

Addition of Citrus Leaf Extract into Frying Oil Prevents Hypertension and Improves Vascular Reactivity in Heated Oil-Fed Rats

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Abstract The use of repeatedly heated frying oil is hazardous to health, due to oxidative process that occurs in the oil. Addition of an additive with antioxidant property could lessen the detrimental effects of repeated frying in the oils. Citrus leaf extract (CLE) supplementation was reported to possess antioxidant and blood pressure-lowering properties. Our study aimed to determine the effects of CLE addition into frying oil in rats fed with repeatedly heated oil diet. Seven groups of male Sprague-Dawley rats were given fresh and heated oil diets (five-time- and ten-time-heated palm oil), with and without the CLE addition for 16 weeks. CLE addition decreased peroxide value and augmented the total phenolics content in the heated oils. CLE addition reversed the negative effects of heated oil on nitric oxide level, systolic blood pressure, and vascular reactivity. In conclusion, these findings show that CLE has the ability to reduce oxidative damage caused by thermal degradation in frying oil and subsequently lowers the risk of hypertension in rats that consumed heated oil.

Keywords: *Citrus hystrix* DC, Rutaceace, kaffir lime, hypertension, nitric oxide, vascular reactivity

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1. Introduction

The practice of recycling used frying oil has been so common in households and among food hawkers without realizing the impact of the practice on health, especially in long terms for the sake of saving cost [1]. During heating, the fat content in the frying oil will change to volatile and non-volatile compounds, which later change the physicochemical properties of the fried foods and frying oil [2]. Frying also degrades the antioxidant content [3] and generates trans fatty acids as well as reactive oxygen species (ROS) in the oil [4].

Over the last decade, the rising prevalence of hypertension (>140/90 mmHg) has caused a public health problem worldwide, both in developed and developing countries [5,6]. Hypertension-related effects caused by the consumption of heated oil have been extensively studied in rats. Repeated heating increases the

peroxide value of the oil [7,8]. Its consumption in rats has been shown to diminish nitric oxide (NO) level and impairs vasorelaxation and augments vasoconstriction responses, leading to endothelial dysfunction which is attributable to the development of hypertension [4,9,10].

Citrus leaf extract (CLE) supplementation, an extract from *Citrus hystrix* D.C., or locally known as kaffir lime, been shown to possess antioxidant properties. It is rich in phenolics such as hesperidin, diosmin, didymin, and lutein [11]. Its intake in animal studies has been reported to prevent the development of hypertension in rats fed with repeatedly heated oil [9,12]. The presence of these phenolics most possibly contributes to the protective effects of the CLE against oxidative stress, which is responsible for vascular damage, leading to the development of hypertension. Based on this information, this study was designed to investigate the effects of CLE addition into frying oils on cardiovascular parameters in rats that consumed repeatedly heated oil.

2. Material and Methods

2.1. Materials

The CLE (No. Patent: US8425969B2) [11] was prepared in 10 times dilution with palm oil by the Institute of Bioscience, Universiti Putra Malaysia, Selangor. The phytochemical profile of the CLE was previously published [12]. Palm oil (Lam Soon Edible Oils Sdn. Bhd. Malaysia) was purchased from a local supermarket.

2.2. Diet Preparation

Fresh and repeatedly heated diets (five-time-heated palm oil, 5HPO; and ten-time-heated palm oil, 10HPO) were prepared accordingly [14]. To prepare CLE-added frying oils, the CLE was added into cooled palm oil prior to the heating process according to the groups at the ratio of 1:10 (CLE: palm oil). The final composition of the added CLE into the frying oil each time was 1%. No fresh oil was added throughout the frying process. Diet was prepared by adding 150 g of fresh or heated oil into 850 g ground rat chow. To prepare fresh palm oil (FPO) diet added with CLE, 135 g fresh oil and 15 g CLE were added into 850 g ground rat chow. The chow was then molded into pellets which were then kept in an air-tight container at 4°C.

2.3. Animals and Experimental Design

Male Sprague-Dawley rats (250-300 g) (Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia). After a week of adaptation, the rats were randomly divided into seven groups (8 rats/group); control (normal diet), FPO, FPO+CLE, 5HPO, 5HPO+CLE, 10HPO and 10HPO+CLE, and fed with the diet ad libitum for 16 weeks. Blood pressure was measured at 4-weekly using tail-cuff non-invasive method (CODA Powerlab, ADInstruments, NSW, Australia). Blood was sampled at weeks 0 and 16. The study was approved by the institutional animal ethical committee (FP/FAR/2015/Kamisah/25-MAR/667-MAR-2015-DEC-2017).

2.4. Measurements of Peroxide Values, Total Phenolic Content and Serum NO

Peroxide value was determined in CLE and palm oil using a standard titration official method (Cd 8-53) [7,15]. Briefly, 5 g sample was added into acetic acid-chloroform mixture (30 ml, 3:2) and saturated potassium iodide (0.5 ml), swirled before addition of distilled water (30 ml). Then, a few drops of 10% starch solution were added into the solution and later titrated against 0.01 N Na₂S₂O₃ until the blue color was gone. The value was expressed as milliequivalents of active oxygen per kilogram (mEq/kg).

Total phenolic content (TPC) was determined in the CLE and oils following an established method [16,17]. Briefly, 0.01 ml sample was mixed with 0.79 ml distilled water and 0.05 ml Folin-Ciocalteu reagent before being incubated for 3 min at room temperature. Then, the mixture was incubated for another 2 h after addition of Na₂CO₃ (0.15 ml, 20%). The absorbance at 760 nm

(ELISA EnSpire, PerkinElmer, Waltham, Massachusetts, USA) was read. The TPC was calculated against gallic acid standard curve and expressed as mg gallic acid equivalent (GAE) per g dry extract.

Serum NO was measured using a previously described method [18]. A volume of 50 µL was added into an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA). and incubated at room temperature in dark for 15 min. Absorbance obtained at 540 nm (ELISA EnSpire, PerkinElmer, Waltham, Massachusetts, USA) was used to calculate the NO content against sodium nitrite standard curve.

2.5. Vascular Reactivity

Vascular response in the precontracted aortic rings with endothelium intact exposed to various concentrations of phenylephrine (PE), acetylcholine (ACh) and sodium nitroprusside (SNP) was determined in descending thoracic aortic rings (3-5 mm ring segments) [19] using PowerLab data acquisition (ADInstruments, Sydney, Australia).

2.6. Aortic Histology

Fixed aortic arches in 10% formalin were embedded in Paraplast embedding medium and then cross-sectioned at 5 µm thickness. The sections were stained with Verhoeff van Gieson (VVG) staining. For each section, four fields were taken for quantitative measurements of tunica intima and tunica media (TI/TM) ratio using an image analyzer (Nikon Eclipse 80i, Melville, NY, USA).

2.7. VCAM-1 Immunohistochemistry

Sections of fixed thoracic aortae were processed according to the published method [20]. The sections were stained with an anti-VCAM-1 primary antibody (1:100) (Abcam, Cambridge, UK) and a secondary antibody of polymerized horseradish peroxidase (1:100) (DakoCytomation, Glostrup, Denmark). Positive expression of the VCAM-1 was shown by brown streaks in the aortic intimal layer. The expression was scored by two blinded assessors using a scoring system [21].

2.8. Statistical Analysis

The results were presented as mean ± standard error of the mean (SEM). A normality test was carried out using Kolmogorov-Smirnov. The comparison among groups were analyzed using one-way ANOVA and repeated-measures ANOVA followed by Tukey's post hoc test, accordingly. Statistical significance was defined as P < 0.05, using Statistical Package for Social Sciences version 20.0 software (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Effects of CLE on Peroxide Value and Total Phenolic Content in Frying Oils

The peroxide values in the extract and FPO were relatively low (Table 1). It was significantly increased in

both heated oil groups (5HPO and 10HPO) when compared to FPO ($P < 0.05$). The addition of CLE into the heated oils significantly reduced the peroxide values, respectively. The total phenolic content in the CLE was about eight times higher than the FPO (Table 1). Its addition into the FPO had further increased the content ($P < 0.05$). Repeated heating of the oils for five and ten times significantly decreased their phenolic content, which was significantly raised by the addition of CLE.

Table 1. Peroxide value and total phenolic content in the citrus leaf extract (CLE) and oils

Groups	Peroxide value (mEq/kg-1)	Total phenolic content (mg GAE/g dry extract)
CLE	1.07 ± 0.53	1639 ± 41
FPO	2.67 ± 0.53	231 ± 15¥
FPO+CLE	2.13 ± 0.53	430 ± 13¥¥
5HPO	9.60 ± 0.00*	93 ± 2*
5HPO+CLE	2.67 ± 0.53#	293 ± 18#¥§
10HPO	11.73 ± 0.53*	80 ± 3*
10HPO+CLE	3.20 ± 0.92#	414 ± 14#¥

Values are mean ± SEM (n=3). *vs FPO ($P < 0.05$), #vs respective groups without CLE ($P < 0.05$), ¥vs CLE ($P < 0.05$), §vs FPO+CLE and 10HPO+CLE ($P < 0.05$).

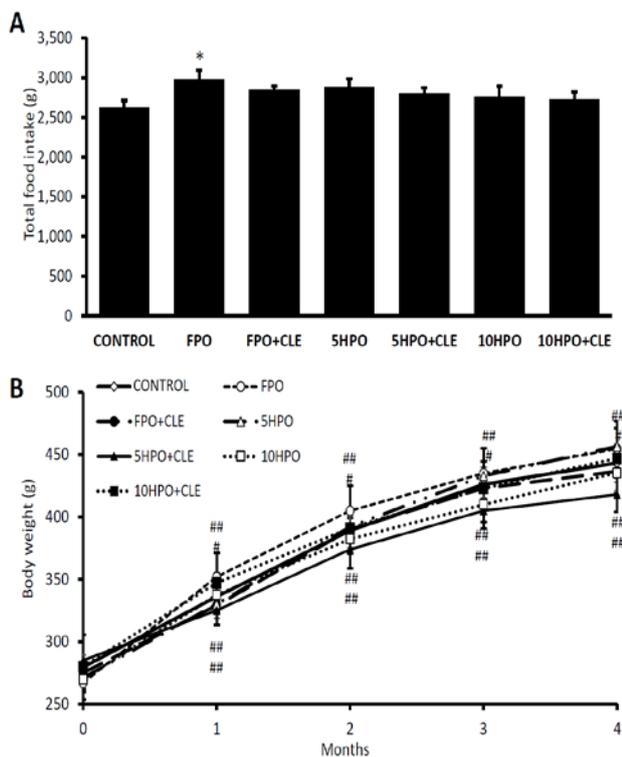


Figure 1. Total food intake (A) and bodyweight (B) in rats fed heated 5HPO and 10HPO, with or without the addition of citrus leaf extract (CLE) into the oils. Data are shown as mean ± SEM (n=8). *vs the control ($P < 0.05$), #vs other weeks ($P < 0.05$)

3.2. Effects of CLE on Food Intake and Body Weight

Total food intake in the FPO-fed rats was significantly higher than the control ($P < 0.05$) (Figure 1A). Dietary heated oil and addition of CLE into the frying oils did not affect the food intake in these rats. There was no remarkable difference in the amount of food intake among

the groups except for the fresh oil diet. There was a consistent and similar increment in body weight for all groups compared to the previous 4-week intervals (Figure 1B). However, no significant difference was noted in the body weight among the groups.

3.3. Effects of CLE on systolic blood pressure and serum NO

The systolic blood pressure in the control and FPO groups had remained consistently low throughout the study (Figure 2A). The rats that were fed 5HPO and 10HPO diets showed a significant increase in the blood pressure compared to the control and FPO groups, starting at week 4. At week 16, the blood pressure in the 10HPO group was significantly higher than the 5HPO group. The heated oil groups with CLE addition exhibited a significant decrease compared to their respective groups without CLE ($P < 0.05$).

The serum NO levels in the control and FPO groups were significantly higher at week 16, compared to their respective week 0 ($P < 0.05$) (Figure 2B). Intake of heated oil diets (5HPO and 10HPO) significantly decreased the serum NO level after 16 weeks. The addition of the CLE into the oils had significantly increased the level compared to their respective diets. However, CLE addition did not affect the level in the FPO group.

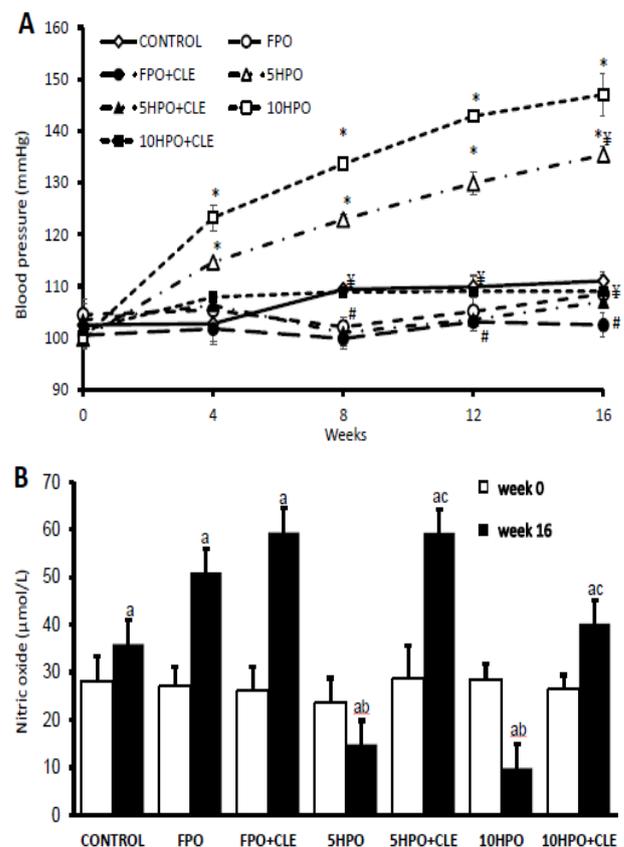


Figure 2. Systolic blood pressure (a) and pre- and post-treatment serum NO (b) in the rats fed fresh (FPO) and heated (5HPO and 10HPO) palm oils, with or without the addition of citrus leaf extract (CLE). Data are shown as mean ± SEM (n=8). *vs the control and FPO at each respective week ($p < 0.05$), #vs 5HPO at each respective week ($p < 0.05$), ¥vs 10HPO at each respective week ($p < 0.05$), ^avs week 0 in the same groups ($p < 0.05$), ^bvs FPO at week 16 ($p < 0.05$), ^cvs 5HPO at week 16 ($p < 0.05$)

3.4. Effects of CLE on Vasoconstriction and Vasorelaxation

The aortic vasoconstriction response to PE was similarly higher ($P < 0.05$) in the 5HPO and 10HPO groups than the control and FPO groups, at concentrations of 10^{-9} M to 10^{-6} M (Figure 3A). Both 5HPO+CLE and 10HPO+CLE groups had a significant decrease ($P < 0.05$) in vasoconstriction response compared to their respective heated oil groups. No significant difference in the aortic vasoconstriction response was observed among the control, FPO, and FPO+CLE groups ($P > 0.05$). The aortic relaxation effects of ACh (10^{-9} - 10^{-6} M) and SNP (10^{-8} - 10^{-6} M) were significantly reduced in the 5HPO and 10HPO groups, in comparison to the control and FPO groups ($P < 0.05$) (Figure 3B and Figure 3C). Both groups of 5HPO+CLE and 10HPO+CLE had significantly higher ($P < 0.05$) vasorelaxation response to ACh and SNP than their respective heated oil groups. Similar vasorelaxation responses to Ach and SNP were seen among the control, FPO, and FPO+CLE ($P > 0.05$).

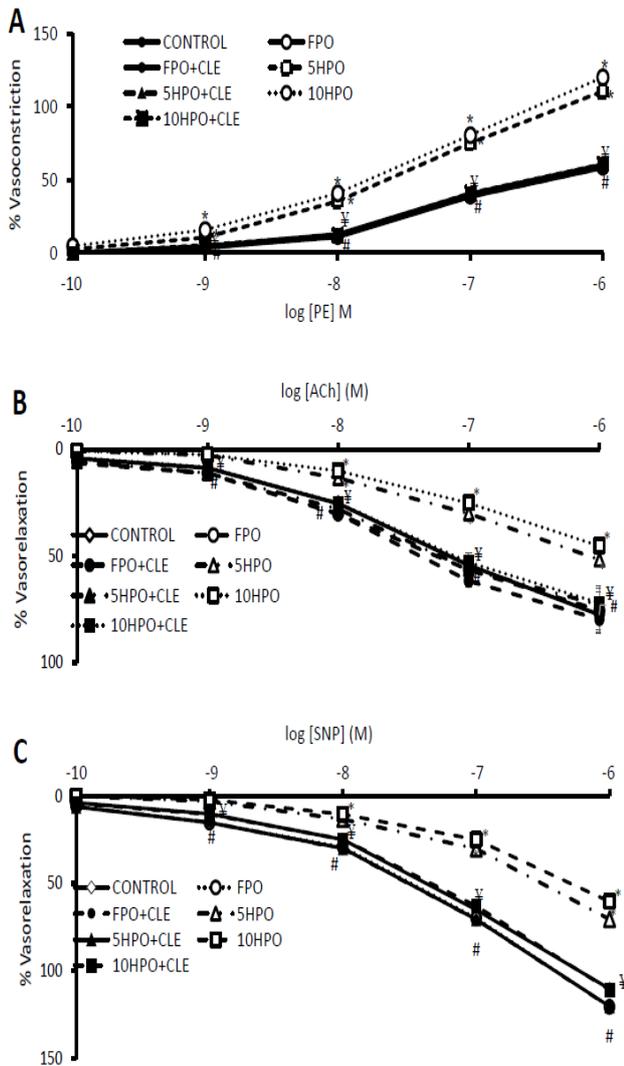


Figure 3. Contraction response to phenylepinephrine (PE) and relaxation response to acetylcholine (ACh) and sodium nitroprusside (SNP) in aortic rings isolated from rats consumed heated palm oil with and without CLE. Data are shown as mean \pm SEM (n=8). *vs the control and FPO ($P < 0.05$), #vs 5HPO at ($P < 0.05$), vs 10HPO ($P < 0.05$), at each respective concentration respectively

3.5. Effects of CLE on Aortic TI/TM Ratio

Figure 4A shows aortic histological sections from each group on TI and TM. The tunica medial elastic lamellae looked organized in the control, FPO and FPO+CLE groups. Disruptions of the lamellae were noted in the groups fed 5HPO and 10HPO diet. The addition of CLE managed to prevent the heated oil-induced disruption of the elastic lamellae. The TI/TM was significantly elevated in the groups fed with 5HPO and 10HPO (Figure 4B). The TI/TM was reduced in the groups fed heated oil and added CLE, when compared to the heated oil-fed groups (5HPO and 10HPO) respectively. In the control and groups given FPO with or without CLE, the ratios were similar.

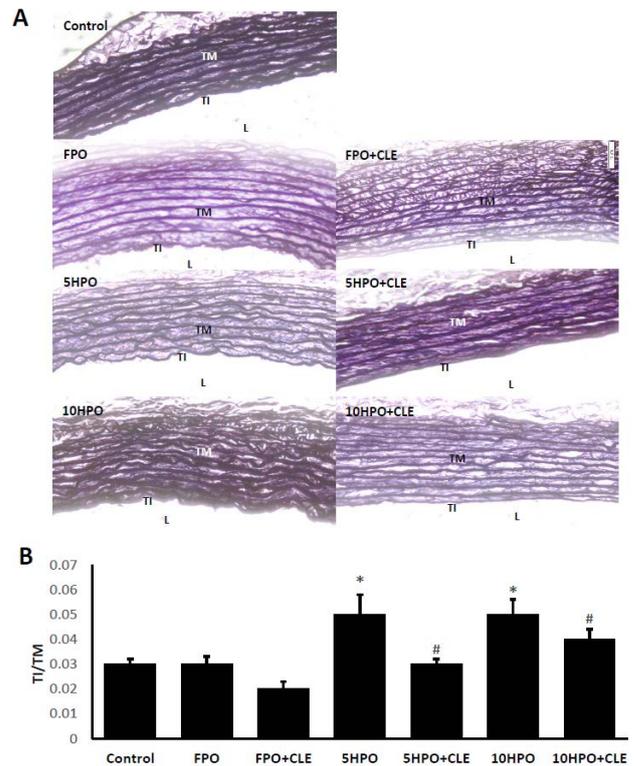


Figure 4. Verhoeff van Gieson staining of the aorta (A) and tunica intima-media ratio (TI/TM) (x200 magnification) (B) in rats fed fresh (FPO), five- (5HPO) or ten-time-heated (10HPO) palm oil added with or without citrus leaf extract (CLE) for 16 weeks. TM, tunica media; TI, tunica intima; L, lumen. Data are shown as mean \pm SEM (n=8). *vs the control and FPO ($P < 0.05$), #vs 5HPO or 10HPO respectively at ($P < 0.05$)

3.6. Effects of CLE on Aortic VCAM-1 Expression

Representative aortic sections from each group that were brownish immunostained for VCAM-1 are shown in Figure 5A. Larger positive-stained area was observed in the groups fed oxidized oil (5HPO and 10HPO). In the groups given oxidized oil added with CLE showed less intensity of the immunostained area. Feeding with 5HPO and 10HPO diets for 16 weeks significantly increased VCAM-1 score compared to the control and FPO (Figure 5B). Addition of CLE into the frying oils (5HPO and 10HPO) reduced the expression of the VCAM-1 ($P < 0.05$). CLE addition however did not affect the expression in the rats fed FPO.

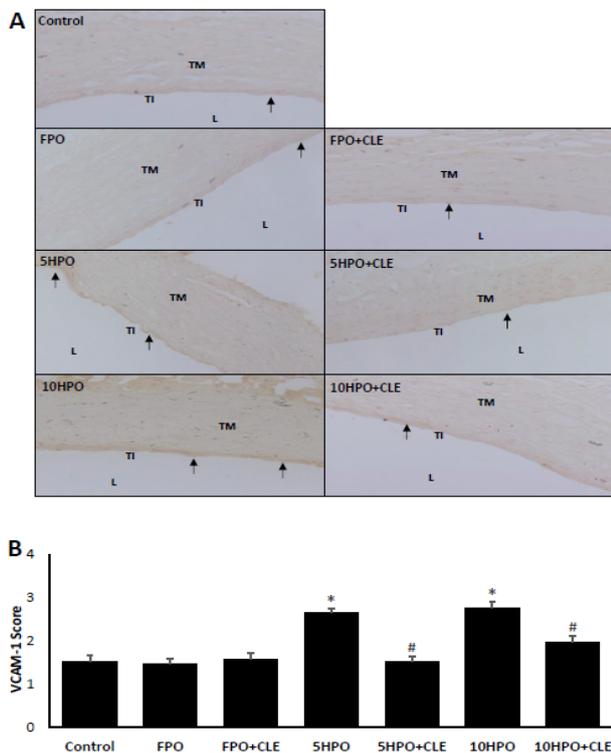


Figure 5. Positive expression of vascular cell adhesion molecule-1 (VCAM-1) at the aortic intimal layer indicated by brownish streaks (arrows) (x200 magnification) (A) and its score (B) in rats fed fresh (FPO), five-time-heated (5HPO) or ten-time-heated (10HPO) palm oil with or without addition of citrus leaf extract (CLE) into heated oil, for 16 weeks. TM, tunica media; TI, tunica intima; L, lumen. Data are shown as mean \pm SEM (n=8). *vs the control and FPO ($P < 0.05$), #vs 5HPO or 10HPO respectively at ($P < 0.05$).

4. Discussion

Total phenolic content is an indicator of antioxidant content in samples. On the other hand, peroxide value is commonly used as an indicator of oil stability and fats [21]. In this study, it was proven that repeated heating of frying oils elevated the degree of lipid peroxidation in the oils. Both heated oil groups had almost or evidently exceeded the upper limit of peroxide value (10 mEq/kg) in foods set by AOCS [15], which was similarly reported by other studies [7,13,22]. Repeated heating diminishes the antioxidant content in the oils [3]. The addition of exogenous antioxidant CLE had obviously prevented the increase in the peroxide value, as well as augmented the phenolic content in the repeatedly heated oils, indicating that CLE incorporation into palm oil prior to frying was able to retard lipid peroxidation, most probably due to the presence of polyphenolic compounds.

There was no remarkable difference in the amount of food intake among the groups except for the fresh oil diet, probably due to rats' preference towards fresh oil [23]. All rats gained similar bodyweight suggesting that heated oils and CLE addition into the oils did not interfere with their growth, as similarly reported by other studies [9,10]. The elevation of blood pressure following prolonged consumption of the heated palm oil starting at week 4, most possibly due to the presence of peroxide radicals and

other ROS in the heated oil. A similar finding was demonstrated in a study [8]. Addition of flavonoid hesperidin which CLE was rich with, into an obesogenic cafeteria diet was shown to significantly suppress the increase in blood pressure after 16 weeks of treatment [24]. Supplementation with a plant extract rich in polyphenols was demonstrated to prevent the development of hypertension in rats administered an NO synthase inhibitor (17). This suggests that prolonged intake of bioactive polyphenolic compounds present in CLE could act as an effective blood pressure-lowering agent.

The rats fed heated frying oil in the current study had an impaired vascular response. Intake of heated oil has been demonstrated to damage the vascular elastic lamellae structure [8,12,20,25]. The heated oil might hinder endothelial relaxation in two ways; either by inactivating endothelium-derived NO or aggravating vascular smooth muscle contraction. The former was more likely. The addition of polyphenol-rich CLE into frying oil has retarded the ROS ability to damage vascular endothelium and improve blood pressure in rats fed oxidized oil diet. The presence of radicals in the heated oils may damage the vasodilating and vasoconstricting capabilities of the vascular, leading to an increase in blood pressure. The reduced vascular response to ACh in the rats fed heated oils indicated an endothelium-dependent impairment of vasorelaxation, in line with the reduced NO level. NO is an endothelium-derived relaxing factor (EDRF). Its synthesis occurs in the endothelial cells [26]. Therefore, damaged endothelial cells would affect the production of NO, hence the vascular response. The intake of 5HPO and 10HPO diets which were high in peroxides might augment oxidative stress in the rats, which later also reduced the level of the serum NO. A similar observation was also noted in other studies [9,10]. Superoxide anion, a commonly generated ROS, reacts with NO to produce peroxynitrite, resulting in reduced bioavailability of the NO [27]. The NO released by the SNP which action was not dependent on the presence of endothelium [10], also did not manage to cause vasodilation, might be due to the effects of the ROS on the released NO. The consumption of heated oil also alters other vasoactive substances. It diminishes plasma prostacyclin and elevates plasma thromboxane levels [8,12].

The blood pressure-lowering effects of CLE that was added into the frying oil might be attributable to the profoundly increased NO level observed in the groups. CLE also attenuated the vascular response towards the induction of vasoconstriction and augmented vasorelaxation response in the rats. However, in a previous report [9], dietary supplementation of CLE in rats fed heated oil did not significantly improve the vascular responses, despite of similar amount of CLE added into the diet. This suggests that the addition of CLE into frying oil was much better than its dietary supplementation. The possible explanation for the discrepancy is the former method retarded the formation of free radicals in the frying oils, while the latter did not completely manage to neutralize the adverse effects of the free radicals which were already present in the oils. The positive effects of the extract most possibly due to the polyphenolic content in the CLE that acted as an antioxidant which decreased the effects of

oxidative stress on NO and preserved the endothelial function.

Consumption of heated oil disrupted the organization of the elastic lamellae, leading to increased intimal media thickness, and then the development of hypertension. It was also associated with increased aortic VCAM-1 expression, an inflammatory biomarker. This adhesion molecule is often detected in early progression of atherosclerosis [20,28]. CLE addition preserved the vascular histomorphometry, reflected by the prevention of the increase in TI/TM ratio induced by the heated oil diet. Similar effect of CLE had been previously demonstrated [9,12]. The extract also prevented the expression of VCAM-1, suggestive of its ability to prevent proatherosclerotic changes in the rats fed heated oil. These findings suggest that addition of CLE into frying oil shows beneficial effects on cardiovascular health.

5. Conclusion

The addition of CLE into the frying oil prevented the development of hypertension, possibly by improving vascular reactivity, augmenting the NO level as well as reducing vascular inflammation in rats fed repeated heated oil.

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