The Effects of the Fractions of *Piper sarmentosum* Leaves on Inhibition of Adipogenesis of 3T3 L1 Preadipocytes

(Kesan daripada Fraksi Daun *Piper sarmentosum* pada Perencatan Adipogenesis pada Sel 3T3L1 Pra-adiposit)

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ABSTRACT

Piper sarmentosum Roxb. was reported to have anti-obesity, hypoglycaemic and anti-oxidant properties. The aim of this study was to identify the fractions of *P. sarmentosum* leaf extract in inhibiting adipogenesis of 3T3L1 preadipocytes. The crude extract of the *P. sarmentosum* leaves was fractionated to produce hexane, dichloromethane, methanol, and aqueous fractions. Various dilutions of the fractions; hexane (0.1 - 1 µg/mL), dichloromethane (9.76 - 97.6 µg/mL), methanol (3.6 - 36 mg/mL), and aqueous (1 - 10 mg/mL), were treated onto the 3T3L1 preadipocytes from 3rd to 15th day of culture. The crude extract (1 - 10 mg/mL) and glycyrrhizic acid (GCA) (0.24 - 2.4 mg/mL) were used as positive controls. The viability of the adipocytes was measured by MTT assay at the 15th day of culture. The content of each fraction was quantified with reference standards of naringin, naringenin, pellitorine, sarmentosine and β-sitosterol by using HPLC. The results showed that 49.1% of the crude extract contained aqueous fraction, 0.12% in hexane fraction, 9.7% in dichloromethane fraction and 36% in methanol fraction. The aqueous fraction and crude extract at the dose of 7 mg/mL and GCA at the dose of 1.92 mg/mL showed potent inhibitory effects on the adipogenesis. However, none of the reference standards were identified from the fractions using HPLC analysis. In conclusion, the aqueous fraction was the main fraction in the crude extract of the *P. sarmentosum* and contributed a significant role in inhibiting adipogenesis of the 3T3L1 preadipocytes.

Keywords: Adipogenesis; aqueous fraction; *P. sarmentosum*; 3T3L1 preadipocytes

ABSTRAK

Piper sarmentosum Roxb. adalah tumbuhan yang mempunyai ciri-ciri anti-obesiti, hipoglisemia dan antioksidan. Tujuan kajian ini adalah untuk mengenal pasti fraksi ekstrak daun *P. sarmentosum* dalam menghalang adipogenesis pada sel 3T3L1 pra-adiposit. Melalui proses fraksinasi, ekstrak daun *P. sarmentosum* ini dapat menghasilkan fraksi heksana, diklorometana, metanol dan akueus. Pencairan fraksi heksana (0.1 - 1 µg/mL), diklorometana (9.76 - 97.6 µg/mL), metanol (3.6 - 36 mg/mL) dan akueus (1 - 10 mg/mL) digunakan untuk merawat sel 3T3 L1 pra-adiposit dalam kultur. Pencairan ekstrak (1 - 10 mg/mL) dan asid gliserizik (GCA) (0.24 - 2.4 mg/mL) digunakan sebagai kawalan positif. Kebolehhidupan sel adiposit pada hari ke-15 kultur diukur dengan menggunakan kaedah asai MTT. Kandungan setiap fraksi dianalisis dengan menggunakan kaedah HPLC untuk mengesan rujukan sebatian piawai naringin, naringenin, pellitorine, sarmentosine dan β-sitosterol. Keputusan menunjukkan 49.1% ekstrak mengandungi fraksi akues, 0.12% fraksi heksana, 9.7% fraksi diklorometana dan 36% fraksi metanol. Fraksi akues dan ekstrak pada dos 7 mg/mL dan GCA pada dos 1.92 mg/mL menunjukkan kesan perencatan yang kuat terhadap adipogenesis. Walau bagaimanapun, tidak terdapat rujukan sebatian piawai pada fraksi ekstrak melalui analisis HPLC. Kesimpulannya, fraksi akues adalah fraksi utama ekstrak *P. sarmentosum* dan ia memainkan peranan yang penting dalam merencat adipogenesis sel 3T3L1 pra-adiposit.

Kata kunci: Adipogenesis; fraksi akues; *P. sarmentosum*; sel 3T3L1 pra-adiposit

INTRODUCTION

In 2016, approximately 39% and 13% of the global adult population suffered from overweight and obesity, respectively (WHO 2020). The worldwide prevalence of the obesity nearly doubled between 1980 and 2013, and tripled between 1975 and 2016 (Ng et al. 2014; WHO 2020). In Malaysia, the prevalence of obesity has markedly increased from 14.0% in 2006 to 33.7% in 2019 (Institute of Public Health of Malaysia 2019, 2006). Obesity is due to the accumulation of body fat and has adverse effects on health (Blüher 2019). Obesity has been linked to many diseases such as cardiovascular, diabetes, sleep apnoea, asthma, osteoarthritis and several cancers (Blüher 2019; Malnick & Knobler 2006).

Energy intake that exceeds energy expenditure contributes to obesity because this surplus energy is stored in adipocytes (Hill et al. 2012). Obesity was found to be associated with increase in the number (hyperplasia) and size (hypertrophy) of the adipocytes (Arner et al. 2010; Spalding et al. 2008). Inhibition of adipogenesis is through impediment in the induction of stem cells into preadipocytes, or through cessation of proliferation of the preadipocytes into adipocytes (Caron et al. 2003; Han et al. 2003, 2002; Harmon et al. 2001; Hua et al. 2004; Suzawa et al. 2003). Previous studies have shown the inhibition of adipogenesis was associated with a decrease in the 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) enzyme activity (Berthiaume et al. 2007; Wang et al. 2006).

The inhibition of the 11βHSD1 enzyme is a novel approach in reducing adipogenesis. Obesity and metabolic syndrome were closely associated with increase in the activity of the 11βHSD1 enzyme (Alberti et al. 2007). Previous studies have shown that intracellular metabolism of glucocorticoids by 11βHSD1 enzyme was important for the development of metabolic syndrome (Thieringer et al. 2004). Chapagain and colleagues (2014) reported elevated levels of 11βHSD1 enzyme associated with elevated gluconeogenesis and lipogenesis, which resulted in reduced glucose tolerance, reduced insulin sensitivity, hyperinsulinemia, dyslipidemia and increased body weight in the rats with chronic kidney disease. Thus, inhibition of the 11βHSD1 enzyme can specifically treat the metabolic syndrome by reducing body weight, reducing the serum cholesterol, and by increasing insulin sensitivity (Chapagain et al. 2014).

Piper sarmentosum Roxb. belongs to the Piperaceae family and contains natural compounds (Rukachaisirikul et al. 2004). It has been reported that *P. sarmentosum* contains essential oil, alkaloids, flavonoids, lignans, and steroids (Sun et al. 2020). It has been used to treat colds, gastritis and rheumatoid joint pain, and had anti-inflammatory, anticancer, antipyretic, anti-osteoporosis, antibacterial, antidepressant, anti-atherosclerotic and hypoglycemic properties. Another study showed that the leaf extract of *P. sarmentosum* was able to reduce adipocyte tissue deposition in the mice by inhibiting 11βHSD1 enzyme (Fairus et al. 2013). However, determination of the active compounds of *P. sarmentosum* leaves and its mechanism in inhibiting the adipogenesis have not been fully elucidated. The purpose of this study was to identify the fractions of the *P. sarmentosum* leaf extract which inhibited the adipogenesis of 3T3L1 preadipocytes.

MATERIALS AND METHODS

PLANTS MATERIAL

Leaves of *P. sarmentosum* (10 kg) were collected from Herbs Garden, Universiti Putra Malaysia. The specimens were deposited at Universiti Kebangsaan Malaysia Herbarium in Bangi with a voucher specimen UKM30058.

EXTRACTION AND FRACTIONATION

The extraction and fractionation processes were based on a method previously described by Hairi et al. (2018). The plant materials were air-dried and ground prior to the extraction process. About 10 kg of the fresh *P. sarmentosum* leaves produced just 2.45 kg of dried *P. sarmentosum* leaves. The dried and ground *P. sarmentosum* leaves were then refluxed with 2% of acetic acid in water at 100 °C for 2 h and filtered. The remaining water content was withdrawn using a freeze-drying technique. The yield of crude extract was 300 g. The crude extract (50 g) were sequentially fractionated using 500 mL of hexane, dichloromethane and methanol as eluting solvents with various compositions. About 0.12% of the hexane, 9.7% of the dichloromethane and 36% methanol fractions were obtained from the *P. sarmentosum* crude extract. While 49.1% of the unfiltered *P. sarmentosum* crude extract from the sequential fractionation with eluting solvents was collected as the aqueous fraction.

HPLC QUANTITATIVE ANALYSIS OF THE FRACTIONS OF THE *P. sarmentosum* LEAVES WITH REFERENCE STANDARDS

Quantitative analysis of the *P. sarmentosum* fractions was based on a method previously described by Joshi et al. (2013) and Ribeiro and Ribeiro (2008). The *P. sarmentosum* fractions were quantified with

the flavonoids naringin and naringenin; the amides, pellitorine and sarmentosine; and the phytosterol, β-sitosterol, by using HPLC. A total of 1 mL of hexane fraction (0.4 mg/mL), dichloromethane fraction (0.25 mg/mL), methanol fraction (1.25 mg/mL), aqueous fraction (10 mg/mL), pellitorine (25 µg/mL), sarmentosine (20 µg/mL), naringin (100 µg/mL), naringenin (100 µg/mL) and β-sitosterol (100 µg/mL) were transferred into the separate HPLC bottles (Waters Corporation, Massachusetts, USA). The different concentrations of each fraction were determined by the amount of the fractions obtained from each of the fractionation process. The lower the polarity index of the solvents used in the fractionation process, the smaller the amount of yield obtained. Therefore, the study utilised different concentrations for each fraction. The compounds in the samples and standards were separated on a Cotecs C18 (4.6 × 100 mm; 2.7 µm) (Waters Corporation, Massachusetts, USA) column at 35 ± 5 °C.

The mobile phase for determination of the amides and flavonoids content consisted of 0.5% phosphoric acid solution (solvent A) and acetonitrile (solvent B). The system was run with the following gradient elution program: 0 min, 95%A/5%B; 10 min, 5%A/95%B; 20 min, 5%A/95%B; 21 min, 95%A/5%B and 25 min, 95%A/5%B. The flow rate was maintained constant throughout the analysis at 1.23 mL/min with an injection volume of 50 µL. On the other hand, the mobile phase for the determination of the phytosterol content consist of only the methanol solvent. The system was run with 100% of methanol for 25 min. The flow rate was maintained constant throughout the analysis at 1.5 mL/min with an injection volume of 20 µL. The flavonoids naringin and naringenin; the amides, pellitorine and sarmentosine; and the phytosterol, β-sitosterol, were detected at the wavelength of 260, 280, and 210 nm, respectively. The system was equilibrated after each run with a buffer for 10 min. The retention time of the peak generated by the hexane fraction, dichloromethane fraction, methanol fraction and aqueous fraction were compared with the retention time of pellitorine, sarmentosine, naringin, naringenin and β-sitosterol.

CELL CULTURE OF THE 3T3L1 PREADIPOCYTES

Cell culture of the 3T3L1 preadipocytes was based on a method previously described by Zebisch et al. (2012) (Figure 1). The 3T3L1 preadipocytes was transferred into the 15 mL tube (Falcon®, Belgium) that contains 9 mL of induction medium solution made up of 89% Dulbecco's Modified Eagle's (DMEM) (ATCC, Virginia, USA), 10% bovine calf serum (BCS) (ATCC, Virginia, USA) and 1% penicillin and streptomycin (Biochrom, Germany). The preadipocytes in the induction medium was transferred into the T25 culture flask (SPL Life Sciences, Korea) with the density of 1×10^4 cells/cm^2 and cultured in an incubator (RS Biotech, United Kingdom) at 37 °C and 5% CO_2 until confluent. The trypsinisation (GIBCO Invitrogen™, USA) process was performed when the 3T3L1 preadipocytes of P0 has reached 80 - 85% confluence. Cell count of the preadipocytes was done by using the haemocytometer (Weber Scientific Int, England). Each of the two T25 flasks added with 1 mL of P0 preadipocytes consist of 1×10^4 cells/cm^2. Each of the T25 flask of the cultured preadipocytes from each passage was transferred into three T25 flasks. While the balance of 2 mL of the P0 preadipocytes in the medium was added to the 1 mL of the cryopreservation medium (ATCC, Virginia, USA). This mixed solution was transferred to the vial and stored in the -80 °C freezer. Each of the revived vial was transferred into two T25 flasks for the next passage. The culture expansion of the preadipocytes was continued until P5. The steps for the culture of the P5 preadipocytes are shown in the Figure 1. Once trypsinisation process of the P4 preadipocytes was completed, the preadipocytes was moved either into the 96-well (Cellstar®, Germany), 6-well (NUNC®, USA) or 35 mm culture plates (Cellstar®, Germany) with the density of the transferred preadipocytes was at 1×10^4 cells/cm^2. The induction medium was added to the preadipocytes in the culture plate and placed into the incubator at 37 °C and 5% CO_2. The preadipocytes reached 80 - 85% confluency on the 3rd day of the culture period. Subsequently the induction medium was discarded and the differential medium I which consists of DMEM, fetal bovine serum (FBS) (GIBCO Invitrogen™,USA), penicillin and streptomycin, insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) (SigmaAldrich, Missouri, USA) was added to the culture plate. On the 5th day of the culture, the differential medium I was discarded and replaced with the differential medium II which consists of DMEM, FBS, insulin, penicillin and streptomycin. The differential medium II was then replaced with culture medium which consists of DMEM, FBS, penicillin and streptomycin on the 7th day of the culture. The culture medium was then changed on the 9th, 11th and 13th days. The fully differentiated adipocytes of the P5 were achieved at 15th day of the culture period and these adipocytes were used to analyse the effects of the *P. sarmentosum* on the adipogenesis. The culture plates were viewed under inverted phase contrast microscope (Axiovert S100, Carl Zeiss, Germany).
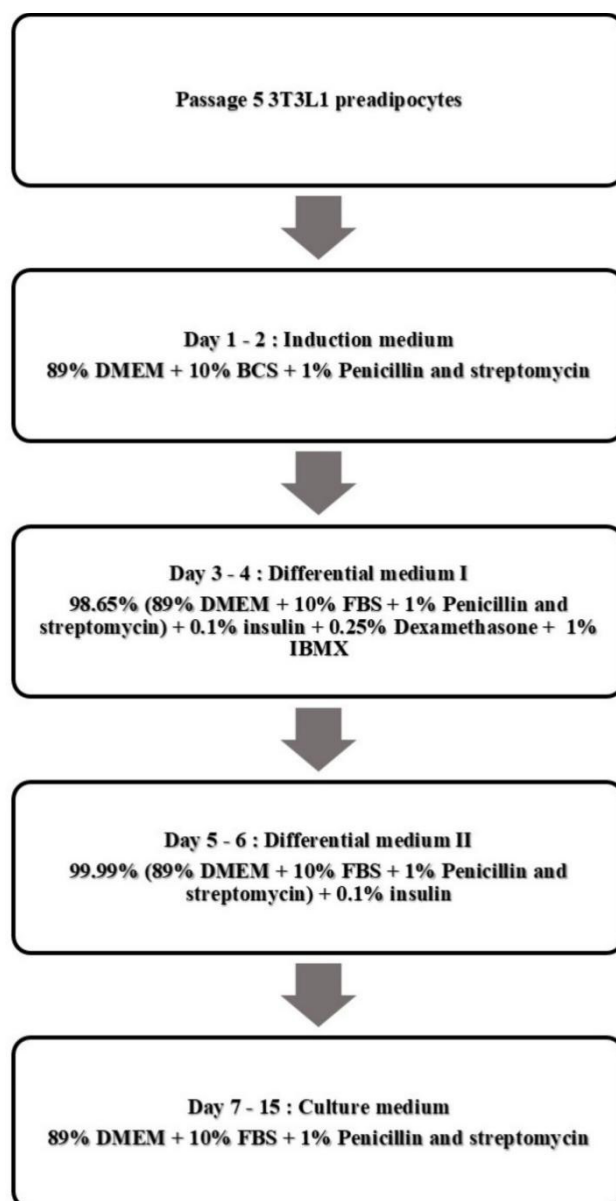
FIGURE 1. *In vitro* induction process of preadipocytes to adipocytes. DMEM = Dulbecco's Modified Eagle's, BCS = bovine calf serum, FBS = fetal bovine serum, IBMX = 3-isobutyl-1-methylxanthine

THE 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

This test was performed to determine the cytotoxicity and the optimal dose of the crude extract and fractions of *P. sarmentosum*, hydrogen peroxide (H_2O_2) and glycyrrhizic acid (GCA) (Sigma Aldrich, Missouri, USA). A total of 10 mg of the crude extract, 1 µg of hexane fraction, 48.8 µg of dichloromethane fraction, 36 mg of methanol fraction and 10 mg aqueous fraction of the *P. sarmentosum* were dissolved in 1 mL of the differential culture media (I and II) to produce 100% solution. About 2.4 mg of the GCA and 18.4 µL of 32.63 M H_2O_2 were dissolved with 1 mL and 2981.6 µL of the differential culture media (I and II) to produce 100% solution of positive and negative controls, respectively. A total of 1×10^4 cells/ cm^2 of the preadipocytes was suspended in 100 µL of the induction medium and then transferred into the 96-well plate. The induction medium was replaced with one fold serial dilution of the crude extract and fractions of *P. sarmentosum*, H_2O_2 and GCA. These treatments were dissolved in the differential medium I on 3rd day and in the differential medium II on 5th day of culture. Subsequently,

the differential medium II was replaced with the one fold serial dilutions of the treatments dissolved in the culture medium on 7th, 9th, 11th, and 13th day. The viability of the adipocytes was measured by MTT assay (Biotium, Sigma Aldrich, Missouri, USA) on the 15th day of the culture based on a method previously described (Yusof et al. 2016). A total of 10 µL of the MTT solution (5 mg/mL) was pipetted into each well and this procedure was performed in a dark area. The plate then was wrapped with aluminium fold and incubated for 4 h at 37 °C and 5% CO_2. Subsequently, 100 µL of dimethyl sulfoxide (DMSO) solution (Friendemann Schimdt, Germany) was added into each well and the colour of the mixture was reduced from a yellow tetrazole to purple formazan. The optical density of the formazan crystals was measured at 570 nm wavelength. The 96-well plate was placed in the microplate reader (Thermo Scientific, Waltham, USA) and analysed using the SkatIt software. The effectiveness of each treatment was based on the percentage of viable adipocytes at the lowest concentration of the crude extract and fractions of *P. Sarmentosum*, H_2O_2 and GCA.

DATA ANALYSIS

Statistical analysis was performed using the GraphPad Prism version 6 (San Diego, USA). The differences of mean values between groups were determined using one-way Anova test and Tukey post-hockey. All data were presented as mean + SEM. P value < 0.05 was regarded as significant.

RESULTS AND DISCUSSION

Figures 2 to 4 showed the chromatography results of the *P. sarmentosum* fractions compared with the amides at 260, flavonoids at 280 and phytosterol at 210 nm, respectively. The peak area was proportional to the amount of the compounds in the fractions of the *P. sarmentosum* and reference standards. However, the retention time of each peak in the fractions of the *P. sarmentosum* were not identical with all the reference standards peaks. These finding were also in agreement with Ugusman et al. (2012) in which the standard compounds were not the contents of the extract, when the retention time of the reference standard did not match the extract. In other words, the standard compounds used in the study were not part of the bioactive compounds present in the fractions.

Figure 5 shows the morphology of the 3T3L1 preadipocytes and its differentiation to mature adipocytes. On the 2nd day of the culture, the preadipocytes attached to the culture plate and transformed into oval-shaped cells (yellow arrow heads). The preadipocytes reached 80 - 85% confluence on the 3rd day of the culture. There was an increased number of fat droplets in the adipocytes (circles). The preadipocytes or fibroblasts differentiated into adipocytes from 3rd day to 7th day of the culture. These multilocular adipocytes were markedly increased in number due to hyperplasia from 7th day to 9th day of the culture. The size of the fat droplets was increased due to hypertrophy of the adipocytes. The increase in size or the hypertrophy of the fat droplets were more noticeable from 9th day to 15th day. Most of the fat droplets found in each multilocular adipocyte were located at the periphery, while the nucleus (N) was located at the centre of the adipocytes. However, a few of the preadipocytes (red arrows) did not change its morphology and remained undifferentiated (resting phase) throughout the culture period. Subsequently, the size of the fat droplets was apparently increased on 15th day of the culture (black arrow heads).

Figure 6 shows viability of the adipocytes at 15th day of culture. These adipocytes received a serial dilution treatment of the crude extract and fractions of *P. sarmentosum* as well as GCA and H_2O_2 from 3rd day to 15th day of culture. There was a reduction in the percentage of the adipocytes after addition of crude extract and aqueous fraction of the *P. sarmentosum* at various dilution doses of 1 - 10 mg/mL compared to control (0 mg/mL) (Figure 6(a) and Figure 6(b)). The percentage of the adipocytes were significantly reduced at 1 to 7 mg/mL of the crude extract and 3 to 7 mg/mL of the aqueous fraction of the *P. sarmentosum* (a, p < 0.05). Thus, the optimal dose of the *P. sarmentosum* crude extract was at 7 mg/mL. However, the aqueous fraction was able to reduce only 48% of the adipocytes at 7 mg/mL. Therefore, both crude extract and aqueous fractions were able to inhibit the adipogenesis at the dose of 7 mg/mL. There were no significant differences in the percentage of the adipocytes after treatment with various dilution doses of the methanol (3.6 to 36 mg/mL), hexane (0.1 to 1.0 µg/mL) and dichloromethane (4.88 to 48.8 µg/mL) compared to untreated adipocytes (p > 0.05) (Figure 6(c), Figure 6(d) and Figure 6(e)). Therefore, methanol, hexane and dichloromethane fractions of the *P. sarmentosum* were unable to inhibit the adipogenesis. This is probably due to the inability of the bioactive compounds in the fractions to reduce the percentage of the adipocytes. He et al. (2015) had shown that inhibition of adipogenesis was correlated with a reduction in the percentage of adipocytes.

The percentage of the adipocytes was reduced after treatment with the GCA at 0.24 to 2.4 mg/mL dilution (Figure 6(f)). There was a significant reduction in the percentage of the adipocytes after treatment with GCA at the doses of 1.92 to 2.4 mg/mL compared to control (0 mg/mL) (a, p < 0.05). As a result, GCA was able to inhibit adipogenesis at the optimal dose of 1.92 mg/

mL. In addition, the percentage of the adipocytes was reduced significantly after treatment with the H_2O_2 at the doses of 10, 50 and 200 mM compared to control (0 mM) (a, $p < 0.05$) (Figure 6(g)). H_2O_2 caused a significant reduction of the adipocytes from 2 to 10 mM due to its toxicity effects.
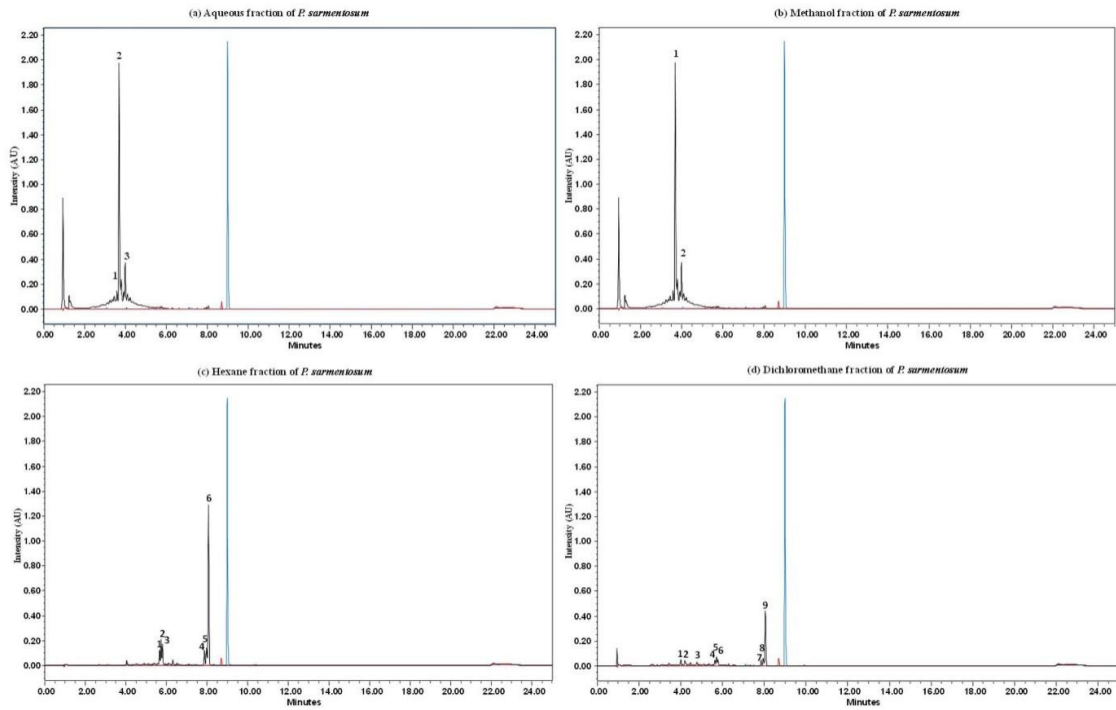

FIGURE 2. The chromatography of the (a) aqueous, (b) methanol, (c) hexane, and (d) dichloromethane fractions of *P. sarmentosum* and amides at 260 nm. The retention time of the peak of the sarmentosine (red) was formed at 8.697 min and that of pellitorine (blue) at 8.993 min
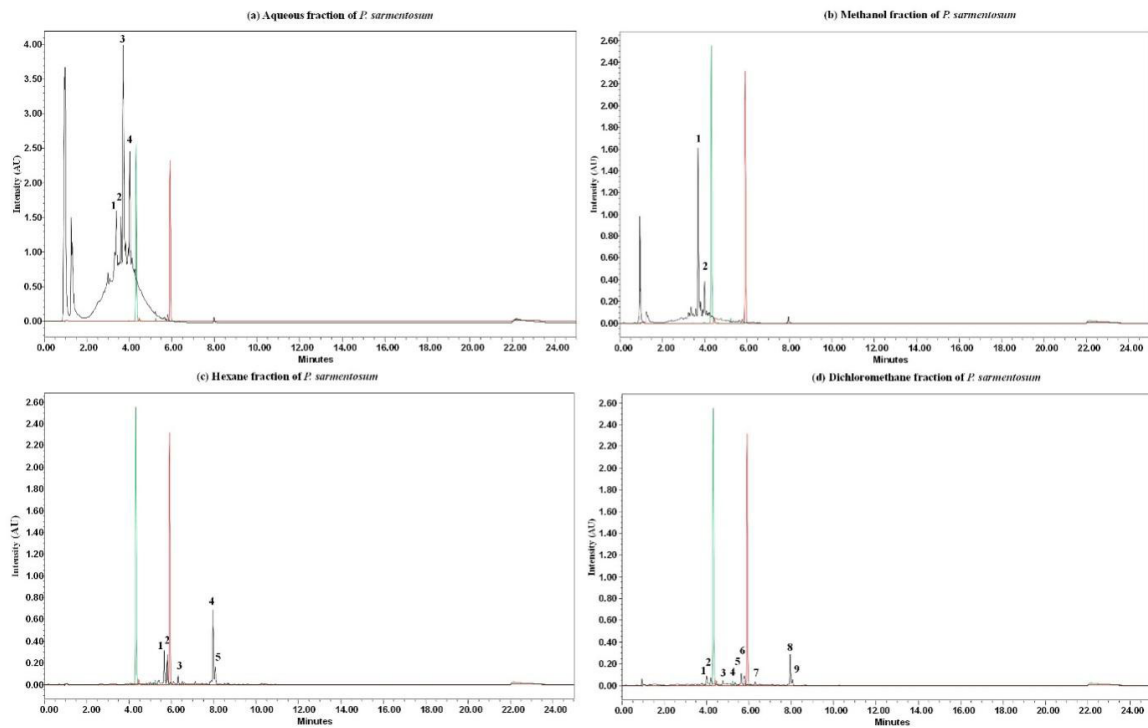

FIGURE 3. The chromatography of the (a) aqueous, (b) methanol, (c) hexane, and (d) dichloromethane fractions of *P. sarmentosum* and flavonoids at 280 nm. The retention time of the peak of the naringin (green) was formed at 4.312 min and that of naringenin (red) at 5.909 min
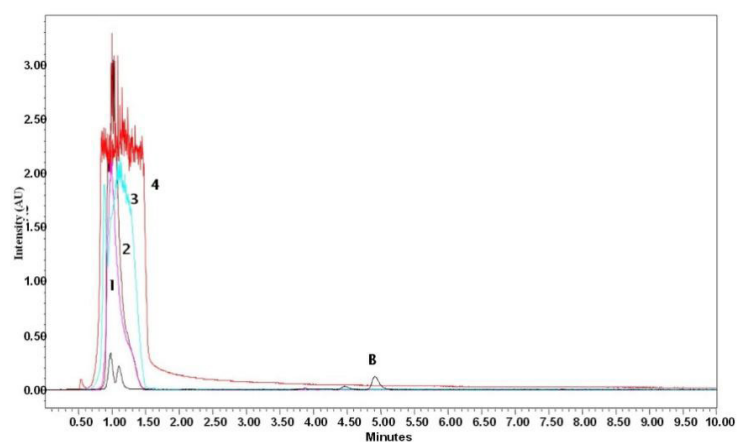
FIGURE 4. The chromatography of the (1) hexane, (2) dichloromethane, (3) methanol, and (4) aqueous fractions of *P. sarmentosum* and phytosterol at 210 nm. The retention time of the peak of the β-sitosterol (B) was formed at 4.911 min
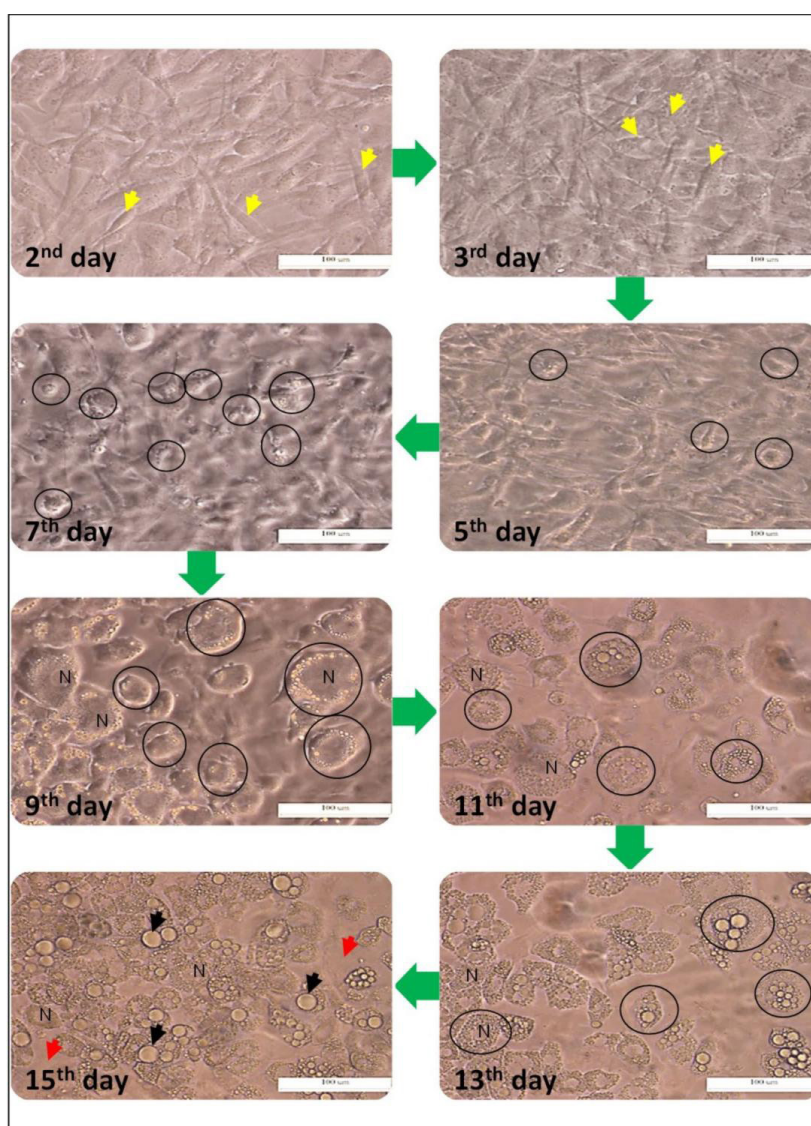

FIGURE 5. Phase contrast micrographs of *in vitro* 3T3L1 preadipocytes differentiation to mature adipocytes from 2nd to 15th day. Adipogenesis of the 3T3L1 preadipocytes involved hyperplasia from 5th to 9th day and hypertrophy from 9th day to 15th day of the culture period. Yellow arrows = preadipocytes, red arrows = non differentiated preadipocytes, black arrow = fat droplets, N = nucleus, circle = multilocular adipocytes. Magnification: 40X, measurement scale = 100 μm
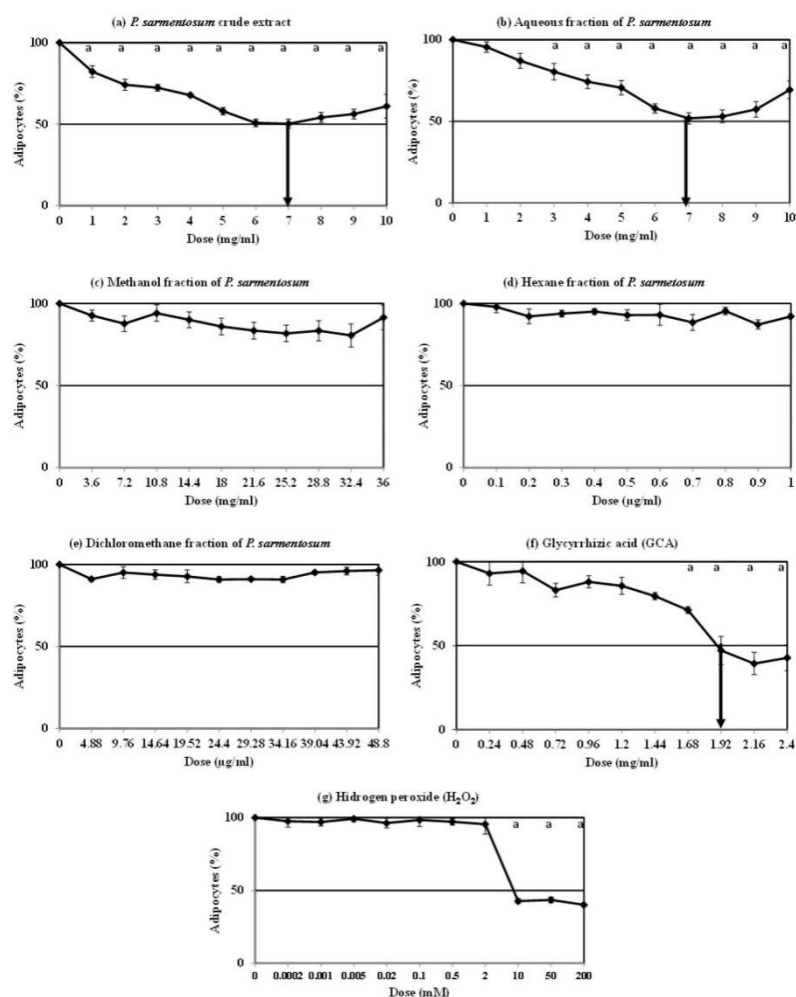
FIGURE 6. Percentage of the adipocytes viability at 15th day of culture. The cultured adipocytes was treated with the serial dilution of the (a) *P. sarmentosum* crude extract (1.0 to 10 mg/mL), (b) aqueous fraction *P. sarmentosum* (1.0 to 10 mg/mL), (c) methanol fraction *P. sarmentosum* (3.6 to 36.0 mg/mL), (d) hexane fraction *P. sarmentosum* (0.1 to 1.0 µg/mL), (e) dichloromethane fraction *P. sarmentosum* (4.88 to 48.80 µg/mL), (f) GCA (0.24 to 2.4 mg/mL), and (g) H_2O_2 (0.0002 to 200 mM). The arrows showed the optimal dose of the *P. sarmentosum* extract (7 mg/mL), aqueous fraction of *P. sarmentosum* (7 mg/mL) and GCA (1.92 mg/mL). 'a' significant different of the treatments of the crude extract and aqueous fraction *of P. sarmentosum* as well as GCA and H_2O_2 compared to the untreated adipocytes (0 mg/mL) ($p < 0.05$)

Adipogenesis is the differentiation of adipocytes from preadipocytes which involved hyperplasia and hypertrophy of the adipocytes. Previous study has shown that the oval-shaped preadipocytes were resting at the G1 phase of the cell cycle (Student et al. 1980). The induction medium induced these preadipocytes to grow, reaching confluence after 48 h of culture. The same findings were reported by Green and Meuth (1974). On the 3rd day of culture, the preadipocytes differentiated into mature adipocytes after supplementing the culture with the differentiation medium I. The differentiation medium I consists of DMEM, FBS, insulin, IBMX, dexamethasone, penicillin, and streptomycin. Previous study has shown that the differentiation process began with the activation of IGF-1 by insulin and cyclic AMP by IBMX (Hamm et al. 2001). The author reported that the IBMX and dexamethasone activated the C/EBPβ and C/EBPδ, respectively. The activation of C/EBPβ and C/EBPδ induced the activations of PPARγ2 and C/EBPα which are important for the formation of the mature adipocytes.

Differentiation of the preadipocytes into adipocytes occurred 16 to 20 h after addition of the differentiation medium I. The same findings were reported by Davis and Zur Nieden (2008). Previous study has shown that the IGF-1 induced mitotic cell division and resulted in differentiation of preadipocytes into adipocytes (Tang et al. 2003). At the same time, there was an increase in triglyceride deposition in the cytoplasm of the preadipocytes. As a result, the morphology of the oval-shaped preadipocytes transformed into multilocular adipocytes. The multilocular adipocytes began with the formation of the small fat droplets which were visible on the 5th day of the culture period. The same findings were reported by Student et al. (1980).

The differentiation medium I was replaced with the differentiation medium II on the 5th day. As a result, there was an increased in the number of multilocular adipocytes and the presence of fat droplets in the cytoplasm from 5th day to 7th day of culture. The same findings were reported by Fan et al. (1983) and Russell and Ho (1976). The increased formation of the fat droplets of various sizes was occurring around the nucleus of the multilocular adipocytes. The hyperplasia of the adipocytes was due to the increased in the number of multilocular adipocytes and their fat droplets. The hyperplasia of the adipocytes continued from 3rd day to 9th day of the culture. The same findings were reported by Brasaemle et al. (2004) and Green and Meuth (1974).

The fat droplets in the multilocular adipocytes increased in size from 9th day until 15th day of the culture due to merging of the fat droplets in the multilocular adipocytes into the unilocular adipocytes. The same findings were reported by Brasaemle et al. (2004) and Fischbach et al. (2004). As a result, hypertrophy of the adipocytes was due to the increase in the size of the fat droplets and the transformation of the multilocular to the unilocular adipocytes. Hypertrophy of the adipocytes occurred from 9th day to 15th day of the culture. The same findings were reported by Zebisch et al. (2012).

This study showed that serial dilutions of the *P. sarmentosum* crude extract and GCA treatments were able to inhibit adipogenesis of the cultured 3T3L1 preadipocytes. The optimal doses of *P. sarmentosum* extract and GCA were 7 and 1.92 mg/mL, respectively. These doses were able to reduce 50% of the adipocytes on 15th day of the culture. Thus, *P. sarmentosum* crude extract showed similar results with positive control of GCA in the inhibition of adipogenesis. Previous studies have shown a reduction in the deposition of the adipose tissue of the rats after receiving either *P. sarmentosum*

crude extract or GCA (Azlina et al. 2009; Fairus et al. 2013). Similarly, other studies have shown that GCA reduced the deposition of adipose tissue in rats by inhibition of the 11β-hydroxysteroid dehydrogenase type 1 enzyme (11βHSD1) (Azlina et al. 2009; Chandramouli et al. 2011; Eu et al. 2010; Fairus et al. 2013; Lim et al. 2009). Therefore, we hypothesised that GCA and *P. sarmentosum* crude extract might be involved in downregulating the glucocorticoid metabolism during adipogenesis. Previous studies have shown that the 11β-hydroxysteroid dehydrogenase type 1 enzyme was involved in regulating glucocorticoid metabolism during adipogenesis (Bujalska et al. 2008, 2002). The author reported that the dehydrogenase enzyme of 11βHSD1 was important in the initiation of adipocyte cell formation through hyperplasia.

This study also showed that serial dilution of the aqueous fraction of *P. sarmentosum* extract was able to inhibit adipogenesis of cultured 3T3L1 preadipocytes. As a result, the aqueous fraction was able to reduce 48% of the adipocytes on 15th day of culture at the dose of 7 mg/mL. The crude extract and main aqueous fractions of *P. sarmentosum* were able to inhibit adipogenesis of the cultured 3T3L1 preadipocytes. However, for the methanol, dichloromethane, and hexane fractions were unable to inhibit adipogenesis. On the other hand, the phytochemical compounds such as polyphenols and phenolic glycosides in the methanol, dichloromethane and hexane fractions may have induced the adipogenesis of the cultured 3T3L1 preadipocytes. Previous study had shown that polyphenols and phenolic glycosides extracted from the roots of *Heracleum dissectum* were able to induce adipogenesis in cultured 3T3L1 preadipocytes (Wang et al. 2018). The percentage of the viable adipocytes was reported to be increased with the increase in the concentration of the *H. dissectum* extract. The increase in the number of adipocytes was correlated with the induction of the adipogenesis.

An increase in the H_2O_2 concentration from 2 to 10 mM resulting in a sharp decrease in the percentage of the adipocyte in this study. Similarly, the study conducted by Yoon et al. (2002) has shown that increase in the H_2O_2 concentration led to increase apoptosis of the adipocytes resulting in a sharp decrease in the percentage of the adipocyte. The reduction in the viability of the adipocytes after treatment with serial dilution of the crude extract and aqueous fraction of *P. sarmentosum* as well as GCA was gradually decreased compared with H_2O_2. Furthermore, a study by Mohd Zainudin et al. (2013) has shown *P. sarmentosum* extract up to 2000 mg/kg/day did not

show any toxicity in the rat. This was 200-folds more compared to the doses that being used in this study. Therefore, the reduction of the adipocytes after treating with the aqueous fraction and the crude extract of the *P. sarmentosum* as well as GCA was not due to its toxicity, but due to the inhibition of adipogenesis of the cultured 3T3L1 preadipocytes.

This study showed that three quarters of the total weight of *P. sarmentosum* leaves contained water. Previous study by Hussain et al. (2009b) has shown that *P. sarmentosum* leaves and stems had higher water content compared to its fruit and roots. As a result, the leaves had a higher anti-oxidant activity compared to the other parts due to its high water content (Agu & Paulinus 2017; Baskar at el. 2007; Gavamukulya et al. 2014). Another study has shown that the plant extract contained polar compounds because it was extracted by using a water extraction method (Ibanez et al. 2003). However, the plant extract produced by a water extraction had different ranges of solubility (Kumar et al. 2010). The isolation or fractionation of the plant water extract into different fractions depends on the tendency or affinity of the solvent used.

The *P. sarmentosum* has been reported to have naringenin, amide and phytosterol in the methanol, ethanol and hexane extracts, respectively (Atiax et al. 2010; Hussain et al. 2010, 2009a; Subramaniam et al. 2003). These bioactive compounds possess anti-obesity, anti-inflammatory, antihypertensive, anti-atherosclerotic, and hypoglycaemic activities (Aslam et. al. 2017). As the result, the naringin and naringenin (flavonoids), pellitorine and sarmentosine (amide) and β-sitosterol (phytosterol) compounds were used as reference standards. Qualitative analysis showed that the HPLC peak of amide, flavonoids and phytosterol of the reference compounds were not identified from the fractions of the *P. sarmentosum*. This may be due to lower activity of the compounds in the water extract of *P. sarmentosum* which made it difficult to detect. Previous studies showed that the phenolic compound derived from the water extract of *P. sarmentosum* had lower activity compared to the methanol extract of *P. sarmentosum* (Lee et al. 2011; Wongsa et al. 2012). Another study conducted by Ugusman et al. (2012) had shown that the naringenin was not identified but flavonoids, rutin, and vitexin were identified in the water extract of the *P. sarmentosum*. This probably due to the activity of the naringenin in the methanol extract was higher than that of the water extract. The liquid chromatography–mass spectrometry (LCMS) analysis should be used to measure the molecular weight of the bioactive compounds in the water extract.

The reference compounds of similar molecular weight are selected as the standard compounds. These standard compounds will be quantified using a HPLC analysis for the detection of the bioactive compounds of the water extract. However, LCMS analysis of the *P. sarmentosum* extract was not performed in this study.

This study showed that both the aqueous fraction and crude extract of *P. sarmentosum* were able to inhibit adipogenesis of the cultured preadipocytes. The main fraction of the crude extract of *P. sarmentosum* was most probably the aqueous fraction because it had a similar effective dose (7 mg/mL) with the crude extract of *P. sarmentosum* in inhibiting adipogenesis of the cultured preadipocytes. Previous study had shown that the content of polyphenols was higher in the water extract compared to the methanol, dichloromethane and hexane extracts (Barchan et al. 2014). In addition, another study has shown the aqueous fraction isolated from plant extract had high amounts of soluble alkaloids and flavonoids (Nune et al. 2009). A study by Sultana et al. (2009) has shown that the plant's water extract, especially its aqueous fraction, had high content and activity of the antioxidant compounds. These findings were supported by Azmi et al. (2021), in the aqueous fraction of *P. sarmentosum* extract was composed of antioxidant activity of the flavonoids, rutin and vitexin. Therefore, we postulate that other antioxidant compounds in the aqueous fraction of *P. sarmentosum* were able to inhibit adipogenesis probably through the inhibition of 11βHSD1 enzyme in the adipocytes. However, further studies are needed to elucidate the mechanisms involved in the inhibition of the adipogenesis. On the other hand, the percentage of the viable adipocytes was increased after treatment with 8 to 10 mg/mL of the *P. sarmentosum*. This probably due to the reduction in the breakdown of the adipocytes which leads to an increase in the percentage of the adipocytes. Previous study had shown that the percentage of the viable 3T3L1 adipocytes was increased when treated with a higher concentration dilution of the *Momordica charantia* extract (Shobha et al. 2017). The concentrations dilution of the *M. charantia* extract was found to be directly proportional with the glycerol release. Glycerol has reduced the phosphorylation of hormone-sensitive lipase, resulting in a reduction of the adipocytes breakdown. Thus, the percentage of the adipocytes was higher after treated with 8 to 10 mg/mL compared to the effective dose (7 mg/mL) of the *P. sarmentosum* extract and aqueous fraction.

This study, however, is subjected to several limitations. The first limitation is the difference in quantity of the fractions produced by various

fractionation processes. This has resulted in the use of different concentrations of each fraction in the study. Thus, the same concentration comparison of the fractions could not be performed. The second limitation is the selection of the standard compounds for the HPLC analysis. The standard compounds in this study were selected based on the literature review. The best way to select the compounds should be based on the LCMS analysis of the *P. sarmentosum* extract, which was not performed in this study.

CONCLUSION

The aqueous fraction was the main fraction of the *P. sarmentosum* crude extract. Both the aqueous fraction and crude extract of *P. sarmentosum* were able to inhibit adipogenesis at the dose of 7 mg/mL. The aqueous fraction of *P. sarmentosum* probably contained other flavonoid compounds that may play a role in inhibition of adipogenesis through modulation of the 11βHSD1 enzyme. However, further studies are needed to elucidate the mechanisms suggested and to determine the active compound of the most polar fraction of *P. sarmentosum*.

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