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Original Article

Effects of *Leucaena leucocephala* (Lam.) de Wit Leaves Extracts in Culture of Human Umbilical Vein Cells

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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 anticholiesterase 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 FeII 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.

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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.[1] 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.[2,3] 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.[4]

Cardiovascular diseases (CVS) contribute to most noncommunicable diseases (NCDs) with 17.5 million in year 2012, and was predicted to increase to 592 million in year 2035.[5] It is known that an impairment of NO production[6,7] and scavenger enzyme such as superoxide dismutase (SOD) can be resulting in oxidative stress.[8] This event is eventually leading to endothelial dysfunction, and tissue injury of vascular cells. Endothelial dysfunction can be found in patients with diabetes,[2,9,10] hyperlipidemia, and atherosclerosis and hypertension.[10,11] 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.[12,13,14] 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.[15] It has been reported for lowering blood glucose, blood pressure and NO level, and providing hypocholesteromic, anti-inflammatory and antioxidant activity.[16,17,18] In previous studies, glucose and LDL can induce endothelial dysfunction of the vessels in diabetic and cardiovascular patients.[9,19,20] Thus, optimal level of *L. leucocephala* may protect vascular cell through reduction of LDL and glucose

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oxidation. Altogether, it leads to objective of the study, to observe antioxidant activity of *L. 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, leave 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.[21] Briefly, 10% Folin-Ciocalteu reagent and 10.75% Na_2CO_3 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.*[22] *L. leucocephala* was incubated in 5% $NaNO_2$ at room temperature for 5 min, and followed by 10% $AlCl_3$ 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.[23] Briefly, leave 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.

$$\text{Radical scavenging activity} = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \times 100$$

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.[24] 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.[25] Briefly, LDL was isolated from fresh plasma of fasting overnight, healthy volunteers. Fresh plasma containing 4 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF) as serine protease inhibitor. Adjusted density of plasma is 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.[26]

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).[27] The reaction was divided into two parts; the preliminary part was divided into three groups, no treated, LDL-Cu_2SO_4 co-incubation and pretreated of *L. leucocephala* and LDL prior to the incubation with Cu_2SO_4. 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 thiobarbituric acid (TBA) in an acidic pH condition at 90°C for 20 min. The reaction was precipitated by centrifucation 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 Cu_2SO_4. Kinetic of LDL oxidation was monitored the changes of conjugated diene at the absorbance of 234 nM as described previously.[28] 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.*[29] 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% CO_2 incubator with 95% O_2 flow for four days. Oxidative stress was induced in HUVCs using different concentration of glucoses (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 amiNO-700 probe (Innovative Instruments, Inc., Tampa, FL), based on direct oxidation of NO to nitrite.[30,31] Briefly, microelectrode probe was calibrated with calibration solution (1M H_2SO_4, 20 mg KI and 18 ml of ddH_2O) prior to use. Picoamparer (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 ($O_2^{\bullet-}$), 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^{0}C for 20 min. SOD activity was determined spectrophotometrically at 450 nm. Calculated of SOD activity was showed in percent inhibition of $O_2^{\bullet-}$ production as the equation and reported in U/ml/mg tissue.

Morphology in HUVCs

HUVCs were sectioned according to embedding paraffin method.[32] 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 was measured using the Folin–Ciocalteu method and aluminum chloride colorimetric method, respectively. The results showed that total phenolic of leaves dry weight of *L. 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 *L. 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.

Anti-lipid peroxidation activity of *L. leucocephala* was observed in Cu_2SO_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

NO concentration

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 $O_2^{\bullet-}$ by SOD enzyme was performed using SOD determination kits-WST (Dojindo) and displayed in percent inhibition of $O_2^{\bullet-}$ 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 results 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.

	Lag phase time (minutes)	MDA (nmol/mg LDL protein)
LDL	>90-120	10.5 ± 0.62
LDL + 10 µm Cu_2SO_4	38-70	29.35 ± 2.62
LDL + 10 µm Cu_2SO_4 + 0.1mg/ml *LL*	65-85	23.45± 1.69

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.

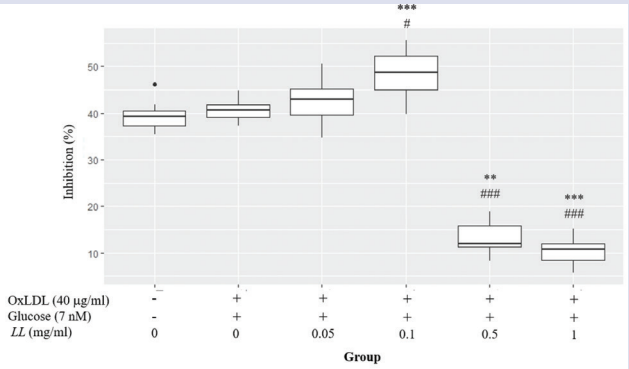

Figure 1: NO concentration under pre-obese induced condition. *Significantly different from control group (* p<0.05), (** p<0.01), (*** p<0.005). #Significantly different from positive control group (# p<0.05), (## p<0.01), (### p<0.005). OxLDL indicated for oxidized LDL. LL indicated for *Leucaena leucocephala*.

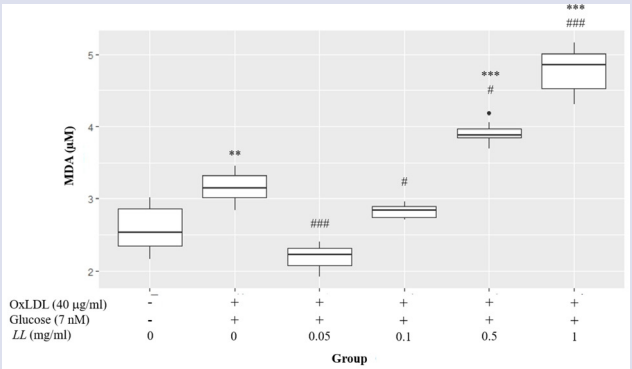

Figure 3: Concentration of MDA, an indicator of lipid peroxidation, in pre-obese induced condition. *Significantly different from control group (* p<0.05), (** p<0.01), (*** p<0.005). #Significantly different from positive control group (# p<0.05), (## p<0.01), (### p<0.005). OxLDL indicated for oxidized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

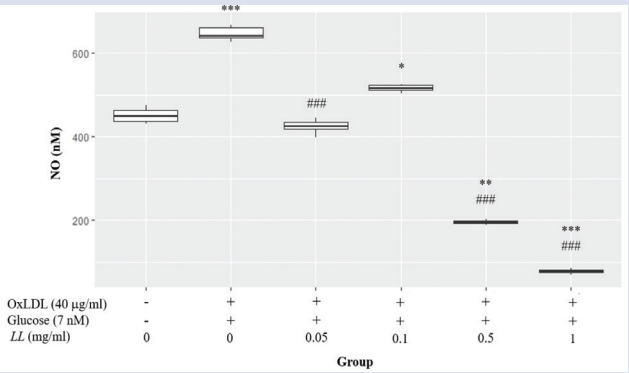

Figure 2: Level of SOD activity reported as percent inhibition of superoxide anion production in pre-obese induced condition. *Significantly different from control group (* p<0.05), (** p<0.01), (*** p<0.005). #Significantly different from positive control group (# p<0.05), (## p<0.01), (### p<0.005).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

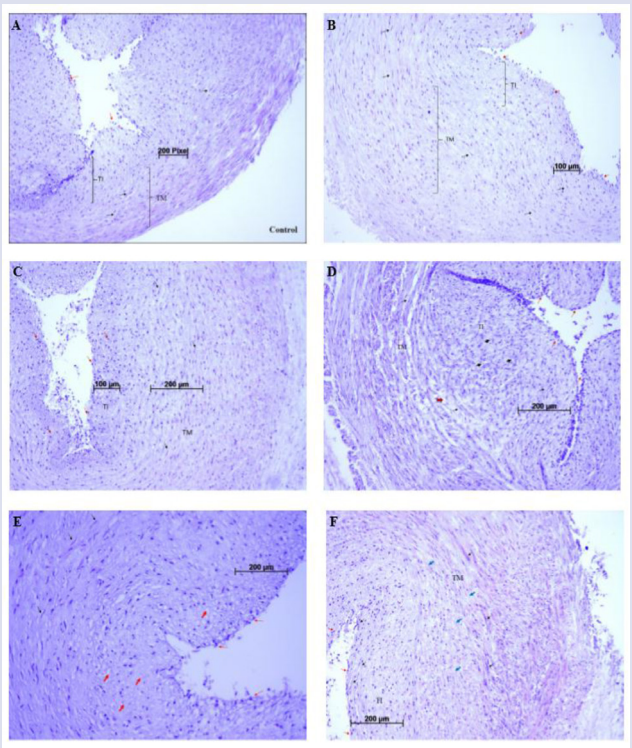

Figure 4: Morphological changes in HUVC. A, control group. B, LDL and glucose induced oxidative stress in HUVC. C, 0.05 mg/ml of *L. leucocephala* treated with LDL and glucose induced oxidative stress in HUVCs. D, 0.1 mg/ml of *L. leucocephala* treated with LDL and glucose induced oxidative stress in HUVCs. E, 0.5 mg/ml of *L. leucocephala* treated with LDL and glucose induced oxidative stress in HUVCs. F, 1 mg/ml of *L. leucocephala* treated with LDL and glucose induced oxidative stress in HUVCs.

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.[1] Thus, to promote reduction of oxidative stress, this study aims to observe antioxidant activity of *L. 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,[33] 37.38 ± 0.49 mg GAE/g,[17] 78.8 g GAE/100g extract,[18] 258.4±7.45 mg GAE/g,[16] 7.51 to 12.06 mg GAE/g,[34] and flavonoid contents at 159.61±12.79 mg QE/g (Quercetin equivalent),[16] 2.80 to 3.59 mg QE/g[34] depended on parts of plant collection and solution extracted. This compon ents suggest antioxidant activities of

L. leucocephala, as confirmed in Table 1. The preliminary study indicated that *L. leucocephala* provides antioxidant activity using DPPH method resulting in 50% scavenging activity at 369.6 µg/ml, which is equivalent to 576.79 ± 16.08 µg of vitamin C equivalent/mg. The result was confirmed by FRAP method showing the reducing power activity of dried extract at 428.54 ±15.32 mM FeII equivalent/g. This result showed higher scavenging activity comparing to previous study [16] that observing the activity in seed extract [17,18], suggested leave extract might provide greater scavenging activity compared with the seed part.

The effects of *L. leucocephala* on HUVCs metabolites were shown in Figure 1 to 4, despite on each category. Figure 1 showed NO production in *L. leucocephala* preincubated HUVCs. The result shown that *L. leucocephala* significantly influenced NO production in HUVC-induced pre-obese condition at the concentration of 0.1 mg/mL, but maintained its level at 0.05 mg/mL. On the other hand, result in Figure 2 showed that induction of SOD activity was gradually increased in parallel with concentrations of leave extract (0.05 and 0.1 mg/mL), before significantly decreasing at dose 0.5 and 1 mg/mL. The alteration of HUVCs activity in high dose of leave extracts might suggest the death of those vascular cells as observed in Figure 4. In assessment of lipid peroxidation activity in HUVCs, MDA concentration, an indicator of lipid peroxidation, was determined in Figure 3. The result showed decreasing of MDA level in 0.05 mg/mL *L. leucocephala* treated group, and gradually in dose response concentration in *L. leucocephala* concentration of 0.1, 0.5 and 1 mg/mL, respectively. These results were verified in culture of HUVCs Figure 4. With histology of vascular cells, the result showed that the vascular cells were obviously dead in HUVC-induced pre-obese condition. In this condition of increasing extracellular glucose, it was suggested to induce endothelial cell dysfunction by increased activation of NADPH oxidase and IL1β,[35,36,37] which might eventually lead to senescence of the cells. When 0.05 mg/mL of leave extracts were pre-incubated in the system, the overall of vascular cells have been found resembling the control group. It suggested the healthy condition of HUVCs as *L. leucocephala* has been found to provide the activity to reduce blood glucose level in diabetic rats [17] by increasing of glucose uptake into cells.[38] In comparison with *L. leucocephala* at concentration of 0.1 mg/mL, smooth muscle cells migration was observed in Figure 4C, suggested this dose may performed the activity of pro-oxidant agent by activating NAPPH oxidase, induces inducible nitric oxide synthase (iNOS) activity of vascular smooth muscle cell.[39] In addition, ROS may also act as vascular endothelial growth factor (VEGF) which induces vascular cells migration and proliferation.[40] With the production of NO, it can interact with superoxide anions ($O_2^{\cdot-}$) and results in peroxynitrite production, which is a radical that can induce endothelial dysfunction and damage cells.[19,41] Although no cell death have been observed. This condition may explain the increasing in NO level and SOD activity Figure 1 and Figure 2, respectively as for the oxidation process have been introduced. The MDA level in Figure 3 also confirmed this probability as the lipid peroxidation was slightly increased.

At the higher concentration of *L. leucocephala*, the result of both NO level and SOD activity were significantly decreased Figure 1 and 2, respectively. These events may suggest endothelial cell dysfunction and damage, because of mimosine, a toxic component of *L. leucocephala*.[42,43,44] With this result, it is suggested that this toxic activity may be applied to the inhibitory effect on proliferation of cancer.[45]

In 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 and 0.1 mg/mL in reducing oxidative stress condition in impaired fasting blood glucose patients. The higher doses of leave extracts may introduce the toxic effect to endothelial cells, which may contribute to a plausible way to inhibit angiogenesis of cancer cell. Nevertheless, the optimal level for toxic activity in inhibition of cancer angeiogenesis should be further investigated. As well as the underlying mechanism of how *L. leucocephala* affects in oxidative stress in condition of having impaired fasting glucose has been performed should be further validated.

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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 picryhydrazyl; **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:** thiobarbituric acid reactive substance; **BCA:** bicinchoninic acid; **TCA:** trichloroacetic acid; **TBA:** thiobarbituric acid; **PUEAs:** polyunsaturated fatty acid; **PBS:** phosphate buffer saline.

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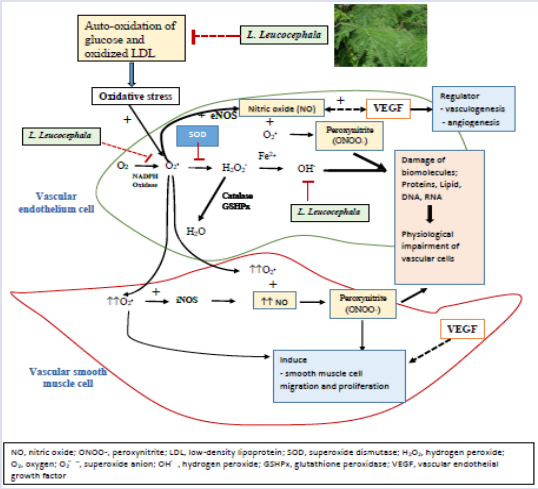
GRAPHICAL ABSTRACT



NO, nitric oxide; ONOO-, peroxynitrite; LDL, low-density lipoprotein; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; O_2, oxygen; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydrogen peroxide; GSHPx, glutathione peroxidase; VEGF, vascular endothelial growth factor

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