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Original Article

In vitro Study of *Cnidoscolus aconitifolius* Leaf Extracts on Foam Cells and their Antioxidant

Bung-on Prajanban[1,*], Niramai Fangkrathok[2]

ABSTRACT

Background: Internalization of oxidized low-density lipoprotein (oxLDL) by macrophages results in lipid-laden foam cell transformation and pro-inflammatory cytokine releasing leading to atherosclerosis which is one of chronic inflammatory diseases. Leaf extracts from Chaya or *Cnidoscolus aconitifolius* (Mill.) I. M. Johnst. were reported to show a potential effect on levels of lipids and lipoproteins in *in vivo*. **Objectives:** This study was to determine the biological activities of crude ethanolic extracts from Chaya leaves on foam cell formation and inflammation. **Materials and Methods:** Extracts of age different leaves were screened the contents of phenolics and flavonoids, antioxidant, cytotoxicity and nitric oxide production by using macrophages (RAW264.7). The effective extract was selected to study on the formation of foam cells, protein production and TNF-α gene expression. **Results:** All extracts showed antioxidative activity and less cytotoxicity to the cells. A mature leaf extract (Ma-CAE) significantly reduced nitric oxide production (IC_{50} of 235.23 ± 21.29 µg/mL). For foam cell formation, Ma-CAE slightly reduced oxLDL-induced foam cell formation. Co-treatment of oxLDL, LPS and Ma-CAE decreased the level of TNF-α production. In addition, co-treatment of 800 µg/mL Ma-CAE and oxLDL as well as co-treatment of 800 µg/mL Ma-CAE, oxLDL and LPS, significantly decreased mRNA levels of TNF-α genes. **Conclusion:** These results indicated that Chaya leaf extracts could slightly decrease foam cell formation and also reduce pro-inflammatory mediators which may further decrease foam cell formation.

Key words: *Cnidoscolus aconitifolius*, Anti-inflammation, oxLDL, TNF-α, Foam cells.

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INTRODUCTION

Atherosclerosis is an important chronic disease that inflammatory process plays role in the pathogenesis. This disease leads several causes of death worldwide such as cardiovascular diseases and ischemic stroke. Low-density lipoprotein (LDL) uptake by monocytes and macrophages leads to atherosclerotic lesions.[1] A foam cell formation is an important process of atherosclerotic development and progression. Macrophages internalize the oxidized LDL (oxLDL) via their scavenger receptors causing the foam cells and subsequently release the pro-inflammatory mediators including reactive oxygen species, nitric oxide (NO) and cytokines such as tumor necrosis factor-α (TNF-α).[2] oxLDL causes the direct and indirect impairments of constitutive release of NO and emphasizes the overexpression of inducible nitric oxide synthase. This effect was combined with overproduction of ROS leading to endothelial cell dysfunction and following development of atherosclerosis.[3] Macrophages uptake oxLDL resulting in foam cell formation, increasing of TNF-α production[4] and enhancing of LPS-induced TNF-α gene expressions.[5] Pro-inflammatory cytokines especially TNF-α are important factors in the atherosclerotic pathogenesis.[6] Decreasing of TNF-α gene expression has been proposed as a therapeutic target for atherosclerosis or inflammatory diseases. Therefore, inhibition of inflammation and oxLDL-induced lipid deposition in macrophages may be a beneficial strategy for prevention of atherosclerosis.[7]

Cnidoscolus aconitifolius (Mill.) I. M. Johnst. Belonging to Euphorbiaceae, commonly called "Chaya", has been used as a leafy vegetable food and a medicinal plant. In Mexico, *C. aconitifolius* has been used as a traditional medicine for several diseases including high blood pressure and diabetes mellitus.[8] It has high nutritional and phytochemical values as well as medicinal potential. Many phytochemicals including phenols, flavonoids, alkaloids, terpenoids, saponins, phlobatannins, tannins, anthraquinones, terpenes, cardiac glycosides and cyanogenetic glycoside have been found in *C. aconitifolius* leaves.[9,10] Phytochemicals from *C. aconitifolius* leaves were reported to have antioxidative,[11] anti-diabetic,[11,12] and anti-inflammatory activities,[13] as well as to reduce the damage of liver and kidney.[14] Furthermore, *C. aconitifolius* was reported to have anti-hypercholesterolemic and anti-hypertriglyceridemic activities in *in vivo*.[15-17] Moreover, the ethanol extract of Chaya leaves increased the HDL level and reduced the LDL,

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VLDL, cholesterol and triglyceride levels in the studied animal serum.[18] It also showed a strong cardioprotective activity and was safe for animals.[19]

Therefore, the phytochemical properties and biological activities of an ethanol extract of *C. aconitifolius* leaves on cytotoxicity, antioxidants, NO production, foam cell formation, TNF-α production and gene expressions of TNF-α were investigated.

MATERIALS AND METHODS

Plant collection and extract preparation

C. aconitifolius leaves were obtained from Burapha University Sakaeo Campus, Wattana Nakorn District, Sa Kaeo Province and surrounding village area (May 2019). This plant was identified and collected as a specimen (voucher specimen No. CN013) by Dr. Chakkrapong Rattamanee, a botanist and lecturer, and kept at the Faculty of Agricultural Technology, Burapha University Sakaeo Campus. The leaves were collected from 3 parts including young or immature leaves (5 leaves on the top part of branch), mature leaves (5 leaves lower than the young leaves) and aging leaves (5 leaves lower than the mature leaves). The leaves (without petiole) were cooked in boiling water for 10 min in order to eliminate toxic hydrocyanic glycosides.[20] The cooked leaves were dried at 60°C for 2 days by using a hot air oven. The dried leaves were blended and macerated in 50% ethanol (ratio of 1:5) for 7 days. After filtration, the remaining debris from extraction was processed again by maceration in 50% ethanol (ratio of 1:5) for 7 days. Extracted liquid was combined, evaporated and then dried by using a freeze dryer. The *C. aconitifolius* extracts were called Im-CAE, Ma-CAE and Ag-CAE for immature leaves, mature leaves and aging leaves, respectively. These extracts gave the yields of 17.34%, 19.69% and 16.34%, respectively.

Total phenolic content

Dissolved extracts (100 µL) and Folin-Ciocalteu reagent (200 µL) were mixed in the test tubes and then added with 2 mL of distilled water. After 3 min, solution was added to 20% w/v sodium carbonate (1 mL) and then incubated at room temperature for 1 hr. The solution absorbance was measured at 765 nm.[21] The results of gallic acid was calculated and plotted a standard curve. The mg gallic acid equivalent/g extract (mg GAE/g) of the extracts were calculated.

Total flavonoid content

Dissolved extracts (100 µL) were added into a 96-well plate. The solution was combined with 2% w/v aluminium chloride (50 µL) and ethanol (50 µL) and then incubated at room temperature for 10 min without light exposure. The solution absorbance was analysed at wavelength of 425 nm. This method was modified from.[22] The extract results were calculated by comparing with a quercetin standard curve. The mg quercetin equivalent/g extract (mg QCE/g) of the extracts were calculated.

Radical scavenging assay by using 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The 0.5 mM DPPH was freshly prepared in ethanol. Each extract was dissolved and diluted in ethanol. Diluted extract (1 mL) was added into DPPH solution (1 mL). The solution was incubated at room temperature for 10 min without light exposure. Mixture solution absorbance was measured at wavelength of 517 nm. This method was modified from.[21] The results were calculated for DPPH inhibition (%) and expressed as 50% inhibitory concentration (IC_{50}). The standard compounds, ascorbic acid and gallic acid, were calculated for IC_{50} and used for effective comparison.

Ferric reducing antioxidative power (FRAP) assay

Each extract was diluted in ethanol and then pipetted (20 µL) into a 96-well plate. The FRAP reagent in this study was the mixture (ratio of 10:1:1) of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM) in HCl (40 mM) and $FeCl_3$ (20 mM) in HCl (20 mM). FRAP reagent (180 µL) was subsequently added. The solution was incubated at room temperature for 10 min without light exposure. Mixture solution absorbance was analysed at wavelength of 593 nm. Extract solution absorbance (no FRAP) was also measured as a background. This method was modified from.[21] The gallic acid result was calculated and plotted a standard curve. The extract results were subtracted its background and calculated by comparing with the standard curve. The mg gallic acid equivalent/g extract (mg GAE/g) of the extracts were calculated.

Determination of cytotoxicity

Macrophages in this study was RAW264.7 cell line which was purchased from Biomedia Thailand Co. Ltd., Thailand and was ATCC® No. TIB-71T™. The cells were cultured in 1% antibiotic-antimycotic solution and 10% fetal bovine serum-contained Dulbecco's Modified Eagle Medium (DMEM). The cells were harvested and seeded into 96-well plate (1×10^4 cells/well) and then incubated in CO_2 incubator at 37°C for 24 hr. Extracts were diluted with culture media, added (100 µL) into the cells and then incubated for 24 hr. MTT assay was used to analysed a cell viability[23] and the extract results were calculated for cytotoxicity and then expressed as IC_{50}.

Determination of NO production

RAW264.7 (1×10^5 cells/well) were cultured in 96-well plate and incubated in CO_2 incubator at 37°C for 24 hr. Each extract was diluted with culture media in the absence or presence of 10 µg/mL lipopolysaccharides (LPS) and then added (100 µL) into the cells and incubated in CO_2 incubator at 37°C for 24 hr. Griess reagent was freshly prepared by using the mixture of sulfanilamide (in 5% phosphoric acid) and 0.01% N-1-napthylethylenediamine dihydrochloride (in distilled water) with a ratio of 1:1. The culture media (50 µL) was pipetted to mix with Griess reagent (50 µL). The reaction absorbance was analysed at wavelength of 540 nm.[24] The results from the presence of LPS were subtracted by the absence of LPS and then calculated for NO inhibition and expressed as IC_{50}.

Determination of foam cell formation

RAW264.7 cells were harvested, adjusted to 2×10^4 cells/well and then seeded into 96-well plate by using serum-free DMEM. The cells were incubated in CO_2 incubator at 37°C for 24 hr. The experiment was divided into 2 conditions of either the absence or presence of oxLDL condition. oxLDL (Invitrogen, USA) was used to induce foam cell formation. The 100 µg/mL oxLDL was diluted in serum-free medium. For oxLDL induction, cell culture medium was removed and then replaced with 50 µL of 50 µg/mL oxLDL, whereas, in the absence of oxLDL the serum-free medium (50 µL) was added instead. Ma-CAE or simvastatin (Svt) was diluted with serum-free medium and then added (50 µL) into the cells in both the absence and presence of oxLDL conditions. After 24 hr incubation, the cell culture medium was removed for further TNF-α production measurement. The treated cells were stained with Oil Red O for lipid accumulation in foam cells.[25] These stained cells were photographed.

Real-Time PCR

The treated cells were harvested and then extracted for total RNA by using Total RNA Kit (E.Z.N.A.®, Omega Bio-tek, GA). The extracted RNA was pre-treated with DNase I. Oligo-dT primers and

FIREScript RT cDNA synthesis kit were used to synthesize the cDNA. Hot FIREPol® EvaGreen ® qPCR Mix Plus (no ROX) was used to prepared the sample. The sample was run on Bio-Rad CFX96 Touch Real-Time PCR Detection System. The TNF-α forward primer, 5′ATGAGCACAGAAAGCATGATC-3' and TNF-α reverse primer, 5'-TACAGGCTTGTCACTCGAATT-3' were used. For β-actin, 5'-TCATGAAGTGTGACGTTGACATCCGT-3' and 5'-CCTAGAAGCATTTGCGGTGCACGATG-3' were used as the forward and reverse primers, respectively.[26] β-actin, a housekeeping gene, was used as an internal control. According to manufacturing protocol, the 20 µL of polymerase chain reaction (PCR) was contained with 10 pmol each forward and reverse PCR primers, 2.5 ng of sample cDNA and 5X HOT FIREPol® EvaGreen ®qPCR Mix Plus. The reaction was performed in triplicate. The genes were amplified by using real-time PCR with amplifying process; initial activation at temperature of 95°C for 12 min, 40 cycles of denaturation at temperature of 95°C for 15 sec, annealing at temperature of 54°C for 20 sec and elongation at temperature of 72°C for 20 sec. The $2^{-\Delta\Delta Ct}$ method was used to calculated for fold changes of gene expression. The calculation methods were as follows:[27]

$$\Delta CT = CT_{(target)} - CT_{(ref.)}$$

$$\Delta\Delta CT = CT_{(test)} - CT_{(control)}$$

Moreover, 1 µL of each cDNA sample as a template was mixed with Tag DNA polymerase recombinant (Invitrogen, Brazil), deoxynucleotide mix and each primer. Amplification was completed using 30 cycles and the PCR amplification followed as described in Tag DNA polymerase recombinant protocol (Invitrogen, Brazil). The PCR products were then confirmed by using 1.5% agarose gel electrophoresis, using FluoroVue™ nucleic acid gel staining (SMOBIO Technology, Inc., Taiwan).

Determination of TNF-α production

The TNF-α production releasing in treated culture medium was measured by using enzyme-linked immunosorbent assay (ELISA) kit. 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. The *p* value lesser than 0.05 showed a significant difference. One-Way ANOVA and Fisher's least significant difference were used for statistical analysis by using SPSS.

RESULTS

Total phenolic and flavonoid contents and antioxidative activity of Im-CAE, Ma-CAE and Ag-CAE

After antioxidative activity and phytochemicals of the extracts were investigated, Ag-CAE had higher phenolic (32.30 ± 0.46 mg GAE/g) and flavonoid contents (21.41 ± 2.49 mg QCE/g) than Ma-CAE and Im-CAE. Moreover, Ag-CAE showed higher antioxidative activity by using DPPH (IC_{50} of 2.27 ± 0.02 µg/mL) and FRAP (7.68±0.15 mg GAE/g) than Ma-CAE and Im-CAE (Table 1).

Cytotoxicity of Im-CAE, Ma-CAE and Ag-CAE and nitric oxide production

In LPS induction, the Im-CAE and Ma-CAE at concentrations of 50-800 µg/mL showed very low cytotoxicity against RAW 264.7 cells (Figure 1). Ag-CAE at concentrations of 50-400 µg/mL showed low cytotoxicity but Ag-CAE at concentration of 800 µg/mL slightly increased cytotoxicity on cells (Figure 1). The Im-CAE and Ma-CAE strongly suppressed NO production whereas Ag-CAE slight reduced NO production (Figure 1). Ma-CAE had higher anti-oxidative activity and phytochemical contents than Im-CAE and Ma-CAE, therefore, Ma-CAE was selected for further studies on foam cell formation, gene expression and cytokine production.

Foam cell formation

Without oxLDL induction, Ma-CAE at low concentration and 40 µM Svt did not induce lipid accumulation in treated cells. However, 400 µg/mL Ma-CAE slightly induced lipid accumulation. Whereas oxLDL addition resulted in lipid accumulation in RAW264.7 cells. In oxLDL and Svt co-treatment, the lipid accumulation was decreased when compared with oxLDL treatment alone. Co-treatment of oxLDL and Ma-CAE (100-200 µg/mL) slightly reduced lipid accumulation in foam cells but 400 µg/mL Ma-CAE decreased lipid accumulation in foam cells comparing with oxLDL treatment alone (Figure 2).

Effects of Ma-CAE on oxLDL and LPS-induced TNF-α gene expression

In Figure 3, without oxLDL induction, Ma-CAE at the concentration of 400-800 µg/mL showed significantly increasing of TNF-α gene expression. In oxLDL induction alone increased the TNF-α expression. In the presence of oxLDL, 40 µM Svt and 400 µg/mL Ma-CAE significantly

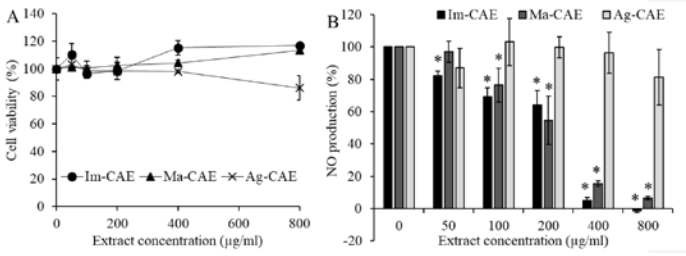

Figure 1: The effect of Im-CAE, Ma-CAE and Ag-CAE extracts on the cell viability (A) and NO production of RAW264.7 cells in the presence of LPS (B). * represents significant difference when compared with control ($p < 0.05$).

Table 1: Antioxidative activity and phytochemical properties of Im-CAE, Ma-CAE and Ag-CAE extracts.

Extracts/compounds	Antioxidative activity		Total phenolic content (mg GAE/g)	Total flavonoid content (mg QCE/g)
	DPPH assay (IC_{50}, mg/mL)	FRAP (mg GAE/g)		
Im-CAE	4.01 ± 0.14	3.39 ± 0.04	23.48 ± 0.44	10.95 ± 0.79
Ma-CAE	2.69 ± 0.06	4.86 ± 0.09	27.79 ± 0.28	18.42 ± 1.09
Ag-CAE	2.27 ± 0.02	7.68 ± 0.15	32.30 ± 0.46	21.41 ± 2.49
Gallic acid (µg/mL)	14.70 ± 0.76	ND	ND	ND
Ascorbic acid (µg/mL)	16.59 ± 0.76	ND	ND	ND

Note: ND represents no determination.

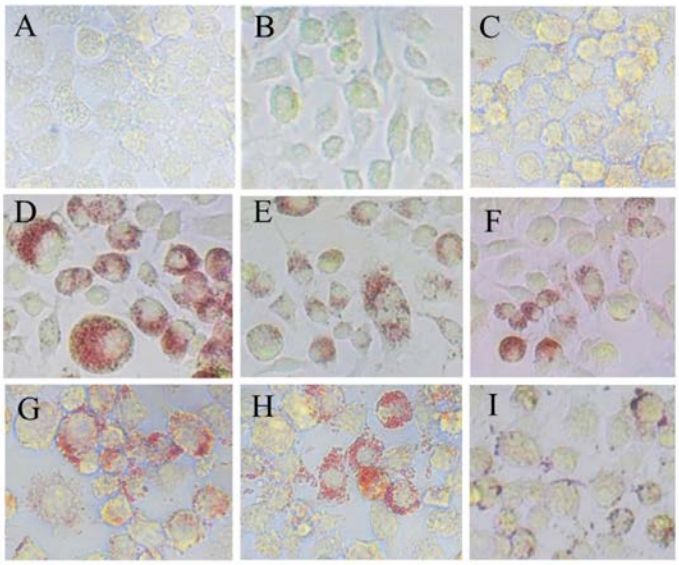

Figure 2: Effect of Ma-CAE and Svt on foam cell formation in the presence or absence of 50 µg/mL oxLDL. A) Control, B) 40 µM Svt, C) 400 µg/mL Ma-CAE, D) 50 µg/mL oxLDL, E) 20 µM Svt + oxLDL, F) 40 µM Svt + oxLDL, G) 100 µg/mL Ma-CAE+oxLDL, H) 200 µg/mL Ma-CAE + oxLDL, and I) 400 µg/mL Ma-CAE + oxLDL. Red color in the cells indicate lipid droplet in foam cells.

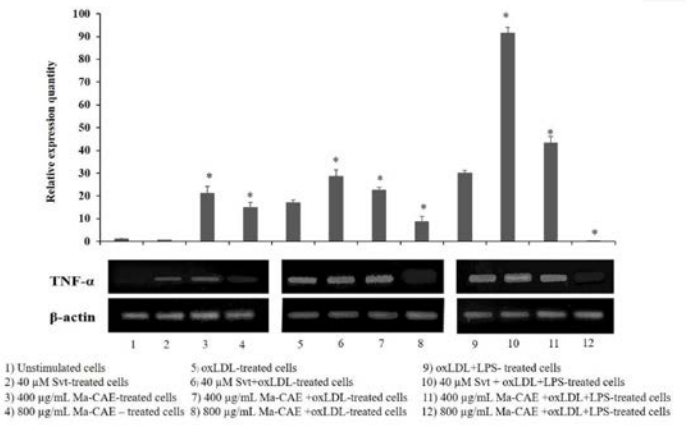

Figure 3: The effect of Ma-CAE and Svt on oxLDL and oxLDL+LPS induced TNF-α expression in RAW 264.7 cells. * represents significant difference between control group of each treatment ($p < 0.05$).

increased the TNF-α expressions whereas 800 µg/mL Ma-CAE caused significant decreasing in the TNF-α expression. Co-treatment of oxLDL and LPS increased the gene expression levels of TNF-α. In the presence of oxLDL and LPS, Ma-CAE (400 µg/mL) as well as Svt (40 µM) caused significant increasing in TNF-α gene expression. Interestingly, 800 µg/mL Ma-CAE significantly decreased the TNF-α gene expressions in dose-dependent manners.

TNF-α production

To confirm the gene expression results, TNF-α production was analysed. With or without oxLDL induction, 40 µM Svt and Ma-CAE (400-800 µg/mL) elevated the TNF-α production when compared with the control (Figure 4). However, in oxLDL and LPS co-treatment, 40 µM Svt and Ma-CAE (400-800 µg/mL) decreased the production of TNF-α (Figure 4).

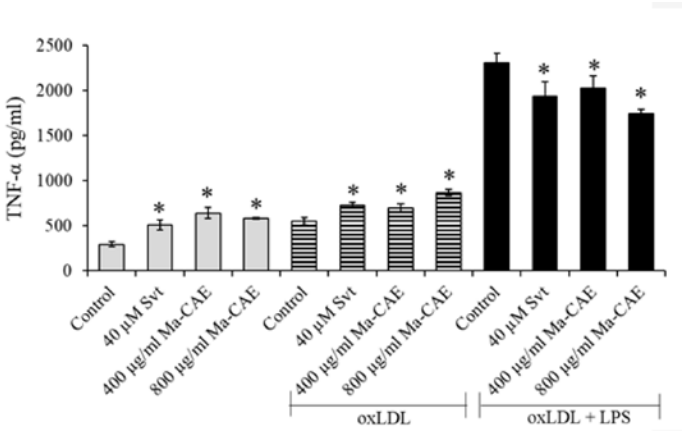

Figure 4: Effect of Ma-CAE and Svt on TNF-α production in the absence or presence of 50 µg/mL oxLDL and in the 50 µg/mL oxLDL and 0.5 µg/mL LPS co-treatment. * represents significant difference between control and treatment in each condition.

DISCUSSION

C. aconitifolius leaves contain many phytochemicals which have been reported to have several pharmaceutical effects. The difference in leaf age affects to active biosynthesis and accumulation of secondary metabolites in leaves resulting in the difference of phytochemical contents and antioxidative activities.[28-31] In this study, Ag-CAE contained the highest total contents of phenolics and flavonoids and showed the highest antioxidative activities, whereas, Im-CAE had the lowest total contents of phenolics and flavonoids and showed the lowest antioxidative activities. The young stage leaf extract of *C. chayamansa* was previously reported to contain both phenolics (71.3 ± 1.7 mg gallic acid equivalent/g extract) and flavonoids (42.6 ± 3.7 mg (+)-catechin equivalent/g extract).[11] Although these phytochemical contents in this study were lesser than that of the previous study, the DPPH radical scavenging activity of the extracts in our study was higher than that previous report (5 mg/mL or 45.5% inhibition).[11] Also, in the present study, Ag-CAE and Ma-CAE contained high polyphenol content e.g. flavonoids and phenolics which might be the bioactive compounds in antioxidative activity of these extracts.

Phenolics and flavonoids are known to have inhibitory effect on NO production.[32-34] We found that the Ma-CAE showed lower total contents of phenolics and flavonoids than Ag-CAE but exhibited stronger inhibitory effect on NO production than that of Ag-CAE effect. Ag-CAE may contain the other phenolic compounds that did not inhibit NO production. There was a report that resveratrol, quercetin, epicatechingallate and epigallocatechin gallate significantly enhanced endothelial NO level, whereas, gallic acid did not increase the NO level on endothelial cells.[35] Antioxidative and NO inhibitory activities appear prominent of phenolics and flavonoids contained in Ma-CAE suggesting their potential for pharmacological application in the prevention or treatment inflammatory diseases, including atherosclerosis.

Ma-CAE and oxLDL co-treatment slightly reduced lipid accumulation in foam cells when compared with oxLDL treatment alone. Polyphenolics and flavonoids have a potential to delay LDL oxidation through their radical-scavenging activity.[36] The *C. chayamansa* extract that contained phytochemicals such as flavonoids, glycosides, and alkaloids showed significant hypolipidemic activity by reducing the LDL, VLDL, cholesterol and triglyceride levels in rat serum.[18] In addition, *C. aconitifolius* leaf extract decreased cholesterol, triglyceride and LDL in *in vivo*.[16,17]

TNF-α have been known as the major pro-inflammatory mediator on lipid metabolism[37] and inflammatory responses.[13] The oxLDL loaded macrophages enhanced LPS-induced TNF-α gene expressions.[5] In our study, 800 μg/mL Ma-CAE co-treated with oxLDL and LPS significantly decreased the TNF-α gene expression and protein production. In the oxLDL, LPS and 800 μg/mL Ma-CAE co-treatment suppressed TNF-α production to a lower degree than those of TNF-α gene expression. Moreover, in oxLDL and 800 μg/mL Ma-CAE co-treatment decreased TNF-α expression while it increased the TNF-α production. These results possibly caused of 1) a turnover rate of mRNA that few copies of mRNA can be translated to dozens of the corresponding protein per mRNA, 2) mRNAs are less stable than proteins, and 3) produced protein is secreted outside the cells and can be accumulated in culture media.[38] Therefore, the TNF-α can be possibly found in higher levels than gene expression levels. Ma-CAE might decrease lipid accumulation, TNF-α gene expression and TNF-α production via its phytochemical contents, antioxidative and anti-inflammatory activities.[13,39] Moreover, flavonoid content in Ma-CAE might play role in suppression of LPS-induced inflammatory gene expression.[34]

CONCLUSION

Leaf age difference of *C. aconitifolius* affected to phytochemical difference in its leaf extracts. Ma-CAE and Ag-CAE had higher phenolic and flavonoid contents than Im-CAE. All extracts showed antioxidative activity and less cytotoxicity to the tested cells. Im-CAE and Ma-CAE had high inhibitory activity on NO production. Ma-CAE showed slightly reducing activity on foam cell formation and could significantly decrease mRNA levels of TNF-α genes that correlated with decreased TNF-α production. Therefore, the Ma-CAE could be a good candidate for functional food development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Ag-CAE: Aging leaf- *C. aconitifolius* extract; **IC_{50}:** Inhibition concentration at 50%; **Im-CAE:** Immature leaf- *C. aconitifolius* extract; **LPS:** Lipopolysaccharide from *Escherichia coli*; **Ma-CAE**: Mature leaf- *C. aconitifolius* extract; **NO:** Nitric oxide; **oxLDL:** Oxidized low-density lipoprotein; **Svt:** Simvastatin; **TNF-α:** Tumor necrosis factor-α.

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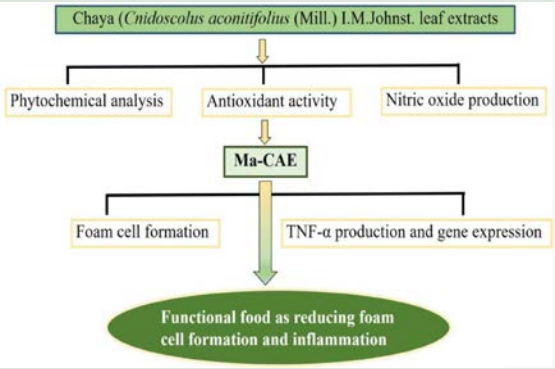
GRAPHICAL ABSTRACT



SUMMARY

This study revealed the suppressive effects of Chaya (*Cnidoscolus aconitifolius* (Mill.) I.M.Johnst.) leaf extracts on foam cell formation and inflammation. Mature leaf extract (Ma-CAE) showed higher antioxidative activity and phytochemicals content than immature leaf extract (Im-CAE) and higher inhibitory activity on nitric oxide production than aging leaf extract (Ag-CAE). Ma-CAE and oxLDL co-treatment slightly reduced lipid accumulation in foam cells when compared with oxLDL treatment alone. In addition, co-treatment of 800 µg/mL Ma-CAE, oxLDL and LPS caused significant decrease in gene expression and protein production of TNF-α. Therefore, the Ma-CAE could be a good candidate for functional food development.

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