Progress in Nutrition 2019; Vol. 21, Supplement 2: 238-245 DOI: 10.23751/pn.v21i2-S.6367 

Original article

# Bottle gourd (*Lagenaria siceraria*) extracts improve glucose and lipid metabolism in 3T3-L1 adipocytes

*Assia Sassi*[1], *Mohammad Muzaffar Ali Khan Khattak*[1], *Mohammad Taher*[2]

[1]Nutrition Department, Kulliyyah of Allied Health Sciences, IIUM Kuantan Malaysia - E-mail: sassia19@gmail.com, [2]Pharmaceutical Technology Department, Kulliyyah of Pharmacy, IIUM Kuantan Malaysia.

**Summary.** The aim of this study was to investigate the effect of bottle gourd which belongs to family of cucurbitaceae (*Lagenaria siceraria* (LS) on hyperglycemia by assessing the cells viability, adipogenesis, adipolysis, glucose uptake, adiponectin and leptin using 3T3-L1 adipocytes as a model. Fresh bottle gourd were washed thoroughly with distilled water and segregated into three parts namely; whole vegetable (LSW), peels (LSP) and seeds (LSS). Each part was blended either with water or ethanol which then labeled as LSWw, LSPw, LSSw LSWe, LSPe and LSSe, respectively. The collected data was compiled and statistical analysis was performed. Mainly, one-way analysis of variance (ANOVA) was performed followed by Tukey's post-hoc test to ascertain differences in the means. The lipid droplet formation was significantly ($P<0.001$) higher in the adipocytes treated with the extracts of LSWw, LSSw, LSWe , LSSe and lower in the extracts of LS peels (LSPw, LSPe) compared to induction medium (MDI)/insulin (control). The same extracts also significantly ($P<0.001$) increased glycerol release during adipolysis compared to the control. It caused a significant ($P<0.001$) increase in adiponectin concentrations for LSPw, LSWw and a decrease in leptin concentrations for the water extracts of the LSPw. The present study showed that there was a hypoglycaemic effect of LS extracts by improving the regulation of adipogenesis through the formation of lipid droplets, adipolysis by increasing the release of glycerol, glucose uptake by increasing the uptake into the cells as well as adiponectin and leptin concentrations which could be of clinical importance in energy regulation which is a key factor in diabetes, obesity and metabolic syndrome.

**Key words**: adipocytes, adipogenesis, adipolysis, glucose uptake, adiponectin, leptin

## Introduction

It is evident that plant-based foods for human diet have no alternatives around the world. Cereals crops, fruits and vegetables are major and excellent sources of nutrients (1). The phytochemicals in these plants play an important role in health and help in the prevention of diseases such as cancer (2), cardiovascular disease (3), obesity (4), Diabetes Mellitus (5) gastrointestinal disorders (6) and many more. Good nutrition helps avoid side effects, long term undesirable effects and the economic burden resulting from the use of drugs (7). Among the biggest challenges of the 21st century diseases is type 2 Diabetes Mellitus (T2DM) regardless of the improved treatment and management options available in clinical settings. The current estimate of individuals suffering from T2DM is 364 million worldwide and the number is estimated to double by the year 2030 (8). Traditional herbal remedies represent a better alternative for treatment due to their extensive use around the world every year. There are over 1200 species claimed to possess antidiabetic properties (9) which led the recent researches to focus more on providing scientific validation of these plant foods and health benefits emitting from their phytochemicals. Among these widely spread medicinal plants generally

in Asia including Malaysia. One such example is the cucurbitaceae family which despite of its abundance and availability, has been poorly investigated for its potential health benefits. The vegetables from this family are also called gourds which are considered as great dieting food due to its higher water and fiber contents as well as a greater source of vitamins, minerals and other antioxidants (10). Limited data on these vegetables in relation to T2DM is evident in the present literature. Therefore, this was an effort to study the effect of a common Malaysian vegetable of a cucurbitaceae family vegetable also known as Bottle Gourd, Opo squash or Calabash for T2DM using adipocytes as experimental model. The effect was investigated for its water and ethanolic extracts (extracted from whole vegetable, peels and seeds) on the cells viability, adipogenesis, adipolysis, glucose uptake, adiponectin and leptin.

## Materials and Methods

Fresh bottle gourd was purchased from a local market and washed thoroughly with distilled water and segregated into three parts which were namely; whole vegetable (LSW), peels (LSP) and seeds (LSS). About 300g of each part was separately blended with distilled (300ml) using an electric juicer (Philips HR 7620). The juice was processed according to the method described by Chen et al., (11). Another 300g of each part was extracted using absolute ethanol as described by Mujahid, et al., (12). The MTT Assay (Seeded cells with test compounds and DMSO as a negative control were incubated for 72h then treated with 10 µl of MTT solution (5 mg/mL) followed by an incubation for 4h in a dark room. The absorbance was measured using a microplate reader (Tecan Infinite 200 PRO NanoQuant) at a wavelength of 570 nm (13). Adipocytes differentiation was assessed using a cocktail of insulin, IBMX and Dexamethasone to differentiate pre-adipocytes seeded in a 96 well plate into mature adipocytes according to the method described by Zebisch et al., (14). The extracts were introduced to the cells prior to the induction to observe the effect on cells differentiation. Once the lipid droplets were visible; the cells were stained using a 0.1% working solution of Oil Red O dye and left for 2 hours at room temperature. The wells were then washed using double distilled water until no reddish color emerged from the washing solution. Triton x-100 in isopropanol was used as a dye extraction solution followed by a measurement of the absorbance at 520 nm using enzyme-linked immunosorbent assay (ELISA) (15). A kit was used for the measurement of glycerol release in the media of fully differentiated adipocytes. The extracts under investigation were introduced to the differentiated cells and incubated for 1h using isoproterenol as a positive control and fresh DMEM as a negative control. The supernatant of each well was then retrieved and transferred into new wells and free glycerol was quantified according to manufacturer's instruction/protocol. The absorbance was measured at 540 nm using a micro plate reader. The glucose uptake of the differentiated 3T3-L1 adipocytes was assessed by incorporating the extracts along with insulin 30 minutes prior addition of radiolabelled glucose as described by Yamamoto et al., (16). In this method, 2-deoxyglucose (0.001 mM) was used together with the radiolabelled 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 uCi/mL. The glucose uptake procedure was terminated by washing the wells three times with ice-cold PBS (pH 7.4) then the cells were washed and lysed with 0.7 mL of 1% Triton X-100 for 40 mn/37°C. The counting of each sample from each lysate was performed using a liquid scintillation counter (Packard Tricarb 2700 TR/SL liquid scintillation analyzer, Packard instrument CO.) Adiponectin and leptin concentration in the cellular supernatants from the mature adipocytes were measured by using the ELISA mouse kits specific for adiponectin and leptin. The assays were performed according to the manufacturer's protocol. This method make use of precoated ELISA plate with a pretitered anti-mouse adiponectin/leptin monoclonal antibodies and also insulin or fresh media. The collected data was compiled and statistical analysis were performed using SPSS statistical software version 20.0. Mainly, one-way analysis of variance (ANOVA) were performed followed by Tukey's post-hoc test to ascertain differences in the means. The results were expressed as means standard deviations of the means. The statistical differences among the means were regarded as significant at 95% confidence interval.

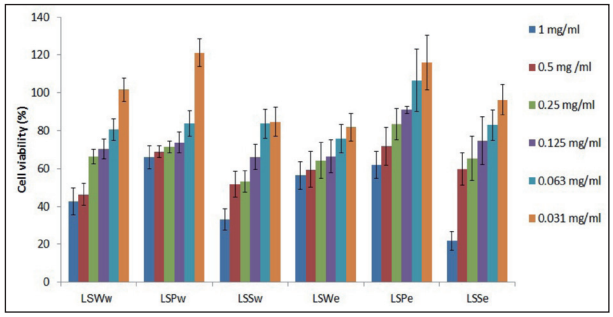


**Figure 1:** Effect of different concentrations of *Lagenaria siceraria* water and ethanol extracts on the proliferation of 3T3-L1 adipocytes. The cells were incubated in the presence of the extracts at decreasing concentrations ranging from 0.031 to 1 mg/ml and the cell viability was evaluated with the MTT assay after 48 hours. The values represent the mean ± SD of three independent readings.

## Results

The results for MTT analysis shows that treatment of 3T3-L1 preadipocytes with LS extracts had a dose-dependent effect on the cellular proliferation: the lower the dose/extract the higher the cell viability. The IC50 value was 0.063 mg/ml for all extracts (Figure 1) whereas cells' growth was stimulated up to 121% for the extracts of the peels (LSPw, LSPe). The MTT assay shows that the most tolerable dose of all extracts (whole, peel and seeds) for both water and ethanol was 0.25 mg/ml.

The intracellular fat accumulation (Figures 2A-2H) showed a stimulation of lipid droplets and higher accumulation in the adipocytes treated with the extracts of LS (Water/ethanol) of the whole vegetable/seeds (LSWw, LSSw, LSWe, LSSe) and lower in the extracts of LS peels (Water/ethanol) (LSPw, LSPe) compared to control (Figures 2 A-2H). Quantitatively, the cell differentiation follow the same pattern being significantly ($P<0.001$) higher for the extracts of Water and ethanol of the LSWw, LSSw, LSWe, LSSe) and lower in the extracts of LSPw, LSPe compared to control (Figures 3).

Furthermore, lipolysis was assessed with glycerol release of 3T3-L1 adipocytes following exposure to the LS extracts for an hour. There was significant ($P<0.001$) enhanced release of glycerol with all extracts but slightly higher in the ethanol extracts (LSWe, LSPe, LSSe) compared to the control (Figure 4).

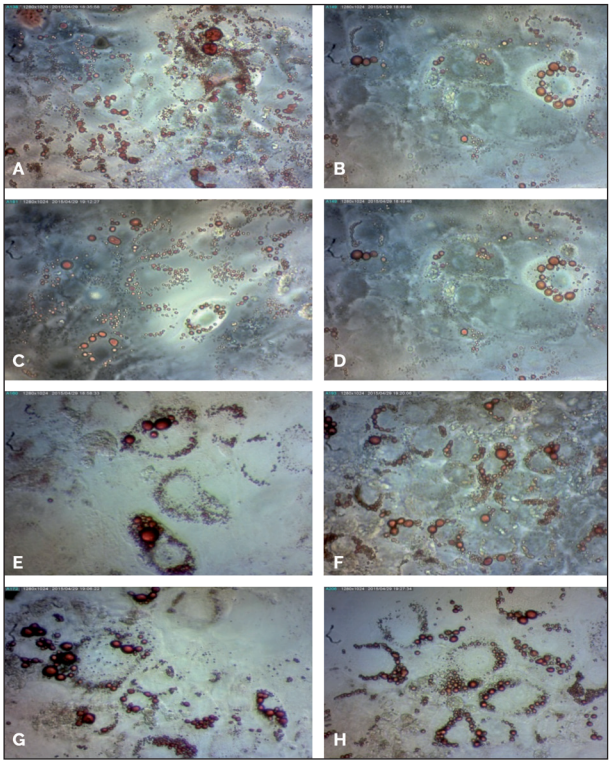


**Figure 2.** 3T3-L1 treated with the extracts for adipogenesis using Oil Red O dye for the intracellular fat accumulation (2A-2H) showing stimulation of lipid droplets accumulation in the adipocytes treated with LS (Water/ethanol) of the whole vegetable/seeds (LSWw, LSSw, LSWe, LSSe) and lower in the extracts of LS peels (Water/ethanol) (LSPw, LSPe) compared to control. A) MDI (induction media) differentiated adipocytes. (A): differentiated 3T3-L1 adipocytes stained with Oil Red O dye used as a positive control (Mag. 100x). B) Mature adipocyte stained with Oil Red O (Mag. 200x). C) Differentiated 3T3-L1 adipocytes treated with water extract of *Lagenaria siceraria* whole vegetable (Mag. 100x). D) Differentiated 3T3-L1 adipocytes treated with ethanol extract of *Lagenaria siceraria* whole vegetable (Mag. 100x). E) Differentiated 3T3-L1 adipocytes treated with water extract of *Lagenaria siceraria* peels (Mag. 100x). F) Differentiated 3T3-L1 adipocytes treated with ethanol extract of Lagenaria siceraria peels (Mag. 100x). G) Differentiated 3T3-L1 adipocytes treated with water extract of *Lagenaria siceraria* seeds (Mag. 100x). H) Differentiated 3T3-L1 adipocytes treated with ethanol extract of *Lagenaria siceraria* seeds (Mag. 100x).

Glucose uptake activity was significantly ($P<0.001$) higher in all water extracts of the whole vegetable (LSWw, LSPw, LSSw) as well as in the ethanol extracts of whole vegetable and peels (LSWe, LSPe) compared to control. Interestingly, the activity of the whole vegetable extract of the ethanolic extract on glucose activity was comparable to insulin (Figure 5).

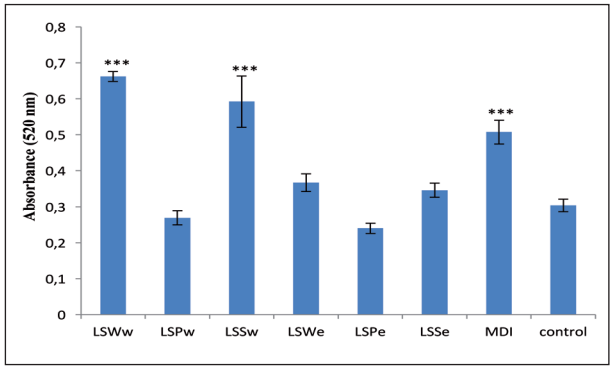


**Figure 3.** Effect of *Lagenaria siceraria* water and ethanol extracts on 3T3-L1 cell differentiation LSWw=0.66**±0.01** LSSw=0.59**±0.07** (n=3, * = p<0.05, ** = p<0.01, *** = p<0.001). The values represent the mean ± SD of three independent readings.

The adipocytes treated with the LS extracts showed a significant (P<0.001) increase in adiponectin concentration in all water extracts compared to the ethanol extracts which were only observable in the seed extract (LSSe) compared to the control (Figure 6). The leptin release was significantly (P<0.05) lower for the water extracts of the peels compared to the control followed by the whole vegetable (LSPw, LSWw). Compared to insulin, the values were significantly (P<0.001) lower for both water and ethanol extracts (Figure 7).

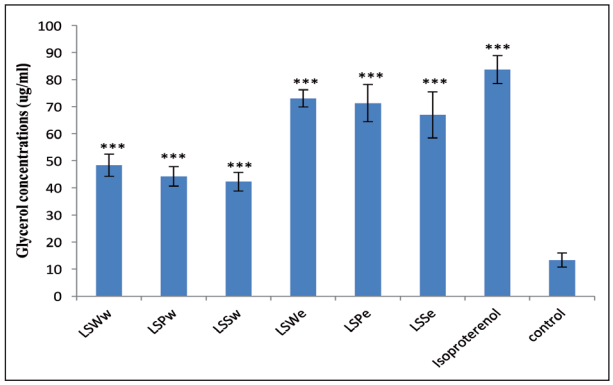


**Figure 4.** Effect of *Lagenaria siceraria* water and ethanol extracts on glycerol release in 3T3-L1 adipocytes. LSWe=73.04±3,14 µg/ml LSPe=71.35±6.88 µg/ml (n=3, * = p < 0.05, ** = p < 0.01, *** = p<0.001). The values represent the mean ± SD of three independent readings.

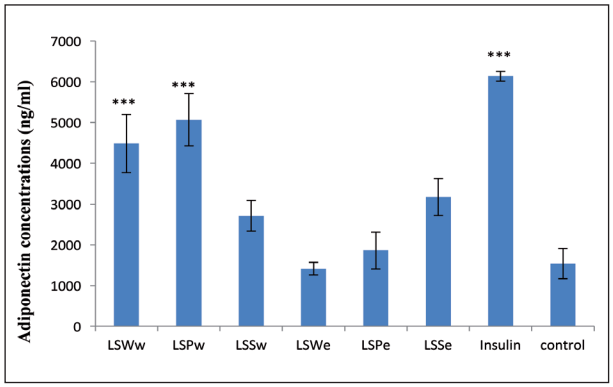


**Figure 6.** effect of *Lagenaria siceraria* water and ethanol extracts on adiponectin concentrations in 3T3-L1 adipocytes LSPw=5405.79±1557.686 ng/ml LSWw=4924.55±1372.88 ng/ml (n=3, * = p<0.05, ** = p<0.01, *** = p<0.001). The values represent the mean ± SD of three independent readings.

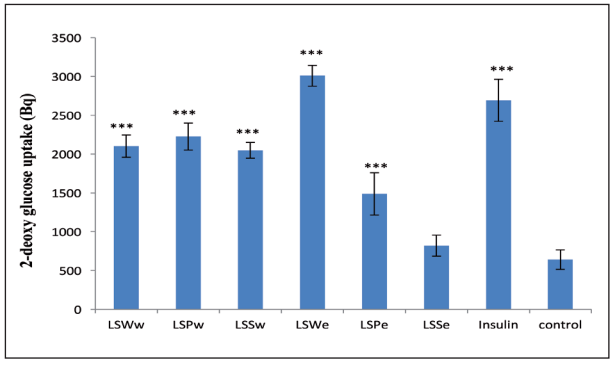


**Figure 5.** Effect of *Lagenaria siceraria* water and ethanol extracts on glucose uptake in 3T3-L1 adipocytes. LSWe=3008±133.06 Bq LSPw=2224.78±174.03 Bq (n=3, * = p<0.05, ** = p<0.01, *** = p<0.001). The values represent the mean ± SD of three independent readings.

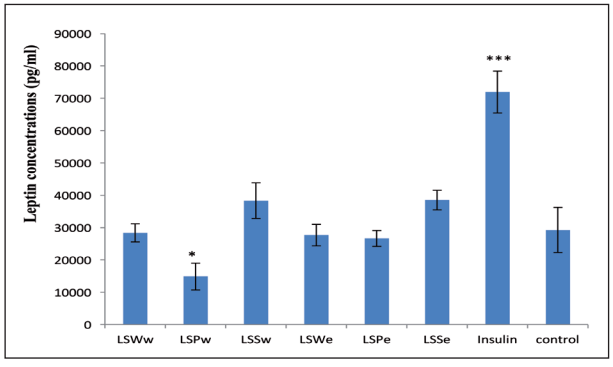


**Figure 7.** Effect of *Lagenaria siceraria* water and ethanol extracts on leptin concentrations in 3T3-L1 adipocytes. LSSw=38344±5500.18 pg/ml LSSe=38544±3011.58 pg/ml LSWw=28336.67±2806.28 pg/ml (n=3, * = p<0.05, ** = p<0.01, *** = p<0.001). The values represent the mean ± SD of three independent readings.

## Discussion

In the present study, MTT assay indicated that there was a concentration-dependent response in 3T3-L1 adipocytes in which higher concentrations of extracts caused lower adipocytes proliferation and vice versa. The adipocytes exhibited lower proliferation with the treatment of higher concentrations of extracts. It is well known that tetrazolium salt is reduced when reacting with the mitochondrial succinate dehydrogenase found in living cells. This forms purple precipitates and the amount of precipitates formed is proportional to the amount/numbers of living cells (17). Interestingly, our LS belong to the cucurbitaceae family which is known to contain some compounds called cucurbitacins, a class of highly oxidized tetracyclic triterpenoids (18). Cucurbitaceae have also been investigated for their triterpenoid content (19) which is believed to affect adipocytes differentiation. We believe that the presence of these cucurbitacins at higher concentrations in the plant are responsible for its bitterness and toxicity (19) toward cell growth as demonstrated by the decrease in adipocytes proliferation when treated with LS extracts. Therefore, it is of extreme importance to have an established dose of cells survival prior to the actual treatment of the cells for the assessments of various parameters.

In the present study, the cell treated with the LS extracts showed remarkable adipogenesis with significant ($P<0.001$) stimulation of adipocytes differentiation in LSWw, LSSw exceeding that of the MDI (induction media) especially in the water extracts of the whole vegetable and seeds (LSWw, LSSw). This is indicative of the LS extracts with water possess insulin like properties. A study by Chen et al., (20) showed that cucurbitacins can bind to a glucocorticoid receptor in order to modify prostaglandin and adrenocorticosteroid synthesis which can cause changes in cell morphology; this in part could explain the adipogenesis stimulating properties of LS extracts. Another study (21) on cucurbitaceae seeds extracts shows the presence of globulins and it is believed to increase adipogenesis and improve glucose uptake. These globulins could act individually or synergistically to stimulate adipogenesis in adipocytes. Various health conditions including diabetes and obesity are excessive fat accumulation which has been linked to the increase in adipocytes number and size of adipocytes. Therefore, the natural plant therapy which can regulate these in adipocytes may provide better therapeutic approaches both in hyperglycemia, hyperlipidaemia and obesity.

In the present study, the extracts also exhibited a significant increase ($P<0.001$) in glycerol release in an action similar to that of isoproterenol. According to Kolditz & Langin (23) the mechanisms by which lipolysis is induced are linked to an increased intracellular cyclic adenosine monophosphate (cAMP) concentration. This is followed by the activation of protein kinase A (PKA) and the phosphorylation of perilipins (also known as lipid droplet-associated protein or PLIN which are necessary for the dispersal of lipid droplets. This involves hormone-sensitive lipase (HSL) which control the rate-limiting step of the enzyme for the mobilization of free fatty acids to lipid droplets and lipolysis. Rayalam et al., (24) suggested that several natural compounds found in cucurbitaceae extracts, such as polyphenols and flavonoids, stimulate lipolysis by increasing cAMP and PKA levels in 3T3-L1 adipocytes, while a study by Zang et al., (25) indicates that the lipid breakdown effect of the extracts is due to their cucurbitacin and triterpenoid contents. This has been confirmed by another study (26) which reports that triterpenoids play lipolysis activity via the AMP-activated protein kinase (AMPK) pathway with a possible stimulation of GLUT4 translocation which is a key factor in adipogenesis, adipolysis and glucose uptake. Other studies suggest that these phytochemicals could affect Adipose triglyceride lipase (ATGL) which plays a major role in adrenergically stimulated triacylglycerol (TAG) breakdown in adipocytes (27, 28).

The observed enhanced glucose uptake with the extract of the LSWe compared to insulin treated adipocytes may be of clinical significance particularly in type diabetes. According to Kumar et al., (29) this glucose uptake enhancing properties are due to the cucurbitaceae lectin content which is believed to lower blood glucose concentrations by acting on peripheral tissues in an action similar to insulin's effects. This was confirmed later by Chowdhury et al., (30) who reports that lectins possess insulin-like activity due to their non-protein-specific linking together to insulin receptors. According to another study (31), cucurbitaceae

phytochemicals are potentially peroxisome proliferator-activated receptors (PPARs) agonists. These are associated with an increase insulin-stimulated glucose uptake by activating PPARγ and PPARγ through a mechanisms which enhance glucose uptake in adipocytes and the up-regulation of GLUT 4 expression mediated by PPAR activation. Since the studies on LS effect on adipocytes are scarce, some studies suggest that the stimulation of glucose uptake can be due to the synergistic effect of various phytochemicals that are yet to be determined which could reveal several new mechanisms (32).

Adipose tissue plays a major role in glucose homeostasis through the secretion of normal levels of adipokines such as leptin and adiponectin which are benefcial toward insulin sensitivity (32). The observed increased release of adiponectin (Figure 5), in the adipocytes, according to a study could be due to their high triterpenoid and cucurbitacins contents that stimulate adipocytes to produce more adiponectin (33). Other nutrients can also play a role in increasing adiponectin concentrations such as polyunsaturated fatty acids (PUFA) and vitamin E (34) since cucurbitaceae extracts possess higher amounts of PUFAs as well as high amounts of monounsaturated fatty acids (MUFA) (35), in addition to vitamin E especially in the seeds. It has been reported that adiponectin plasma concentration is under the influence of PPARγ, where stimulation of this nuclear receptor potentiates its direct binding with the peroxisome proliferator-activated receptor response element (PPRE) in the promoter region of the adiponectin gene, thus, enhancing the production and secretion of this cytokine (36). Furthermore, it has also been also recorded that cucurbitaceae induced-glucose- uptake is accompanied by an increased adiponectin secretion which is the communication between adipose tissue and skeletal muscle (36, 37). This correlates with the results previously mentioned earlier, therefore, substances that enhance adiponectin levels and glucose uptake can be a promising therapeutic strategy for the prevention and treatment of diseases such as Type 2 Diabetes. On the other hand, water extracts of LSPw significantly ($P<0.05$) decreased leptin concentrations (Figure 7). According to Singh & Sexena (38), this could be due to the presence of hexosamines which can be found in various cucurbitaceae family. Chen et al., (11) suggested that decreased leptin levels could rather be due to the synergistic effect of more than one bioactive compound present in cucurbitaceae vegetables such as triterpenoids and cucurbitacins. A study by Jeong, Yoo, Seo, & Shin (39) suggested that the decrease in leptin levels in 3T3-L1 adipocytes could be due to the suppression of the mRNA expression of PPARγ caused by the presence of triterpenoids which are abundant in cucurbitaceae vegetables. Other extracts such as water and ethanol extracts of the seeds (LSSw, LSSe) increased leptin concentrations which was similar to a study by Tsuda et al., (40) which showed that flavonoids (such as anthocyanin) induce an increase in leptin levels in cell medium of isolated rat adipocytes. Additional studies need to be performed on the effect of cucurbitaceae extracts on leptin regulation which could clarify the specific mechanisms by which it is regulated. In the presents study, the compounds present in this vegetable were not isolated. It appears that the seeds have considerable amount of cucurbitacins which aids to the effectiveness in the glucose regulations. Further investigations are required to analyze seeds for various components.

## Conclusion

The present study data showed that there was a hypoglycaemic effect in adipocytes treated with the *Lagenaria siceraria* extracts by enhancing adipogenesis, adipolysis and glucose uptake. This effect can be mainly observed in the seeds extract of the plant. Furthermore, the observed adipocytes concentrations of adiponectin and leptin could be of clinical significance in energy regulation which is a key factor in diabetes, obesity and metabolic syndrome.

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Correspondence:
Assia Sassi
Nutrition Department, Kulliyyah of Allied Health Sciences, IIUM Kuantan Malaysia
E-mail: sassia19@gmail.com