ABSTRACT
Oxidative stress can induce vascular endothelial dysfunction in diabetic and hyperlipidemia patients. Leucaena leucocephala (Lam.) de Wit has been reported of possessing antioxidant, antidiabetic and anticholesterol activity; as well as the toxic substance called mimosine. Aims: To determine antioxidant effects of Leucaena leucocephala (Lam.) de Wit Leave extracts in oxidative stress induced vascular endothelial function. Methods and Material: Leave extracts were determined for antioxidant activity. Either or both of oxidized low density lipoprotein (oxLDL) and glucose were applied to induce oxidative stress condition in human umbilical vein cultured (HUVCs) to observe superoxide dismutase (SOD) activity, nitric oxide (NO) level and morphological changes. Results: Total polyphenol and flavonoid were 51.04 ± 0.91 mg GAE/g and 0.13 ± 0.01 mg catechin/g of dried weight (DW), respectively. Free radical reduction efficiency of crude extract observed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay showed IC$_{50}$ value of 329.6 μg of vitamin C equivalent/mg of extracts. Frap value was showed 428.54 ± 15.32 mM Fe(II) equivalent/g of DW. The result observing in HUVCS showed that comparing to the control, SOD activity, NO and MDA level were maintained in 0.05 mg/mL of L. leucocephala treated group, but NO and MDA level were lowered when comparing with oxLDL and glucose-induced oxidative stress. No change was observed in 0.05 mg/mL of L. leucocephala treated group, comparing with control group. Conclusion: This study has been performed to exhibit the antioxidant activity of L. leucocephala in endothelium functions and has been found to have an appropriate concentration at 0.05 mg/mL in reducing oxidative stress condition in impaired fasting blood glucose patients. Nevertheless, the optimal level for toxic activity in inhibition of cancer angiogenesis should be further investigated.

Key words: Leucaena leucocephala (Lam.), De Wit, Antioxidant activity, Nitric oxide, Superoxide dismutase, Vascular endothelial.

INTRODUCTION
Impaired fasting glucose (IFG) is a type of prediabetes that found in those with increasing of fasting blood glucose but not yet develop diabetes mellitus. Several studies have suggested that rising of blood glucose can be induced by the elevation of oxidative stress, through the production of reactive oxygen species (ROS) that influence insulin resistance and eventually result in beta-cell dysfunction and diabetes mellitus. The ROS are also initiate a chain reaction, and result in reduction of nitric oxide (NO) availability and presentation of several inflammation markers. Altogether, these events lead to dysfunction of endothelial cell and development of cardiovascular disease. Cardiovascular diseases (CVS) contribute to most noncommunicable diseases (NCDs) with 17.5 million in year 2012, and was predicted to increase to 25.2 million in year 2035. It is known that an impairment of NO production and scavenger enzyme such as superoxide dismutase (SOD) can be resulting in oxidative stress. This event is eventually leading to endothelial dysfunction, and tissue injury of vascular cells. Endothelial dysfunction can be found in patients with diabetes, hyperlipidemia, and atherosclerosis and hypertension. Other than NO and SOD, the reported potential biomarkers of endothelial dysfunctions is oxidized low-density lipoprotein (oxLDL), as well as malondialdehyde (MDA) that have been expressed in vascular diseases. Several Thai traditional herbal medicines have been suggested as providing antioxidant activities, and one of them are Leucaena leucocephala (Lam.) de Wit. L. leucocephala has been reported of processing antioxidant activities by providing important substances such as flavonoids, terpenes and coumarins, sterols. It has been reported for lowering blood glucose, blood pressure and NO level, and providing hypcholesteromic, anti-inflammatory and antioxidant activity. In previous studies, glucose and LDL can induce endothelial dysfunction of the vessels in diabetic and cardiovascular patients. Thus, optimal level of L. leucocephala may protect vascular cell through reduction of LDL and glucose.

oxidation. Altogether, it leads to objective of the study, to observe antioxidant activity of *Leucaena leucocephala* in endothelium function using impair fasting glucose in human umbilical vein cultured (HUVCs) model.

**MATERIAL AND METHOD**

**Extracts preparation**

Fresh leaves of *L. leucocephala* were collected from Sakolnakorn province, Thailand. Leaves were dried at 60°C and then immersed in 90% ethanol for 3 days. The solution was evaporated using rotary evaporator (BossTech, Scientific Instruments) followed by lyophilization. Prior to the experiment, leaf extract preparing in 50% ethanol was filtrated by membrane filter (0.45 µm-pore size) and diluted to different concentrations (0.05, 0.1, 0.5 and 1 mg/ml).

**Determination of antioxidant contents**

The antioxidant properties of total phenolic content (TPC) are potential for inhibiting lipid peroxidation. TPC concentration was estimated with Folin-Ciocalteu reagent using gallic acid as standard, described previously. Briefly, 10% Folin-Ciocalteu reagent and 10.75% Na₂CO₃ were mixed with *L. leucocephala*. TPC product was measured at 760 nm spectrophotometrically and was calculated from standard curve of gallic acid and expressed as mg GAE/g of sample dried weight. Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method according to Zhishen J et al. *L. leucocephala* was incubated in 5% NaNO₂ at room temperature for 5 min, and followed by 10% AlCl₃ for 5 min and 1M NaOH. The mixture was incubated at room temperature for 15 min and measured absorbance at 510 nm with UV-spectrophotometer. TFC was calculated from standard curve as catechin and expressed as mg of catechin/g of sample dry weight.

**Antioxidant activity**

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH assay was performed to measure scavenging activity. Briefly, leaf extracts were dissolved in 0.5 nM DPPH to final concentration as following, 6.125 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. The solution was incubated at room temperature for 30 min and analyzed spectrometrically at 517 nm. DPPH radical scavenging activity was calculated as percent inhibition. IC50 determined 50% inhibition of DPPH radical formation and capacity of DPPH radical scavenging activity was determined compared with vitamin C as standard antioxidant.

\[
\text{Radical scavenging activity} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

where Ablank and Asample were absorbance data of control and sample, respectively.

**Ferric reducing antioxidant power (FRAP)**

Reducing scavenging activity was observed using FRAP method according to Amarowicz. Briefly, extracts were mixed with frap reagent containing 300 mM acetate buffer, 10 mM TPTZ and 20 mM ferric chloride, and incubated at 37°C for 30 min in the dark condition. Ferrous tripyridyltriazine product were measured using spectrophotometer at 593 nm. FRAP value was expressed as mmol ferrous sulfate equivalent/g of sample dried weight.

**Isolation of LDL**

Process of human LDL isolation was approved by Mahasarakham University Ethics committee. LDL was isolated using floating sequential ultracentrifugation method, described previously. Briefly, LDL was isolated from fresh plasma of fasting overnight, healthy volunteers. Fresh plasma containing 4 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF)asein protease inhibitor. Adjusted density of plasma 1.21 g/ml with solid potassium bromide (KBr). High density plasma was loaded to the lower part of ultracentrifuge tube, which was overlaid by gradient salt solutions, 1.063, 1.019 and 1.005 g/ml, respectively. LDL was isolated by ultracentrifugation at 286,000 g at 4°C for 24 h. The yellowish LDL fraction was collected between 1.019-1.063 g/ml densities and dialyzed using phosphate buffer saline (pH 7.38-7.42) with nitrogen flow at 4°C for 24 h. LDL was filtered through 0.22µm pore size membrane and then measured using bicinchoninic acid (BCA) assay.

**Assessment of lipid peroxidation**

**Thiobarbituric reactive substance (TBARs)**

Malondialdehyde (MDA) is indicated as a product of lipid. MDA level was determined based on the reaction with thiobarbituric acid (TBA). The reaction was divided into two parts; the preliminary part was divided into three groups, no treated, LDL-CuSO₄ co-incubation and pretreated of *L. leucocephala* and LDL prior to the incubation with CuSO₄. The experiment part was the observation of anti-lipidperoxidation activity in HUVC. The assessment of lipid peroxidation activity was determined by incubation of the mixture with trichloroacetic acid (TCA) and thio-barbituric acid (TBA) in an acidic pH condition at 90°C for 20 min. The reaction was precipitated by centrifugation at 4,000g for 10 min at 4°C. MDA level was read using UV spectrophotometer at 532 nm. TBARs value was calculated using 1, 1, 3, 3-tetraethoxypropane as MDA standard curve.

**Conjugated diene formation**

Conjugated diene developed in LDL through oxidation of poly-unsaturated fatty acids (PUFAs) was observed. One milligram protein of LDL was incubated with 0.01 mg/ml *L. leucocephala* solution for 1 h at room temperature. oxLDL was induced using 10 µM CuSO₄. Kinetic of LDL oxidation was monitored the changes of conjugated diene at the absorbance of 234 nm as described previously. The absorbance curve was comprised of three phases; lag phase, propagation phase and decomposition phase. Initial stage of LDL oxidation is lag phase time in which oxidant primary interacted with LDL. Prolong of lag phase time was referred to antioxidant activity of LDL. Lastly, decomposition phase is where LDL is completely oxidized, and OD value is stable.

**HUVCs cultured**

Endothelial cells function, and antioxidant capacity of vascular cells were determined in HUVC system. The process was approved by Mahasarakham University Ethics Committee. Umbilical veins were dissected from human umbilical cord of normal labor. Umbilical cord was processed within 2 h after vaginal delivery. With some modifications, umbilical veins were cultured according to Friedman R et al. Briefly, umbilical veins were dissected into ring with range 3-4 millimeters under strictly sterile technique and the rings are placed in 24 well plate cultured containing 2 ml of RPMI 1640 culture media (GIBCO) supplemented with 10% FCS with 100 µg/ml gentamycin and 10 µg/ml ampicillin. Cultures were maintained at 37°C in 5% CO₂ incubator with 95% O₂ flow for four days. Oxidative stress was induced in HUVCs using different concentration of glucose (7, 14, 50 mM) and/or 40 µg/ml oxLDL in each group. Concentration 0.1, 0.5 and 1 mg/mL of *L. leucocephala* were subsequently incubated in the culture for two days. HUVCs were rinsed with phosphate buffer saline (PBS) pH 7.37-7.47 before determination of SOD activity and NO level of vascular cells.
Real-time measurement of NO production

Real time production of NO in vascular endothelial cells was monitored using model inNO-T, NO measuring system with an aminoNO-700 probe (Innovative Instruments, Inc., Tampa, FL), based on direct oxidation of NO to nitrite. Briefly, microelectrode probe was calibrated with calibration solution (1M H$_2$SO$_4$, 20 mg KI and 18 ml of ddH$_2$O) prior to use. Picoamperae (pA) was adjusted to zero and calibrated by adding nitrite at different concentrations as following: 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM. NO concentration was reported in nM.

SOD activity

SOD, an important scavenging enzyme for superoxide anion (O$_2^-•$), was determined for its activity using xanthine oxidase method. HUVCs were homogenized and ultracentrifuged at 12,000 g for 15 min. Aqueous of homogenate was aspirated and determined SOD activity using SOD determination kit (Fluka). The solutions and standard tube were mixed and incubated at 37°C for 20 min. SOD activity was determined spectrophotometrically at 450 nm. Calculated of SOD activity was showed in percent inhibition of O$_2^-•$ production as the equation and reported in U/ml/mg tissue.

Morphology in HUVCs

HUVCs were sectioned according to embedding paraffin method. Briefly, the tissue was fixed, dehydrated and embedded in paraffin. The paraffin embedded tissue was then sectioned and deparafinized on a slide, followed by staining process using hematoxylin and eosin. The morphological changes were observed under light microscope.

Statistical analysis

All variables were summarized using means and standard deviations (SD). Statistical analysis was carried out to compare between groups by Kruskal-Wallis tests, and followed by Dunn’s method for multiple comparison. Statistical significant was consider when p-value is less than 0.05. (R 3.4.0).

RESULTS

Antioxidant contents

TPC and TFC contents were measured using the Folin–Ciocalteu method and aluminum chloride colorimetric method, respectively. The results showed that total phenolic of leaves dry weight of Leucaena leucocephala at 1 g was found 51.04 ± 0.91 mg GAE/g. Whereas, TFC was showed 0.13 ± 0.01 mg catechin/g.

Antioxidant and anti-lipid peroxidation activity of Leucaena leucocephala

Antioxidant activities were determined by using DPPH and FRAP methods. IC50 concentration of leave extract measured by DPPH method was 369.6 µg/ml, that is 576.79 µ 16.08 µg of vitamin C equivalent/mg of leave extract. The reducing power activity using FRAP method exhibited dried extract at 428.54 ± 15.32 mM FeII equivalent/g.

Antioxidant and anti-lipid peroxidation activity of L. leucocephala was observed in Cu$_2$SO$_4$-induced diene formation in isolated LDL. The result showed lag phase time was slightly longer in 0.1 mg/ml of L. leucocephala treated condition, in contrast with 44 ± 3.3% approximately lower of MDA concentration Table 1.

Effect of L. leucocephala on vascular cell of Human Umbilical Vein Cultured

Influence of L. leucocephala in NO production was conducted under model in NO-T with amino-700 probe. The experiments were observed in HUVC-induced pre-obese condition using 40 µg/ml oxLDL and 7 nM glucose. Compared with positive control (without L. leucocephala), the result showed that NO concentration was significantly reduced when leave extracts at concentration of 0.05, 0.5 and 1 mg/mL were added Figure 1. However, only the leave extract at concentration of 0.1 mg/mL significantly increase NO level (p < 0.05).

Superoxide dismutase (SOD) activity

Scavenging of O$_2^-•$ by SOD enzyme was performed using SOD determination kits-WST (Dojindo) and displayed in percent inhibition of O$_2^-•$ production Figure 2. The experiments were demonstrated using pre-obese condition. Adding 0.1 mg/ml of L. leucocephala, SOD activity was significantly increased compared with control and positive control (p < 0.005 and p < 0.05, respectively). In contrast, 0.5 and 1 µg/ml of leave extracts significantly reduced SOD activity compared with both control (p < 0.001 and p < 0.005, respectively) and positive control (p < 0.005).

Assessment of lipid peroxidation

Figure 3 have shown MDA concentration (mmol/mg), an indicator for lipid peroxidation. The experiment was performed using TBAR assay to indicate level of MDA in the pre-obese induced system. The result showed that only L. leucocephala at concentration 0.05 mg/ml can maintain level of lipid peroxidation compared with control group. When leave extract concentration was increased, the result also exhibited the dose response relationship of the extract and MDA concentration Figure 3.

Morphology of HUVCs

Morphology of HUVCs were observed under 20x light microscope Figure 4. The result showed migration of smooth muscle cells in LDL and glucose induced oxidative stress comparing with control group Figure 4A and 4B. Moreover, intact of vascular cell was clearly reduced, in parallel with death of vascular smooth muscle cells and endothelial cells. In contrast, 0.05 mg/ml of L. leucocephala treated with LDL and glucose induced oxidative stress showed similarity of HUVCs morphology compared with control Figure 4A and 4C. When concentration of extract was increased, at dose 0.1 mg/ml leave extract resulted in increase of smooth muscle cells density and migration Figure 4D. Interestingly, at dose 0.5 and 1 mg/ml leave extracts, the result showed obviously reduction in vascular cells and change in nucleus morphology Figure 4E and 4F.

Table 1: Effect of L. leucocephala in LDL oxidation. Lag phase is determined in the initial step of diene formation curve.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lag phase time (minutes)</th>
<th>MDA (nmol/mg LDL protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>&gt;90-120</td>
<td>10.5 ± 0.62</td>
</tr>
<tr>
<td>LDL + 10 µm Cu$_2$SO$_4$</td>
<td>38-70</td>
<td>29.35 ± 2.62</td>
</tr>
<tr>
<td>LDL + 10 µm Cu$_2$SO$_4$ + 0.1mg/ml LL</td>
<td>65-85</td>
<td>23.45± 1.69</td>
</tr>
</tbody>
</table>

Note: LDL oxidation was determined of final concentration at 0.1 mg/ml of L. leucocephala (LL) (n =5). Data were express as Mean ± SD.
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Figure 1: NO concentration under pre-obese induced condition. *Significantly different from control group (* p<0.05), (** p<0.01), (*** p<0.005). #Significantly different from positive control group (# p<0.05), (## p<0.01), (### p<0.005). OxLDL indicated for oxidized LDL. LL indicated for Leucaena leucocephala.

Figure 2: Level of SOD activity reported as percent inhibition of superoxide anion production in pre-obese induced condition. *Significantly different from control group (* p<0.05), (** p<0.01), (*** p<0.005). #Significantly different from positive control group (# p<0.05), (## p<0.01), (### p<0.005). OxLDL indicated for oxidized LDL. LL indicated for Leucaena leucocephala.

Figure 3: Concentration of MDA, an indicator of lipid peroxidation, in pre-obese induced condition. *Significantly different from control group (* p<0.05), (** p<0.01), (*** p<0.005). #Significantly different from positive control group (# p<0.05), (## p<0.01), (### p<0.005). OxLDL indicated for oxidized LDL. LL indicated for Leucaena leucocephala.

Conclusion and discussion

Impaired fasting blood glucose is caused by increasing of glucose concentration in fasting state, but the patients with IFG have not already develop diabetes mellitus. Those with IFG might eventually develop CVS by glucose inducing oxidative stress production. Thus, to promote reduction of oxidative stress, this study aims to observe antioxidant activity of Leucaena leucocephala in endothelium function using impair fasting glucose in human umbilical vein cultured (HUVCs) model. Total phenolic and flavonoid contents was determined, and the result showed 51.04 ± 0.91 mgGAE/g and 0.13 ± 0.01 mg of catechin/g content in dry leave extract, respectively. Likewise, other studies that found phenolic at 3.21 mg GAE/g, 37.38 ± 0.49 mg GAE/g, 78.8 g GAE/100g extract, 258.4±7.45 mg GAE/g, 7.51 to 12.06 mg GAE/g, and flavonoid contents at 159.61±12.79 mg QE/g (Quercetin equivalent), 2.80 to 3.59 mg QE/g depended on parts of plant collection and solution extracted. This components suggest antioxidant activities of Leucaena leucocephala.
**REFERENCES**


**ACKNOWLEDGEMENT**

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**CONFLICTS OF INTEREST**

The authors declare that there is no conflict of interest regarding the publication of this article.

**ABBREVIATION USED**

LL: Leucaena leucocephala; LDL: low-density lipoprotein; oxLDL: oxidized low-density lipoprotein; HUVCs: human umbilical vein cultured; SOD: superoxide anion; NO: nitric oxide; GAE: gallic equivalent; DW: dried weight; DPPH: 1,1-diphenyl-2-picrylhydrazyl; MDA: malondialdehyde; IFG: impaired fasting glucose; ROS: reactive oxygen species; CVS: cardiovascular diseases; NCDs: noncommunicable diseases; TPC: total phenolic content; TFC: total flavonoid content; FRAP: ferric reducing antioxidant power; TBAR: thiorbituric acid reactive substance; BCA: bicinchoninic acid; TCA: trichloroacetic acid; TBA: thiorbituric acid; PUEAs: polyunsaturated fatty acid; PBS: phosphate buffer saline.


