glucosidase and AR Inhibitory Activities of Hispolon, Hispidin, and Inotilone Isolated from the Fruiting Body of PM[a]

species and positive controls	IC_{50} (μg/mL) α-glucosidase inhibitor	IC_{50} (μg/mL) AR inhibitor
acarbose	637.04 ± 0.56	not detected
quercetin	12.35 ± 0.35	8.54 ± 0.14
hispolon	12.38 ± 0.13	9.47 ± 0.52
hispidin	297.06 ± 2.06	48.26 ± 2.48
inotilone	18.62 ± 0.23	15.37 ± 0.32

[a] Values represent means ± SEs of three parallel measurements ($P < 0.05$).

numbers indicating higher qualities of enzymatic inhibition. The IC_{50} of α-glucosidase inhibitory activity in the extracts of the fraction of the EPM ranged from 9.25 to 332.55 μg/mL and increased as in the following order: *n*-BuOH-soluble fraction (9.25 ± 0.41 μg/mL) > EtOAc-soluble fraction (9.34 ± 0.23 μg/mL) > suspension between *n*-BuOH- and H_2O-soluble fraction (14.35 ± 0.42 μg/mL) > H_2O-soluble fraction (231.59 ± 10.14 μg/mL) > *n*-hexane-soluble fraction (332.55 ± 16.35 μg/mL). The BuOH-soluble fraction had the highest α-glucosidase inhibitory activity. The positive controls against α-glucosidase were acarbose (IC_{50} = 637.04 ± 0.56 μg/mL) and quercetin (IC_{50} = 12.35 ± 0.35 μg/mL).

Compounds **A**, **B**, and **C** (Figure 1) are highly oxygenated and functionalized aromatic compounds that possess the unique basic structural unit, namely, hispidin, hispolon, and inotilone, respectively. Inhibitory activities against α-glucosidase of compounds **A**, **B**, and **C** were evaluated. For α-glucosidase, PNP-glycoside was used as the substrate and cofactor, respectively. As shown in Table 2, the inhibitory activity of hispolon (compound **B**) (IC_{50} = 12.38 ± 0.13 μg/mL) against α-glucosidase was stronger than that of hispidin (compound **A**) (IC_{50} = 297.06 ± 2.06 μg/mL) and inotilone (compound **C**) (IC_{50} = 18.62 ± 0.23 μg/mL). Quercetin showed strong inhibition against α-glucosidase (IC_{50} = 12.35 ± 0.35 μg/mL) activity, while acarbose (IC_{50} = 637.04 ± 0.56 μg/mL) did not inhibit at all.

Phenolic compounds in plants have long been recognized to inhibit the activities of digestive enzymes because of their ability to bind with proteins.[18] Various in vitro assays have shown that many plant phenols possess carbohydrate hydrolyzing enzyme inhibitory activities. These compounds include green tea polyphenols that inhibit the activities of α-glucosidase and sucrase,[19] sweet potato polyphenols,[15] and berry polyphenols that inhibit α-glucosidase and α-amylase activities.[20]

α-Glucosidase inhibitors are currently the most commonly used oral agents for improving postprandial hyperglycemia due to the lack of a hypoglycemic threat and, more importantly, the prospect of blood glucose control without hyperinsulinemia and body weight gain.[21] Inhibition of α-glucosidase and α-amylase should result in delayed carbohydrate digestion and glucose absorption with attenuation of postprandial hyperglycemic excursions. It has been reported that α-glucosidase inhibitors usually do not alter the total amount of carbohydrate absorbed and, therefore, do not cause any net nutritional caloric loss, although they slow carbohydrate digestion. Quercetin is an oral agent for good penetration of sorbitol through cellular membranes, fast metabolism of sorbitol by sorbitol dehydrogenase, and, more importantly, the therapeutic prospect of patient treatment associated with such diabetic complications as retinopathy, cataracts, neuropathy, and nephropathy.[22] In this regard, hispolon and inotilone may be used as a lead compound for the development of antidiabetic therapeutics, and the inhibitory activity of hispolon and inotilone was lower than that of acarbose and quercetin. The IC_{50} of positive control for α-glucosidase inhibitor (acarbose) is found to be much higher in the present assay, which is similar to the previous literature.[23,24] When compared to acarbose as the control, only mammalian enzyme was inhibited. This was expected since acarbose has been shown to be a potent inhibitor of mammalian sucrase and maltase and inactive against yeast and bacterial forms.[25]

Measurement of AR Activity in Vitro. AR, the principal enzyme of the polyol pathway, has been shown to play an important role in the complications associated with diabetes. The percentages of AR inhibitory activities of the five fractions from EPM are shown in Table 1. The IC_{50} of the every fraction from EPM on AR inhibitory activities ranged from 7.57 to 240.36 μg/mL and increased as in the following order: EtOAc (7.57 ± 0.36 μg/mL) > *n*-BuOH (14.12 ± 1.53 μg/mL) > suspension between *n*-BuOH and H_2O (33.90 ± 5.57 μg/mL) > H_2O (104.53 ± 9.01 μg/mL) > *n*-hexane (240.36 ± 1.37 μg/mL). EtOAc had the highest AR inhibitory activity.

Compounds **A**−**C** with inhibitory activities against AR was evaluated (Figure 1). For AR, DL-glyceraldehyde and NADPH were used as the substrate and cofactor, respectively. As shown in Table 2, inhibitory activities against AR of compounds **A**, **B**, and **C** were evaluated. The inhibitory activity of hispolon (compound **B**) (IC_{50} = 9.47 ± 0.52 μg/mL) against AR was stronger than that of hispidin (compound **A**) (IC_{50} = 48.26 ± 2.48 μg/mL) and inotilone (compound **C**) (IC_{50} = 15.37 ± 0.32 μg/mL). The positive control of quercetin showed stronger inhibition against AR (IC_{50} = 8.54 ± 0.14 μg/mL) activity than hispolon (**B**). It has been previously reported that hispidin (**A**) also has AR inhibitory activity. The IC_{50} of AR inhibitory activity hispidin (**A**) was about 12.45 μM.[26]

Many natural compounds have been tested for AR inhibitory activity. Medicinal plants are particularly likely to be nontoxic and may be useful for the prevention and treatment of diabetes-related complications.[27] In addition to its antioxidant properties, quercetin has an inhibitory effect on the formation of advanced glycation end products.[28] Furthermore, quercetin has been shown to decrease blood glucose and glycated hemoglobin levels ($HbA1_C$) and increase the glucagon/insulin ratio in type 2 diabetic animals.[29]

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It has been well acknowledged that plant-derived extracts and phytochemicals are potential alternatives to synthetic inhibitors against AR. [30−32] In this study, the components isolated from PM against AR were identified as the hispidin, hispolon, and inotilone, although the inhibitory responses varied with the chemicals and concentrations tested. It has been reported that the PM-derived materials including phenols compounds have antioxidative, [33] antitumor, [34] and anti-inflammatory effects. [35] It might be expected, then, that the active components isolated from PM have a range of pharmacological actions for antidiabetic principles.

AR inhibitors are the most commonly used oral agents for good penetration of sorbitol through cellular membranes and fast metabolism of sorbitol by sorbitol dehydrogenase. More importantly, they are considered as prospective therapeutics for treatment of diabetic complications such as retinopathy, cataracts, neuropathy, and nephropathy.[22] Hispolon and inotilone may be used as lead compounds for the development of antidiabetic therapeutics.

Plant-derived extracts and phytochemicals are potential alternatives to synthetic inhibitors against AR and α-glucosidase.[36] Currently, α-glucosidase inhibitor and AR inhibitor compounds isolated from plants are classified as diterpene-, triterpene-, and flavonoid-related compounds.[21] The EtOAc-soluble fraction and BuOH-soluble fraction contain great inhibitory activity against α-glucosidase and AR with different compounds.[26] In this study, the active component isolated from PM against α-glucosidase inhibitor and AR was identified as hispidin, hispolon, and inotilone, although the inhibitory responses varied with concentrations tested.

In conclusion, the results from in vitro experiments include α-glucosidase inhibition and AR inhibition (Table 1 and 2). These results indicate that PM materials have inhibitory effects in vitro against α-glucosidase and rat lens AR. On the basis of our limited data and some earlier findings, the inhibitory action of PM-derived phenol compounds confirms their potential utility as antidiabetic agents, although their use in vivo and the clinical efficacies remain to be evaluated.

AUTHOR INFORMATION

Corresponding Author

*Tel: +886 2 3366-1671. Fax: +886 2 2362-6359. E-mail: yhkuo@ntu.edu.tw (Y.-H.K.). Tel: +886 4 2205 3366-5508. Fax: +886 4 2208 3362. E-mail: gjhuang@mail.cmu.edu.tw (G.-J.H.).

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