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PINEAPPLE HONEY INHIBITS ADIPOCYTES PROLIFERATION AND REDUCES LIPID DROPLET ACCUMULATION IN 3T3-L1 ADIPOCYTES

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ABSTRACT

Honey has potential in controlling obesity by reducing excess weight gain and other obesity parameters such as triglyceride levels. However, its effects on the cells that stores lipid (adipocytes) is still unclear. This study was performed to observe the effects of pineapple honey on the growth and lipid accumulation of adipocytes *in vitro*. Pineapple honey was standardised according to its total phenolic and flavonoid contents prior to treating on differentiated 3T3-L1 adipocytes. Proliferation of adipocytes was observed using 3-(4,5 dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide (MTT) assay while lipid accumulation and droplet size were determined using oil red O staining and *Feret's* diameter. Pineapple honey exhibited 0.0379 ± 0.001 mg/100 mL GAE of total phenolic content and 0.098 ± 0.001 mg catechin/kg of total flavonoid content. It significantly inhibited adipocytes' proliferation starting from 6.25% of pineapple honey concentration. In addition, the honey also significantly reduced lipid droplet size by 33.78% to 70.36% and reduced lipid accumulation compared to the control. These findings suggest that pineapple honey may affect the storage of lipids in adipocytes. Future investigation involving the biomarkers of adipogenesis is required to confirm whether the reduction in lipid accumulation is attributed to the effect of honey on these pathways.

Key words: 3T3-L1 adipocytes, adipocyte size, pure honey, pineapple honey

INTRODUCTION

Obesity is one of the major health problems of the 21st century, especially in high-income and developing countries (Power *et al.,* 2013). In Malaysia, the National Strategic Plan reported a 250% increase in the local obesity prevalence during 1996 to 2006, for Non-Communicable Diseases (Ismail *et al.,* 2004; Teo *et al.,* 2014). The most affected age groups were adult and children, with 60% of Malaysians aged eighteen and above had a body mass index (BMI) over 25 and an increase of 30% in childhood obesity was observed (Teo *et al.,* 2014).

Various types of treatments are available for obesity, including drugs such as Orlistat (Xenical) and through bariatric surgery intervention (Nwobodo, 2015). However, both treatments have risks and adverse side effects. Thus, in order to treat and prevent obesity, researchers are now looking towards functional foods or drugs without negative side effects. Furthermore, present trends are also focused on functional foods as a proactive approach to healthcare (Sharma *et al*., 2016).

Honey is one of the functional foods that have been long known for its nutritional and medicinal value (Bogdanov *et al.,* 2012). It contains micro-nutrients, antioxidants and phytochemical constituents that are believed to be one of the remedies for weight loss (Alvarez-Suarez *et al.,* 2010). Several varieties of monofloral honey are available in

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Malaysia and one of them is pineapple honey. It is produced mainly from the floral source of *Ananas comosus* species (common local name is Nenas) trees (Hussein *et al.,* 2011). Although it can be found in pineapple plantations especially in the southern region of Peninsular Malaysia, surprisingly very few studies have been conducted using this honey compared to a more popular honey variety such as Tualang honey.

Previous studies have indicated that honey may have potential in reducing weight gain. Consumption of honey for a long period decreased triacylglycerol levels and improved lipid profiles in patients with hypertriglyceridemia (Yaghoobi *et al.,* 2008). This is corroborated by a recent study which proved that consumption of honey for four weeks improved obesity-related parameters and reduced weight gain of Sprague-Dawley rats fed with high fat diet (Samat *et al.,* 2017). Results obtained from Chepulis and Starkey (2008), Nemoseck *et al*. (2011) and Ajibola *et al*. (2013) have also shown the potential benefits of honey in controlling weight gain and obesity. However, these *in vivo* studies do not address how honey could affect weight gain and obesity at the cellular level.

At present, there is no *in vitro* study conducted using honey on the adipocytes, also known as fat cells, which is responsible for the storage of lipids. Although the cells are connected with the incidence and development of obesity (Rizzatti *et al.,* 2013), no direct observation on the actions of honey towards the cell growth and its lipid storage have been reported. Thus, this study was conducted to observe how honey could directly affect the adipocytes and its lipid accumulation. This could serve as a first step towards understanding whether honey has a direct effect in controlling adipocytes growth and subsequently inhibiting adipogenesis.

MATERIALS AND METHODS

Pineapple honey and cell culture preparation

Pineapple honey concentrations were prepared using serial dilutions (2-fold dilution) with culture medium before filtered using a 0.20 µm sterile filter. Meanwhile, 3T3-L1 murine pre-adipocytes acquired from the American Type Culture Collection (ATCC) were cultured in Dulbecco's Modified Eagle's Media (DMEM). Cells were later differentiated in medium with different supplements such as insulin, dexamethasone (DMX), and 3 isobutyl-1-methylxanthine (IBMX) following the procedures described by Mohd-Radzman *et al.* (2013).

Determination of total phenolic content (TPC)

In assessing the total phenolic content of honey, Folin-Ciocalteu procedure was used. Briefly, 1 mL of pineapple honey (0.2 g/mL) was mixed with Folin-Ciocalteu reagent (1 mL). After 3 minutes, 1 mL of sodium carbonate solution (10%) was added to the mixture. Then, the mixture was incubated for 90 minutes in alkaline condition in the presence of sodium carbonate (Na_2CO_3). The intensity of blue colour reflects the quantity and strength of phenolic compounds prior to measurement using spectrophotometer (Almey *et al.,* 2010).

Determination of total flavonoids content (TFC)

The total flavonoid content of pineapple honey was measured using the colourimetric assay (Jia *et al*., 1999). Briefly, 1 mL of diluted honey was mixed with 4 mL of distilled water. Then, sodium nitrite was added to the honey mixture. Next, aluminium chloride was added to the mixture and incubated for 5 minutes before the addition of 2 mL of sodium hydroxide (NaOH, 1 M). The volume was adjusted to 10 mL with distilled water prior to agitation. The absorbance was then read at 510 nm using a calibration curve (a standard solution of catechin at the concentration of 20, 40, 60, 80 and 100 µg/mL; $r^2 = 0.998$). The results were displayed as mg catechin equivalents (CE) per kg of honey.

MTT assay

3T3-L1 adipocytes were cultured in a 96-well plate at a density of 2×10^4 cells/well and were pre-treated with pineapple honey at different concentrations (0-100%) for 24 hours. Cell proliferation (viable cells) was assessed through 3-(4,5 dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide (MTT) assay. In the MTT assay, mitochondrial dehydrogenase enzyme in viable cells reduces the salt of the assay into a coloured formazan product. This product can be measured directly using spectrophotometer at the wavelength of 590 nm.

Oil Red O staining

Oil Red O staining is an assay designed to stain and detect mature adipocytes (Mohd-Radzman *et al.,* 2013; Rizzatti *et al.,* 2013). The cells were grown in 6-well/60 mm plates prior to washing with phosphate buffer saline (PBS). Then, the cells were fixed with 10% formalin in PBS at pH 7.4 and stained with 0.5% Oil Red O (Sigma, St. Louis, MO, USA). In order to quantify lipid accumulation in cells as a result of differentiation, the stain was eluted with 100% isopropanol and measured spectrophotometrically at 520 nm (Rizzatti *et al.,* 2013).

Image and Feret's Diameter determination

Cells images were observed using trinocular inverted microscope Olympus CK 40 (Japan) equipped with a Dino-I camera (Taiwan). The images were analysed using the software Dino

capture version 2.0 and Sigma Scan Pro 5. Each image of maximum Feret's Diameter (MFD) of untreated and treated adipocytes was measured. The Feret's diameter (FD) is generally used in optical microscopy to measure the size of irregularly shaped particles and cells (Rizzatti *et al.,* 2013).

Statistical analysis

Data were presented as mean ± standard error mean (SEM). Statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Dunnet's post-hoc tests, using the Sigma Plot version 12. A level of probability of $p<0.05$ was set as statistically significant.

RESULTS

Pineapple honey exhibited a total phenolic content (TPC) of 0.0379 ± 0.001 mg/100 mL GAE based on the linear calibration curve of gallic acid in mg/mL of honey. Meanwhile, the honey had a total flavonoid content (TFC) of 0.098 ± 0.001 mg catechin/kg of honey. Pineapple honey inhibited 3T3-L1 adipocytes' proliferation in a dose-response manner (Figure 1). Significant reduction in cell viability can be observed starting from 6.25% up to 100% of the honey concentration.

Effects of pineapple honey on the lipid droplets in the cells were observed using oil red O staining (Figure 2). At honey concentrations of 25% to 100%, the lipid droplets shrunk and finally were inhibited. This is further confirmed when the stain concentration was measured spectrophotometrically at 520 nm (Figure 3B), in which a significant reduction in lipids ($p<0.05$) was observed at similar honey concentration. Meanwhile, the effect of pineapple honey in inhibiting the lipid droplet size was determined using Feret's diameter analysis. A significant decrease ($p<0.05$) by 33.78% to 70.36% in the size of adipocytes was observed starting from 12.5% to 100% of pineapple honey concentration (Figure 3C). Results in Feret's diameter showed that no significant difference was observed in the diameter of lipid droplets treated with lower concentrations of pineapple honey compared with untreated adipocytes. This is in line with the significant reduction of total lipid accumulation (Figure 3B) and lipid droplet size (Figure 3C) measured by oil red O staining when higher concentration of honey was used.

DISCUSSION

Previous studies using animal model and clinical trial on human have demonstrated the potential of honey in controlling obesity (Chepulis & Starkey, 2008; Mushtaq *et al.,* 2011; Nemoseck *et al*., 2011; Ajibola *et al.,* 2013; Alvarez-Suarez *et al.,* 2013). Nemoseck *et al*. (2011) showed that clover honey could help in improving weight regulation and reducing triglyceride level in rats. Another study by Chepulis and Starkey (2008) showed that honey reduced visceral adiposity of rats supplemented with honey compared to rats fed with low dose-diet of golden syrup. A similar finding by Ajibole *et al*. (2013) revealed that rats in the honey-fed group significantly displayed lower visceral adiposity compared to the rats fed with sucrose. Meanwhile,

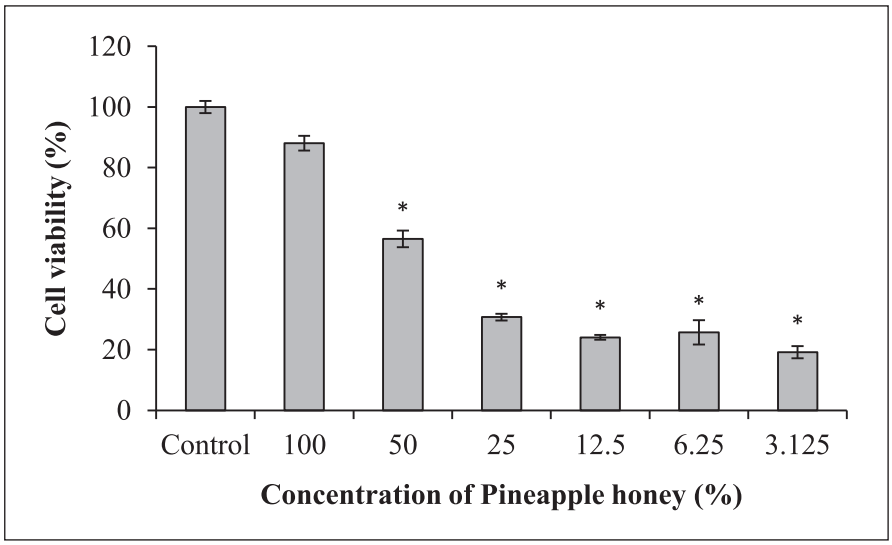

Fig. 1. Effects of different concentration of pineapple honey on adipocytes' proliferation for 24 hours. The results are expressed as mean ± SEM of triplicate determinations. *$p<0.05$.

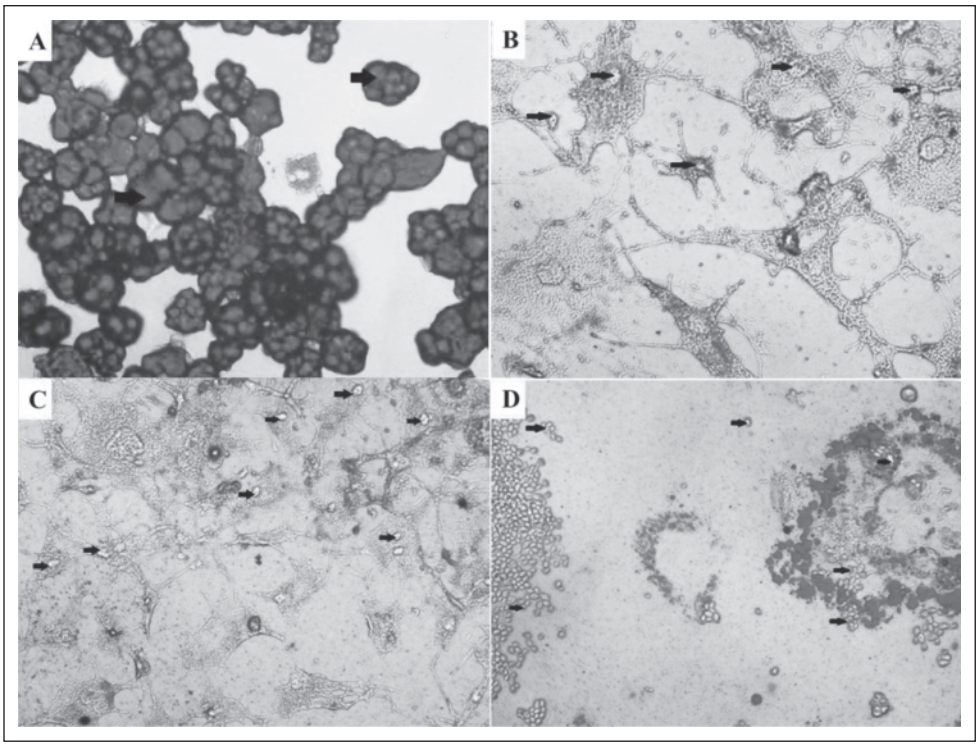

Fig. 2. Effect of pineapple honey on lipid accumulation in 3T3-L1 adipocytes after staining with Oil Red O. The arrow (➡) showed varying sizes of lipid droplets; (A) larger and more spherical in untreated adipocytes, (B, C and D) smaller lipid droplets in adipocytes treated with pineapple honey.

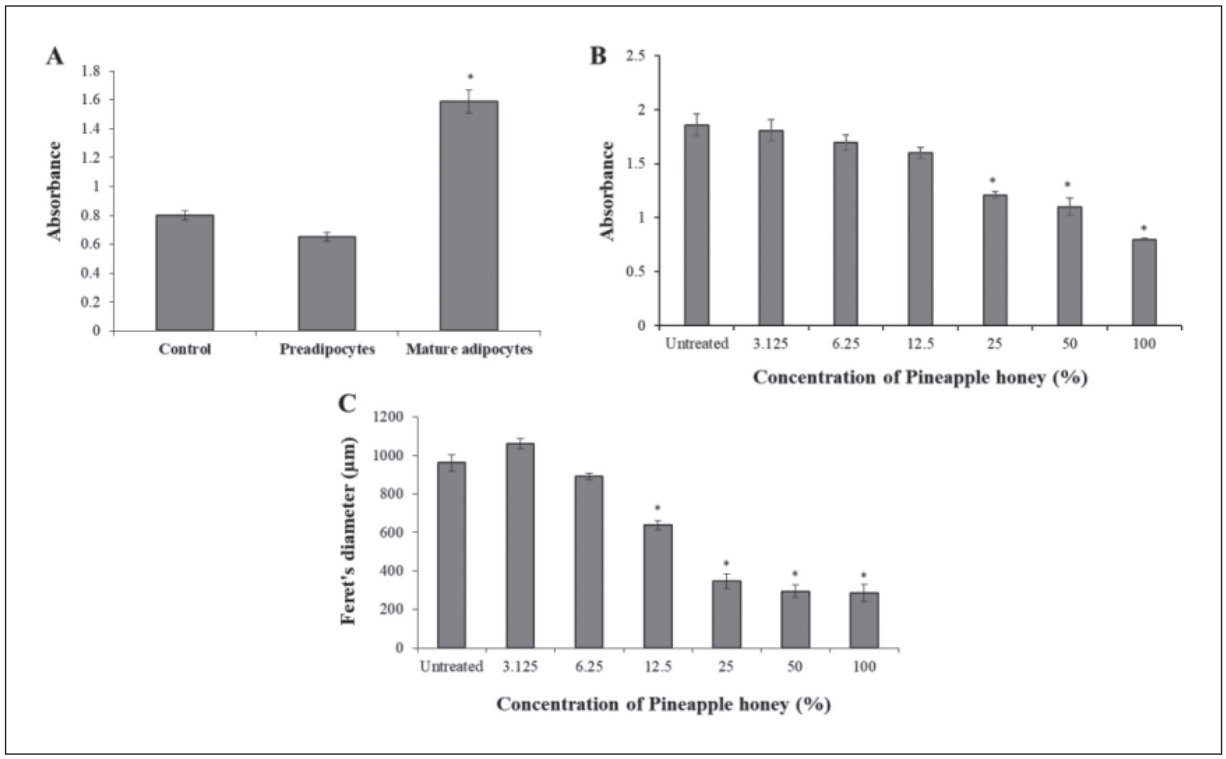

Fig. 3. Effects of pineapple honey on the lipid droplets in differentiated 3T3-L1 adipocytes for 24 hours. (A) Differences in lipid accumulation between pre-adipocytes and mature adipocytes. (B) Lipid droplet accumulation in 3T3-L1 adipocytes. (C) Size of lipid droplet in 3T3-L1 adipocytes. The results are expressed as mean ± SEM of triplicate determinations. *p<0.05.

a clinical study conducted in different ethnic groups and different genders in Pakistan proved that consumption of honey demonstrated a prominent reducing effect on total cholesterol, low-density lipoprotein (LDL) and triacylglycerol of both genders, and improving effect on lipid profiles in obesity (Mushtaq *et al.,* 2011).

Present study showed that pineapple honey from concentrations as low as 6.25% can inhibit adipocyte proliferation followed by significant reduction in lipid droplet accumulation and size. The *in vitro* data is in agreement with the *in vivo* study conducted by Romero-Silva *et al*. (2011). They reported that rats fed with honey-fat based diet showed significant reduction in adipocyte size after two months of treatment compared with those fed without honey-supplemented diet. Honey may also involve down-regulation of the adipogenic transcription factors that play a crucial role in adipocytes differentiation as reported using blueberry extract (Song *et al.,* 2013).

The exact components of honey that displayed the effects are still unknown. However, high antioxidant properties in honey particularly polyphenols are believed to play a role. Polyphenols are known to acts as the key factor in weight regulation, reducing blood glucose levels as well as increasing high-density lipoprotein (HDL) cholesterol as reported by previous studies (Wang *et al.,* 2014). Moreover, synergetic effects of bioactive compounds in honey including flavonoids, phenolics and other polyphenols, minerals, vitamins and other components may be involved in reducing the lipid accumulation.

CONCLUSION

Pineapple honey is able to inhibit adipocytes and reduce lipid droplet accumulation and size even at low concentration. Future investigation involving the biomarkers of adipogenesis is required to confirm whether the reduction in lipid accumulation is attributed to the effect of honey on these pathways.

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