Research Article

ผลของสารสกัดเอธานอลใบชายาต่อการเกิดโฟมเซลล์และผลของอายุใบและฤดูกาลเก็บเกี่ยวต่อปริมาณสารพฤกษเคมี ฤทธิ์ต้านอนุมูลอิสระ ความเป็นพิษต่อเซลล์ และการสร้างไนตริกออกไซด์

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บทคัดย่อ

ผลของสารสกัดเอธานอลใบชายาต่อการเกิดโฟมเซลล์และผลของอายุใบและฤดูกาลเก็บเกี่ยวต่อปริมาณสารพฤกษเคมี ฤทธิ์ต้านอนุมูลอิสระ ความเป็นพิษต่อเซลล์ และการสร้างไนตริกออกไซด์

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ใบของต้นชายา (*Cnidoscolus aconitifolius* (Mill.) I.M.Johnst.) อยู่ในวงศ์ Euphorbiaceae ถูกนำมาใช้เป็นอาหารและยาเพื่อลดความดันโลหิต ส่วนโฟมเซลล์เกี่ยวข้องกับการเกิดโรคหลอดเลือดแดงแข็งตัวซึ่งสัมพันธ์กับระดับแอลดีแอลคอเลสเทอรอลและความดันโลหิตสูง วัตถุประสงค์การศึกษาครั้งนี้เพื่อศึกษาผลของสารสกัดจากใบชายา (CAE) ที่สกัดด้วยเอทานอลที่มีต่อการเกิดโฟมเซลล์ ไนตริกออกไซด์และ TNF-α และยังศึกษาความแตกต่างทางพฤกษเคมีของใบชายาที่ได้รับอิทธิพลจากฤดูกาลที่เก็บเกี่ยวและอายุใบ **วิธีการศึกษา:** ปริมาณฟีนอลิคและฟลาโวนอยด์รวมทั้งหมดถูกศึกษาด้วยการทำปฏิกิริยากับโฟลิน-ไซโอแคลทูและอลูมิเนียมคลอไรด์ตามลำดับ วิเคราะห์ปริมาณแคมพ์เฟอรอลด้วยวิธี HPLC ฤทธิ์ต้านอนุมูลอิสระถูกศึกษาด้วยวิธี DPPH และ FRAP ส่วนความเป็นพิษของสารสกัด การสร้างไนตริกออกไซด์และการสร้าง TNF-α ถูกศึกษาในเซลล์ RAW264.7 ด้วยวิธี MTT, Griess reaction และ ELISA ตามลำดับ และศึกษาการสร้างโฟมเซลล์ด้วยการย้อมสี Oil Red O **ผลการศึกษา:** ความแตกต่างของอายุใบและฤดูกาลในการเก็บเกี่ยวมีผลกระทบต่อสารพฤกษเคมีและฤทธิ์ต้านอนุมูลอิสระของใบ โดยใบที่เก็บเกี่ยวในฤดูแล้งมีฤทธิ์ต้านอนุมูลอิสระ ปริมาณฟีนอลิคและฟลาโวนอยด์รวมทั้งหมดมากกว่าใบที่เก็บเกี่ยวในฤดูฝน ส่วนใบที่แก่มีความเป็นพิษต่อเซลล์น้อยกว่าใบอ่อนและใบสมบูรณ์ สารสกัดทุกชนิดสามารถยับยั้งการสร้างไนตริกออกไซด์ในเซลล์ที่กระตุ้นด้วย LPS ได้ สารสกัดจากใบที่เก็บเกี่ยวในฤดูแล้งสามารถยับยั้งการสร้างไนตริกออกไซด์ได้มากกว่าใบจากฤดูฝน และสารสกัดใบสมบูรณ์ที่เก็บเกี่ยวในฤดูแล้งมีฤทธิ์ในการยับยั้งการสร้างไนตริกออกไซด์ (IC_{50} เท่ากับ 66.16 ± 1.13 µg/mL) ได้มากกว่าสารสกัดอื่นๆ ในสภาวะที่มีสารสกัดจากใบสมบูรณ์ที่เก็บเกี่ยวจากฤดูแล้งร่วมกับการกระตุ้นด้วย oxLDL พบว่า สารสกัดจากใบสมบูรณ์ที่เก็บเกี่ยวจากฤดูแล้งสามารถลดการสะสมของไขมันในโฟมเซลล์ได้เมื่อเปรียบเทียบกับสภาวะที่มี oxLDL เพียงอย่างเดียว และยังสามารถลดการสร้าง TNF-α เมื่อถูกชักนำด้วย oxLDL ได้อีกด้วย ผลการศึกษานี้ชี้ให้เห็นว่า CAE โดยเฉพาะสารสกัดจากใบสมบูรณ์ที่เก็บเกี่ยวจากฤดูแล้งสามารถลดการสะสมไขมันในโฟมเซลล์ที่ถูกชักนำด้วย oxLDL โดยอาจจะผ่านการลดการสร้างไนตริกออกไซด์และ TNF-α **สรุปผลการศึกษา:** ใบชายาที่สมบูรณ์ที่เก็บในฤดูแล้งมีฤทธิ์ต้านอนุมูลอิสระได้ดี มีพิษน้อยและสามารถลดการสร้างไนตริกออกไซด์ ลดการเกิดโฟมเซลล์และลดการสร้าง TNF-α ที่เกี่ยวข้องกับการเกิดโฟมเซลล์ได้ ดังนั้น สารสกัดใบชายาจึงสามารถนำมาเป็นตัวเลือกหนึ่งในการพัฒนาผลิตภัณฑ์เพื่อสุขภาพได้

คำสำคัญ: ชายา, พฤกษเคมี, ต้านอนุมูลอิสระ, ไนตริกออกไซด์, โฟมเซลล์

Effect of ethanol extracts from *Cnidoscolus aconitifolius* leaves on foam cell formation and effect of leaf age and harvesting season on phytochemicals, antioxidant, cytotoxicity and nitric oxide production

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Abstract

Effect of ethanol extracts from *Cnidoscolus aconitifolius* leaves on foam cell formation and effect of leaf age and harvesting season on phytochemicals, antioxidant, cytotoxicity and nitric oxide production

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Cnidoscolus aconitifolius (Mill.) I.M.Johnst. or Chaya (Euphorbiaceae) leaf has been used as a food and a medicinal plant for treatment of hypertension. Foam cells are involved with an atherosclerosis that related with high level of LDL cholesterol and hypertension. Therefore, aims of this study were to investigate the effects of *C. aconitifolius* ethanolic extracts (CAE) on foam cell formation, nitric oxide (NO) and TNF-α. The differences in phytochemicals influencing from harvesting season and age stage of its leaves were also studied. **Methods:** Total phenolic and flavonoid contents were studied by using Folin-Ciocalteu and aluminum chloride reaction, respectively. Amount of kaempferol was analysed using HPLC. Antioxidative activity was determined by using DPPH and FRAP assay. In RAW264.7 cells, cytotoxicity, NO and TNF-α production were studied by using MTT assay, Griess reaction and ELISA, respectively. Foam cell formation was determined by using Oil Red O staining. **Results:** The difference in leaf age and harvesting season affected to its phytochemicals and antioxidative activity. Leaves that were harvested in dry season showed higher antioxidative activity, total phenolic and total flavonoid contents than those of leaves that were harvested in rainy season. Aging leaf extracts showed lower cytotoxicity than young and mature leaf extracts. All extracts exhibited inhibitory activity on NO production in LPS-induced RAW264.7 cells. Extracts that were harvested in dry season showed greater inhibitory activity on NO production than those of rainy season. Mature leaf extract harvested in dry season showed higher inhibitory effect on NO production (IC_{50} of 66.16 ± 1.13 µg/mL) than those of the others. Co-treatment between mature leaf extract harvested in dry season and oxLDL could lower lipid accumulation and TNF-α production in foam cells than those of oxLDL treatment alone. These results indicate that CAE, especially mature leaf extract harvested in dry season, may reduce lipid accumulation in oxLDL-inducing foam cells via suppression of NO and TNF-α production. **Conclusion:** Mature leaf extract of *C. aconitifolius* that harvested in dry season had high antioxidants, low cytotoxicity, inhibitory activities on NO production, foam cell formation and TNF-α production. Therefore, *C. aconitifolius* leaf extract can be an alternative choice for health beneficial product development.

Keywords: *Cnidoscolus aconitifolius*, Phytochemicals, Antioxidant, Nitric oxide, Foam cells

Introduction

Atherosclerosis is a chronic and an inflammatory disease that is cause of coronary heart disease and ischemic stroke leading to sudden death in adulthood worldwide. Low- density lipoprotein (LDL) uptake by monocytes and macrophages result in atherosclerotic lesions (Ross, 1999). An internalization of oxidized LDL (oxLDL) by scavenger receptors on macrophages to form foam cells (lipid- laden macrophages) is a hallmark of atherosclerotic development and progression (Maiolino *et al*., 2013). The action of macrophages plays an important role in atherogenesis that involves with their proinflammatory mediators including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) (Maiolino *et al.*, 2013). Macrophages uptake oxLDL via scavenger receptors and then release TNF-α (Krieger, 2001; Jovinge *et al.*, 1996) resulting in foam cell transformation (Ross, 1999). This increased secretion of TNF-alpha from oxLDL-induced macrophages may be associated in the formation of atherosclerotic lesions (Jovinge *et al*., 1996).

Cnidoscolus aconitifolius (Mill.) I.M.Johnst. or Chaya, a plant in the family Euphorbiaceae, has been used as a food and a medicinal plant. It has been used as a traditional medicine for treatment of cancer, weight loss, high blood pressure, ulcers, diabetes mellitus and kidney diseases in Mexico (Jiménez-Arellanes *et al.*, 2014). *C. aconitifolius* is rich in nutritional and phytochemical values as well as medicinal potential. *C. aconitifolius* was reported to contain numerous phytochemicals including phenols, flavonoids, alkaloids, terpenoids, saponins, phlobatanins, tannins, anthraquinones, terpenes, cardiac glycosides and cyanogenetic glycoside (Orji *et al.*, 2016; Roy *et al*., 2016). Pillai *et al.* (2012) reported that flavonoids, especially kaempferol, are a major bioactive compound in *C. aconitifolius* leaves. There are several reports about pharmacological properties of *C. aconitifolius* such as antioxidant (Loarca-Piña *et al.*, 2010), antidiabetic (Loarca-Piña *et al.*, 2010; Samuel *et al.*, 2014), antiinflammation (Onasanwo *et al.*, 2011), reduction of liver and kidney damage (Oyagbemi and Odetola 2013). In addition, *C. aconitifolius* was reported to have antihypercholesterolemic and antihypertriglyceridemic activities in *in vivo* (Miranda-Velasquez *et al.*, 2010; Olaniyan *et al.*, 2017; Achi *et al.*, 2017). Therefore, *C. aconitifolius* is a good candidate for further nutraceutical product development. However, intrinsic factors (e.g. age, maturity, cultivar) and extrinsic factors (e.g. harvesting season, location, extraction solvent) affect the compositional quality and quantity of phytochemicals in plants as well as pharmacological properties (Agamou *et al.*, 2015; Nobossé *et al.*, 2018). Therefore, this study aims to investigate the phytochemical properties of different leaf age from ethanolic extract of *C. aconitifolius* leaves. In addition, this study also reports the plant extract effect on cytotoxicity, antioxidant, NO production, foam cell formation and TNF-α production.

Methods and materials

Extract preparation

C. aconitifolius leaves were collected from 20 tree in 4 different areas (Burapha University Sakaeo Campus and 3 village areas) in Wattana Nakorn District, Sa Kaeo Province on May 1, 2019 (Dry season) and June 19, 2019 (Rainy season). The plant was identified by Dr.Chakkrapong Rattamanee, a botanist and lecturer in Faculty of Agricultural Technology, Burapha University Sakaeo Campus. Plant voucher specimen (No. CN013) was made by Dr. Chakkrapong Rattamanee and kept at Faculty of Agricultural Technology, Burapha University Sakaeo Campus. The leaves were separated into 3 parts including young leaves (5 leaves on top part of branch), mature leaves (5 leaves lower than 5 young leaves) and aging leaves (5 leaves lower than mature leaves) (Figure 1). Raw leaves (without petiole) were cooked in boiling water for 10 min in order to eliminate toxic hydrocyanic glycosides. The cooked leaves were dried at 60°C for 2 days by using hot air oven. The dried leaves were blended and macerated in 95% ethanol for 7 days. After filtration, the remain debris from extraction was repeated by maceration in 95% ethanol for 7 days. Extracted liquid was combined and then evaporated the solvent by using rotary evaporator. The concentrated extract was dried by using freeze dryer. The *C. aconitifolius* extract was called CAE.

Figure 1 Habit of *C. aconitifolius* tree (A) and its young (B), mature (C) and aging leaves (D).

Determination of antioxidative activity

DPPH assay

DPPH or 2,2-diphenyl-1-picrylhydrazyl (0.5 mM) was dissolved in ethanol. Each extract was dissolved and diluted in ethanol. Diluted extract (1 mL) was added into DPPH solution (1 mL) and then incubated at room temperature in the dark for 10 min. Mixture solution was measured absorbance at 517 nm. The results were calculated for DPPH inhibition (%) and expressed as 50% inhibitory concentration (IC_{50}). Ascorbic acid and gallic acid were used as a standard compound.

Ferric reducing antioxidant power (FRAP) assay

FRAP reagent was freshly prepared by mixing of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (in 40 mM HCl) and 20 mM $FeCl_3$ (in 20 mM HCl) in ratio of 10:1:1. Each extract was diluted in ethanol and then pipetted 20 µL into 96-well plate. FRAP reagent (180 µL) was subsequently added and then incubated at room temperature in the dark for 10 min. Mixture solution was measured absorbance at 593 nm. Extract solution (no FRAP) was also measured the absorbance as a background. The extract results after subtraction its background were calculated by comparing with a standard curve of standard compound, gallic acid. The results were expressed as mg gallic acid equivalent/g extract (mg GAE/g).

Determination of phytochemical properties

Total phenolic content

Extracts were dissolved in ethanol and then pipetted 100 µL to mix with Folin-Ciocalteu reagent (200 µL) and distilled water (2 mL). After incubation at room temperature for 3 min, mixture solution was added with 20% w/v sodium carbonate (1 mL). After incubation at room temperature in the dark for 1 h, the solution was measured the absorbance at 765 nm. The extract results were calculated by comparing with a standard curve of standard compound, gallic acid. The results were expressed as mg gallic acid equivalent/g extract (mg GAE/g).

Total flavonoid content

Extracts were dissolved in ethanol and then pipetted 100 µL into 96-well plate. The solution was added with 2% w/v aluminum chloride (50 µL) and ethanol (50 µL). After incubation at room temperature in the dark for 10 min, the solution was measured absorbance at 425 nm. The extract results were calculated by comparing with a standard curve of standard compound, quercetin. The results were expressed as mg quercetin equivalent/g extract (mg QCE/g).

Quantitation of kaempferol

Reverse phase C18 column was used in high-performance liquid chromatography (HPLC) system. Mobile phase system was 0.1% phosphoric acid: MEOH in ratio

of 45:55 with flow rate 1.0 mL/min and detected at UV 366 nm. CAE was dissolved in ethanol at a concentration of 10 mg/mL. Standard kaempferol was diluted to various concentration for calibration curve. CAE or kaempferol (20 µL) was injected. The areas under the curve of each extract was compared with calibration curve of kaempferol.

Determination of cytotoxicity

Macrophages (RAW264.7 cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% antibiotic-antimycotic solution and 10% fetal bovine serum (FBS). Cells (1×10^4 cells/well) were seeded into 96-well plate and incubated at 37°C, 5% CO_2 for 24 h. Extracts were diluted with culture media and then added (100 µL) into the cells. After incubation for 24 h, the cell viability was analysed by using MTT assay (Mosmann, 1983). The results were calculated for cell viability and expressed a cytotoxicity as IC_{50}.

Determination of NO production

RAW264.7 (1×10^5 cells/well) were seeded into 96-well plate and incubated at 37°C, 5% CO_2 for 24 h. Extracts were diluted with culture media in the absence or presence of 10 µg/mL lipopolysaccharides (LPS) and then added (100 µL) into the cells. After incubation for 24 h, 50 µL of culture media was pipetted to mix with 50 µL of Griess reagent (1% Sulfanilamide: 0.01% N-1-napthylethylenediamine dihydrochloride in ratio of 1:1 under acidic condition). The pink color of reaction was measured absorbance at 540 nm. The results were compared between with or without LPS induction and then calculated for NO inhibition. The results were expressed as IC_{50}.

Determination of form cell formation

oxLDL, a modified LDL, is highly up-taken by macrophages though the action of scavenger receptors. Then the cells become lipid-loaded foam cells which is a hallmark of atherosclerotic disease. Therefore, in order to induce foam cell formation, oxLDL (Invitrogen, USA) was used in this study. RAW264.7 cells (2×10^4 cells/well) were cultured in 96-well plate by using serum-free DMEM and incubated at 37°C, 5% CO_2 for 24 h. The experiment was divided into 2 conditions including the absence or presence of oxLDL condition. The oxLDL was diluted with serum-free medium to concentration of 100 µg/mL. In the presence of oxLDL condition, cell culture medium was removed and then added with 50 µL of 50 µg/mL oxLDL whereas in the absence of oxLDL the serum-free medium (50 µL) was added instead. CAE or simvastatin (Svt) was diluted with serum-free medium and then added (50 µL) into the cells in both absence or presence of oxLDL condition. After incubation at 37°C, 5% CO_2 for 24 h, the cell culture medium was removed for further TNF-α measurement. The treated cells were analysed for lipid accumulation in foam cells by using Oil Red O staining (Zhang, 2015). The stained cells were photographed.

Determination of TNF-α production

The treated culture medium was measured by using enzyme-linked immunosorbent assay (ELISA) kit for mouse TNF-α quantification. TNF-α production was analysed as described in DuoSet® ELISA kit standard protocol.

Statistical analysis

The results in this study were performed in triplicate and represented in mean ± S.D. Significant difference was p value ≤ 0.05. One-Way ANOVA and multiple comparison (LSD) were used for statistical analysis by using SPSS.

Result and Discussion

The results of antioxidative activity and chemical properties of extracts in Table 1 showed that the difference in leaf age and harvesting season affected to phytochemicals in leaves resulting in difference in antioxidative activity. Mostly leaf extracts harvested in dry season showed higher antioxidative activity, phenolics and flavonoids than those of leaf extracts harvested in rainy season. Although the extracts did not show so strong antioxidative activity, aging leaf extracts showed higher antioxidative activity than those of mature leaf and young

leaf extracts, respectively. For total phenolic and flavonoid contents, the extracts showed slightly different except mature leaf extract harvested in rainy season that contained higher total flavonoid content than the others. In this study, young leaf extract harvested from rainy season contained higher amount of kaempferol (3.14 ± 0.02 µg/10 mg extract) than the other extracts. Leaves harvested from dry season, except aging leaf, had lower amount of kaempferol than leaves harvested from rainy season. These results indicated that there was variation in kaempferol and flavonoid contents in *C. aconitifolius* leaf but it tended to decrease upon aging and environmental stress. Stage of maturity is an important factor that influences phytochemicals in plant leaves (Agamou *et al.*, 2015; Nobossé *et al.*, 2018). Environmental stress (e.g. heat stress, moisture stress, UV radiation) affects to biosynthesis and accumulation of phytochemicals in plant in order to defense the stresses such as phenolic compounds (Ncube *et al.*, 2011; Daniels *et al.*, 2015). For example, young *Cosmos caudatus* aqueous leaf extracts showed higher antioxidant activity than mature and old leaves (Dian-Nashiela *et al.*, 2015). Whereas, aqueous extract of *Moringa oleifera* mature leaves showed higher antioxidant activity than young leaves (Sreelatha and Padma, 2009). And study in *Aquilaria beccariana* leaf extract, level of phenolic and flavonoids in mature leaves of *A. beccariana* was higher than the young and old leaves but there was no significant difference between phenolic and flavonoids in mature leaves, whereas, level of phenolic in the young and old leaves was higher than the flavonoids (Anwar *et al.*, 2017). Ben Ahmed and colleagues (2017) reported that secondary metabolite content and antioxidant activities of *Pistacia atlantica* leaves were more influenced by harvest time and growing region. They found that total phenolic content and reducing power capacity of *P. atlantica* leaf extracts were decreasing from spring to autumn whereas flavonoids, tannins and radical scavenging activity did not show a seasonal pattern (Ben Ahmed *et al.*, 2017). In the study of seasonal variation in leaves of *Cyclocarya paliurus*, it was found that *C. palliurus* leaf extracts showed variation in phenolic content and antioxidant activity throughout the year but that leaf extract that harvested from early November, Autumn, in Nanjing, China had highest phenolic compounds and antioxidant activity (Cao *et al.*, 2019). Similar with our research, mostly leaf extracts harvested in dry season showed higher antioxidative activity, total phenolics and total flavonoids although variation in phytochemical contents in leaf were found.

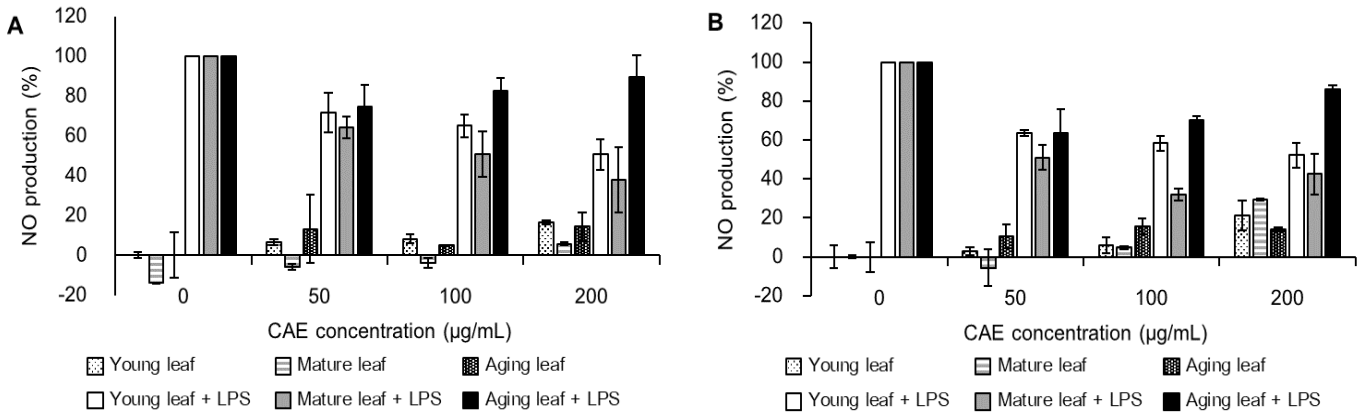

Figure 2 Effect of CAE on NO production from RAW264.7 cells in the absence and presence of LPS. (A) CAE from rainy season harvested leaves, (B) CAE from dry season harvested leaves.

Table 1 Antioxidative activity and phytochemical properties of extracts.

Extracts/Standard compounds		DPPH assay (IC_{50}, mg/mL)	FRAP (mg GAE/g)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QCE/g)	Kaempferol (µg/10 mg)
Leaves	**Season**					
Young	Rainy	4.55 ± 0.37^{a}	0.83 ± 0.22^{a}	21.08 ± 2.21^{a}	16.09 ± 2.62^{a}	3.14 ± 0.02^{a}
	Dry	3.69 ± 0.09^{b}	2.03 ± 0.09^{b}	25.43 ± 3.99^{ab}	17.98 ± 3.48^{a}	1.84 ± 0.13^{b}
Mature	Rainy	3.25 ± 0.06^{c}	1.14 ± 0.44^{a}	24.57 ± 2.31^{ab}	24.80 ± 3.35^{b}	2.72 ± 0.54^{ac}
	Dry	2.50 ± 0.07^{d}	2.88 ± 0.35^{c}	28.20 ± 3.11^{b}	16.03 ± 2.08^{a}	2.49 ± 0.00^{c}
Aging	Rainy	2.73 ± 0.04^{d}	1.85 ± 0.57^{b}	25.72 ± 1.78^{b}	14.49 ± 4.35^{a}	1.83 ± 0.01^{bd}
	Dry	2.09 ± 0.05^{e}	3.22 ± 0.26^{c}	26.45 ± 1.10^{b}	16.26 ± 1.33^{a}	2.24 ± 0.50^{bcd}
Gallic acid (µg/mL)		14.70 ± 0.76	ND	ND	ND	ND
Ascorbic acid (µg/mL)		16.59 ± 0.76	ND	ND	ND	ND

Note: ND is no determination.

a-e represents significant difference between extracts in each parameter ($p \leq 0.05$).

Table 2 Effect of extracts on cytotoxicity and inhibition of NO production.

Extracts		Cytotoxicity (IC_{50}, µg/mL)	NO production (IC_{50}, µg/mL)
Leaves	**Season**		
Young	Rainy	530.75 ± 67.89^{a}	186.54 ± 29.32^{a}
	Dry	539.04 ± 131.43^{a}	179.90 ± 31.32^{a}
Mature	Rainy	479.89 ± 61.10^{a}	141.05 ± 37.47^{a}
	Dry	489.54 ± 136.48^{a}	66.16 ± 1.13^{b}
Aging	Rainy	>800	>200
	Dry	>800	>200

Note: a-b represents significant difference in IC_{50} between extracts in each column ($p \leq 0.05$).

In Table 2, the aging leaf extracts showed lower cytotoxicity against RAW264.7 cells. And mature leaf extracts showed higher cytotoxicity than those of young leaf extracts. Based on its cytotoxic results, concentration of extracts (lesser than 200 µg/mL) was used afterward. In the absence of LPS, CAE at 50-100 µg/mL did not induce NO production from RAW264.7 cells but CAE at 200 µg/mL stimulate NO production from cells (Figure 2). In the presence of LPS, leaf extracts harvested in dry season suppressed NO production greater than those of leaf extracts harvested in rainy season (Figure 2 and Table 2). Mature leaf extract harvested in dry season exhibited higher NO inhibitory activity (IC_{50} of 66.16 ± 1.13 µg/mL) than young and aging leaf extracts, therefore, this extract was selected for further studies on foam cell formation and cytokine production. These results correlated with the effect of harvesting season on phytochemicals (Table 1) which might be due to plant response against environmental stress (Ncube *et al.*, 2011; Daniels *et al.*, 2015). Flavonoids are well known to have anti-inflammatory activity and immunomodulation. Flavonoids, particularly kaempferol and their glycosides, can potently scavenge reactivity of ROS and NO and also inhibit inducible NO synthase gene expression and NO production in both *in vitro* and *in vivo* (Kim *et al.*, 2005; Kim *et al.*, 2015; Wang *et al.*, 2018; Rostoka *et al.*, 2010). The inhibitory effect of CAE in this study may due to the activity of antioxidants and flavonoids in the extracts.

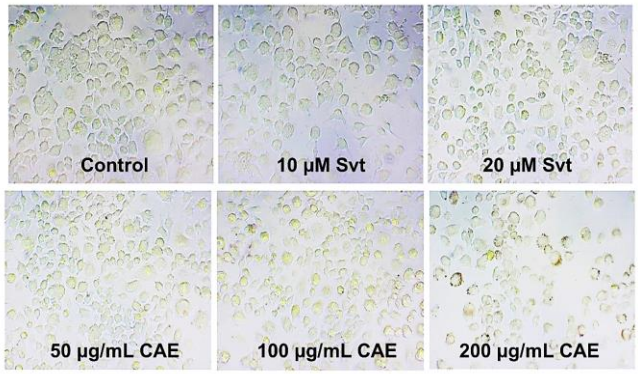

Figure 3 Effect of CAE and Svt on foam cell formation in the absence of oxLDL.

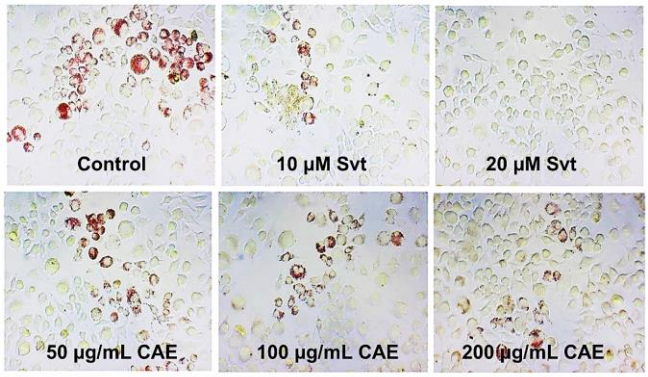

Figure 4 Effect of CAE and Svt on foam cell formation in the presence of 50 µg/mL oxLDL.

Without oxLDL induction, 10 and 20 µM Svt and CAE at concentration of 50 and 100 µg/mL did not induce lipid accumulation in treated cells. However, 200 µg/mL CAE slightly induced lipid accumulation (Figure 3). Whereas, addition of oxLDL resulted in lipid accumulation in RAW264.7 cells (Figure 4). In oxLDL and Svt co-treatment, the lipid accumulation was decreased in dose dependent manner compared with oxLDL alone. Mature leaf extract, CAE, slightly reduced lipid accumulation in foam cells compared with oxLDL alone (Figure 4). This result may support the previous *in vivo* studies. Ethanolic and aqueous extracts of *C. aconitifolius* leaves (400 mg/kg BW) decreased cholesterol, triglyceride and LDL in plasma of hypercholesterolemic rabbits (Olaniyan *et al.*, 2017). Similarly, methanolic extract of *C. aconitifolius* leaves (500 mg/kg BW) reduced cholesterol and triglyceride in streptozotocin induced diabetic rats (Achi *et al.*, 2017), whereas, aqueous extract from this plant leaves could also lower cholesterol in hypercholesterolemic mice (Miranda-Velasquez *et al.*, 2010). Kaempferol was reported to inhibit oxLDL uptake by macrophages. Kaempferol and kaempferol 7-O-methyl ether decreased lipid accumulation and increased cholesterol efflux from THP-1-derived macrophages by reducing gene expression and protein level of cell surface receptor, CD36, resulting in decreasing of foam cell formation (Tu *et al.*, 2007; Li *et al.*, 2013). Kaempferol was also found in *C. aconitifolius* leaf extract in this study and it might be a bioactive compound that played role in this inhibitory activity. However, kaempferol amount in the CAE was very small compared with the total flavonoid content. Therefore, it is possible that kaempferol or other flavonoids in CAE might play an important role in suppression of ox-LDL-induced foam cell formation in RAW264.7 cells in this study.

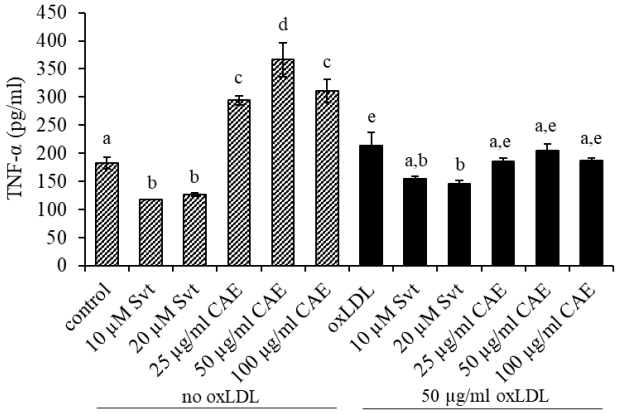

Figure 5 Effect of CAE and Svt on TNF-α production in the absence or presence of oxLDL.
When a-e show significant difference of TNF-α production from the cells ($p \leq 0.05$).

In TNF-α producing study, oxLDL could significantly increase the production of TNF-α compared with control (Figure 5). Svt could significantly decrease this cytokine production in both with and without oxLDL treatments. CAE slightly decreased TNF-α production compared with oxLDL treatment alone. Lipid accumulation in macrophage results in lipid-laden foam cell formation leading to pro-inflammatory cytokine releasing (e.g. IL-1β, IL-6, TNF-α) and further promote inflammation (Nguyen *et al.*, 2019). CAE might

decrease lipid accumulation and TNF-α production via its anti-inflammatory activity (Onasanwo *et al.*, 2011). There were reports in anti-inflammatory activity of *C. aconitifolius* and *C. chayamansa* leaf extracts and their phytochemicals such as terpenoids, flavonoids and polyphenols in the plant extracts play role in this anti-inflammatory activity (Pérez-González *et al.*, 2017; Pérez-González *et al.*, 2018). Kaempferol was reported to have *in vitro* and *in vivo* anti-inflammatory activity by direct suppression upstream signaling and activation of NF-ΚB and AP-1 that involved with inflammation (Kim *et al.*, 2015; Kadioglu *et al.*, 2015). Phenolics, flavonoids and/ or kaempferol in CAE might suppress ox-LDL-induced TNF-α production that involved with their anti-inflammatory activity. However, we also found that CAE increased TNF-α and NO production in the absence of oxLDL and LPS, respectively. Although it is still unclear, there were possibly some phytochemicals in CAE that might induce the production of TNF-α and NO. In the presence of ox-LDL, cells increased AP-1 levels but did not increase NF-ΚB level (Ares *et al.*, 1995). The oxidation of LDL is associated with enhancing of macrophage to release TNF-α that is mediated by AP-1 activation. Moreover, oxidation of LDL is associated with reactive oxygen species, therefore, the increasing of NO may contribute with AP-1 activation and lead to increasing of TNF-α production. It is possible that some phytochemicals in the extract may directly activate NO and TNF-α production. Further molecular studies are needed for better understanding.

Conclusion

In conclusion, results in this study indicate phytochemical difference influencing from harvesting season and age stage of *C. aconitifolius* leaves. In addition, CAE has antioxidative activity, NO inhibitory activity in the presence of LPS and also shows inhibitory effect on foam cell formation and TNF-α production in oxLDL induction. However, TNF-α and NO productions were increased in the absence of stimuli. These effects are still unclear, therefore, consumption of high amount of *C. aconitifolius* leaves should be careful. In addition, further molecular studies are needed to explain the mechanism of action.

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