Original article

Herbal extracts exhibit anti-diabetic activities in 3T3-L1 adipocytes model

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Summary. The prevalence and suffering of diabetes and obesity has been increasing among the various communities of the world including Malaysia. The cost of treatment is also rising. Therefore, it is important to explore non pharmacological regimens that are non-invasive and with less health risks and cost burden to the patients, healthcare professionals and nations. The objective of this study was to evaluate efficacy of the traditionally used antidiabetic herbs. *Methods:* Water extracts of *Andrographis paniculata*, *Lagerstroemia speciosa* and *Orthosiphon stamineus* (Cat whisker) were prepared and evaluated for their effects on cell proliferation, adipogenesis, glucose uptake, quantification of mRNAs for Ppary, Glut4, adiponectin and leptin using Real-Time Polymerase Chain Reaction (qRT-PCR) in 3T3-L1 preadipocytes. *Result and Discussion:* There were significant ($P<0.01$, $P<0.05$) increase in adipogenesis activity for cells treated with insulin, *A. paniculata* and *O. stamineus* compared to control. There was significant ($P<0.01$, $P<0.05$) increase in glucose uptakes in the cells treated with Sodium Orthovanadate, *L. speciosa*, *A. paniculata* and *O. stamineus* compared to control. *Pparγ* transcriptional levels in cells treated with *A. paniculata* extract was similar to control. However, *L. speciosa* extract ($P<0.01$) showed significantly lower levels of *Pparγ* mRNA expression compared to control. Gene expression analysis demonstrated that *A. paniculata* and *L. speciosa* extracts significantly ($P<0.01$, $P<0.05$) induced Glut4 mRNA transcription compared to control in 3T3-L1 adipocytes. *Conclusions:* The present study suggests that the extracts of *A. paniculata* and *O. stamineus* possess insulin-mimicking effects.

Key words: Herbal extracts, 3T3-L1 adipocytes, adipogenesis, glucose uptake, Glut4

Introduction

The number of individuals suffering from diabetes mellitus is exponentially increasing around the world. The rapid increase in prevalence is of greater concern to the countries and global communities. World Health Organization (WHO) estimated that 30 million people suffered from diabetes in year 1985 and the number increased to more than 171 million in year 2000. The incidence is expected to be 366 million by year 2030 (1, 2). Therefore, an emphasis should be given on the alternative treatment/prevention of diabetes which would be less expensive and easily available that is manufactured from local ingredients. World Health Organization

Expert Committee on diabetes has recommended that traditional herbs and plants be further investigated (3). Modern drugs usually give greater and quicker effects than medicinal plants but pose a higher degree of side effects whereas plants sources are considered as natural with no adverse effects (4, 5). Plants sources have the ability to delay the complications of diabetes (6) and the efficacy of more than 1200 plants sources have been documented in ethno-pharmacological survey reports (7, 8). Most of these plants have been evaluated using animal models in order to confirm their hypoglycemic effect (9, 10) as well as in human and some of these plants have also been studied for their bioactive compounds (11-13). Therefore, this project was designed to study the effects of herbs namely *Andrographis paniculata* (Hempedu bumi), *Lagerstroemia speciosa* (Banaba/ bungur) and *Orthosiphon stamineus* (Cat whisker) for the efficacy of diabetes in *in vitro* model based on their traditional uses in Malaysians.

Materials and Methods

Dried herbs namely *Andrographis paniculata*, *Lagerstroemia speciosa* and *Orthosiphon stamineus* were purchased from a local supplier in Kuantan, Pahang, Malaysia. The cell line namely 3T3-L1 preadipocyte (derived from mouse embryonic fibroblast) were purchased from American Type Culture Collection (ATCC) 10801, University Boulevard, Manassas, VA 20110 USA. Dried powdered herbs materials were extracted using distilled water and stirred using incubator shaker for two days at 1000 rpm and 60°C. This procedure was repeated for three times using the same materials. The extracts were then combined and filtered through Whatman filter paper. The extracts were placed in a freezer at 4°C overnight followed by freeze drying for 1 week or until dried to yield crude extracts. The crude extracts were weighed and stored at -80°C for further analysis.

Cell viability was assessed by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously with slight modification by (14). The 3T3-L1 adipocytes were treated with a concentration of 100 µg/ml of samples. Herbal extract treated cells were incubated for a day at 37°C in humidified 5% CO_2 atmosphere. The adherent cells were washed two times with Phosphate buffered saline (PBS). Then, 20 µl of MTT stock solution (5 mg/ml) was added to each well and the plates were further incubated for 4 hours at 37 °C. Then, 100 µl of DMSO was added to each well. Cells were left for one hour and the absorbance was measured at a wavelength of 570 nm and reference wavelength 630 nm with a micro plate reader (Dynatech MR5000).

Measurement of adipogenesis and lipid droplets were performed by cultured and maintained 3T3-L1 cells as previously described by Taher, (Ref.15). Cells were seeded and differentiated at 3 x 10^4 in 96-well plate containing 0.1 ml (100 µl) medium until reached confluence. Induction of differentiation was performed at day-2 post confluence. After three days the induction, the cells were treated with insulin alone for another two days. Then, after 5 days of induction, the medium was replaced in every 48 hours until day-10. The visible accumulation of lipid droplets was monitored with microscope. On the day 11 after the induction, the medium was replaced with the extracts concentration of 0.25 mg/ml. At day 13, the medium was replaced with 75 µl of lipid droplets assay fixative in each well and incubated for 15 minutes. After that, the wells were washed with 100 µl of wash solution twice for five minutes and allowed to dry completely. Then, 75 µl of Oil Red *O* working solution was added to all wells and incubated for another 20 minutes. After that, all Oil Red *O* solution was removed and the cells were washed with distilled water. This was followed by washing the wells with 100 µl of washing solution twice for 5 minutes. At this point, microscopic images were taken to visualize pink to red oil droplets stained in the differentiated cells. Lastly, 100 µl of dye extraction solution was added and the cells were gently mixed for 15-30 minutes and the absorbance was measured at 490-520 nm using ultraviolet spectrophotometer.

Glucose uptake activity was analyzed by measuring the uptake of radiolabelled glucose. This was performed based upon previously described methods with modification by Roffey et al. (16). Differentiated cells were washed twice with serum-free DMEM and incubated in 1 ml/well serum-free DMEM for 3 hours at 37°C. Then, washed three times with Krebs-Ringer HEPES (KRPH) buffer (118 mM NaCl, 5 mM KCl,

1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 30 mM HEPES, pH 7.4) and incubated with 1 ml/well of KRPH buffer for 30 minutes at 37 °C. Plant extracts, metformin and sodium orthovanadate were added 30 minutes prior to addition of 2-deoxy-D-[1, 2-3H]-glucose. For the measurement, 2-deoxyglucose (0.001 mM) was used together with the radiolabeled tracer, 2-deoxy-D-[1,2-3H]-glucose (0.037 MBq) to give a concentration of 0.2 mM (0.5 mCi/mmol) yielding an activity of 0.1 μCi/ml. After 60 minutes at 37°C, glucose uptake was terminated by washing the cells three times with 3 ml/well of ice-cold phosphate-buffered saline (pH 7.4). Cells were then washed and lysed with 0.7 ml of 1% Triton X-100 for 40 minutes at 37°C. Samples from each lysate were counted using liquid scintillation counter (Packard Tricarb 2700 TR/SL liquid scintillation analyzer, Packard Instrument Co.).

The total RNA was extracted from the earlier treated 3T3-L1 adipocytes with the plant extracts using Trizol® reagent kit. The quantity and quality of RNA in this study was measured by using the NanoDrop UV-VIS spectrophotometer. The amount of each RNA samples used in this procedure was initially standardized. Reverse-transcription PCR was conducted using QuantiTect® Reverse Transcription (Qiagen) kit according to the manufacturer's instruction. Quantitative real-time PCR reaction was performed by using Quantitect PCR SYBR® Kit (Qiagen) according to manufacturer's instructions. Quantitative real-time PCR was performed using Corbett real-time PCR Thermocycler. To confirm the amplification of specific transcripts, melting curve profiles were produced at the end of each PCR. The relative expression level of the *Glut4* and *Pparγ* mRNA was normalized by the amount of β-actin, a housekeeping gene.

The collected data was compiled and statistically analysed using SPSS (Version 15.0). One-way analysis of variance (ANOVA) was used wherever appropriate. The data was considered statistically different at 95% confidence interval.

Result and Discussion

The extract product yield for *Andrographis paniculata*, *Lagerstroemia speciosa* and *Orthosiphon stamineus* was 6.4, 4.9 and 5.4 percent of dried powder. Most of the chemically synthesized drugs are based on plant extracts, for example, the traditional use of the Goat's rue (*Galega officinalis*) which led to the development of the oral hypoglycemic drug metformin, a biguanide for type 2 diabetes mellitus (17 and 18). The method used for extraction in the traditional medicine is based on decoction. The extracts are believed to contain numerous different compounds with broad range of polarity that makes it suitable for screening purposes. Water extraction provides several advantages such as inexpensive, easy to prepare and requires no special expertise in preparing the extract. However, the disadvantage is that all therapeutically useful constituents may not be extracted (19). Therefore, it is utmost important to extract herbs with water in order to justify the use of plants/herbs in a traditional way. This procedure actually mimics the preparation of hot tea where the leaves of tea are left for a certain period of time in hot water in order to extract their polar constituents. In the present study, it was observed that the effective concentration of extracts was 0.25 mg/ml (Fig. 1). However, when the highest concentrations up to 1.0 mg/ml were used in the cell culture, the number of live cells decreased. The concentration of 0.25 mg/ml extracts resulted in the maximum cell growth with a 90% of survival rate. The MTT calorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation of the cells. The main advantages of the colorimetric assay are its rapidity and precision, convenient and the lack of any radioisotope (14, 20). The adipocytes viability with increasing concentrations of the extracts reduces the cell viability (21). Therefore, in this study, a concentration of 0.25mg/ml was chosen in order to avoid toxic effect to the adipocytes and to maximum cell growth of approximately.

Replacing insulin with extracts exhibited an activity by promoting cell differentiation which was indicated by accumulation of the lipid droplets. There was significant increase in adipogenesis activity for cells treated with insulin ($p<0.01$), *A. paniculata* ($p<0.05$)

and *O. stamineus* ($p<0.01$) extracts compared to the control. The insulin caused highest lipid droplets formation followed by *O. stamineus, A. Paniculata* (Fig. 2).

After day 11, preadipocytes differentiation was terminated and stained with Oil Red *O and* the lipid droplets were observed in the induced cells. The stage-wise performance of the adipocytes for the lipid droplets formation is indicated in the Figg. 3 and 4.

When the cells were incubated in a medium containing 2-deoxy-D-[1, 2-^{3}H]-glucose in combination with 2-deoxyglucose, there were significant ($P<0.01$, $P<0.5$) increase in glucose uptake in cells treated with Sodium Orthovanadate, *L. speciosa*, *A. paniculata* and *O. stamineus as* compared to control (Fig. 5).

The RNA was extracted from 3T3-L1 adipocytes treated with extracts (0.25 mg/ml) by using Trizol. The ratio of absorbance 260/280 nm and 260/230 nm indicates the quality and purity of RNAs. A ratio greater than 1.8 indicates better quality of RNA as described by Manchester, (22). Results showed that the quality of RNA collected was within the acceptable range in 3T3-L1 adipocytes (Table 1). Average yield was measured by means of two independent concentrations.

Gene expression analysis showed that the levels of *Pparγ* mRNA were significantly ($p<0.01$) higher in the cells treated with insulin compared to control in 3T3-L1 adipocytes. *Pparγ* transcriptional levels in cells treated with *A. paniculata* extract was similar to control. Conversely, *L. speciosa* extract caused significantly ($P<0.01$) lower level of *Pparγ* mRNA expression compared to control (Fig. 6). In the undifferentiated adipocytes *Pparγ* and *Glut4* mRNA were barely expressed (Figg. 6, 7). Gene expression analysis showed no effect in the expression of adiponectin and leptin mRNA in cells with insulin, *L. Speciosa* and *A. paniculata* extracts compared to control in 3T3-L1 adipocytes. (Figg. 8, 9).

The observed effect of the extracts on adipogenesis and glucose uptakes in the adipocytes is clinically important for glucose homeostasis and energy storage (see summary of the effects Table 2. Thus, it might lead to more effective strategies for the treatment of diabetes and metabolic disease (23). The 3T3-L1 preadipocytes undergo differentiation process known as adipogenesis which is the conversion of preadipocytes to mature adipocytes to perform highly specialized function.

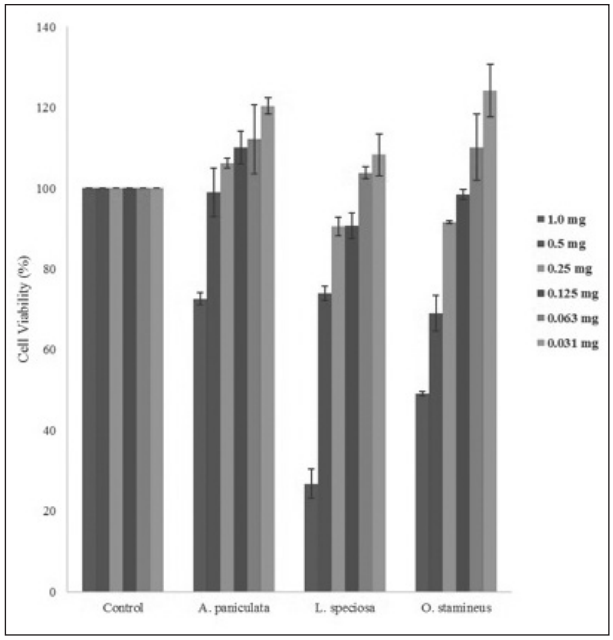

Figure 1. Effect of extracts on the adipose cells (3T3-L1 adipocytes) viability at a concentration range of 0.031–1.00 mg/ml assessed by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

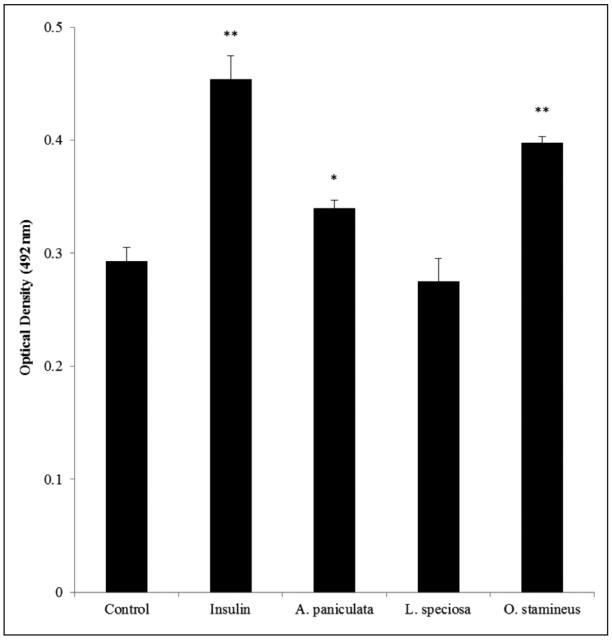

Figure 2. The effect of herbs extracts on the adipogenesis in 3T3-L1 adipocytes. Data are means ± S.D. of three observations.

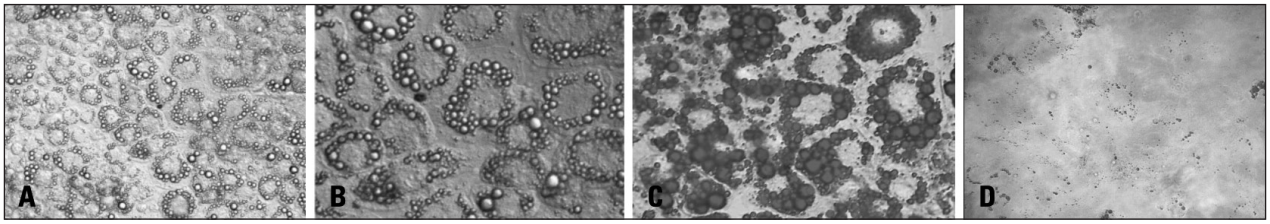

Figure 3. The lipid droplets accumulation in the fully differentiated preadipocytes (A) without Oil Red O staining at magnification 20X and (B) at magnification 40X. (C) Positive control (insulin), (D) Negative control (without treatment).

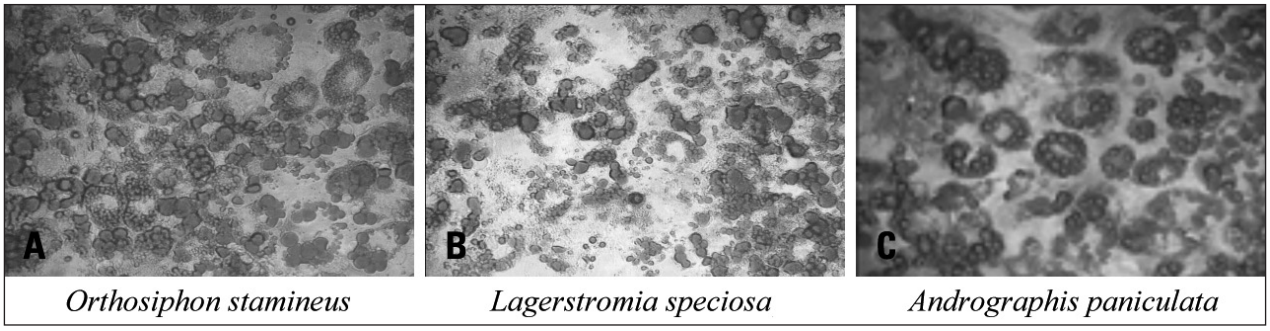

Figure 4. The lipid droplets accumulation in the fully differentiated preadipocytes treated with extracts after Oil Red O staining at magnification 20X. The red areas show the lipid droplets formation in the adipocytes.

Conversion to adipocytes can happen spontaneously but the process is enhanced by the addition of combination of dexamethasone, isobutylmethylxanthine (IBMX) and insulin (24). Within 3 days exposure to inducers, the cells undergo mitotic clonal expansion or two rounds of mitosis which are required for differentiation (25). The adipocytes adopt a rounded phenotype and within 5 - 8 days begin to accumulate lipids in the form of lipid droplets. Dexamethasone acts to activate the transcriptional factor enhancer-binding protein β and binds to the glucocorticoid receptor. The IBMX inhibits soluble cyclic nucleotide phosphodiesterases which results in increased intacellular cAMP levels. Insulin or insulin-like growth factor-1 promotes adipocytes differentiation by activating P1-3 kinase (Phosphatidylinositol 3-Kinase), Akt (Protein Kinase B) activity and binds to insulin receptor. These three pathways are terminated by the activation of PPARγ genes which activate adipocyte specific genes encoding secreted factors, insulin receptor and proteins involved in the synthesis and binding of fatty acids that compose Oil Red *O* stain lipid droplets.

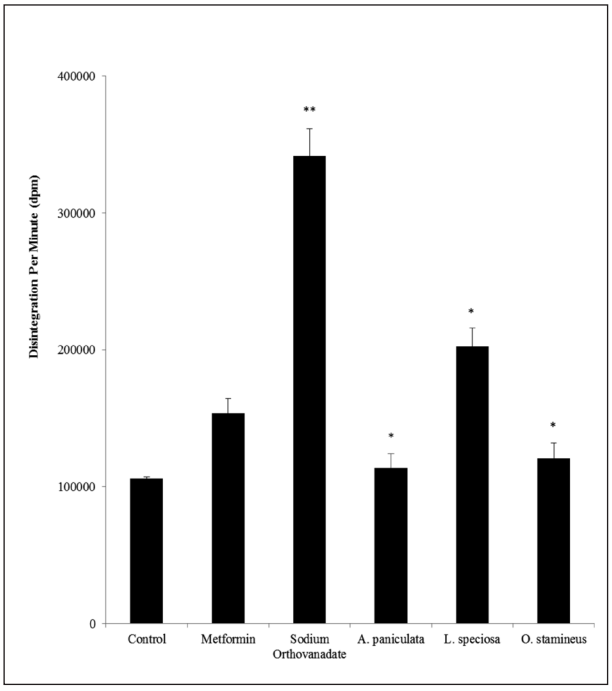

Figure 5. The effect extracts on glucose uptake in the adipocytes assessed with radiolabeled glucose. Data are the means ± S.D of three observations, expressed as disintegration per minute (DPM).

Table 1. RNAs Concentrations, Total Yield and Purity.

Sample	µg/µl	A260/ A280 (nm)	A260/ A230 (nm)
1. Differentiated cell	1.6	1.9	0.9
2. Undifferentiated cell	1.0	2.0	1.2
3. Insulin	2.1	2.0	1.1
4. *Andrographis Paniculata*	1.7	2.0	2.1
5. *Lagerstroemia speciosa*	1.5	2.0	1.3

The quantity of lipid in the adipocytes stained with Oil Red *O* was appeared to be proportional to the level of cell differentiation. It shows that the Oil Red *O* is specifically stains both triglycerides and cholesteryloleate, as reported by Ramirez et al., (26). Thus, high dense colour suggests increased differentiation as well as triglycerides accumulation. Conversely, less colour intensity indicates decreased lipid accumulation and inhibition of differentiation. Differentiation is accompanied by an increase of 10 to 50 fold in the specific activities of enzymes which involved in fatty acid and triglycerides synthesis. Usually specific insulin receptors already present in cells before the onset of differentiation but the number is increase to 25 fold during conversion to adipocytes (27).

In our study, replacing insulin with extracts exhibited an activity in promoting cell differentiation which was indicated by the accumulation of lipid droplets. Statistically, insulin, *A. paniculata* and *O. stamineus* extracts showed significant increase of adipocytes differentiation compared to the control. As mentioned earlier, insulin gave the highest lipid droplets formation followed by *O. stamineus* and *A. paniculata* extracts (Figg. 3, 4). This effect indicates that insulin acts as an essential regulator of the differentiation of 3T3-Ll adipocytes and accelerates the differentiation process (27-29). *L. speciosa* extract showed a decrease

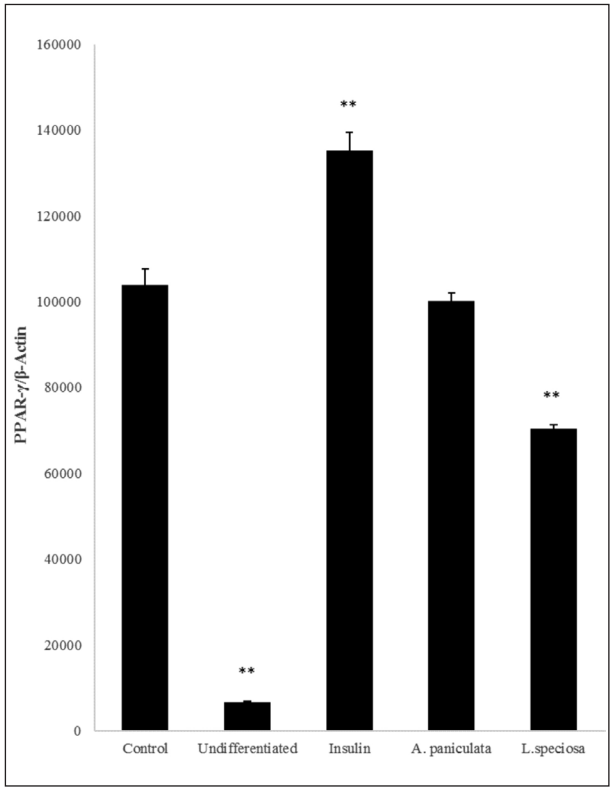

Figure 6. Real time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of adipogenesis related factor (PPARγ) mRNAs were quantified using β-Actin as an internal standard. The results are means ± SD of three observations.

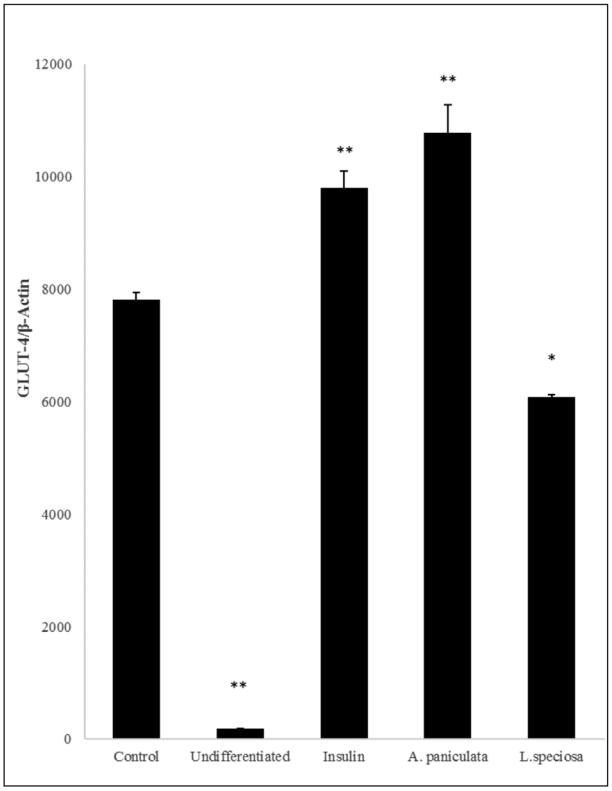

Figure 7. Real time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of glucose-transporter 4 (GLUT4) mRNAs were quantified using β-Actin as an internal standard. The results are means ± SD of three observations.

Table 2. Summary of the results from various activities.

Activities	*A. paniculata*	*L. speciosa*	
1. Adipogenesis	↑*	↓	↑**
PPARγ mRNA	↑*	↓**	N.T.
2. Glucose uptake	↑*	↑*	↑*
Glut4 mRNA	↑**	↑*	N.T

** = P<0.05 and **=P <0.01*

↑ = Increase activity, ↓ = Decrease activity, N.T. = Not tested

in lipid accumulation as compared to control. This finding is consistent with a previous study that shows that *L. speciosa* extract inhibits the adipocyte differentiation activity in adipocytes (30). This might be due to the presence of ellagitannins, an active compound from water extract of *L. speciosa* in inhibiting adipocyte differentiation in 3T3-L1 cells (31). In the undifferentiated cell no differentiation occurs as exhibited by the absorbance and lack of lipid droplets formation. This observation is also consistent with Bernlohr & Simpson, (32).

The observed effects of the extracts on the radiolabeled glucose uptake of *A. paniculata*, *L. speciosa* and *O. stamineus* extracts is also of clinical importance which has been reported elsewhere in 3T3-L1 adipocytes (30). The compound in water extract of *L. speciosa* works as an activator of glucose transport in fat cells (33). Similarly, Cheng and Liu, (34) revealed that *O. stamineus* extract which are rich in phenolic compound namely caffeic acid, has been reported to increase glucose uptake in rats. However, there is lack of studies on adipocytes. Previous study on andrographolide, which is a major active compound in *A. paniculata* extract, has been shown to be involved in an increased basal glucose uptake of adipocytes in dose dependent and time-dependent manner and is further, increased when adipocytes are treated with a combination of extract and insulin compared to insulin (35). In the present study, glucose uptake activity induced by the extracts was compared with metformin and sodium orthovanadate known as insulin mimetic agents. The findings showed that sodium orthovanadate significantly elevated glucose uptake in adipocytes but not metformin. This effect suggests that vanadate induced the recruitment of Glut4 in the plasma similarly to that of insulin (36). Metformin has been shown to increase glucose uptake in human adipocytes in a dose and time dependent manner in a study performed by Grisouard et al., (37). The study reveals that an increases cellular glucose uptake after 24 and 48 hours treatment with metformin but does not increase glucose uptake in 3 hours incubation time. Furthermore, incubation with 0.001 and 0.1 mM metformin also does not potentiate the glucose uptake (37).

The effect of herbal extracts on the aforementioned activities in adipocytes was followed by gene expression analysis by quantitative Real-time Polymerase Chain Reaction (qRT-PCR. In order to avoid bias, there is a need for internal control genes or housekeeping gene (HKG) which does not fluctuate during treatments under normal and patho-physiological conditions (38). The HKGs are expressed in all cells of organism required for the maintenance of basic cellular function (39). In this study, the amount of mRNA extraction from the samples was low and therefore, only β-actin was used as HKG due to its stability. Aanother reason was the fewer numbers of genes to be analized. This was previously reported by Suzuki et al., (40) that almost 90% of the RNA transcription analyses published just use one reference gene. However, some studies suggested to use probably two or three housekeeping genes whose expression has been shown to be unaffected by experimental conditions in order to produce more reliable results in *in vitro* experiments (41). Yet, another study also reports that, if it is only just a few genes to test or a sample set with low diversity such as cell culture, it is not practical to run multiple housekeeping genes (42). After that, the effect of herbs and spices extracts on the expression of *Pparγ*, Glut4, adiponectin and leptin mRNA in 3T3-L1 adipocytes under basal condition, were determined using quantitative real time PCR (qRT-PCR) over the internal control of β-*actin* mRNA.

Pparγ plays an important role in adipocytes differentiation, glucose metabolism as well as regulating fatty acid storage. The increase in activity of *Pparγ* mRNA simultaneously increases adipocyte differentiation or adipogenesis. Our results, clearly shows that the expression of *Pparγ* mRNA was significantly (P<0.01) elevated in cells treated with insulin. This

finding is consistent with a previous study on isolated adipocytes which revealed that insulin and corticosteroids show a synergistic effect to induce transcriptional level of *Pparγ* mRNA (43). Furthermore, cells treated with *A. paniculata* extract also shows a significant (P<0.01) increase in *Pparγ* transcriptional level compared to the control (Fig. 6). But, this extract received a minimal attention on molecular level. Thus, we are not able to compare our finding with previous study. However, the expression of *Pparγ* mRNA in cells with *L. speciosa* extracts showed significant (P<0.01) reduction when compared to control. However there was no effect on the mRNAs studied for adiponectin and leptin (data not shown). Previous study report that *L. speciosa* does not induce adipocyte differentiation and show reductions of peroxisome *Pparγ* mRNA level in mature cells containing extracts (30).

Another gene studied in this research was insulin-stimulated glucose-transporter 4 or (Glut4) mRNA. Herman and Kahn (44) reported that Glut4 recruitment to the cell surfaces of adipose tissue stimulated by insulin, indepedent of transcription or translation. This initiate distinct signalling mechanisms that enhance glucose uptake activity. Consistent with the result in this study, it was shown that the cells treated with insulin significantly increase the expression of *Glut4* mRNA. Another study also demonstrated that insulin induces the translocation of the Glut4 from intracellular membrane compartments to the plasma membrane where the glucose metabolism happen which causes glucose uptake into adipose cells (45). Study by Garvey et al. (46) showed that Glut4 is the only one that responds to insulin by facilitating the transport of glucose into cells and probably this happens in response to insulin action. Extracts of *L. speciosa* and *A. paniculata* showed a significant (P<0.5, P<0.01) increase in transcriptional level of *Glut4* mRNA levels compared to control (Fig. 7). Previous study revealed that tannic acid, a major component of tannins which contain in *L. speciosa* extract induces translocation of Glut4, protein factors involved in the signaling pathway of insulin-mediated glucose transport (47). This study shows similar effect of *L. speciosa* extract of enhanced glucose uptake activity which might be due to increased transcriptional level as well as translocation of Glut4 mRNA. Furthermore, studies of Subramanian et al., (48) and Zhang, (49) report that *A. paniculata* extract stimulate Glut4 translocation in order to increase glucose utilization which is consistent with the finding of this study.

Conclusion

Based on the present study, *A. paniculata* and *O. stamineus* water extracts display insulin-like activity in *in vitro*. These extracts increase the adipocytes differentiation (adipogenesis) with concurrent increase in the expression of *Pparγ* mRNA (which is a key regulator in adipocytes differentiation). This study also suggested that *A. paniculata*, *L. speciosa* and *O. stamineus* extracts increase glucose uptake activity which happens through the enhanced Glut4 transcription. The present study suggests that the extracts of *A. paniculata* and *O. stamineus* possess insulin-mimicking effects.

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Conflict of interest

With this submission I also undertake and declare that the contribution of the authors as mentioned in the authorship of this research paper has directly participated in the planning, execution, or analysis of this study. All authors of this paper have read and approved the final version submitted. The authors involved in this research project declare and agreed to publish this article in the present sequence of authorship. Furthermore, there is competing interest among the authors and the fund provider of this research project.

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