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ORIGINAL ARTICLE

Enhancing Glucose Uptake by *Astraeus odoratus* and *Astraeus asiaticus* Extracts in L6 Myotubes

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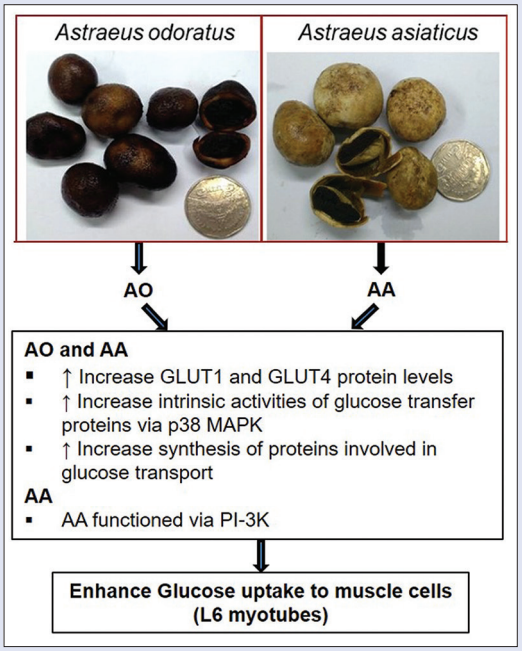
ABSTRACT

Background: Mushrooms, including *Astraeus* spp., are known for hypoglycemic properties. *Astraeus odoratus* is a delicious edible mushroom, while *Astraeus asiaticus* is less popular due to its unpleasant texture. Both mushrooms have not been reported for the glucose uptake activities yet. **Objectives:** The aim of this study is to describe the enhancing of glucose uptake and related mechanisms *in vitro* of the extracts from two *Astraeus* spp. **Materials and Methods:** The extracts of *A. odoratus* and *A. asiaticus* (AO and AA, respectively) were assayed for the stimulation of glucose uptake in L6 myotubes. The mechanism of actions was proved by using specific inhibitors and determined for the expression of glucose transporters type 1 and 4 (GLUT1 and GLUT4) by quantitative real-time polymerase chain reaction and Western blotting. **Results:** The extracts of both mushrooms enhanced glucose uptake in the muscle cells L6 myotubes at the level of the function of GLUT1 and GLUT4, which involved the partial stimulation of their intrinsic activities through p38 mitogen-activated protein kinase and increased GLUT1 and GLUT4 protein levels. AO increased both GLUT1 and GLUT4 protein, while AA increased mainly GLUT4 protein and stimulated GLUT4 translocation through phosphatidylinositol 3-kinase. **Conclusion:** The results supported the hypoglycemic activity of *A. odoratus* and *A. asiaticus* and suggested their potential use for hypoglycemic purposes.

Key words: *Astraeus asiaticus, Astraeus odoratus*, glucose uptake, hypoglycemic mushroom, L6 myotubes

SUMMARY

- *Astraeus odoratus* and *A. asiaticus* were potential sources for hypoglycemic purposes
- *Astraeus odoratus* extract (AO) and *A. asiaticus* extract (AA) enhanced glucose uptake through insulin dependent pathway which involved the function of glucose transport protein intrinsic activities via p38 mitogen-activated protein kinase
- AO increased both glucose transporters type 1 and glucose transporters type 4 (GLUT4) proteins, while AA affected phosphatidylinositol 3-kinase and increased mainly GLUT4 protein.



Abbreviations used: GLUT1: Glucose transporter protein type 1; GLUT4: glucose transporter protein type 4; AO: Hexane layer from ethanolic extract of *Astraeus odoratus*; AA: Hexane layer from ethanolic extract of *Astraeus asiaticus*; P38 MAPK: P38 mitogen-activated protein kinase; PI-3K: Phosphatidylinositol 3-kinase

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disease caused by hyperglycemia from insulin insufficiency and/or insulin resistance (insulin insensitivity). This condition leads to a high risk of cardiovascular diseases, kidney dysfunction, blindness, and other diseases. Diabetes medicines used to control blood glucose level include insulin releasers, insulin sensitizers, and alpha-glucosidase inhibitors. However, these medicines have mild-to-severe side effects such as hypoglycemia, lactic acidosis, and increased cardiovascular disease risk.[1] On the other hand, prediabetic

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conditions, in which the blood sugar level is not high enough to be classified as diabetes are a high risk of type 2 diabetes. In this situation, it is still possible to prevent or delay the development to diabetes by exercise, diet, functional foods, and the use of antidiabetic drugs.[2,3] The consumption of herbs that regulate blood sugar levels has also become an option to prevent the progress of the prediabetic condition to diabetes.

Mushrooms are not only good sources of nutrients but also potential sources for health products due to their pharmacological activities in preventing diseases such as diabetes, hypercholesterolemia, cancer, and hypertension. Mushrooms with hypoglycemic activity include *Ganoderma lucidum*,[4] *Lentinus edodes*,[5] and *Astraeus hygrometricus*.[6] The bioactive compounds of mushrooms that reduce blood glucose level are mainly polysaccharides such as beta-glucan and glucuronoxylomannan.[7,8] Triterpenoids from mushrooms are also reported to have hypoglycemic activity such as dehydrotrametenolic acid from *Poria cocos*[9] and ergosterol.[10] Although hypoglycemic herbs and mushrooms have been used as diabetic functional foods worldwide, there is still a need for the demonstration of efficacy and mechanism of action.

Astraeus is a mushroom of the family Diplocystaceae. There are approximately 10 species described and used for cooking in many countries, including India, Japan, Laos, and Thailand.[11] In Thailand, *Astraeus odoratus* Phosri, Watling, M.P. Martín & Whalley is an edible mushroom that is a favorite of Thai people, whereas *Astraeus asiaticus* Phosri, M.P. Martín & Watling, is also edible but has an unpleasant texture and has been discarded. Both *Astraeus* species are generally found in the northern and northeastern regions of Thailand[12] and contain triterpenoids as the main chemical components.[13-17] To date, although there have been no reports of antidiabetic biological activities for of both mushrooms, the presence of many triterpenoids, including the bioactive compound ergosterol in *Astraeus*, brought researchers attention to investigate for their hypoglycemic activities.

Glucose uptake is an important process to decrease blood glucose in skeletal muscle, adipocytes, and liver. Uptake is stimulated by insulin in insulin-sensitive tissue such as muscle cells and by insulin-independent mechanisms in vital organs such as the brain and red blood cells. As the muscle cell is the major target for glucose uptake, rat L6 myotubes were used for activity assays of the extracts from both *Astraeus* mushrooms in this study. Enhancing glucose uptake in muscle cells involves glucose transporters type 1 and 4 (GLUT1 and GLUT4) with regard to the translocation (for GLUT4), the intrinsic activity and affinity to glucose,[18] and the increase in GLUTs levels. GLUT1 is located at the cell surface, while GLUT4 is distributed in the cytosol. Insulin causes GLUT4 to undergo translocation from the cytosol to the plasma membrane for collecting glucose and results in a lower blood glucose level.[19] GLUT4 translocation is inhibited by the phosphatidylinositol 3-kinase (PI-3K) inhibitor wortmannin while the intrinsic activity of GLUT4 is inhibited by the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB203580. The synthesis of proteins responsible for the glucose uptake process could be proved by using the protein synthesis inhibitor cycloheximide.

To date, numerous mushrooms have not been studied for their hypoglycemic effect. Although mushrooms are potential sources of anti-diabetic compounds,[20] the market for mushroom-based hypoglycemic products remains restricted to a few mushrooms, such as *Agaricus blazei* and *Grifola frondosa*.[21] Since the activity and mechanism related to the insulin signaling pathway of *Astraeus* spp. have not been described and *Astraeus* mushrooms were proposed to possess hypoglycemic activities, this study is the first to assess *in vitro* glucose uptake activities and mechanisms of *A. odoratus* and *A. asiaticus* in L6 myotubes. These findings could suggest their potential use as new sources of hypoglycemic products for diabetes prevention and lead to added value for the distasteful *A. asiaticus*.

MATERIALS AND METHODS

Chemicals and reagents

All general solvents and chemicals were analytical grade. Ergosterol (purity >98%) was purchased from Biopurity, China. Alpha-minimum essential medium (α-MEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, horse serum (HS), penicillin-streptomycin, and trypan blue were obtained from GibCo, USA. Human insulin solution was purchased from Santa Cruz, USA. The glucose (GO) assay kit, wortmannin, cycloheximide, cytochalasin B, and SB203580 were purchased from Sigma-Aldrich, USA. The Superscript III first-strand synthesis system for reverse transcription-polymerase chain reaction (PCR), 1 Kb plus DNA ladder, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Trizol reagent were obtained from Invitrogen, USA. Prestained protein marker and SsoAdvanced Universal: SYBR Green Supermix was purchased from Bio-Rad, USA. Anti-GLUT1 antibody and anti-GLUT4 antibody were obtained from Millipore, Germany. Anti-actin antibody anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody, anti-mouse IgG HRP-linked antibody, and enhanced chemiluminescent (ECL) Western blotting substrate kit were purchased from GE Healthcare, USA. RIPA buffer was from Thermo Scientific, USA.

Astraeus materials

A. odoratus was purchased from a local market in Khon Kaen province and *A. asiaticus* was purchased from a local market in Mahasarakham province, Thailand. They were macroscopically differentiated and were confirmed by comparing the physical appearance and microscopy of spores to the literature.[12,22] The spores were stained using Melzer's reagent[23] and investigated by light microscopy.

Preparation of *Astraeus odoratus* and *Astraeus asiaticus* extracts

The mushrooms were washed, dried at 50°C, and ground. Dried powders were macerated three times with 95% EtOH. The extracts were dried using a rotary evaporator and freeze-dried. They were then kept at −20°C until use.

The ethanolic extract was suspended in water and centrifuged. The pellet was freeze-dried and then was partitioned between 2% aqueous MeOH and hexane. The hexane layer was dried using a rotary evaporator and freeze-dried to obtain the hexane fractions which were used in this study. Those fractions from *A. odoratus* and *A. asiaticus* were named AO and AA, respectively.

Thin-layer chromatography of *Astraeus* extracts

Thin-layer chromatography (TLC) of both *Astraeus* was performed using silica gel GF_{254} precoated plates (Merck, Germany), a mobile phase of hexane-EtOAc (8.5:1.5), visualization under UV 254 nm and spraying with 10% H_2SO_4. Ergosterol was used as a biomarker.

High-performance liquid chromatography of Astraeus extracts

High-performance liquid chromatography (HPLC) pattern of *Astraeus* extracts (1 mg/mL) was determined on an Agilent 1220 series HPLC (Agilent Technologies). The separation was carried out on a VertiSep C30 column (4.8 mm × 250 mm, 5 µm, Vertical Chromatography, Thailand). A solvent system consisted of H_2O (solvent A) and MeOH (solvent B) with a gradient elution as followed: 0–5 min, 0%B; 15 min, 80%B; 35 min, 100%B; and 35–70 min, 100%B. The flow rate was 1.0 mL/min, and the injection volume was 20 µL. The UV detector was used at 280 nm.

Cell cultures

L6 myoblast (American Type Culture Collection [ATCC], CRL-1458) was purchased from ATCC, USA. Cells, 3×10^5 cells, were grown in 100 mm x 20 mm cell culture dish containing α-MEM, 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO_2 and were subcultured into 24-well plates (0.1×10^5 cells/well). After reaching 70%–80% confluence, cells were differentiated by replacing 10% FBS in the culture media with 2% HS. The medium was changed every 48 h until 80%–90% myotubes were obtained.

Cell viability assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay)

Cell viability was assessed using the MTT assay[24] to obtain the optimal concentration of extracts that provided cell viabilities not <80% of basal levels (untreated control). L6 myotubes were treated with extracts at various concentrations for 48–50 h at 37°C, 5% CO_2. Then, the media were discarded and the cells were incubated with 0.5 mg/mL MTT for 2 h. After washing the cells with PBS, the formazan crystals were dissolved in 500 µL dimethylsulfoxide and absorbance measured at 570 nm. The percentage cell viability was calculated as follows:

Percentage cell viability = $(A_{sample}/A_{basal}) \times 100$

where A_{basal} was the absorbance of the untreated cells and A_{sample} was the absorbance of the treated cells.

Glucose uptake assay in L6 myotubes

Extracts (400 µL) in α-MEM containing 2% HS were used to treat L6 myotubes in 24 well plates for 50 h at 37°C and 5% CO_2. The culture media were collected, and the glucose content was determined using a glucose assay kit according to the manufacturer's protocol. The glucose content was calculated from a glucose standard graph. The percentage of decreasing media glucose from that of basal levels at 50-h incubation time was considered as percentage stimulation of glucose uptake, calculated as follows:

Percentage stimulation of glucose uptake = $([glucose_{basal}\text{-}glucose_{sample}] \times 100)/glucose_{basal}$

where $glucose_{basal}$ was the glucose content of the basal media and $glucose_{sample}$ was the glucose content in the media of treated samples.

EC_{50} was the concentration of extracts or insulin that provided a reduction in medium glucose by 50% from that of basal levels at 50 h, indicating 50% stimulation of glucose uptake to L6 myotubes. A treatment of 25-50 nM insulin was used as a positive control, and the basal level was used as a negative control.

Effect of inhibitors on the glucose uptake activities of the extracts

To study the mechanism of action of the glucose uptake activity by AO and AA in L6 myotubes, the inhibitors were added to the treated samples as follows: 3 µM wortmannin was added after cells had been incubated with extracts for 1 h; 2 µM cytochalasin B and 20 µM SB203580 were added after cells had been incubated with the extracts for 4 h; and 3.5 µM cycloheximide was added together with the extracts.

Quantitative real-time polymerase chain reaction

The 10 µL quantitative real-time PCR (qPCR) reaction consisted of cDNA equivalent to 12 ng of total RNA, SsoAdvanced Universal: SYBR Green Supermix and 0.2 µM of the forward and reverse primers. The primers were: GADPH forward: 5′ GAAGGTCGGTGTGAACGGAT 3′; GADPH reverse: 5′ ACCAGCTTCCCATTCTCAGC 3′; GLUT1 forward: 5′ ATAGGGGTCCAGGCTCCATT 3′; GLUT1 reverse: 5′ GAGTGTCCGTGTCTTCAGCA 3′; GLUT4 forward: 5′ GGTTGTCTTGACCCCTCCAG 3′; and GLUT4 reverse: 5′ TTCGGGTTTAGCACCCTTCC 3′. The qPCR was run in a real time PCR machine (CFX96 Touch Real-Time PCR, Bio-Rad, USA). The PCR cycle was predenaturation at 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 57°C for 20 s and 72°C for 30 s. The melting curve analysis was 95°C for 5 s, 65°C for 5 s, and 95°C for 5 s to verify the amplification product. Negative controls consisted of the reaction mixture with water instead of cDNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used. The cycle threshold (Ct) values were used to calculate relative changes in gene expression using the $2^{-\Delta\Delta Ct}$ method.[25]

Western blotting analysis

Protein was extracted from L6 myotubes using RIPA buffer according to the manufacturer's protocol. The concentration of protein was measured by the Lowry method[26] using bovine serum albumin as the standard. Protein (20-100 µg: for GLUT1 100 µg, GLUT4 40 µg, and actin 20 µg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes by wet blotting overnight (30 volt at 4°C). The membrane was blocked with 5% skim milk and incubated overnight in 1:1000 primary antibody against actin, GLUT1 or GLUT4 at 4°C. The membrane was washed with tris-buffered saline with Tween 20 and blotted with HRP secondary antibody for 1 h. After incubation with ECL Western blotting substrate, the specific protein was detected using X-ray film (Kodak).

Statistical analysis

The experiments were performed in triplicate and presented as average ± standard deviation the significance between groups was determined using one-way ANOVA (IBM SPSS Statistics 23, USA) and in-group with Duncan's multiple range test. The difference between two groups was tested using the independent sample *t*-test. $P < 0.05$ and $P < 0.01$ were considered as indicating significant differences.

RESULTS

Identification of *Astraeus* fruiting bodies and characterization of the extracts

Although both *Astraeus* mushrooms were similar, they could be identified by physical appearances. The morphology of *A. odoratus* and *A. asiaticus* fruiting bodies and the microscopy of spores were in agreement with the literature,[12,22] which confirmed the correct *Astraeus* species identification. TLC analysis of the ethanolic extract of both mushrooms and their hexane layer (AO and AA in this study) after spraying with 10% H_2SO_4 showed red to purple color bands of compounds [Figure 1b] indicated the presence of triterpenoids. Moreover, TLC chromatogram of both *Astraeus* was clearly different under UV 254 nm from the two additional bands as shown in Figure 1a (arrows) which were observed in the extracts of *A. odoratus* [Figure 1] lane 1 and 3, compared to those of *A. asiaticus* [Figure 1] lane 2 and 4. Ergosterol was presented in all extracts of both mushrooms.

HPLC chromatogram of the extracts from both *Astraeus* appeared similar patterns, which involved nearly the same significant peaks and contained ergosterol at retention time (RT) 62.8 min [Figure 2]. AO and AA, which were the hexane layers from ethanolic extracts demonstrated more proportion of the main peaks (RT 38.1–40.0 min) to the polar compounds (RT 11.0–22.1 min), which indicated the successive removal of those parts by the partitioning process.

Glucose uptake of *Astraeus odoratus* and *Astraeus asiaticus* extracts

Glucose uptake is one of the main processes for lowering blood glucose, which occurs at 80% in muscle cells. In basal conditions, glucose uptake occurred normally and resulted in lower medium glucose. The reduction in medium glucose to a lower level than the basal level after the addition of AO and AA to L6 myotubes indicated the stimulation of glucose uptake activities of those extracts.

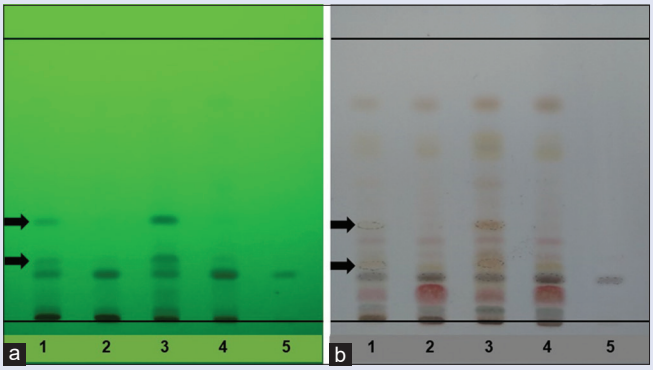

Figure 1: Thin-layer chromatography chromatogram of *Astraeus odoratus* and *Astraeus asiaticus* extracts. Thin-layer chromatography was detected under UV 254 nm (a); and by spraying with 10% H_2SO_4 and heated (b). Two additional bands of *Astraeus odoratus* extracts were shown (arrows). Lane 1: 95% ethanolic extract of *Astraeus odoratus*; lane 2: 95% ethanolic extract of *Astraeus asiaticus*; lane 3: hexane layer of *Astraeus odoratus* or AO in this study; lane 4: hexane layer of *Astraeus asiaticus* or AA in this study; lane 5: ergosterol

The results showed that AO and AA stimulated glucose uptake in a dose-dependent manner [Figure 3]. The concentration that decreased medium glucose to 50% lower than that of basal (EC_{50}) for AO and AA was 81–144 μg/mL, whereas insulin, the positive control, had an EC_{50} of 6.6 nM. The dose-dependent range of AO and AA was 25–100 μg/mL and 12.5–200 μg/mL, respectively and reaching a plateau effect at 200–400 μg/mL. Increased cell viability ($P < 0.01$) was found at up to 200 μg/mL and 400 μg/mL of AO and AA, respectively. The time course of glucose uptake in L6 myotubes on the incubation of AO and insulin resulted in an increased percentage of glucose uptake in a time-dependent manner [Figure 4] and reached the maximum effect at 50 h. Therefore, AO 100 μg/mL and AA 200 μg/mL at the incubation time of 50 h were used for further studies.

Effect of inhibitors on the glucose uptake activities of the extracts

To explain the mechanism of action related to that of insulin, inhibitors of the enzymes involved in the insulin signaling pathway, such as cytochalasin B, cycloheximide, wortmannin, and SB203580, were added to L6 myotubes in the presence of extracts at concentrations and times that did not cause a % cell viability <80% of basal. The reduction in glucose uptake in the presence of each inhibitor hence indicated the action of extracts involved with that enzyme. The results revealed partly different mechanisms for both extracts, as shown in Figure 5. The stimulation of glucose uptake by AO and AA was completely inhibited by cytochalasin B [Figure 5c] and obviously reduced by cycloheximide [Figure 5e]. Only AA action was reduced by wortmannin [Figure 5a]. The partial inhibition by SB203580 could be observed at the higher dose of AO and AA [Figure 5b] and [Figure 5d]. In summary, AO action was inhibited by cytochalasin B, cycloheximide, and SB203580, while

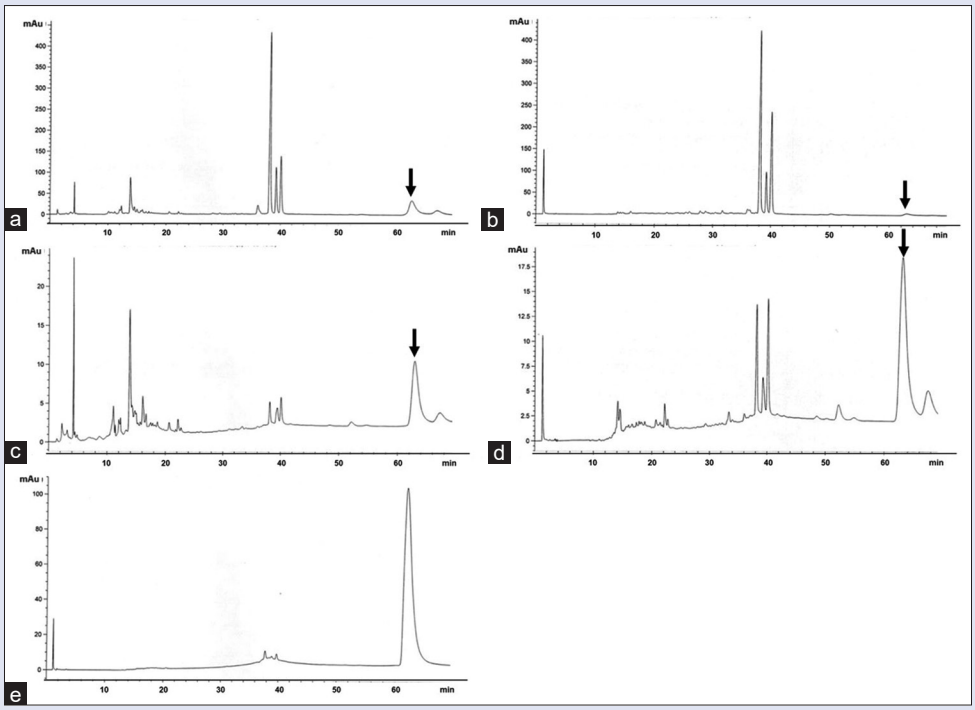

Figure 2: HPLC chromatogram of the *Astraeus* extracts consisting of ergosterol as a biomarker. (a) ethanolic extract of *Astraeus odoratus*; (b) hexane layer from the ethanolic extract of *Astraeus odoratus* (AO in this study); (c) ethanolic extract of *Astraeus asiaticus*; (d) hexane layer from the ethanolic extract of *Astraeus asiaticus* (AA in this study); (e) ergosterol. The arrow in a-d was pointed at the peak of ergosterol

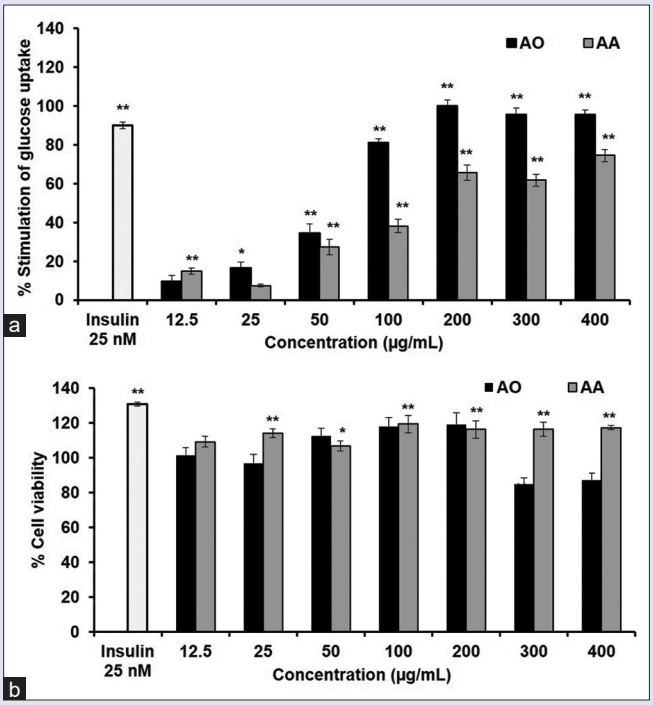

Figure 3: Glucose uptake stimulation activities of *Astraeus odoratus* and *Astraeus asiaticus* extracts (AO and AA, respectively) (a) and % cell viability of L6 myotubes (b). *P <0.05; **P <0.01 versus basal, n = 3

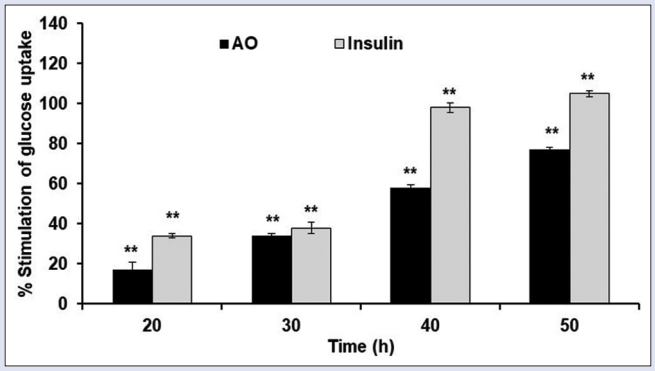

Figure 4: Time course of glucose uptake to L6 myotubes by 200 μg/mL *Astraeus odoratus* extract (AO) and 50 nM insulin. **P <0.01 versus basal, n = 3

AA action was significantly decreased by wortmannin, cytochalasin B, cycloheximide, and SB203580. These results indicated the enhancing of glucose uptake by AA and AO were included the function of glucose transporter type 1 and 4, which related to the increasing of their intrinsic activities via p38 MAPK and the synthesis of proteins involved in the glucose uptake processes. The mechanism of action of AA was related to PI-3K in addition.

Gene expression analysis of glucose transporter type 1 and glucose transporter type 4

At 50 h, the maximal glucose uptake stimulation was achieved from insulin, AO, and AA as 77.6%, 77.6%, and 76.9%. The upregulation of GLUT1 mRNA from the basal level (P < 0.01) was seen in insulin and AO, while that of AA was increased nonsignificantly [Figure 6a]. On the other hand, the mRNA level of GLUT4 of insulin, AO, and AA was lower than that of basal [Figure 6b], which indicated the downregulation of GLUT4 expression after reaching very low glucose content in media. For basal, the glucose uptake process was still kept on going; therefore, the expression of both GLUT1 and GLUT4 was still presented.

Western blot analysis

The protein level of GLUT1 and GLUT4 was detected at 50 h treatment of insulin, AO, and AA, which all of them provided the glucose uptake stimulation of 78%. The basal expression of GLUT4 protein, which was more intense than that of GLUT1 [Figure 7a] indicated a more abundant GLUT4 than GLUT1 in muscle cells. The increasing of both proteins intensities to those of basal were presented [Figure 7b]. The increasing of GLUT1 by insulin, AO, and AA was 32.9-, 9.6-, and 1.5-fold of basal, respectively, and the increasing of GLUT4 was 2.5-, 2.3-, and 2.3-fold of basal, respectively [Figure 7c]. The results were clearly indicated that the induction of GLUT1 and GLUT4 protein synthesis was due to insulin, AO, and AA, which AO gave more potent effects to GLUT1 synthesis than AA. The remained higher GLUT4 protein levels by insulin, AO and AA than that of basal, although the mRNA expression was downregulated [Figure 6b] suggested the stimulation of GLUT4 gene expression had occurred before detection time at 50 h and followed by induction of protein synthesis.

DISCUSSION

Astraeus odoratus and *A. asiaticus* fruiting bodies have a very similar morphology and both of them have a closely phylogenetic relationship.[27] However, they could be differentiated by physical appearances and microscopy of spores. In this study, it was found that the TLC chromatogram under wavelength 254 nm revealed an interesting different pattern. The extracts from *A. odoratus* presented two clear additional bands which those from *A. asiaticus* did not have. Therefore, this method could be used to identify between these mushrooms fruiting bodies in the form of crude extract or powder.

TLC and HPLC chromatograms revealed the consisting of ergosterol as a biomarker of *Astraeus* extracts in this study [Figure 1]. Ergosterol possesses glucose uptake activity in L6 cells, enhancing GLUT4 translocation and upregulating GLUT4 expression and the phosphorylation of protein kinase B (Akt) and protein kinase C.[10] From our preliminary study, the hexane layer of partitioning ethanolic extract (AO and AA, respectively) had a more potent glucose uptake ability than their ethanolic parts; therefore, they were used in this study. The increasing of activities after the removal of higher polar components of the extracts suggested that the active compounds of both mushrooms might belong to less polar substances.

The reduction of glucose content in the basal medium in the presence of the extracts or insulin was interpreted as a glucose uptake stimulation effect of the samples. A time course for AO was performed to investigate the incubation time to reach the maximum glucose uptake of *Astraeus* extracts. Although the maximum accumulative reduction in medium glucose by AO was at 50 h, while that of insulin was at 40 h [Figure 4], the incubation time of 50 h was used in this study to achieve the highest effect for the *Astraeus* extracts. Since the chronic effect of insulin and tested samples has been reported after treatment for 6 h,[28] 12 h,[29] or 24 h to 72 h,[30] the incubation time of 50 h in this study might reflect the glucose uptake and mechanism of action of chronic exposure to insulin in muscle cells.

GLUT1 and GLUT4 are present in the insulin-sensitive tissues fat and muscle. GLUT1 functions in basal cellular activity, which requires a low level of glucose,[31,32] while GLUT4 responds to insulin or other stimuli for higher levels of cellular glucose transport. Normally, GLUT1 is at very

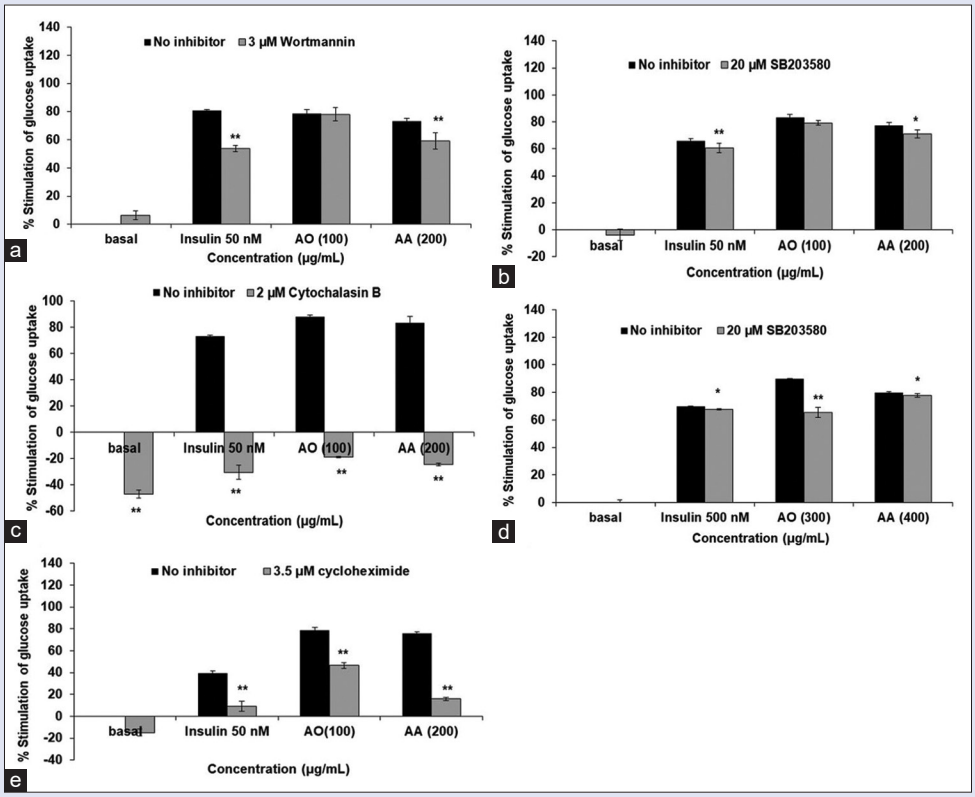

Figure 5: Effects of specific inhibitors on the stimulation of the glucose uptake activity of *Astraeus odoratus* and *Astraeus asiaticus* extracts (AO and AA, respectively). L6 myotubes were treated with 100 µg/mL AO and 200 µg/mL AA (a-c, e) or 300 µg/mL AO and 400 ug/mL AA (d) and 50 nM insulin was used as positive control. (a) 3 µM Wortmannin was added after a 1 h treatment; (b and d) 20 µM SB203580 was added after treatment at 4 h; (c) 2 µM cytochalasin B was added after treatment at 4 h; and (e) 3.5 µM cycloheximide was added together with treatment. **P* <0.05, ***P* <0.01 compared the activity without inhibitor versus with inhibitor; *n* = 3

low abundance in muscle cells, whereas GLUT4 is much more expressed in this tissue,[18] which could also be seen from the Western blotting of protein extracts of untreated L6 myotubes in this study [Figure 7a]. The basal GLUT1 protein appeared as faint bands, but the GLUT4 protein band was very intense.

Since GLUT4 is related to the insulin response and GLUT1 is independent of insulin, AO might stimulate both insulin-dependent and insulin-independent mechanisms of glucose uptake while the major effect of AA was based on insulin-dependent action. This could also be confirmed by the slightly changed level of GLUT1 both mRNA expression and protein level, in the presence of AA [Figures 6a and 7c].

The higher GLUT1 protein level to that of basal which were detected by Western blotting were along with the upregulation of GLUT1 mRNA, whereas increasing of GLUT4 protein from basal was shown in contrast to downregulation of GLUT4 mRNA expression in the presence of insulin, AO, and AA. This could be explained that at 50 h incubation, glucose uptake took place until the culture medium was almost glucose-free, and hence, it was not available for further glucose uptake and therefore, the mRNA expression of GLUT4 was downregulated, whereas the non-insulin dependence continued to be stimulated by insulin and AO and AA as reflected by the higher mRNA level of GLUT1 compared to the basal level. The other possible reason was the effect of the chronic exposure of insulin, AO, and AA that led to a decrease in the mRNA expression of GLUT4. For the cells chronically treated with insulin, where glucose uptake reaches the maximum level at 50 h, the blockage of GLUT4 protein loss and reduction in the mRNA of GLUT4 were described.[29] The intense protein band of GLUT4 remained higher than that of the basal level [Figure 7a and b], although the transcription process was already terminated because the half-life of GLUT4 protein ($t_{1/2}$) is 18 h and after the reaching minimum point at 36 h, the GLUT4 protein level will rise again at 36–40 h and reach constantly at 50 h incubation.[33]

To investigate the insulin-dependent mechanisms of AO and AA, the glucose uptake levels for those extracts after the addition of specific inhibitors were determined. The optimal incubation time with inhibitors and their concentration that do not cause cell viabilities lower than 80% of basal levels were considered before the experiments. Based on previous knowledge, the glucose uptake activity of insulin was inhibited by all of the selected inhibitors in this study; that is, wortmannin, cytochalasin B, SB203580, and cycloheximide.

GLUT4 translocation in rat skeletal muscle, L6 muscle cells and 3T3-L1 adipocytes and the intrinsic activity of the translocated GLUT4 are essential to achieve the maximum glucose uptake by insulin.[34] Wortmannin inhibits glucose uptake through PI-3K, which is responsible for the translocation of glucose transporters to the plasma membrane.[35] Our results showed that AA activity could be inhibited by wortmannin, while it was not affected to that of AO. These results revealed that AA transports glucose through PI-3K.

SB 203580 is a selective p38 MAPK inhibitor. p38 MAPK responds to insulin, which results in the activation of transporter intrinsic activity in muscle cell culture as well as in rat skeletal muscle.[36] SB203580 decreases the glucose uptake of insulin by interfering with an insulin-derived signal that leads to activation of GLUT4[34] and decreases glucose uptake by insulin in 3T3-L1 adipocytes and L6 muscle cells.[37] The effect

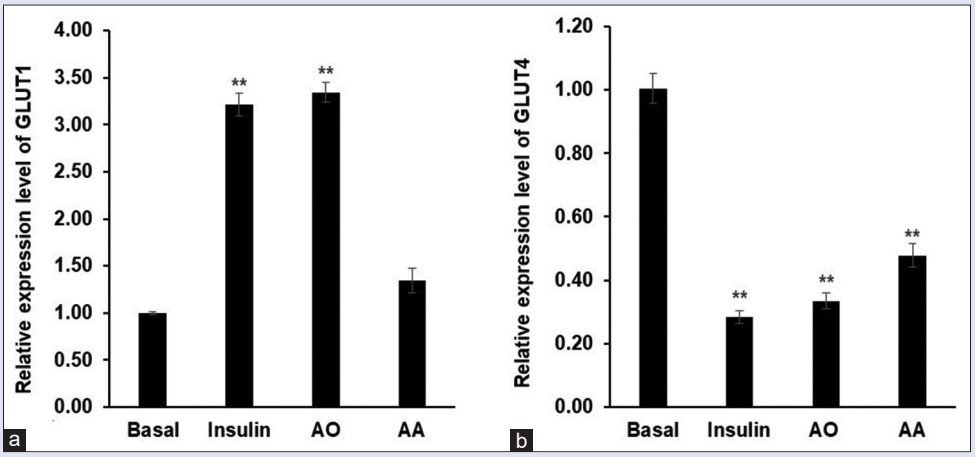

Figure 6: mRNA expression of glucose transporter type 1 (a) and glucose transporter type 4 (b) in L6 myotubes. Glucose uptake stimulation at 50 h in the presence of 50 nM insulin, 100 µg/mL *Astraeus odoratus* extract (AO) and 200 µg/mL *Astraeus asiaticus* extract (AA) were 77.6%, 77.6% and 76.9%, respectively, higher than that of basal. $^{**}P$ <0.01 versus basal, $n = 3$

on glucose uptake by a *Coccinia indica* extract in L8 myotubes was also inhibited by SB203580.[38] In this study, SB203580 partially decreased the glucose uptake effect of insulin, AO and AA. Although the inhibitory effect on insulin [Figure 5] has been demonstrated as being unrelated to p38 MAPK inhibition,[39,40] it might differ in the case of AO and AA. Therefore, it could be suggested that AO and AA partially activated the intrinsic activity of GLUT4 and might have a function related to p38 MAPK.

The lower level of glucose uptake by AO than that of insulin at the incubation time of 20 h [Figure 4] indicated the slower action and lack of an activated translocation effect but partially stimulated intrinsic effect,[41] in agreement with the noninhibitory effect of wortmannin [Figure 5a] and the partial inhibitory effect by SB203580 [Figure 5d].

Cytochalasin B inhibits the insulin-dependent glucose transporter GLUT1 and GLUT4 activity,[42-44] and completely inhibits the glucose uptake function of insulin.[44] From our results, glucose uptake activities by insulin, AO, and AA were completely inhibited in the presence of cytochalasin B [Figure 5c], which reflects glucose uptake through the function of GLUT1 and GLUT4. The percentage of stimulation of glucose uptake by L6 myotubes in the presence of cytochalasin B that were <0 indicated that the inhibition effects appeared until a greater medium glucose content than that of basal at 50 h was obtained. Since the basal level generated insulin-independent glucose uptake by GLUT1, therefore this result confirmed the inhibition of GLUT1 activity by cytochalasin B and indicated that glucose uptake also occurred in the basal (the untreated L6 myotubes).

Cycloheximide inhibits protein biosynthesis by the termination of peptide elongation in the protein translation process.[45,46] From our results, the addition of 3.5 µM cycloheximide dramatically decreased the glucose uptake action of insulin, AO, and AA [Figure 5e]. This might result from suppressing the synthesis of some proteins[47] such as the insulin receptor, which is responsible at the initiation process of glucose uptake and the inactivation of the insulin receptor.[48]

In summary, the inhibition of glucose uptake by the specific inhibitors [Figure 5] demonstrated that the mechanism of action of AO and AA actions were driven through the increase in GLUT1 and GLUT4 proteins [Figure 7] and increased the intrinsic activity of GLUT4 through p38 MAPK. AA action is primarily mediated through GLUT4 by increasing protein synthesis and via PI-3K, which elicits the translocation of GLUT4 from within the cell to the plasma membrane. The results showed that AA provided a broader insulin-mimetic effect than AO.

There have been not many studies of the mechanism of action of mushrooms on glucose uptake and this is the first report for *Astraeus* extracts. The mushroom extracts that were previously characterized for the mechanism of glucose uptake include a *G. lucidum* methanolic extract, which stimulated glucose uptake through PI-3K and 5´ adenosine monophosphate-activated protein kinase;[49] and beta-glucan from *Pleurotus sajor-caju* (Fr.) Sing., which stimulated glucose uptake in L6 myotubes through PI-3K/Akt and p38 MAPK.[7] Both of these provided an action similar to that of AA.

CONCLUSION

It has been confirmed that *Astraeus odoratus* and *A. asiaticus* possessed *in vitro* glucose uptake stimulation activity. The effects of both AO and AA occurred through the function of GLUT1 and GLUT4. They had slightly different mechanisms in that AO increased glucose uptake by the promotion of GLUT1 and GLUT4 synthesis and might be related to both insulin-dependent and insulin-independent pathways, while the effect of AA mainly involved GLUT4, which is related to the insulin-dependent pathway and revealed both inducing GLUT4 protein synthesis and increasing its activity through the translocation upon PI-3K. These results indicated the possibility of using both mushrooms for blood glucose lowering through glucose uptake stimulation. This will lead to added value for both mushrooms, especially *A. asiaticus*, for developing health products.

Finally, since both mushrooms are categorized as mycorrhizal species favorable to dipterocarpus trees such as *Dipterocarpus alatus*, *Dipterocarpus tuberculatus,* and *Shorea roxburghii*, the results from this study might be an inspiration to cultivate those trees to produce more *Astraeus* mushroom as a raw material for health products.

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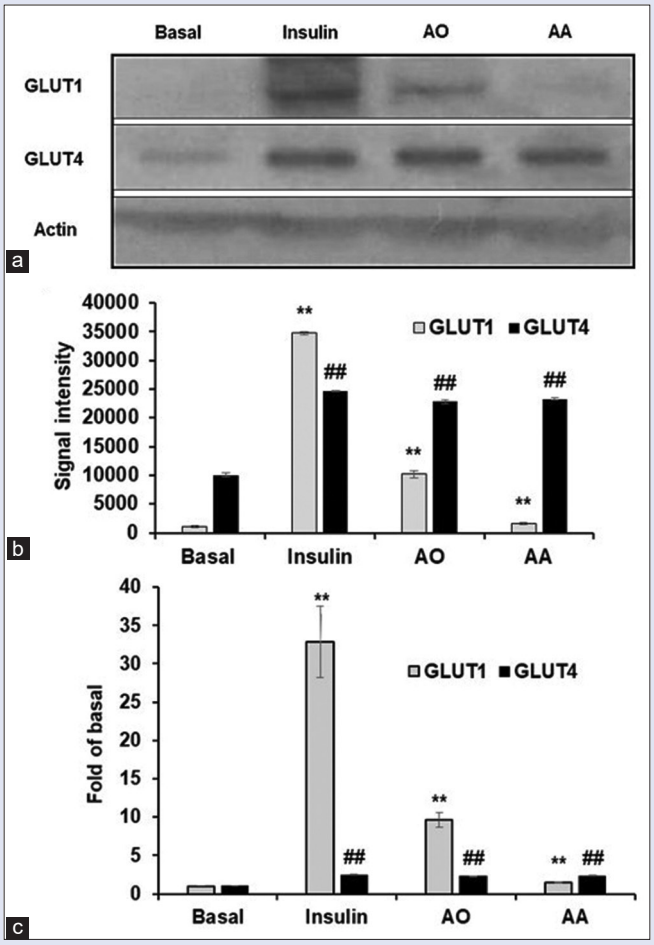

Figure 7: Glucose transporter type 1 and glucose transporter type 4 protein expression by insulin, *Astraeus odoratus* extract (AO) and *Astraeus asiaticus* extract (AA). Glucose uptake stimulation in the presence of 50 nM insulin, 100 µg/mL AO, and 200 µg/mL AA were 78.80%, 78.76%; and 78.4%, respectively, higher than that of basal. (a) Western blotting of glucose transporter type 1 and glucose transporter type 4 protein of basal and the treatments of 50 mM insulin, 100 µg/mL AO, and 200 µg/mL AA. Actin was used as a control. (b) Signal intensity of glucose transporter type 1 and glucose transporter type 4; (c) Signal intensity of glucose transporter type 1 and glucose transporter type 4 presented as fold of basal. $^{**}P < 0.01$ versus basal glucose transporter type 1 at 50 h; $^{\#\#}P < 0.01$ versus basal glucose transporter type 4 at 50 h; $n = 3$

Conflicts of interest

There are no conflicts of interest.

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