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Research article

Antioxidant activities of plant extracts and essential oil-cyclodextrin complexes and their effect on lipid accumulation in porcine adipocytes *in vitro*

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Abstract

Fat deposition is important for pig production because excessive fat decreases the economic value of meat production. Plant extracts have been used as feed additive in pig diets to improve oxidative stability of meat. However, only a few studies had investigated the effect of plant extracts on lipid accumulation of porcine adipocytes. Therefore, this study examined antioxidative potential of plant extracts and essential oil-cyclodextrin complexes and their effect on lipid accumulation in porcine adipocytes. Porcine preadipocytes were isolated from dorsal subcutaneous adipose tissue of a 5-month-old female pig. Preadipocytes were differentiated in the presence of extracts, then lipid accumulation was determined using oil red O staining. Results showed that green tea and grape seed extracts had the highest total phenolic and total flavonoid contents and also possessed the highest antioxidant activities. Among essential oil-cyclodextrin complexes, thyme oil had the highest antioxidant activities by all assays. With a regard to the lipid accumulation, all of plant extracts and essential oil-cyclodextrin complexes decreased lipid accumulation in adipocytes from 32.61 to 91.71% compared with the control. Green tea extract, thyme and galangal oil products decreased lipid accumulation about 60% lower than that of the control. In conclusion, green tea and grape seed extracts, and thyme oil powder illustrated high antioxidant potentials, while lipid accumulation in porcine adipocytes was greatly reduced by green tea extract, thyme and galangal oil products. The research finding is beneficial in the development of natural feed additives to improve oxidative stability and reduce excessive subcutaneous fat in pig production.

Keywords: Antioxidant activities, Essential oil-cyclodextrin complexes, Lipid accumulation, Plant extracts, Porcine adipocytes

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INTRODUCTION

In pig production, fat accumulation play a pivotal role because the excessive subcutaneous fat (backfat) decreases the economic efficiency of meat production (Suzuki et al., 2013). The backfat thickness is an important parameter used to evaluate carcass quality (Lucas et al., 2017), and it inversely correlated with meat percentage in pig carcasses (Knecht and Duziński, 2016). The reduction of fat percentage in pig carcasses has been a major selection goal to improve feed efficiency during fattening. This selection goal was in line with consumer preferences, which need the carcasses with low fat percentage and high meat percentage (Catillo et al., 2021). Dietary supplementation of conjugated linoleic acid (CLA), which is a fatty acid found in meat and dairy products, has been reported to improve feed efficiency, decrease backfat thickness and improve meat quality (Dunshea et al., 2002; Wiegand et al., 2001). Pigs have high levels of polyunsaturated fatty acids (PUFA), including the long chain (C20-22) PUFA in adipose tissue and muscle (Wood et al., 2008). However, PUFA are particularly susceptible to oxidize and could affect meat quality and stability (Jiang et al., 2017). Synthetic antioxidants have been used to minimize oxidative process in meat and meat products but the customers concern about these synthetic antioxidants because of their potential toxicological effects. Therefore, the demand for natural antioxidants, especially of plant origin has increased mainly in the recent years (Shah et al., 2014).

Nowadays, natural products are interesting for various purposes (Bootyothee et al., 2022). Plant extracts contain variety of organic compounds produced that are considered as the secondary active ingredients (Liu et al., 2013). Polyphenols are major bioactive compounds found in plant extracts (Efenberger-Szmechtyk et al., 2021). Essential oils contain those of volatile components such as those of the terpene groups. Consequently, they can be easily degraded by light, heating, oxidation and volatilization if they are exposed to the environments (Asbahani et al., 2015). Besides, they are poorly soluble in aqueous systems. Cyclodextrins, which are non-toxic cyclic oligosaccharides obtained from the enzymatic degradation of starch, have been used to formed inclusion complexes with essential oils in order to improve their solubility and stability, and enhance bioactivities (Kfoury et al., 2019; Wadhwa et al., 2017). The profile of secondary metabolites in plants is the influence of genetics (Sobeh et al., 2019; Zengin et al., 2018), seasonality (De Macêdo et al., 2018), harvest times (Ozkan et al., 2010), and growing location (Yahyaoui et al., 2019). The variation and bioavailability are also as the results of extracting solvents (Bandara et al., 2018; Ruamrungsri et al., 2016) and plant part used (Malsawmtluangi et al., 2016). Several plant extracts and essential oils have shown the potential benefit on antioxidant activities, such as grape seed extract (Mandic et al., 2008), green tea extract (Farooq and Sehgal, 2019), *Caesalpinia sappan* wood extract (Arjin et al., 2020), thyme essential oil (Youdim et al., 2002), ginger ang galangal essential oils (Ivanović et al., 2021). These six natural products were selected to use in this study because their antioxidant potential, and they are available in the local market. In addition, it is generally known that phenolic compounds, total phenolic and total flavonoid contents contributes to the antioxidant potentials of the extracts (Farooq and Sehgal, 2019; Mandic et al., 2008; Zhou et al., 2019). Many natural products derived

from plants have been utilized as feed additive in pig diets to improve growth performance (Li et al., 2012; Liu et al., 2013; Sampath et al., 2020; Yan et al., 2010), and oxidative stability in meat (Cheng et al., 2017; Rossi et al., 2013). Besides, essential oils have been viewed as the alternative to antibiotics in pig production (Omonijo et al., 2018). It was also found that the functional properties and stability of essential oils in feed additive can be improved by encapsulation (Asbahani et al., 2015; Bilenler et al., 2015). There are only a few studies to investigate the effects of plant extracts and essential oils on lipid accumulation of porcine adipocytes. The lower lipid accumulation in porcine adipocytes can reduce the backfat thickness, improve the carcass quality, and increase the economic value. Previous works reported that ramie leaf extract inhibited lipid accumulation in porcine adipocytes (Lee et al., 2016) and Korean medicinal plant extracts modified the differentiation of porcine preadipocytes *in vitro* (Choi et al., 2010). Therefore, this study aimed to investigate the effect of plant extracts and essential oil-cyclodextrin complexes on antioxidant activities and lipid accumulation in porcine adipocytes.

MATERIALS AND METHODS

This experiment was designed to investigate the effects of plant extracts and essential oil-cyclodextrin complexes on antioxidant activities and lipid accumulation in porcine adipocytes. The flow chart for this experiment was shown in Figure 1.

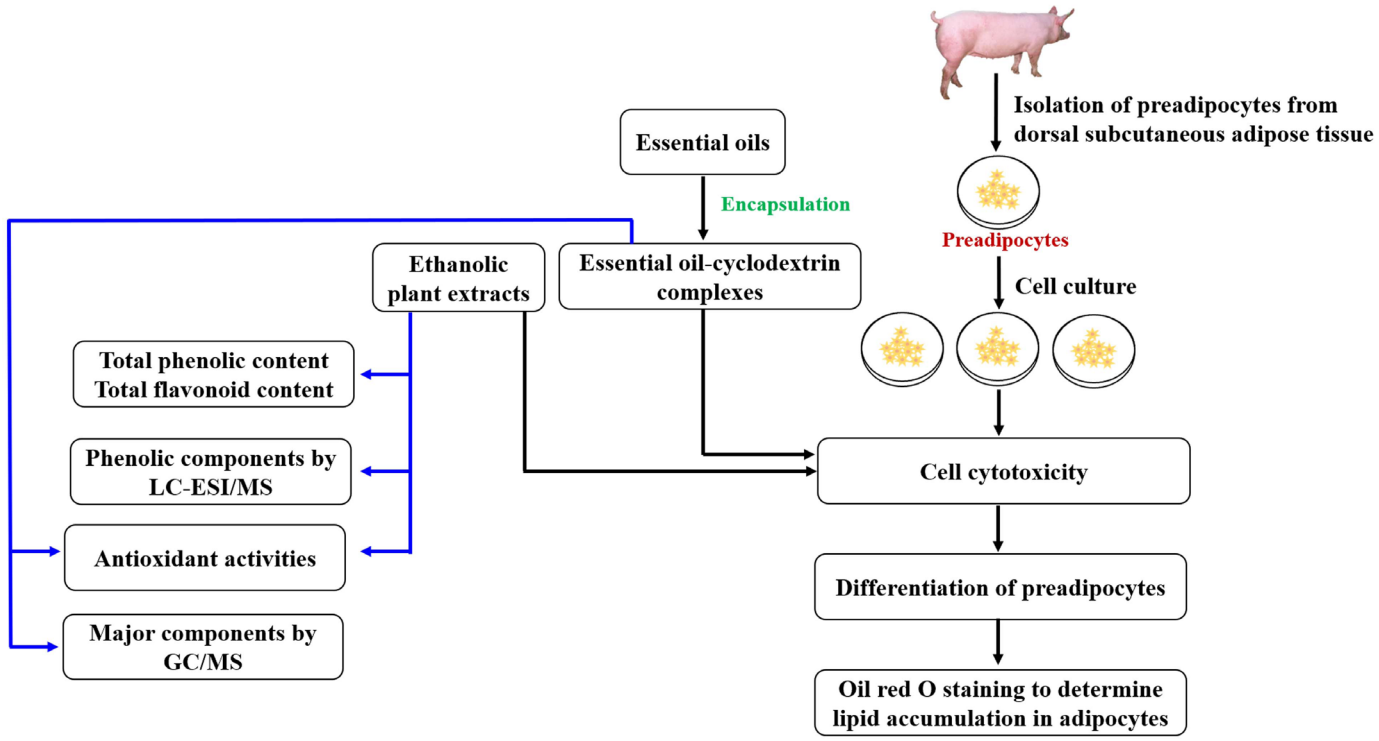

Figure 1 Flow chart for investigating the effects of plant extracts and essential oil-cyclodextrin complexes on antioxidant activities and lipid accumulation in porcine adipocytes.

Chemicals

All chemicals were of analytical grade or HPLC grade. Ethanol, methanol, chloroform, sodium hydroxide, acetonitrile, dimethyl sulfoxide (DMSO), formalin and isopropanol were purchased from RCI Labscan Ltd., Thailand. β-carotene, linoleic acid, Tween 20, ABTS [2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], potassium persulfate, DPPH (2,2-Diphenyl-1-picrylhydrazyl), Foline-Ciocalteu's phenol reagent, sodium carbonate, sodium nitrite, aluminum chloride hexahydrate, catechin, formic acid, HEPES, dexamethasone, oil red O, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and insulin were obtained from Sigma-Aldrich, USA. Gallic acid was produced by Fluka Chemical Co., Switzerland. β-cyclodextrin was supplied by Yimming Biological Product Co., Ltd., China. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), type-1 collagenase and penicillin/streptomycin were purchased from Gibco, USA.

Plant extracts and essential oils

Three plant extracts and three essential oils were used in this study. Ethanolic sappan wood (*Caesalpinia sappan*), green tea, and grape seed extracts were purchased from Specialty Natural Product Co., Ltd., Thailand. Thyme, ginger, and galangal essential oils were obtained from Thai-China Flavours and Fragrances Industry Co., Ltd., Thailand. The essential oils were encapsulated in β-cyclodextrin before used.

Preparation of β-cyclodextrin inclusion complexes of essential oils

The inclusion complexes of essential oils were prepared according to the method of Hill et al. (2013) with some modifications. Essential oil was added to an ethanolic solution of β-cyclodextrin at equimolar concentration of essential oil: β-cyclodextrin (16 mmol/L: 16 mmol/L). The mixture was then magnetically stirred in a sealed container for 24 h at room temperature to allow complex formation. After that, the solution was evaporated by a rotary evaporator, then frozen and lyophilized at -90 °C and 0.1 mbar in a Freeze-Dryer Beta 2-8 LSCbasic (Martin Christ, Osterode, Germany) until all moisture had been sublimated. The lyophilized powder was stored in sealed containers at -20 °C until used.

Total phenolic content

The phenolic content was analyzed according to the modified method of Nantitanon et al. (2010). The extract was dissolved in distilled water prior test. A 12.5 µL of aqueous solution of each extract was mixed with 50 µL of distilled water and 12.5 µL of Folin-Ciocalteu reagent. After 6 min, 125 µL of 10% w/v sodium carbonate and 100 µL of distilled water were added. The mixture was then incubated at room temperature for 1.5 h. After that, the absorbance was measured at 750 nm using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA). Gallic acid was used as a standard to prepare the calibration curve. The total phenolic content was expressed as milligram gallic acid equivalents (GAE) per gram of dried sample.

Total flavonoid content

The total flavonoid content was determined according to the method of Sunanta et al. (2020). The extract was dissolved in methanol prior test. A 25 µL of the extract solution was mixed with 125 µL of distilled water, and then 7.5 µL of 5% sodium nitrite was added. The mixture was permitted to react at room temperature for 5 min before adding 15 µL of 10% Aluminum chloride hexahydrate. After incubating for 6 min, 50 µL of 1 M of sodium hydroxide and 27.5 µL distilled water were added. The absorbance was measured at 510 nm using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA). Catechin was used as a standard to prepare the calibration curve. The total flavonoid content was expressed as milligram catechin equivalents (CE) per gram of dried sample.

β-carotene bleaching assay

In this test, antioxidant activity was determined using the method of Kulisic et al. (2004) with some modifications. The β-carotene-linoleic acid emulsion was prepared as follows: 0.25 mg β-carotene was dissolved in 1 mL of chloroform, then 23 µL of linoleic acid and 400 µL of Tween 20 were added. The chloroform was totally evaporated using a rotary evaporator. Afterwards, 50 mL of oxygenated distilled water was added and sonicated for 3 min to form emulsion. A 200 µL of extract solution in ethanol was mixed with 5 mL of β-carotene-linoleic acid emulsion in test tubes. Tubes containing 200 µL of ethanol and 5 mL of β-carotene-linoleic acid emulsion were prepared as a control. Besides, a blank consisting of 23 µL of linoleic acid, 400 µL of Tween 20 and 50 mL of oxygenated distilled water was prepared for setting zero. The absorbance of all samples was instantly measured (t=0) at 470 nm using a C30M portable spectrophotometer (PG instrument Ltd, Leicestershire, UK) and then heated in a water bath for 120 min at 50 °C before measuring the absorbance once again (t=120). The percentage inhibition was calculated as the following equation;

$$\text{Inhibition (\%)} = [(AS(120) - AC(120) / (AC(0) - AC(120)] \times 100$$

Where AS(120) is the absorbance of extract sample at t=120 min, AC(120) is the absorbance of control at t=120 min, and AC(0) is the absorbance of control at t=0 min.

ABTS radical scavenging activity

The ABTS radical scavenging activity was assayed as the method described by Binsan et al. (2008) with slight modifications. A stock solution was produced by mixing 7.4 mM ABTS solution with 2.6 mM potassium persulfate solution in equal quantities, and allowing the mixture to react for 12 h at 25 °C in darkness. Afterwards, ABTS working solution was prepared by diluting 1 mL of the stock solution with 65 mL of methanol to get an absorbance 0.7 ± 0.2 units at 734 nm. A 10 µL of extract solution in methanol was mixed with 190 µL of the ABTS working solution in a 96-well plate, and then incubated for 10 min at 25 °C in darkness. Wells containing 10 µL of methanol and 190 µL of the ABTS working solution, without extract, were used as a control. The absorbance was subsequently measured at 734 nm

using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA). The ABTS radical scavenging activity of extract were expressed as the percentage inhibition, which was calculated by below equation;

$$\text{Inhibition (\%)} = [(AC - AS)/ AC] \times 100$$

Where AS is the absorbance of extract sample and AC is the absorbance of control.

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined using the modified method of Kulisic et al. (2004). A 50 µL of extract solution in ethanol was mixed with 200 µL of 0.6 mM DPPH solution in a 96-well plate, and then incubated for 30 min at 25 °C in darkness. Wells containing 50 µL of ethanol and 200 µL of 0.6 mM DPPH solution, without extract, were used as a control. After incubating, the absorbance was measured at 517 nm using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA). The DPPH radical scavenging activity of extract were reported as the percentage inhibition, which was calculated by below equation;

$$\text{Inhibition (\%)} = [(AC - AS)/ AC] \times 100$$

Where AS is the absorbance of extract sample and AC is the absorbance of control.

Quantitative Analysis of phenolic components by Liquid Chromatography–Electrospray Ionization/Mass Spectrometry (LC-ESI/MS)

The phenolic components of ethanolic sappan wood, green tea, and grape seed extracts were performed on LC-ESI/MS. The extracts were dissolved in 95% methanol to a final concentration of 20 mg/mL. Stock solution (10 mg/mL) of 17 standards including gallic acid, caffeic acid, phytic acid, ferulic acid, chlorogenic acid, hydroxybenzoic acid, rosmarinic acid, ρ-coumaric acid, o-coumaric acid, catechin, epicatechin, epigallocatechin gallate, naringin, naringenin, quercetin, rutin and kaempferol were individually prepared by dissolving standards in ethanol. An intermediate mixed stock solution containing all the 17 standards (0.1 mg/mL) was prepared in ethanol and diluted with the same solvent to obtain working solutions with different concentrations from 3.125 to 50 µg/mL, which were used for preparing standard curves.

The analytical liquid chromatography (LC) was performed using an Agilent 1260 Infinity II series, coupled with an electrospray ion (ESI) quadrupole mass spectrometry 6130 (Agilent Tech., Santa Clara, CA, USA). Separation was executed using a Reverse-phase column chromatography was performed using the Restek Ultra C18 column (250 x 4.6 mm, 5 µm, Restek, USA). The running protocol was followed the method of Mighri et al. (2019) with slight modification. The mobile phase consisted of A (0.2% acetic acid in 95% water and 5% methanol) and B (0.2% acetic acid in 50% water and 50% acetonitrile) with a linear gradient elution: 0-45 min, 10-20% B; 45-85 min, 20-55% B; 85-97 min, 55-100% B; 97-110 min, 100% B; the initial conditions

were hold for 10 min as a re-equilibration step. The flow rate of the mobile phase was 0.5 mL/min. The column temperature was maintained at 40 °C and the injection volume was 20 µL. The spectra were operated in the negative selected ion monitoring (SIM) as a followed condition: a capillary voltage of -3.5 V, a nebulizing gas flow of 1.5 L/min, a dry gas (N2) flow rate of 12 L/min, a DL (dissolving line) temperature of 250 °C, a block source temperature of 400 °C, fragmentor voltage 70 V and the full scan spectra from 100-1200 m/z with 250 ms/spectrum. The spectra processed using OpenLab software (Agilent Tech., Santa Clara, CA, USA).

Gas Chromatography/Mass Spectrometry (GC/MS) analysis

The major components of essential oil-cyclodextrin complexes were performed on a gas chromatograph-mass spectrometer detector using a Scion 436-MS (Bruker, CA, USA). An injector operating in the split mode (10:1) at 250 °C and a fused silica capillary column (30 m x 0.25 mm ID x 0.25 µM film thickness; Rxi®-5Sil MS (Restek, PA, USA); 5% diphenyl/95% dimethyl polydimethylsiloxane) were used. The oven temperature was programmed at 40 °C for 1 min, increased to 80 °C at 3 °C/min, then increased to 260 °C at 5 °C/min and held at 260 °C for 10 min. Helium was used as carrier gas at a flow 1 mL/min and injection volume of each sample was 1 µL. The temperatures of transfer line and ion source were maintained at 290 °C and 320 °C, respectively. In the full-scan mode, electron ionization mass spectra in the range 35-500 m/z were recorded at 70 eV electron energy. The identification of components was assigned by comparison of their mass spectra with those of NIST and Wiley libraries.

Cell isolation

Porcine preadipocytes were isolated from dorsal subcutaneous adipose tissue of a 5-month-old female pig in accordance with the modified method of Choi et al. (2010). Briefly, adipose tissue was washed once with 70% ethanol, and washed twice with a sterile buffer containing serum-free Dulbecco's modified Eagle's medium (DMEM) and 2% Penicillin/Streptomycin. Then, adipose tissue was finely minced and digested by gently shaking at 37 °C for 1 h in a digestion buffer consisting of DMEM, 20 mM HEPES, 2% penicillin/streptomycin, and 700 U type-1 collagenase/mL. The digested tissue was subsequently filtered through a stainless-steel mesh (250 µm) and centrifuged at 3,000 rpm for 10 min. The pellet was collected and washed twice with the sterile buffer, then centrifuged at 2,000 rpm for 10 min. The cells were resuspended in the culture medium containing DMEM, 10% FBS, and 2% penicillin/streptomycin. After that, the cells were seeded into 6-well plates and cultured in an incubator at 37 °C with 5% CO_2 atmosphere.

Cell cytotoxicity assay

The cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to the method of Arjin et al. (2020) with some modifications. Briefly, preadipocytes were seeded in 96-well plates at 2.5 x 10^4 cells/well and incubated at 37 °C with 5% CO_2 atmosphere for 24 h. Then, the medium was removed and substituted by plant extract that was diluted in the medium. Wells comprising only medium, without

extract, were used as a control. Incubation was subsequently continued for 24 h. After incubation, the medium was cautiously removed and 200 µL of MTT solution (0.5 mg/mL) was added to each well. After that, the plates were incubated in optimum atmosphere at 37 °C for 2 h. The metabolically active cells converted MTT to purple formazan crystals. Then, the MTT solution was replaced with 200 µL of DMSO in order to dissolve the formazan crystals, and the plates were incubated for 5 minutes. The absorbance was measured at 550 nm using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA), and compared with the control. Results were assessed as CC_{80} (the concentration of tested extract that there was an 80% of cell viability). The CC_{80} of each extract was used to differentiate the cells for studying lipid accumulation.

Differentiation of preadipocytes

The differentiation of preadipocytes was performed according to the method of Suzuki et al. (2013) with some modifications. Preadipocytes were seeded at 2.0 x 10^4 cells/well in 96-well plates and incubated at 37 °C with 5% CO_2 atmosphere until they reached confluence. At confluence (Day 0), the medium was replaced with adipogenic medium containing DMEM, 10% FBS, 0.25 µM of dexamethasone, 5 µg/mL of insulin, and extract at CC_{80} to induce differentiation. Wells containing adipogenic medium, without extract, were used as a control. And wells with culture medium containing DMEM, 10% FBS, and 2% penicillin/streptomycin were used as a negative control. The cells were cultured to differentiate for 8 days, then subjected to oil red O staining.

Lipid accumulation using oil red O staining

Lipid accumulation in adipocytes was observed by oil red O staining. The lipid droplets of differentiated cells were stained with oil red O solution in accordance with the modified method of Kim et al. (2017). Briefly, the medium was removed and cells were rinsed once with phosphate buffered saline (PBS). After that, the cells were fixed with 10% buffered formalin for 1 h and washed twice with PBS. After incubating in 60% isopropanol for 2 minutes, the cells were stained with oil red O solution at room temperature for 1 h, and washed three times with PBS. The oil red O-stained lipid droplets were microscopically inspected and each well was taken a photo for color extraction using a MulticolorEngine (TinEye, Ontario, Canada). The extracted red color of lipid droplets in each well was compared with the control. Lipid accumulation was reported as percentage of control, which was calculated by below equation;

$$\text{Lipid accumulation (\% of control)} = [(ERS)/ ERC] \times 100$$

Where ERS is the extracted red color of lipid droplets in well treated with extract and ERC is the extracted red color of lipid droplets in control.

Statistical analysis

All experiments were performed with at least two replicates, and expressed as mean ± standard deviation (SD). The data of antioxidant activities, total phenolic content, and total flavonoid content were statistically analyzed using a one-way analysis of variance and Turkey's post hoc test in the SPSS 23.0 software (SPSS Inc., Chicago, IL, USA), with a P-value < 0.05 considered statistically significant.

RESULTS

Total phenolic and total flavonoid content

The total phenolic and total flavonoid content of plant extracts are shown in Table 1. Among the plant extracts, green tea extract had significantly the highest total phenolic content (323.63 ± 4.77 mg GAE/g sample), followed by grape seed extract (201.77 ± 3.10 mg GAE/g sample). The total flavonoid content was significantly the highest in grape seed extract (211.47 ± 7.02 mg CE/g sample), followed by green tea extract (112.79 ± 7.00 mg CE/g sample). The lowest total phenolic and total flavonoid content were significantly observed in sappan wood extract (25.08 ± 0.35 mg GAE/g sample and 19.15 ± 0.43 mg CE/g sample, respectively).

Antioxidant activities

In this study, the antioxidant activities were determined using the β-carotene bleaching assay, ABTS and DPPH radical scavenging activity. The results were assessed as IC_{50} (half maximal inhibitory concentration). The low IC_{50} value indicates the high antioxidant activity of sample. The antioxidant activities of three plant extracts are illustrated in Table 1. The IC_{50} value with β-carotene bleaching test showed that grape seed extract had significantly the highest antioxidant activity (1.22 ± 0.01 mg/mL), followed by green tea extract (1.97 ± 0.07 mg/mL) and sappan wood extract (8.23 ± 0.02 mg/mL) respectively. Concerning ABTS and DPPH radical scavenging activities, grape seed extract and green tea extract had significant lower IC_{50} value than those of sappan wood extract (Table 1). Taking three antioxidant tests into consideration, the antioxidant activity of grape seed extract was similar to green tea extract. Among the essential oil-cyclodextrin complexes, the IC_{50} value derived from the β-carotene bleaching test, ABTS and DPPH radical scavenging activity of thyme oil had significantly the highest antioxidant activities, followed by galangal oil and ginger oil respectively (Table 2).

Table 1 Total phenolic content, total flavonoid content and antioxidant activities of plant extracts

Extract	Total phenolic (mg GAE/g)	Total flavonoid (mg CE/g)	Inhibition (IC_{50}, mg/ml)		
			β-carotene bleaching	ABTS	DPPH
Sappan wood	25.08 ± 0.35[c]	19.15 ± 0.43[c]	8.23 ± 0.02[a]	3.75 ± 0.21[a]	1.63 ± 0.04[a]
Green tea	323.63 ± 4.77[a]	112.79 ± 7.00[b]	1.97 ± 0.07[b]	0.057 ± 0.00[b]	0.052 ± 0.00[b]
Grape seed	201.77 ± 3.10[b]	211.47 ± 7.02[a]	1.22 ± 0.01[c]	0.048 ± 0.00[b]	0.051 ± 0.00[b]
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

[a, b, c] Means in the same column with different superscripts differ significantly ($P < 0.05$); IC_{50}, half maximal inhibitory concentration; GAE, gallic acid equivalents; CE, catechin equivalents. Results were expressed as means ± SD (n=3 for total phenolic and total flavonoid; n=2 for antioxidant activities)

Table 2 Antioxidant activities of essential oil-cyclodextrin complexes

Essential oil	Inhibition (IC_{50}, mg/ml)		
	β-carotene bleaching	ABTS	DPPH
Thyme oil	10.57 ± 1.36[c]	7.73 ± 0.54 [c]	97.37 ± 0.06[c]
Ginger oil	58.54 ± 0.39[a]	165.22 ± 11.60[a]	212.20 ± 0.50[a]
Galangal oil	45.20 ± 0.10[b]	106.83 ± 4.92[b]	201.64 ± 4.34[b]
P-value	<0.001	<0.001	<0.001

[a, b, c] Means in the same column with different superscripts differ significantly ($P < 0.05$); IC_{50}, half maximal inhibitory concentration. Results were expressed as means ± SD (n=2)

Major components

Table 3 shows the phenolic components in sappan wood, green tea, and grape seed extracts, which are analyzed by LC-ESI/MS. The major components of sappan wood extract were naringenin (84.78 ± 4.26 mg/100 g of extract) and kaempferol (82.57 ± 8.15 mg/100 g of extract). While epigallocatechin gallate (193.36 ± 1.42 mg/100 g of extract) and epicatechin (64.36 ± 0.35 mg/100 g of extract) were dominant in green tea extract. As for grape seed extract, the major components were hydroxybenzoic acid (28.97 ± 1.67 mg/100g of extract), chlorogenic acid (27.29 ± 0.09 mg/100 g of extract) and catechin (6.51 ± 0.04 mg/100g of extract). Regarding the essential oil-cyclodextrin complexes, the components were identified by GC/MS and shown in Table 4. The components of thyme oil contained the highest content of cymene (39.26 ± 0.63%), followed by thymol (23.08 ± 0.66%) and γ-terpinene (18.87 ± 0.22%), respectively. While zingiberene (26.96 ± 1.43%), geranial (15.41 ± 0.16%) and β-sesquiphellandrene (12.69 ± 0.30%) were dominant in ginger oil. The major components of galangal oil were 1,8-cineole (43.57 ± 0.03%), α-bisabolene (18.05 ± 0.63%) and β-sesquiphellandrene (10.60 ± 0.04%).

Table 3 Phenolic components of plant extracts

No.	Component (mg/ 100 g of extract)	Plant extract		
		Sappan wood	Green tea	Grape seed
1	Gallic acid	6.98 ± 0.19	22.52 ± 0.46	6.23 ± 0.06
2	Rosmarinic acid	2.20 ± 0.01	ND	2.75 ± 0.01
3	Chlorogenic acid	25.70 ± 0.39	25.72 ± 1.46	27.29 ± 0.09
4	Hydroxybenzoic acid	ND	ND	28.97 ± 1.67
5	Catechin	ND	17.15 ± 0.89	6.51 ± 0.04
6	Epicatechin	5.11 ± 0.27	64.36 ± 0.35	2.98 ± 0.01
7	Epigallocatechin gallate	ND	193.36 ± 1.42	3.54 ± 0.01
8	Quercetin	12.61 ± 1.35	10.60 ± 0.20	5.99 ± 0.13
9	Rutin	ND	7.09 ± 0.42	3.16 ± 0.03
10	Kaempferol	82.57 ± 8.15	9.93 ± 0.16	ND
11	Naringin	6.61 ± 0.08	ND	ND
12	Naringenin	84.78 ± 4.26	ND	ND

ND, not detected. Results were expressed as means ± SD (n=2)

Table 4 Major components of essential oil-cyclodextrin complexes

No.	Component (% of total)	CAS Number	RT	Essential oil-cyclodextrin complex		
				Thyme oil	Ginger oil	Galangal oil
1	γ-Terpinene	99-85-4	12.812	18.87 ± 0.22	ND	ND
2	Cymene	25155-15-1	13.135	39.26 ± 0.63	ND	ND
3	1,8-Cineole	470-82-6	13.496	1.00 ± 0.04	ND	43.57 ± 0.03
4	Bergamiol	115-95-7	16.404	2.81 ± 0.01	ND	ND
5	Geranial	5392-40-5	22.098	ND	15.41 ± 0.16	ND
6	Thymol	89-83-8	22.784	23.08 ± 0.66	ND	ND
7	β-Caryophyllene	87-44-5	26.427	2.32 ± 0.03	ND	ND
8	trans-α-Bergamotene	13474-59-4	26.770	ND	ND	9.70 ± 0.05
9	α-Curcumene	644-30-4	27.974	ND	12.14 ± 0.16	ND
10	Zingiberene	495-60-3	28.335	ND	26.96 ± 1.43	ND
11	(Z,E)-alpha-Farnesene	26560-14-5	28.550	ND	9.87 ± 0.06	ND
12	α-Bisabolene	25532-79-0	28.659	ND	7.38 ± 0.17	18.05 ± 0.63
13	β-Sesquiphellandrene	20307-83-9	29.048	ND	12.69 ± 0.30	10.60 ± 0.04
14	α-Sinensal	17909-77-2	29.125	ND	ND	5.66 ± 0.04

RT, retention time; ND, not detected. Results were expressed as means ± SD (n=3).

Cell Cytotoxicity

The cytotoxicity of plant extracts and essential oil-cyclodextrin complexes were performed on the viability of porcine preadipocytes, and results were expressed as CC_{80} (the concentration of tested extract that there was 80% of cell viability). The CC_{80} value indicates the potency of sample. The CC_{80} value of plant extracts and essential oil-cyclodextrin complexes ranged from 0.026 ± 0.00 to 3.64 ± 0.94 mg/mL (Figure 2). The thyme oil had the highest CC_{80} value (3.64 ± 0.94 mg/mL), followed by the galangal oil (1.37 ± 0.16 mg/mL), the ginger oil (1.29 ± 0.02 mg/mL), sappan wood extract (0.59 ± 0.03 mg/mL), grape seed extract (0.032 ± 0.00 mg/mL), and green tea extract (0.026 ± 0.00 mg/mL), respectively. These observed CC_{80} values showed that green tea extract had the highest cytotoxicity, while the thyme oil had the lowest cytotoxicity. The CC_{80} of each extract was used to differentiate the cells for studying lipid accumulation.

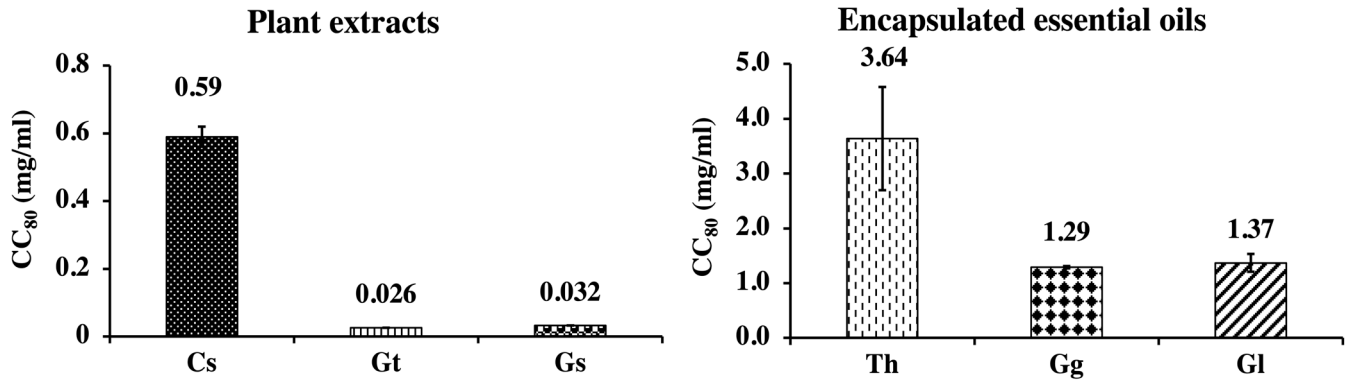

Figure 2 Cytotoxicity of plant extracts and essential oil-cyclodextrin complexes on porcine preadipocytes determined by MTT assay. Cs, sappan wood extract; Gt, green tea extract; Gs, grape seed extract; Th, thyme oil; Gg, ginger oil; Gl, galanga oil. CC_{80}, the concentration of tested extract that there was an 80% of cell viability. Results were expressed as CC_{80} (n=2).

Lipid accumulation

Porcine preadipocytes were differentiated for 8 days and then stained with oil red O solution. Microscopic observation of the oil red O-stained lipid droplets indicated the lipid accumulation in the extract-treated cells, in comparison with that observed in the control (Figure 3). Results showed that all of plant extracts and essential oil-cyclodextrin complexes decreased lipid accumulation in adipocytes. The percentage of lipid accumulation in adipocytes, which was treated with plant extracts and essential oil-cyclodextrin complexes, ranged from 32.61 ± 4.80% to 91.71 ± 16.70% (Figure 4). The thyme oil was observed to have the greatest potential on the decrease of lipid accumulation, with the percentage of 32.61 ± 4.80%. Besides, green tea extract and the galangal oil were also shown to have a great potential on the reduction of lipid accumulation, with the percentage of 33.42 ± 3.12% and 35.87 ± 4.21%, respectively.

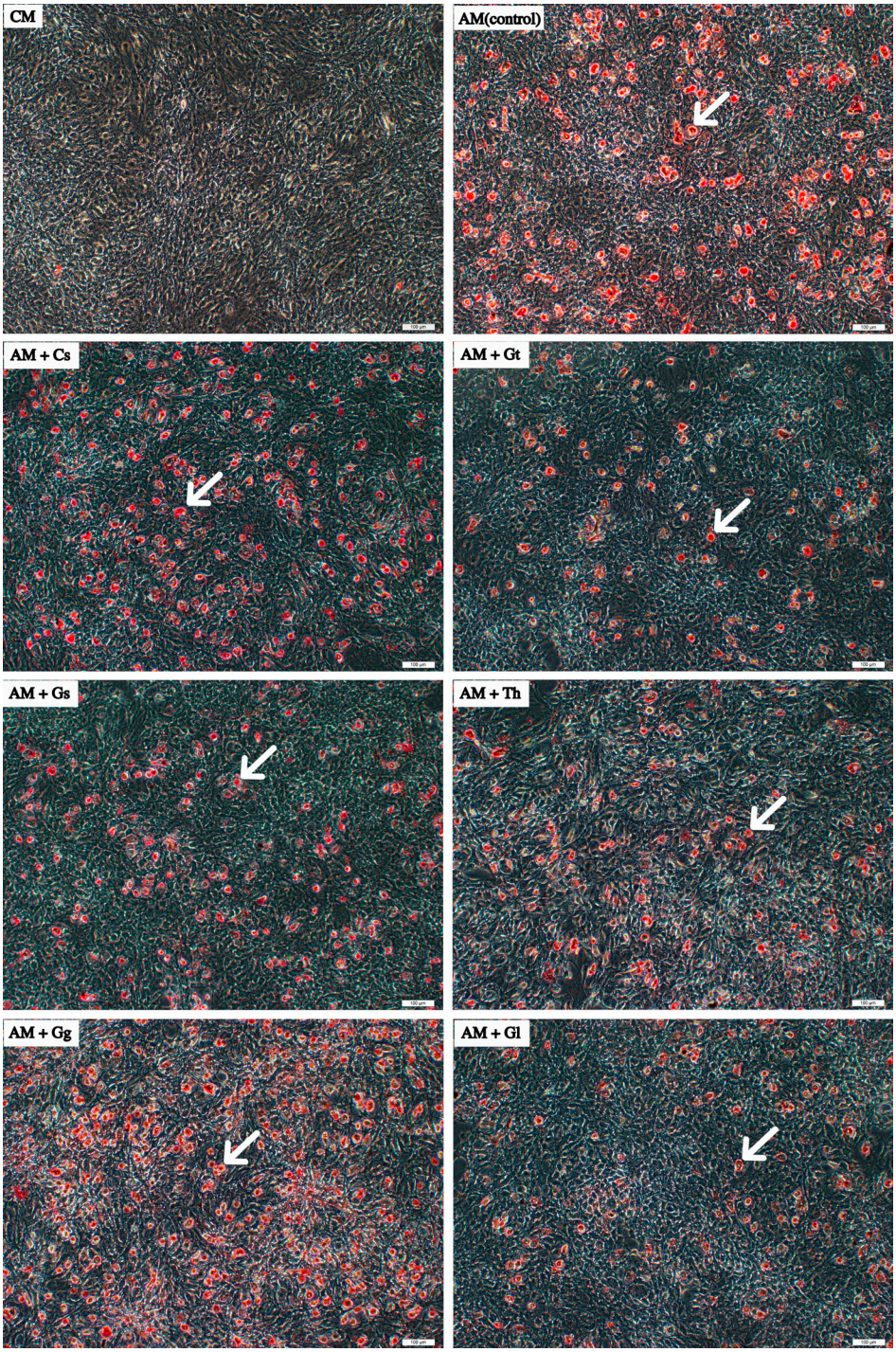

Figure 3 Lipid droplets were observed by oil red O staining in porcine adipocytes that were differentiated for 8 days with various medium, plant extracts and essential oil-cyclodextrin complexes. CM, culture medium; AM, adipogenic medium; Cs, sappan wood extract; Gt, green tea extract; Gs, grape seed extract; Th, thyme oil; Gg, ginger oil; Gl, galangal oil. Arrows indicate the lipid droplets stained with oil red O. Scale bar represents 100 μm.

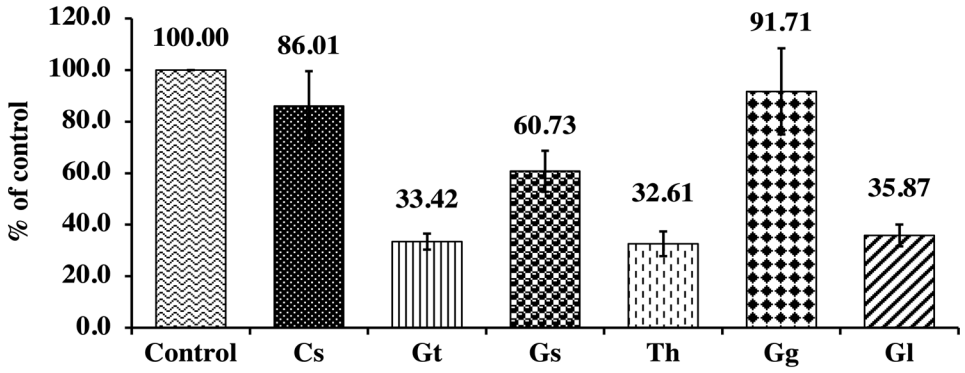

Figure 4 Lipid accumulation in porcine adipocytes treated with plant extracts and essential oil-cyclodextrin complexes. Cs, sappan wood extract; Gt, green tea extract; Gs, grape seed extract; Th, thyme oil; Gg, ginger oil; Gl, galangal oil. Results were expressed as the percentage of control (n=4).

DISCUSSION

The total phenolic and total flavonoid contents varied broadly among all extracts. Green tea extract had the highest total phenolic content, followed by grape seed extract. While the total flavonoid content was the highest in grape seed extract, followed by the green tea extract. This result agreed with Rababah et al. (2004), who reported that total phenolic content differed among different types of plant extracts, which green tea and grape seed extracts displayed higher amount of total phenolic content than those of ginger, ginko, gotu kola and fenugreek extracts. Farooq and Sehgal (2019) reported that green tea had higher total phenolic and total flavonoid contents as compared with the extract of *Ocimum gratissimum*. While Al-Dalain (2020) showed that grape seed extract had high total phenolic and total flavonoid contents. Significant difference in total phenolic and total flavonoid contents was also observed among different plant species, cultivars, extracting solvents, harvest times, growing locations and plant part used (Bakkalbasi et al., 2005; Malsawmtluangi et al., 2016; Ozkan et al., 2010; Yahyaoui et al., 2019; Zengin et al., 2018)

Considering three antioxidant tests in this study, grape seed and green tea extracts showed the great potential for antioxidant activities. While thyme oil had the highest antioxidant activities among essential oil-cyclodextrin complexes. The results agreed with Rababah et al. (2004), who reported that green tea and grape seed extracts illustrated high antioxidant activities against lipid oxidation. The strong antioxidant activities of essential oils obtained from several thymus species had been also reported (Bilenler et al., 2015; Khan et al., 2019; Tepe et al., 2005; Youdim et al., 2002). The total phenolic and total flavonoid content are considered as the indicators of antioxidant capacity because the redox properties of phenolic compounds which are of great reducing agents via hydrogen donors and radical scavengers (Weerawatanakorn et al., 2018). A strong correlation between total phenolic content and total flavonoid contents and antioxidant activities was observed in green tea (Farooq and Sehgal, 2019). Moreover, the correlation between total flavan-3-ol content and antiradical efficiency was also found highly significant in grape seed (Bakkalbasi et al., 2005). Regarding the antioxidant properties of thyme oil in

previous study, it has been attributed mainly to their terpenoid phenols, especially thymol and carvacrol. It is believed that hydroxy groups on the aromatic rings of thymol and carvacrol give the antioxidant activity by acting as a powerful hydrogen donor (Youdim et al., 2002). The antioxidant activity of thymol and carvacrol has also been reported in several studies (Aeschbach et al., 1994; Ruberto and Baratta, 2000).

The major components of sappan wood extract were naringenin and kaempferol. While epigallocatechin gallate and epicatechin were dominant in green tea extract. As for grape seed extract, the major components were hydroxybenzoic acid, chlorogenic acid and catechin. Regarding the essential oil-cyclodextrin complexes, the major components of thyme oil were cymene, thymol and γ-terpinene. While zingiberene, geranial and β-sesquiphellandrene were dominant in ginger oil. The major components of galangal oil were 1,8-cineole, α-bisabolene and β-sesquiphellandrene. These results were in agreement with previous studies, which indicated the major components in *Caesalpinia sappan* extract (Arjin et al., 2021), extract of green tea (Lee et al., 2014), thyme oil (Khan et al., 2019), ginger oil (Ravi Kiran et al., 2013) and galangal oil (Mallavarapu et al., 2002). However, the major components of grape seed extract found in this study were slightly different with Rababah et al. (2004) and Mandic et al. (2008), who reported that the main phenolic components in grape seed extract were epicatechin and catechin. The phenolic components of plant extracts can be affected by species (Zengin et al., 2018), harvest time (Ozkan et al., 2010) and growing location (Ghasemzadeh et al., 2015) and also the solvent choice (Bandara et al., 2018). Therefore, the different results of grape seed extract in this study compared to the previous studies might be as a result of any of these factors.

Prior to determining the effects of plant extracts and essential oil-cyclodextrin complexes on lipid accumulation, the cell cytotoxicity assay was determined to find the concentration that can be used to avoid cells death. The cytotoxicity results were expressed as CC_{80}, which is the concentration of tested extract that there was an 80% of cell viability. These observed CC_{80} values showed that green tea extract had the highest cytotoxicity, while the thyme oil had the lowest cytotoxicity. Lao et al. (2015) investigated the cytotoxicity of green tea polyphenols and epigallocatechin in 3T3-L1 preadipocytes cell line derived from mouse, and found that epigallocatechin gallate (the most major component found in green tea) at a concentration of 68 µg/mL decreased cell viability to 78 ± 5.1% of control, while 100 µg/mL of green tea polyphenols did not significantly affect cell viability until the concentration at 1000 µg/mL (< 80% of control). The CC_{80} of green tea extract in this study was 0.026 ± 0.00 mg/mL, which was lower than in the study of Lao et al. (2015). The lower concentration of green tea extract affecting cell viability in this study might be due to the different kind of preadipocyte used, which this study used porcine preadipocytes, while the previous study used 3T3-L1 preadipocytes derived from mouse.

This study illustrated that all plant extracts and essential oil-cyclodextrin complexes decreased lipid accumulation in adipocytes, with the percentage of lipid accumulation ranged from 32.61 ± 4.80% to 91.71 ± 16.70%. Green tea extract, thyme oil and galangal oil decreased lipid accumulation about 60%

lower than that of the control. The results were in-line with the finding of Choi et al. (2010), who reported that many Korean medicinal plants that had been extracted by ethanol and water could reduce lipogenesis of adipose tissue and differentiation of porcine preadipocytes *in vitro*. Lao et al. (2015) reported that green tea polyphenols and epigallocatechin gallate reduced lipid accumulation during 3T3-L1 preadipocytes differentiation by inhibiting the expression of CCAT element binding protein α (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory element-binding protein-1c (SREBP-1c), which are transcription factors involved in the regulation of adipogenesis pathway. Essential oil obtained from *Alpinia galanga* rhizomes (Liang et al., 2018), and Ethanolic *Alpinia officinarum* extract (Jung et al., 2012) were also indicated to inhibit lipid accumulation by downregulating the expression of C/EBPα, SREBP-1c and PPARγ genes during 3T3-L1 preadipocytes differentiation. Furthermore, several studies demonstrated that plant extracts inhibited lipid accumulation and reduced the expression of PPARγ and C/EBPα in 3T3-L1 preadipocytes (Kim et al., 2017; Ogawa et al., 2010; Popovich et al., 2010). Regarding thyme oil, this study is the first to report its effects on lipid accumulation in adipocytes. Nevertheless, thymol, which is a monoterpene phenolic compound found in thyme oil, could downregulate the expression level of SREBP-1c and reduced lipid accumulation in kidney of mice fed by high fat diet (Saravanan and Pari, 2016). To understand the mechanism of thyme oil on reducing lipid accumulation in adipocytes, further study is indeed still needed. It is worth to notice a relation between antioxidant activities and lipid accumulation in our work. Green tea extract and thyme oil had high antioxidant potential and reduced lipid accumulation about 60% lower than that of the control. While grape seed extract, which had also high antioxidant activities, could reduce lipid accumulation to about 40% lower than that of the control. Concerning galangal oil, its antioxidant activities placed the second among the essential oil products, and it reduced lipid accumulation to percentage that was similar to that of the green tea extract and thyme oil. Previous studies suggested that reactive oxygen species (ROS) facilitated adipocyte differentiation (Lee et al., 2009) and lipid accumulation increased in parallel with ROS production in adipocytes (Lee et al., 2013). In the study of Choi et al. (2016) found that 7,8-dihydroxyflavone, belonged to the flavonoid exhibited high DPPH radical activity, reduced lipid accumulation and attenuated ROS accumulation by inducing antioxidant enzymes during differentiation of 3T3-L1 preadipocytes. Besides, several plant extracts, which exhibited high antioxidant activities, served as ROS scavenger (Barizão et al., 2016; Sittisart and Chitsomboon, 2014). The relation between antioxidant activities and lipid accumulation observed in this study might be explained by the potent antioxidant activities of plant extracts and essential oils on reducing intracellular ROS production during differentiation of porcine preadipocytes, which leaded to reduce lipid accumulation in adipocytes. As the results observed in this study, the products that had high antioxidant activities might be able to use as feed additive in pig diets to reduce the backfat thickness for improving the carcass quality.

CONCLUSION

Based on the results of this study, it can be concluded that green tea and grape seed extracts, and thyme oil powder had high antioxidant potential. Lipid accumulation in porcine adipocytes can be reduced by green tea extract, thyme and galangal oils. This study was initially investigating the effects of plant extracts and essential oil-cyclodextrin complexes on lipid accumulation of porcine adipocytes. These potential products including green tea and grape seed extracts, thyme oil and galangal oil powders need to be further tested for the *in vivo* study.

AUTHOR CONTRIBUTIONS

Conceptualization, R.N. and K.S.; methodology, R.N., C.A., and A.S.; validation, N.C. and W.R.; data curation, R.N. and A.S.; formal analysis R.N. and C.A.; investigation, R.N., C.A., and A.S.; project administration, K.S.; resources, S.M. and K.S.; writing-original draft, R.N. and C.A.; writing-review and editing, R.N., C.A., S.M., W.R., S.R.S. and K.S. All authors read and agreed with the final manuscript.

CONFLICT OF INTEREST

We all declare that we have no conflict of interest.

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