



Solid-substrate Fermentation of Wheat Grains by Mycelia of Indigenous *Ganoderma* spp. Enhanced Adipogenesis and Modulated PPAR γ Expression in 3T3-L1 Cells

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ABSTRACT

Adipocyte dysfunction is strongly associated with the progression of cardiovascular risk factors and diabetes. Appropriate regulation of adipogenesis and adipokine expression, notably adiponectin, are known to prevent or delay the onset of these disorders. The present study evaluated the insulin-like properties of ethanol extract of indigenous *Ganoderma australe* (KUM60813) and *Ganoderma neo japonicum* (KUM61076) through adipogenic, anti-lipolytic and adipogenesis targeted gene expression in differentiated 3T3-L1 adipocytes. *Ganoderma lucidum* (VITA GL) (a commercial strain) was included in the study as a reference. 3T3-L1 adipocytes treated with ethanol extract of wheat grains fermented with *G. neo japonicum* mycelia, significantly stimulated adipogenesis and exerted relatively mild anti-epinephrine induced lipolytic activities. In general, the expressions of target genes such as adiponectin, peroxisome proliferator-activated receptor gamma (PPAR γ), glucose transporter 4 (GLUT4) and hormone sensitive lipase (HSL) were up-regulated by the ethanol extract of wheat grains fermented with *Ganoderma* spp. mycelia. Taken together, these results suggest that *Ganoderma* especially *G. neo-japonicum* has insulin-like properties and may be useful as potential therapeutic agent in the management of type 2 diabetes mellitus.

Keywords: adipocyte, adipogenesis, *Ganoderma*, mycelia, type 2 diabetes

1. INTRODUCTION

Diabetes mellitus (DM) has become one of the most common public health disorders, throughout the world. Diabetes mellitus can be described as a complex metabolic disorder

caused by an inherited or acquired deficiency in insulin secretion, insulin action or both [1,2]. Diabetes mellitus is characterized by elevated blood glucose level (hyperglycemia), insulin

resistance, autoimmune destruction of pancreatic β -cell and the symptoms include thirst, polyuria, blurring of vision, and weight loss [2-4]. There are three major forms of DM: Type 1 DM (Insulin Dependent Diabetes Mellitus), Type 2 DM (Non Insulin Dependent Diabetes Mellitus) and Gestational DM. Among the three major types of DM, Type 2 DM is the most common form of diabetes which contributes to more than 90-95 % of all cases of diabetes worldwide [2] and this form of DM is closely associated with adipocyte dysfunction and obesity. Insulin, a metabolic hormone has a predominant role in regulating blood sugar level, maintaining homeostasis of energy metabolism and coordinating the storage and utilization of fuel molecules in adipose tissue, liver and skeletal muscle [5]. Besides enhancing the storage of triglycerides in adipocytes by increasing fatty acid uptake and stimulating differentiation of preadipocytes to adipocytes, insulin also promotes glucose transport through GLUT4 as well as inhibits lipolysis [3]. In addition, insulin also stimulates lipogenesis through the induction of key lipogenic enzymes such as fatty acid synthase and glycerophosphate dehydrogenase [4,6]. Thus, understanding the mechanisms involved in the regulation of preadipocyte proliferation, differentiation, lipogenesis, lipolysis, as well as uptake of glucose into adipocytes are essential for the treatment of diabetes mellitus as well as obesity [7].

Mushroom research and production have received an increased attention in recent years because of the recognition that mushrooms are nutritious food with health-stimulating and therapeutic properties [8]. As it generally takes more than half a year to complete a fruiting body culture in solid-state fermentation, several efforts are being made to obtain useful cellular materials or to produce valuable substances from a

submerged mycelia culture [9,10]. Although the process of submerged culture for *G. lucidum* mycelia growth has been well established, the components of the mycelia obtained from different types of cultures have been demonstrated to possess significant variations, which affect the related medicinal properties [9]. Furthermore, *G. australe* and *G. neo-japonicum* used in this study are very rare in nature and the amount found in the wild is not sufficient for scientific work and commercial exploitation. Therefore, the mycelia produced through solid-substrate fermentation technique deserve further evaluation for medicinal properties. Recently, biomolecules including polysaccharides, sterols, triterpenes and many other bioactive metabolites isolated from mushrooms and their cultured mycelia, scientifically have been proven successful in diabetes treatment [11]. The antidiabetic potential of the *G. lucidum* fruiting bodies have been shown by several studies [11-13]. However, *G. australe* and *G. neo-japonicum* which are also considered as varieties of *G. lucidum* (Ling Zhi), have not been studied extensively, neither for their biochemical composition nor biological activities especially for the potential insulin-like properties in the management of hyperglycaemia or diabetes.

3T3-L1 preadipocyte cell line is proven to be an excellent and cost effective model for preliminary screening of various bioactive compounds as potential anti-diabetic and anti-obesity agents; particularly glucose metabolism, as these cells can differentiate from preadipocyte fibroblastic form to adipocyte under appropriate culture conditions [7]. 3T3-L1 cells have a fibroblast-like morphology but under appropriate conditions, the cells differentiate into mature adipocyte-like phenotype [14]. Since dietary management is a starting point for the treatment of DM and obesity, it is pertinent

to investigate the effects of ethanol extract of unfermented and fermented wheat grains by indigenous *Ganoderma* spp. mycelia on proliferation and differentiation of 3T3-L1 preadipocytes into adipocytes. This might give an insight into the potential use of the extracts in the prevention and attenuation of the above-mentioned metabolic syndromes.

2. MATERIALS AND METHODS

2.1 Mushroom Samples

The strains of *G. australe* (KUM60813) and *G. neo-japonicum* (KUM61076) were obtained from Mushroom Research Center Fungal Collection, University of Malaya. The commercial strain, *G. lucidum* (VITA GL) was obtained from Ganofarm Sdn. Bhd., Tanjung Sepat, Selangor, Malaysia.

2.2 Reagents and Equipments

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin EDTA, penicillin/streptomycin mixture, 1-methyl-3-isobutylxanthine (IBMX), 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Oil Red O dye and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA) whereas all cell culture plastic wares were purchased from TPP (Trasadingen, Switzerland). Glycerol and dexamethasone were purchased from ICN (Aurora, Ohio). EnzyChrom™ Lipolysis Assay Kit (Bio Assays, USA). RNAqueous-4PCR Kit, High Capacity cDNA Reverse Transcription Kit and TaqMan® gene expression assay kits were purchased from Applied Biosystems, USA. Spectrophotometric measurements were performed using PowerWave X 340 Microtiter-Plate ELISA Reader (Bio-Tek Instruments®, Inc. USA).

2.3 Mycelia Biomass Production and Ethanol Extract Preparation

The axenic mycelia cultures were grown on glucose-yeast-malt-peptone (GYMP) agar at 25 ± 2 °C for 7 days [15]. Wheat grains obtained from a local supermarket were washed and then soaked in distilled water overnight at room temperature. The soaked grains were distributed into each (50 g) of several 250 mL Erlenmeyer flasks and sterilized at 121 °C, 15 psi for 20 minutes. The flasks were cooled overnight and then inoculated with five 7-mm diameter plugs of seven-day old mycelia grown on GYMP medium. The inoculated flasks were incubated at room temperature (25 ± 2 °C) for 14 days in the dark. Wheat grains without the mycelia plugs served as control. Samples were lyophilized prior to extraction. The crude ethanol extracts were prepared by soaking the freeze-dried wheat grain samples in ethanol (1:2 w/v) at 25 °C on a rotary shaker for seven days [16]. The ethanol extracts were filtered through Whatman No. 1 filter paper and vacuum concentrated (178 kPa, at 50 °C) using Büchi Rotavapor R-114 (Switzerland) before freeze drying. The dried crude ethanol extracts were weighed and stored in glass vials at -20 °C for further tests.

2.4 Cell Culture and Differentiation

3T3-L1 mouse preadipocytes were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS at 37 °C in a humidified atmosphere of 5% CO₂ and 95% room air. Adipogenesis was induced in confluent 3T3-L1 preadipocyte culture by incubating them in differentiation medium 1 (DM1) containing 0.5 mM IBMX, 1 μ M dexamethasone, and 10 μ g/mL insulin in

growth media (GM) for 48 hours. Then, the medium was changed to differentiation media 2 (DM2) containing mycelia ethanol extracts at various concentrations (20 - 100 $\mu\text{g}/\text{mL}$) and incubated for another 48 hours [7]. The final concentration of dimethylsulfoxide (DMSO) used was 1%. As for the basal control, 1% DMSO was used to replace the ethanol extract. The medium was changed every 2-3 days and replaced by fresh GM. All experiments, unless otherwise indicated, were performed in triplicates.

2.5 MTT Colorimetric Assay

The effect of ethanol extract of unfermented and fermented wheat grains by *Ganoderma* spp. on the viability of 3T3-L1 preadipocytes was evaluated by MTT assay. Preadipocytes were seeded at a density of 10000 cells/well in 96-well tissue culture plate and were allowed to attach for 24 hours prior to the proliferation assay. Subsequently, the preadipocytes were treated with ethanol extract at different concentrations and incubated for 48 hours before MTT reagent was added into each well. As for the basal control, 1% DMSO was used to replace the ethanol extract. After 4 hours, the culture medium containing MTT was carefully removed. Subsequently, 100 μL of DMSO was then added into each well and left on a plate shaker at room temperature for 5 minutes to make sure all formazan crystals were dissolved completely. The absorbance of formazan was measured at 560 nm.

2.6 Oil Red O Quantification Assay

3T3-L1 adipocytes were fixed in 0.5% (v/v) paraformaldehyde after various treatments and stained with freshly prepared 0.5% (v/v) Oil Red O for 15 min as described previously [17]. For quantitative

analysis of Oil Red O retention in these cells, stained adipocytes were extracted with absolute isopropanol and absorbance was measured at 510 nm. Results were expressed in percentage of difference as compared to untreated cells.

2.7 Glycerol Quantification Assay

The lipolysis activities were assessed using Enzychrom™ Lipolysis assay kit, Bioassay Systems, CA, USA. It directly measured the glycerol release in spent media of completely differentiated adipocytes. The linear detection range was 0-100 $\mu\text{g}/\text{mL}$ glycerol released. Results were expressed in percentage of difference as compared to untreated cells.

2.8 Gene Expression using Real Time - RT-PCR

Total RNA was extracted from the cells using RNAqueous-4PCR Kit, according to the manufacturer's instructions. The purity of recovered total RNA was estimated by calculating the ratio of absorbance reading at 260nm and 280nm. Two micrograms of purified total RNA with an A_{260}/A_{280} ratio between 1.8-2 was used to synthesize complementary DNA (cDNA) using high-capacity cDNA reverse transcription kit. Gene expression levels were analyzed by real time RT-PCR [17], using the StepOnePlus™ Real Time PCR system (Applied Biosystems, Foster City, CA). The cDNA was denatured at 95°C for 10 s, followed by 40 cycles of three-step PCR (95°C, 5 s; 60°C, 31 s). Table 1 shows the list of genes investigated in this study and the corresponding accession numbers. Endogenous control used in this study was eukaryotic 18S rRNA with FAM/MGB probe. All TaqMan® probes used in this study were labelled with FAM™ reporter dye at the 5' end and a MGB quencher at the 3' end.

Table 1. Selected genes for gene expression study in 3T3-L1 adipocyte.

NO.	Gene name and abbreviation	Assay ID	Accession number
1	Glucose transporter 4 (GLUT4)	Mm00436615_m1	NM_009204.2
2	Hormone sensitive lipase (HSL)	Mm00495359_m1	NM_010719.5
3	Lipoprotein lipase (LPL)	Mm00434770_m1	NM_008509.2
4	Peroxisome proliferator-activated receptor gamma (PPAR γ)	Mm01184322_m1	NM_011146.3
5	Adiponectin	Mm00456425_m1	NM_009605.4

General abbreviation of genes selected for this study and corresponding assay ID and accession information available on Applied Biosystems website. Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan[®] probe mix. Assay ID with “Mm” prefix is defined as “Mus musculus”. All Gene Expression Assay kits indicated here is FAM/MGB probed.

2.9 Statistical Analysis

All experiments were performed in triplicates, unless otherwise stated and the data were calculated as mean \pm standard deviation (SD). One-way analysis of variance with Duncan’s multiple range tests (DMRT) was used to determine the significant differences between means. Statistical significance was accepted at $p < 0.05$. Statistical analyses and graphs were prepared with Microsoft Office 2003 EXCEL, STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ, USA) and GraphPad Prism (version 5.02 for Windows, GraphPad Software Inc. California USA).

3. RESULTS AND DISCUSSION

3.1 Effect of Ethanol Extract of Unfermented and Fermented Wheat Grains on Viability of 3T3-L1 Preadipocyte

The MTT assay was used to determine the best cell density for subsequent use that includes preadipocytes proliferation, adipogenesis, lipolysis and gene expression studies. To determine the cytotoxicity of the ethanol extracts of unfermented and

fermented wheat grains by *Ganoderma* spp., the 3T3-L1 preadipocyte was treated with the extracts at various concentrations (20 - 100 $\mu\text{g}/\text{mL}$) and the cell viability was measured using the MTT assay. No significant preadipocyte toxicity was observed when the cells were treated with unfermented and fermented wheat grain extracts up to 100 $\mu\text{g}/\text{mL}$, the cell viability was $< 90\%$ relative to the control (Figure 1). The ethanol extracts of *Ganoderma* spp. fermented wheat grains significantly ($p < 0.05$) stimulated 3T3-L1 preadipocyte proliferation (10 - 35 % stimulation) compared to the unfermented wheat grain extracts.

3.2 Effect of Ethanol Extract of Unfermented and Fermented Wheat Grains on Adipogenesis in 3T3-L1 Preadipocyte

Oil Red O quantification assay was carried out to identify the adipogenic effect of ethanol extract of unfermented and fermented wheat grains by *Ganoderma* spp. on 3T3-L1 preadipocytes. Highly differentiated adipocytes have more lipid accumulation in the cytoplasm compared to the undifferentiated adipocytes. In order to

measure the level of adipogenesis, lipid globules accumulated in adipocytes were stained with Oil Red O dye. Oil Red O dye stained lipids were clearly visible as red globules under an inverted microscope. The results of Oil Red O quantification assay indicated that all the fermented wheat grain extracts tested, stimulated adipogenesis dose-dependently (Figure 2). The ethanol extract of wheat grains fermented with *G. neo-japonicum* exerted the highest adipogenic activity which was approximately 70 % increase in adipogenesis compared to experimental blank (cells treated with 1% DMSO) at a concentration of 100 $\mu\text{g}/\text{mL}$; followed by *G. australe* and *G. lucidum* mycelium extracts

which stimulated adipogenesis by 48% and 35% respectively. In addition, the adipogenic activity of these fermented wheat grains was significantly higher as compared to the unfermented wheat grain, where the maximum lipogenesis achieved was 13 % only (at extract concentration of 40 $\mu\text{g}/\text{mL}$); and the activity attenuated with increasing concentration (Figure 2). The experimental positive control, insulin, at 1 μM , stimulated adipogenesis in adipocytes by 96 % when compared to the experimental blank. These results suggest that fermentation with *Ganoderma* spp. mycelia especially *G. neo-japonicum* significantly ($p < 0.05$) enhanced the insulin-like properties of wheat grains.

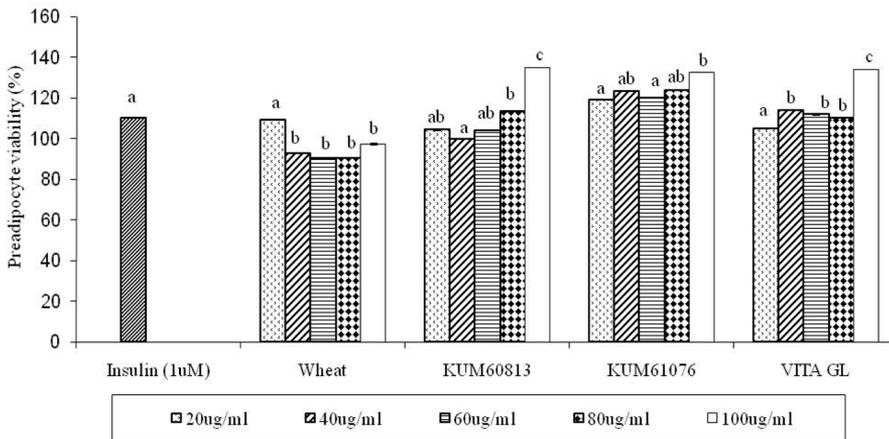


Figure 1. Effects of Insulin and ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability.

Y-axis indicates the mean percentages of preadipocyte proliferation \pm SD of quadruplicate assays compared to control values (cells treated with 1% DMSO). A negative value indicates inhibition of preadipocyte proliferation. Preadipocyte (10 000 cells / well) were incubated with various concentrations of ethanol extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grain (20, 40, 60, 80, and 100 $\mu\text{g}/\text{mL}$) for 48 hours prior to MTT assay. Means with different alphabets within an extract are significantly different ($p < 0.05$, ANOVA).

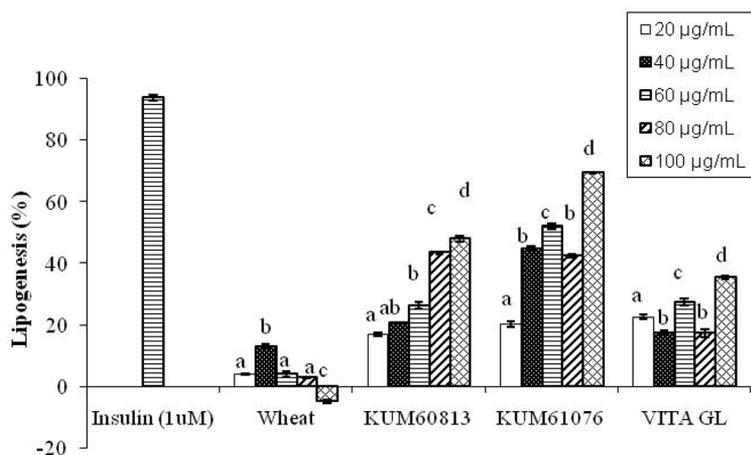


Figure 2. Effect of Insulin and ethanol extract of unfermented and fermented wheat grains on adipogenesis in 3T3-L1 preadipocyte.

Y-axis indicates the mean percentages of lipogenesis \pm SD of triplicate assays compared to control values (cells treated with 1% DMSO). Preadipocyte (80 000 cells / well, in a 24 well plate) were induced to differentiate with various concentrations of ethanol extracts of unfermented and fermented wheat grains prior to Oil Red O quantification assay. Means with different alphabets within an extract are significantly different ($p < 0.05$, ANOVA).

The ethanol extracts of *Ganoderma* spp. fermented wheat grains especially of *G. neo-japonicum* have been discovered to contain higher total phenolics compared to the unfermented wheat grains in our previous investigation [18]. Wheat kernels contain a number of phenolic compounds that are typically found conjugated with sugars, fatty acids or proteins [19,20]. During the fermentation process, different hydrolytic enzymes produced by the mycelia release phenolic compounds from their conjugates [21]. This maximizes the yield of phenolics of cereal grains after fermentation [22]. Numerous *in vitro* and *in vivo* studies support the beneficial effects of polyphenols and polyphenol-rich plant extracts on glucose homeostasis and hence, prevention of type 2 diabetes [23,24]. Thus, it is feasible to speculate that the high phenolic content is an important contributing factor to the enhancement of insulin-like properties i.e.

adipogenic activity of these fermented wheat grain extracts.

3.3 Effect of Ethanol Extract of Unfermented and Fermented Wheat Grains on Lipolysis in 3T3-L1 Adipocyte

After screening for cell viability and adipogenic activity, the ethanol extracts were tested for lipolytic activity in fully differentiated adipocytes. In this assay, epinephrine, 1 μ M was used as experimental positive control to stimulate lipolytic activity in adipocytes. Concentration of epinephrine used was determined based on a preliminary dose-response study (data not shown). The lipolytic activity exerted by the ethanol extracts were assessed by comparing the amount of glycerol released to that induced by epinephrine. Based on our preliminary dose-response studies on lipolysis, 100 μ g/mL was found to be the ideal concentration at which a wide spectrum of lipolytic and/or anti-lipolytic

activities could be observed (data not shown). When adipocytes were incubated with epinephrine the release of glycerol increased by 78.19 % ($p < 0.05$) as compared to the experimental blank (Figure 3). The fermented wheat grains extracts significantly ($p < 0.05$) induced lipolysis (50 - 55%) in adipocytes compared to the experimental blank (<1% DMSO). Nevertheless, the amount of glycerol released by both the crude extracts of unfermented and fermented wheat grain were significantly lower (20.17 - 54.53 %) as compared to the amount of glycerol released by epinephrine (Figure 3). The extracts were also screened for inhibitory effects against epinephrine induced lipolysis in mature adipocyte. When co-incubated with epinephrine (1 μM), the extracts significantly inhibited 60 - 80 % of epinephrine induced lipolysis in adipocyte (Figure 3). The data obtained showed that the ethanol extracts of wheat grain fermented with *G. Neo-japonicum* have insulin-like effect in inhibiting epinephrine-induced lipolysis in spite of being moderately lipolytic by itself.

Increasing lipolysis in adipocyte may be a potentially useful therapeutic target for treating obesity [6]. However, high levels of fatty acids in the blood, typically observed in obesity, are correlated with many detrimental metabolic consequences such as insulin resistance, cardiovascular disease and hypertension [25]. Although anti-epinephrine induced lipolysis is one of the insulin's characteristic, strong anti-epinephrine induced lipolytic activity is not favourable since it inhibits lipid mobilization that might pose a problem for obesity and insulin resistance [26]. Thus, based on the basal and anti-epinephrine induced lipolysis results obtained, ethanol

extract of wheat grains fermented with *G. neojaponicum* mycelia is more suitable for the development of anti-diabetic agent since it stimulated lipolysis when sufficient lipid is present in mature adipocytes while enhancing lipid accumulation (lipogenesis) in differentiating adipocytes when energy intake is adequate [17].

3.4 Ethanol Extract of Fermented Wheat Grains Modulated the Expression of PPAR γ Targeting Genes in Adipocyte

The highly complex genetic reprogramming that occurs during adipogenesis is under the tight control of hormones, cytokines, nutrients, and signalling molecules that change the expression and/or activity of a variety of transcription factors, which in turn finely regulate the magnitude of the adipose conversion process. Today many pro- and anti-lipogenic transcription factors are known to regulate adipogenesis [27]. PPAR γ has been identified as a central adipogenic regulator/switch and its agonists are transcription factors that regulate expression of genes involved in glucose and lipid metabolism [28]. For example, PPAR γ agonists like rosiglitazone and troglitazone are used widely in the treatment of Type 2 DM. The results in this study showed that the ethanol extracts of fermented wheat grains by *Ganoderma* spp. mycelia stimulated adipogenesis dose-dependently (Figure 2), while inhibiting epinephrine induced lipolysis in mature adipocytes (Figure 3). To investigate this further, the relative expression of five selected genes, representing selective key points in the insulin and epinephrine pathways were examined in adipocytes treated with fermented wheat grain extracts.

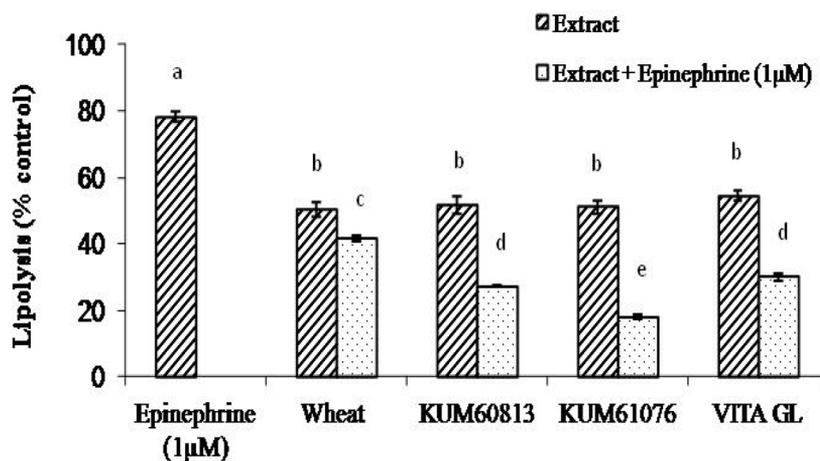


Figure 3. Effect of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte in the absence and presence of Epinephrine (1µM).

Adipocytes cultured (fully differentiated) on 24-well plates were treated with ethanol extracts of *Ganoderma* spp. mycelia and/or epinephrine (1 µM) for 24 hours. The glycerol released was measured spectrophotometrically at 570 nm using standard kit, Enzychrom™ Lipolysis Assay kit. Epinephrine, 1 µM was used as the experimental positive control. Data was referenced to experimental control (1% DMSO carrier blank). Each value is expressed as mean ± SD (n=3). Means with different alphabets are significantly different (p < 0.05, ANOVA).

Figure 4 shows that adipocytes treated with 100 µg/mL (w/v) of ethanol extract of wheat grains fermented with *G. neo-japonicum* mycelia significantly elevated the expression of PPARγ, GLUT4 and adiponectin genes [4.41±0.05-fold (p < 0.05), 3.56±0.29-fold (p < 0.05), and 11.02±0.48-fold (p < 0.05), respectively] compared to treatments with other ethanol extracts. Previous studies have shown that PPARγ and C/EBPα cross-regulate each other to maintain their gene expression and also modulate the expression of other adipogenic marker genes such as aP2,

GLUT4, and LPL, during preadipocyte differentiation [29,30]. Therefore, it is pertinent to suggest that ethanol extract of *G. neo-japonicum* fermented wheat grains stimulated 3T3-L1 preadipocyte differentiation via the modulation of PPARγ. These data also correlate well with the microscopic examination and the Oil red O assay that revealed significant accumulation of lipid bodies in the cells treated with ethanol extract of wheat grains fermented with *G. neo-japonicum* mycelia.

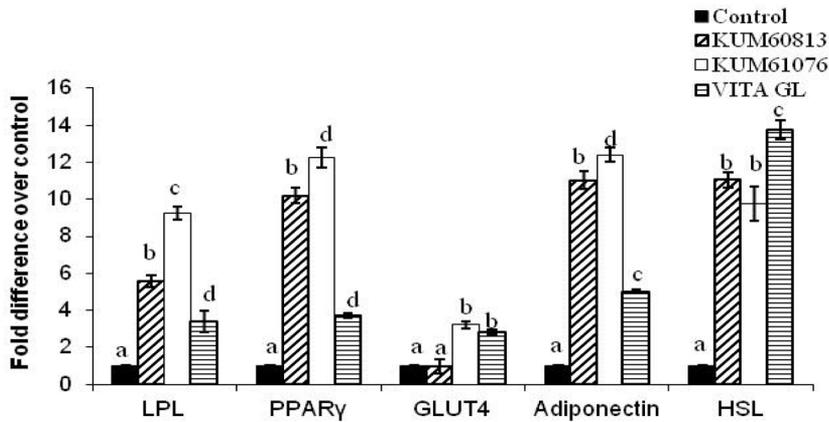


Figure 4. Effect of ethanol extracts of unfermented and fermented wheat grains on gene expression in 3T3-L1 adipocytes.

Preadipocytes were treated with 100 $\mu\text{g}/\text{mL}$ of ethanol extracts of fermented wheat grains for 48 hours and differentiated as stated in *Section 2.4*. Total RNA was extracted and cDNA were synthesized. RT-PCR was performed and the relative expression of the genes was calculated using the delta-delta CT method. The values were normalized with 18S (control) eukaryotic rRNA. Results are expressed as n-fold difference over control. Fold variation values lower than 1 indicates down-regulation. The results presented are mean \pm SD. Statistical significance was calculated based on the mean $\Delta\Delta\text{CT}$ values by one way ANOVA; ($p < 0.05$). Means with different alphabets within a gene are significantly different ($p < 0.05$, ANOVA).

Lipolysis in adipocytes is highly dependent on hormone sensitive lipase (HSL), which is regulated by insulin and catecholamines [31,32]. Epinephrine (a catecholamine) is well known to stimulate lipolysis by promoting adenylate cyclase activity to increase cAMP level leading to the activation of HSL [33]. However, the activation of cAMP phosphodiesterase (PDE) and stimulation of protein phosphatase-1 by insulin may inhibit the HSL activation [31]. As shown in Figure 4, there was a significant increase in expression of HSL gene in adipocytes differentiated with all three ethanol extracts relative to control ($p < 0.05$). The decreasing order of HSL expression among the ethanol extracts was as follows: *G. lucidum* > *G. neo-japonicum* > *G. australe*. Adipocytes treated with 100 $\mu\text{g}/\text{mL}$ (w/v) of *G. lucidum* mycelia ethanol extract showed elevated expression of HSL

by 13.74 ± 0.51 -fold ($p < 0.05$) compared to *G. australe* and *G. neo-japonicum* ethanol extracts treatment. This data correlates well with the previous experiments (Oil Red O and glycerol quantification assay) where the ethanol extract of *G. lucidum* stimulated adipogenesis moderately while strongly stimulating basal and epinephrine-induced lipolysis in adipocytes.

4. CONCLUSIONS

Based on our current findings, it is feasible to conclude that ethanol extract of *G. neo-japonicum* mycelia fermented wheat grains has insulin-like potential and the bioactive compound(s) present might potentially serve as adjuvant or substitute for insulin in treating DM as compared to the other two species of *Ganoderma* tested; since it mimics insulin's action in adipose cells. However, further studies are required

to identify the bioactive compound(s) and the precise metabolic action(s) of the *G. neojaponicum* mycelia extract on the key players of those molecular pathways leading to adipogenesis in 3T3-L1 cells.

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