**Effects of citrus leaf extract on aortic vascular reactivity in repeatedly heated vegetable oil-induced hypertensive rats.**

<table>
<thead>
<tr>
<th>Field</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Journal</td>
<td><em>Applied Physiology, Nutrition, and Metabolism</em></td>
</tr>
<tr>
<td>Manuscript ID</td>
<td>apnm-2018-0175.R2</td>
</tr>
<tr>
<td>Manuscript Type</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author</td>
<td>30-Aug-2018</td>
</tr>
<tr>
<td>Complete List of Authors</td>
<td>Nordin, Siti Hawa; Universiti Sultan Zainal Abidin Fakulti Perubatan Dan Sains Kesihatan, Department of Basic Medical Sciences Kamisah, Yusof; Pusat Perubatan Universiti Kebangsaan Malaysia, Dept of Pharmacology Mohamed, Suhaila; University Putra Malaysia, Institute of Bioscience Jaarin, Kamsiah; Universiti Pertahanan Nasional Malaysia, Pharmacology</td>
</tr>
<tr>
<td>Keyword</td>
<td>citrus, rutaceae, vegetable oil, vascular reactivity, nitric oxide, hypertension</td>
</tr>
<tr>
<td>Is the invited manuscript for consideration in a Special Issue?</td>
<td>Not applicable (regular submission)</td>
</tr>
</tbody>
</table>
Effects of citrus leaf extract on aortic vascular reactivity in repeatedly heated vegetable oil-induced hypertensive rats.

Hawa Nordin Siti a,b, Yusof Kamisah a, Suhaila Mohamed c, Kamsiah Jaarin a,d*

a Department of Pharmacology, Faculty of Medicine, UKMMC, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras Kuala Lumpur, Malaysia.
b Department of Basic Medical Sciences, Faculty of Medicine, Universiti Sultan Zainal Abidin, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Terengganu, Malaysia.
c Institute of Bioscience, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Selangor, Malaysia.
d Faculty of Medicine, National Defence University of Malaysia (UPNM), Kem Sungai Besi, 57000 Kuala Lumpur, Malaysia.

*Corresponding author:
Prof Dr Kamsiah Jaarin
Faculty of Medicine, National Defence University of Malaysia (UPNM)
Kem Sungai Besi,
57000 Kuala Lumpur,
Malaysia.
Tel: 603-90513041; Fax: 603-90513042
Email address: kamsiahjaarin@gmail.com
Abstract

The prolonged intake of diet containing repeatedly heated vegetable oil can cause hypertension in the long run. In this study, the effects of citrus leaf extract (CLE) supplementation on vascular reactivity, plasma nitrite, and aortic structure in repeatedly-heated vegetable oil-induced hypertension rats were investigated. Male Sprague-Dawley rats (n = 56) were divided into seven groups corresponding to the respective diets. For 16 weeks, one group was given standard rat chow (control) while other groups were given diets containing 15% w/w of palm oil, fresh palm oil (FPO), five-time-heated palm oil (5HPO) and ten-time-heated palm oil (10HPO), with or without 0.15% w/w CLE incorporation (FPO+CLE, 5HPO+CLE or 10HPO+CLE). Plasma nitrite levels were measured before and at 16 weeks of treatment. After 16 weeks, the rats were sacrificed and aortae were harvested for measuring vascular reactivity and for microscopic study. CLE supplementation had significantly reduced the loss of plasma nitrite and attenuated the vasoconstriction response to phenylephrine in the 5HPO group but not in the 10HPO group. However, CLE had no significant effect on the vasorelaxation response to acetylcholine and sodium nitroprusside. The elastic lamellae of tunica media in 5HPO, 10HPO, and 10HPO+CLE groups appeared disorganised and disrupted. Obtained findings suggested that CLE was able to enhance nitric oxide bioavailability that might dampen the vasoconstriction effect of phenylephrine.

Keywords

citrus; rutaceae; vegetable oil; vascular reactivity; nitric oxide; hypertension
Introduction

The practice of using repeatedly-heated vegetable oil repeatedly to save money is common. Based on a study conducted among the night market food outlet operators in Kuala Lumpur, 63 percent admitted that they had reused cooking oil repeatedly despite 69 percent of them being aware that the usage of repeatedly-heated cooking oil could be detrimental to health (Azman et al. 2012). This habit is widely practiced in developing and under-developed countries (Phiri et al. 2006; Totani et al. 2006).

Repeatedly-heated vegetable oil has been reported to significantly increase blood pressure and promote oxidative stress as well as lipid peroxidation (Adam et al. 2008; Adam et al. 2009; Perez-Herrera et al. 2013). Oxidative stress and lipid peroxidation cause endothelial dysfunction which may contribute to cardiovascular diseases, particularly, hypertension (Ng et al. 2012). It has been previously demonstrated that chronic intake of repeatedly-heated vegetable oil is associated with both impaired endothelium-dependent and endothelium-independent vasorelaxation as well as augmented vasoconstriction responses (Leong 2009; Nurul-Iman 2013).

Endothelium plays a significant role in regulating the vascular smooth muscle tone by releasing vasoactive mediators. Among the endothelium-derived vasodilators, nitric oxide (NO) is the main mediator. NO induces vasorelaxation by activating soluble guanylate cyclase (sGC) and subsequently increases cyclic guanosine monophosphate (cGMP) level which finally causes the vascular smooth muscles to relax (Archer et al. 1994). Oxidative stress reduces NO bioavailability by triggering the reaction of superoxide anions with NO to form peroxynitrite (Forstermann and Li 2011; Nurul-Iman et al. 2013). Previous studies have shown that chronic ingestion of repeatedly-heated vegetable oil is associated with reduction in the plasma NO level (Leong 2009; Nurul-Iman 2013).

Citrus leaf extract (CLE, patent number: US8425969B2) is an extract derived from citrus species’ leaves consisting of the combinations of C. hystrix, C. aurantifolia, C. microcarpa and C. sinensis. CLE was acquired using validated extraction methods that properly isolated polyphenols, as described in the patent (Mohamed and Mohd Nor 2013). We have previously reported the phytochemical profile of CLE that contains polyphenols specifically flavonoids which includes diosmin, lutein, obacunone, isoquercitrin, hesperidin, didymin, eriocitrin, neocriocitrin, narirutin, naringin, neohesperidin and 7-OH flavone based on high performance liquid chromatography (HPLC) profile, fourier transform infrared spectroscopy (FTIR) spectra, and liquid
chromatography-mass spectrometry (LC-MS) elucidation (Siti et al. 2017). An earlier study showed that CLE added to frying oil reduced blood pressure and oxidative stress in an atherosclerotic rat model (Sukalingam et al. 2016). A dietary supplementation of CLE also reduced the rise in blood pressure and vascular remodelling in repeatedly heated vegetable oil-induced hypertensive rats (Siti et al. 2017). It was also reported that CLE supplementation reduced thromboxane and angiotensin converting enzyme (ACE) while increased heme oxygenase enzyme which further suggested that CLE could ameliorate the imbalance between vasoactive mediators and blood pressure-regulating enzymes (Siti et al. 2017). However, the functional aspect of vessels in terms of vasodilation and vasoconstriction responses remains to be ascertained. Therefore, this study aims to evaluate the effects of flavonoid-rich CLE on aortic vascular reactivity. Additionally, plasma nitrite levels were measured as an index of NO status in rats. The histological observation was also conducted to observe the aortic elastic fibers which might be structurally affected.

**Materials and methods**

**CLE and palm oil**

The patented CLE (patent number: US8425969B2) was obtained from the Institute of Bioscience, Universiti Putra Malaysia, Selangor, Malaysia. Palm oil was bought from Lam Soon Edible Oils, Selangor, Malaysia. The results of the phytochemical profile of CLE based on high performance liquid chromatography (HPLC), fourier transform infrared spectroscopy, (FTIR) spectra and liquid chromatography-mass spectrometry (LCMS) elucidation have been published and therefore can be referenced (Siti et al. 2017).

**Drugs and chemicals**

Phenylephrine-HCl, acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, USA), sodium nitroprusside, and Krebs salts (BDH Limited and BDH Laboratory Supplies, Poole, England) were used for the vascular reactivity study. Modified Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was used for plasma nitrite measurement.
Diet preparation

The palm oil was heated 5 times (5HPO) or 10 times (10HPO) following the method described by Leong et al. (2009). The diets were prepared according to the protocol previously described (Siti et al. 2017). Briefly, ground standard rat chow was mixed with 15% w/w of palm oil either FPO, 5HPO or 10HPO with or without CLE. For diets containing CLE supplementation namely FPO+CLE, 5HPO+CLE, and 10HPO+CLE, an amount of 1.5 g CLE was added to 148.5 g of respective oils before being mixed with 850 g ground standard rat chow. The final composition of CLE in the diet was 0.15% w/w.

Experimental design

Male Sprague-Dawley rats weighing 250-300 g were obtained from the Laboratory Animal Resource Unit, Universiti Kebangsaan, Malaysia. The animals were housed in stainless-steel cages and kept at a room temperature of 27 ± 2°C in a 12-hour light cycle animal room. All rats had free access to food and tap water throughout the experiment. The animals were acclimatized for one week prior to the treatment period. Fifty-six rats were divided into seven groups comprising eight rats each, namely control, fresh palm oil (FPO), fresh palm oil with CLE (FPO+CLE), five-time-heated palm oil (5HPO), five-time-heated palm oil with CLE (5HPO+CLE), ten-time-heated palm oil (10HPO) and ten-time-heated palm oil with CLE (10HPO+CLE). The control group was given a standard rat chow diet without any addition of oil or CLE. Other groups were given a standard diet fortified with 15% weight of total oil composition/ total weight of basal diet (w/w). The respective diets were given for 16 weeks. The non-haemolyzed blood was collected before and after treatment through the orbital sinus under diethyl ether anesthesia in tubes containing ethylene diamine tetraacetic acid (EDTA). The collected blood was immediately kept in ice before being centrifuged at 3000 rpm for 10 min at 4°C to obtain plasma which was stored at -70 to -80°C for nitrite measurement. The rats were killed after 16 weeks of treatment by means of overdose of intraperitoneal ketamine/xylazine combination. The aortae were harvested for the measurement of vascular reactivity. The handling and experimental protocols were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with approval number PP/Far/2014/Kamsiah/22-Jan./571-Mar.-2014-Feb.-2016. The results of the
systolic blood pressures and some blood pressure-regulating mediators including ACE, heme oxygenase-1, prostacyclin and thromboxane as well as lipid peroxidation and aortic histomorphometry which were obtained the same set of experiments using the same set of animals, have been reported in our previous article and therefore are acknowledged (Siti et al. 2017).

Body weight and dietary intake

The body weight measured after the acclimatization period was taken as the baseline body weight and subsequently measured every four weeks using a digital weighing machine (Tanita, Japan). The dietary intake was measured daily using a digital weighing machine (Tanita, Japan).

Plasma nitrite level

Nitrite is a metabolite used as an index of NO status. Nitrite is measured following the method described by Leong et al. (2009). A volume of 50 μl plasma of each rat and standard solutions were pipetted in a microtiter plate and mixed with equal volumes of modified Griess reagent (Sigma-Aldrich, St. Louis, MO, USA). The filled plate was incubated for 15 min at room temperature in a dark environment and the nitrite concentration was measured spectrophotometrically at 540 nm on an Emax ELISA microplate reader using SoftMax Pro Software (Molecular Devices, Sunnyvale, CA, USA). The nitrite concentrations were determined through the standard curve constructed using known concentrations of sodium nitrite (Sigma-Aldrich, St. Louis, MO, USA).

Aortic rings preparation and vascular reactivity

The aortic rings were processed according to the method described by Ajay and Mustafa (2006). Each 3–5 mm length ring segment was suspended in individual 25-mL organ baths filled with Krebs solution containing the following components (mM): NaCl (118.0), KCl (4.7), CaCl$_2$·2H$_2$O (2.5), KH$_2$PO$_4$ (1.2), MgSO$_4$ (1.2), glucose (11.7) and NaHCO$_3$ (25.0). The Krebs solution was kept warm at 37°C and continuously bubbled with a mixture of oxygen and carbon dioxide in a ratio of 95:5 percent. Isometric tension of the aortic rings (g) was recorded using a
force-displacement transducer (F-D-Transducer FT03E, Grass Instruments, West Warwick, RI, USA) connected to a MacLab computerized system (model 8S, AD Instruments, Castle Hill, NSW, Australia). The aortic rings were equilibrated at a basal tension of 1 g for 30 min. During this period, the bathing solution was changed every 15 min, and the resting tension of the aortic rings were readjusted to a basal tension of 1 g whenever needed. Following the equilibration period, vascular reactivity experiments were conducted by determining the reference contractile response to an isotonic KCl solution (high K⁺, 80 mM). Then the rings were washed out of the responses to high K⁺. To confirm the presence of endothelium, the rings were then constricted with phenylephrine (PE 10⁻⁷ M) followed by a single addition of acetylcholine (ACh 10⁻⁵ M) to relax them, following protocol by Leong et al. 2009. The endothelial-intact rings with more than 50% relaxation to ACh were advanced to the next experimental steps to ensure that the rings used were not eroded due to technical faults. The rings were then washed again. Next, the cumulatively increasing concentration of constriction responses to PE (10⁻¹⁰ M to 10⁻⁵ M) were recorded. Furthermore, the relaxation responses to the cumulatively increasing concentration of endothelium-dependent vasodilator ACh (10⁻¹¹ M to 10⁻⁵ M) and 10⁻¹¹ M to 10⁻⁶ M of endothelium-independent vasodilator sodium nitroprusside, (SNP) were recorded for PE (10⁻⁶ M) pre-contracted aortic rings.

Aortic wall histology

The aortic arches were fixed in 10% phosphate buffered formalin solution for 24–48 hours before being embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) and, 5 µm thickness tissue cross sections were obtained using microtome (Leica RM2235, Walldorf, Germany) and affixed to the glass slide. Thereafter, the tissues were stained with Verhoeff-van Gieson (VVG) to identify elastic tissues. All aortic sections were examined under a light microscope at x200 magnification.

Statistics

All results were expressed as mean ± SEM (standard error of mean). A normality test for the data was conducted using the Kolmogorov-Smirnov test. All results were analysed using repeated measures analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Differences (HSD) post-hoc test except for plasma.
nitrite level which was analysed using repeated measures mixed model ANOVA. A value of \( p < 0.05 \) was considered as statistically significant. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 24.0 (SPSS Inc, Chicago, IL, USA).

Results

Body weight and dietary intake

Fig. 1 shows mean body weight for all groups throughout the study period. Body weight was significantly increased compared to the baseline weight (week 0) from week 4 until the end of study \( (p < 0.05) \). There was no difference in weight gain among the treatment groups at any point of time. Fig. 2 shows the mean dietary intake per week for all groups. There was no significant difference in the mean dietary intake per week in all experimental groups.

Plasma nitrite

Fig. 3 shows plasma nitrite level pre-treatment (week 0) and post-treatment (week 16) for all experimental groups. At week 16, plasma nitrite was significantly reduced in 5HPO and 10HPO groups compared to the FPO group as well as versus their respective week 0 levels \( (p < 0.05) \). Plasma nitrite at week 16 was significantly increased in the 5HPO+CLE group compared to the 5HPO group \( (p < 0.05) \). However, plasma nitrite at week 16 in the 10HPO+CLE group was not significantly different compared to the 10HPO group. There was no significant difference in plasma nitrite at week 16 among the control, FPO, FPO+CLE, 5HPO+CLE, and 10HPO+CLE, groups.

Vasoconstriction response to phenylephrine

As can be seen in Fig. 4, phenylephrine (PE) elicited a concentration-dependent vasoconstriction in all the studied aortic rings. The 5HPO and 10HPO groups had significantly increased vasoconstriction response to PE compared to the control and FPO groups from a PE concentration of \( 10^{-6} \) to \( 10^{-5} \) M \( (p < 0.05) \). At the highest PE
concentration \( (10^{-5} \text{ M}) \), the vasoconstriction responses in 5HPO and 10HPO groups were 198 ± 14% and 172 ± 18%, respectively compared to the control (113 ± 10%) and the FPO (109 ± 11%) groups. At PE concentration \( 10^{-5} \text{ M} \), the vasoconstriction response was significantly lower in the 5HPO+CLE group (146 ± 10%) than the 5HPO group \( (p < 0.05) \) but not in 10HPO+CLE group (160% ± 8.4) compared to the 10HPO group.

**Vasorelaxation response to acetylcholine**

As shown in Fig. 5, acetylcholine (ACh) elicited a concentration-dependent vasorelaxation in all the studied aortic rings. The 5HPO and 10HPO groups had a significantly reduced vasorelaxation response to ACh with a concentration of \( 10^{-6} \) to \( 10^{-5} \text{ M} \) \( (p < 0.05) \) compared to the control and the FPO groups. At the highest ACh concentration \( (10^{-5} \text{ M}) \), vasorelaxation responses in the 5HPO and 10HPO groups were 53 ± 4% and 50 ± 5%, respectively compared to the control (100 ± 11%) and the FPO (107 ± 10%) groups. CLE supplementation did not cause significant changes in vasorelaxation response to ACh in 5HPO and 10HPO groups at all ACh concentrations.

**Vasorelaxation response to sodium nitroprusside**

In Fig. 6, sodium nitroprusside (SNP) elicited a concentration-dependent vasorelaxation in all aortic rings studied. 5HPO and 10HPO groups had a significantly reduced vasorelaxation response to SNP of concentration \( 10^{-7} \) to \( 10^{-6} \text{ M} \) \( (p < 0.05) \) compared to the control and FPO groups. At the highest SNP concentration \( (10^{-6} \text{ M}) \), vasorelaxation responses in the 5HPO and 10HPO groups were 101 ± 1% and 104 ± 1%, respectively compared to the control (141 ± 6%) and the FPO (132 ± 4%) groups. CLE supplementation did not cause any significant changes in the vasorelaxation response to SNP in the 5HPO and 10HPO groups at all SNP concentrations.

**Aortic histology**

There were discontinuations of the elastic lamellae of tunica media in 5HPO, 10HPO, and 10HPO+CLE groups. The elastic lamellae in these groups appeared disorganized. Meanwhile, aortic structure in the control, FPO,
FPO+CLE, and 5HPO+CLE groups looked homogenous and parallel without disruption of the elastic lamellae (Fig. 7).

**Discussion and conclusion**

This study shows that CLE provides some potential benefit to prevent vascular dysfunction caused by prolonged consumption of repeatedly heated vegetable oil. Therefore, this study could at least guide future of research towards finding a novel frying oil additive or supplementation to reduce the detrimental effect of repeatedly-heated vegetable oil intake.

In this study, the amount of food intake was not different among all the groups suggesting that the degrees of exposure to the respective diet were equal. The same amount of food intake could also be the reason for the unremarkable difference in the body weight among all groups. Furthermore, all experimental groups showed significant body weight increment from their baseline weight throughout the study period which suggest that repeatedly-heated palm oil as well as CLE did not retard their growth performance. Previous studies also have also shown that repeatedly-heated vegetable oil did not affect body weight (Leong et al. 2009; Ng et al. 2012; Nurul-Iman et al. 2013).

We have previously reported that the supplementation of flavonoid-rich CLE had significantly attenuated the blood pressure-raising effect of 5HPO but not of 10HPO (Siti et al. 2017). Although the observed blood pressure-lowering effect in CLE-supplemented 10HPO group was moderate and statistically insignificant, their potential to lower the blood pressure is of particular interest since reduction by only 20 mm Hg of the usual systolic blood pressure may bring down the occurrence of death from ischemic heart disease and stroke by at least twofold (Lewington et al. 2002). Furthermore, referring to our previous report, both FPO and CLE did not cause hypotension in the normotensive group (Siti et al. 2017).

Five- and ten-time-heated palm oil reduced plasma nitrite therefore indirectly suggesting that there was a reduction in the plasma NO level in 5HPO and 10HPO groups. This finding could be due to oxidative stress event (Leong et al. 2010). During oxidative insult, NO will react with superoxide to form peroxynitrite therefore reducing NO bioavailability (Szabo et al. 2007). CLE supplementation increased plasma NO metabolites in 5HPO but it was not effective enough in 10HPO. The antioxidant effects of CLE against the reaction of NO with superoxide could be
the possible mechanism behind the improvement in NO bioavailability. It was reported that other extracts containing polyphenol such as virgin coconut oil was able to reduce systolic blood pressure possibly by augmenting plasma NO level in the 5HPO-induced hypertension rat model (Nurul-Iman et al. 2013). Previous studies have shown that the degree of lipid peroxidation increases with the frequency of the oil being reheated (Adam et al. 2008; Leong et al. 2012). It was later shown that the supplementation of CLE was able to significantly reduce plasma lipid peroxidation in rats fed with 5HPO but not in 10HPO (Siti et al. 2017). Therefore, the higher degree of oxidative stress in 10HPO compared to 5HPO could be the reason why CLE was not effective enough to improve nitrite bioavailability in the 10HPO group.

Phenylephrine is an \(\alpha_1\)-adrenoreceptor agonist which can indirectly reduce the release of endothelial NO. The NO, in turn, weakens the vasoconstriction effect of PE (Dora et al. 2000). Acetylcholine acts agonistically on the muscarinic receptor (M\(_3\)) in the endothelium thereby inducing the endothelium to produce endogenous NO. SNP on the other hand, is a NO donor which releases NO spontaneously via redox process (Stamler et al. 1992). NO is the vasodilator responsible for which activating soluble guanylate cyclase (sGC) in the smooth muscle cells and eventually causes vasorelaxation. Therefore, ACh requires intact endothelium to exert its vasodilation effect (endothelium-dependent) while SNP does not (endothelium-independent) (Archer et al. 1994; Stamler et al. 1992). Although the aortic rings have been preliminarily screened for the presence of endothelium via incubation with PE \(10^{-7}\) M and ACh \(10^{-5}\) M indicated by more than 50% relaxation, the later dose-response results demonstrated that the vasorelaxation response to the highest ACh concentration (\(10^{-5}\) M) in 5HPO and 10HPO groups were 53 \(\pm\) 4% and 50 \(\pm\) 5%, respectively. The reason for this was unclear. Moreover, the difference in the dose of PE and the protocol make it incomparable. The former screening protocol used a single addition of ACh \(10^{-5}\) M following the addition of PE \(10^{-7}\) M while the later dose-response protocol used the cumulative doses of ACh, supplied as a cumulative regimen from Ach \(10^{-11}\) to \(10^{-5}\) M against the PE \(10^{-6}\) M pre-contracted rings. Perhaps, more study needs to be done focusing on this finding to rule out the deterioration in ACh-mediated dilatation due to nitrate tolerance.

Most likely, the blood pressure-raising effect of repeatedly-heated palm oil (Siti et al. 2017) was associated with the increased vasoconstriction response to PE and reduced vasorelaxation response to both ACh and SNP which suggested that heated oil increased vascular reactivity and total peripheral resistance which then increased blood pressure. These findings were similar to previous findings (Nurul-Iman et al. 2013; Leong et al. 2010). Although the impaired endothelium-dependent vasodilator response is usually associated with normal endothelium-
independent vasodilator response in other models of hypertension and metabolic diseases (Al-Tahami et al. 2011; Lockette et al. 1986; Yamamoto et al. 2008), this association does not appear to be a general rule (Luscher et al. 1992). In the oxidative stress state, peroxynitrite is formed from the reaction of superoxide with NO (Szabo et al. 2007). The increase in peroxynitrite generation may in turn contribute to NO tolerance due to oxidation and desensitization of sGC and eventually reduces vasodilation (Cabassi et al. 2000; Stasch et al. 2006). This phenomenon may explain the impairment in both endothelium-dependent and endothelium-independent vasorelaxation due to prolonged intake of the repeatedly-heated vegetable oil.

CLE supplementation attenuated the vasoconstriction response to PE but not the vasorelaxation response to ACh and SNP in 5HPO group. The reason for this was not clear. The reduction in blood pressure and vasoconstriction response to α1-adrenoreceptor agonist in CLE-supplemented 5HPO group might partly be due to the increase in NO bioavailability as the increased release of endothelial NO may dampen the vasoconstriction effect of PE (Dora et al. 2000). However, the percentage increase in NO concentration in the CLE-supplemented 5HPO group was probably too small to improve the vasorelaxation response to ACh. In addition, the significant improvement of plasma NO in 5HPO+CLE without significant improvement in ACh-mediated vasodilatation suggests that other mediators like prostanoid which may play a part in ACh-mediated vasorelaxation (Morand et al. 2011) may not be affected by CLE and therefore may not contribute to the overall improvement in endothelium-dependent vasorelaxation. Our previous study showed that CLE did not affect the plasma prostacyclin level (Siti et al. 2017) which could therefore possibly support this postulation. Another possible postulation is that CLE may not able to reverse the desensitization of sGC by oxidative insult (Cabassi et al. 2000; Stasch et al. 2006) and therefore cause no improvement in the vasorelaxation response to both ACh and SNP.

Previous studies have shown polyphenol-rich virgin coconut oil (VCO) attenuate the vasoconstriction response to PE in aortic rings of repeatedly-heated vegetable oil-induced hypertensive rats (Nurul-Iman et al. 2013). Furthermore, among flavonoids that tested as being able to improve vascular reactivity and functions includes hesperidin and quercetin (Ajay et al. 2006; Morand et al. 2011). The possible mechanism is probably the ability of flavonoids to enhance NO bioavailability via activation of endothelial NO synthase (Loke et al. 2010; Machha et al. 2007; Yamamoto et al. 2008).

The disruption and disorganization of the elastic lamella structure of 5HPO, 10HPO, and 10HPO+CLE are probably due to mechanical adaptation of the vascular wall towards hypertension. At physiologic pressures, elastic
lamellae are mostly straight. At these pressures, elastic lamellae classically show non-linear mechanics due to load bearing and stiffness (Wagenseil and Mecham 2012). In addition, it is well known that hypertension can accelerate the degradation of elastic fibres (Arribas et al. 2006). The assessment of elastic lamella structure in this study is qualitative and not quantitative which therefore becomes a major limitation. It is uncertain whether the qualitative changes observed on the aortic wall are due to repeatedly-heated palm oil or CLE per se. So far there are no arterial mechanics data to support or refute this.

In conclusion, the present study showed that CLE supplementation was able to attenuate the blood pressure-raising effects of 5HPO but not 10HPO, possibly by improving plasma NO concentration and reducing vascular reactivity towards $\alpha_1$-adrenoreceptor agonist-induced vasoconstriction. CLE had no effect on vasorelaxation and only protected against increased vasoconstriction in the 5HPO group.

Acknowledgements

This study was supported by the Universiti Kebangsaan Malaysia grant GUP-2013-059. The authors would like to thank Mr. Fadlullah Zuhair and Mrs Juliana Hamid for their technical assistance.

Conflict of interest

All authors declare that there is no conflict of interest.

References


https://mc06.manuscriptcentral.com/apnm-pubs


Figure captions

Fig. 1. Rat’s body weight (g) throughout the study period. Data are expressed as mean ± SEM (n = 8). FPO, fresh palm oil; FPO+CLE, fresh palm oil with CLE; 5HPO, five-time-heated palm oil; 5HPO+CLE, five-time-heated palm oil with CLE; 10HPO, ten-time-heated palm oil; 10HPO+CLE, ten-time-heated palm oil with CLE. *significant difference compared to week 0 (p < 0.05); repeated measures ANOVA.

Fig. 2. Mean dietary intake (g) per week. Data are shown as mean ± SEM (n = 8). FPO, fresh palm oil; 5HPO, five-time-heated palm oil; 10HPO, ten-time-heated palm oil; repeated measures ANOVA.

Fig. 3. Plasma nitrite level (µmol/L) pre-treatment (week 0) and post-treatment (week 16) in all experimental groups. Bar is shown as mean ± SEM (n = 8). FPO, fresh palm oil; FPO+CLE, fresh palm oil with CLE; 5HPO, five-time-heated palm oil; 5HPO+CLE, five-time-heated palm oil with CLE; 10HPO, ten-time-heated palm oil; 10HPO+CLE, ten-time-heated palm oil with CLE. *significant difference compared to FPO at week 16 (p < 0.05) and †significant difference compared to 5HPO at week 16 (p < 0.05). ‡significant difference between week 0 and week 16 within the same group (p < 0.05); repeated measures mixed model ANOVA.

Fig. 4. Vasoconstriction response (%) against cumulative increase of phenylephrine (PE) concentration. Points represent mean ± SEM (n = 8). FPO, fresh palm oil; FPO+CLE, fresh palm oil with CLE; 5HPO, five-time-heated palm oil; 5HPO+CLE, five-time-heated palm oil with CLE; 10HPO, ten-time-heated palm oil; 10HPO+CLE, ten-time-heated palm oil with CLE. *significant difference compared to control and FPO (p < 0.05), †significant difference compared to FPO+CLE (p < 0.05) and ‡significant difference between 5HPO+CLE and 5HPO (p < 0.05); repeated measures ANOVA.

Fig. 5. Vasorelaxation response (%) against cumulative increase of acetylcholine (ACh) concentration. Data are shown as mean ± SEM (n=8). FPO, fresh palm oil; FPO+CLE, fresh palm oil with CLE; 5HPO, five-time-heated palm oil; 5HPO+CLE, five-time-heated palm oil with CLE; 10HPO, ten-time-heated palm oil; 10HPO+CLE, ten-
time-heated palm oil with CLE. *significant difference compared to control, FPO and FPO+CLE (p < 0.05); repeated measures ANOVA.

**Fig. 6.** Vasorelaxation response (%) against cumulative increase in sodium nitroprusside (SNP) concentration. Data are shown as mean ± SEM (n = 8). FPO, fresh palm oil; FPO+CLE, fresh palm oil with CLE; 5HPO, five-time-heated palm oil; 5HPO+CLE, five-time-heated palm oil with CLE; 10HPO, ten-time-heated palm oil; 10HPO+CLE, ten-time-heated palm oil with CLE. *significant difference compared to control, FPO and FPO+CLE (p < 0.05); repeated measures ANOVA.

**Fig. 7.** Aortic arch sections stained with VVG representing (a) control, (b) FPO, (c) FPO+CLE, (d) 5HPO, (e) 5HPO+CLE, (f) 10HPO and (g) 10HPO+CLE at magnification x200. Heavy arrows show fragmentation and discontinuation of elastic lamillae observed in 5HPO, 10HPO and 10HPO+CLE groups (Figures 7(d), 7(f) and 7(g)). Light arrows show the elastic lamellae in 5HPO, 10HPO and 10HPO+CLE groups which appeared less parallel compared to the control (Figures 7(d), 7(f) and 7(g)). Abbreviation, TI, tunica intima; TM, tunica media; TA, tunica adventitia; L, lumen.
Graphical abstract

Citrus leaf extract (CLE) supplementation is able to attenuate the blood pressure-raising effects of repeatedly heated vegetable oil, possibly by improving plasma nitric oxide bioavailability and subsequently reducing vascular reactivity towards $\alpha_1$-adrenoreceptor agonist-induced vasoconstriction and therefore reducing the total peripheral resistance.