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Beneficial effects of citrus leaf extract supplementation on renal vasoactive substances in rats fed with repeatedly heated palm oil diet

Derick Zee Yang Tan[1], Hawa Nordin Siti[1], Mohammed SM Saleh[1], Mohamed Azhar Nur-Amalina[1], Nur Izzatul Afiqah Mohd Shakri[1], Jie Lei Chong[1], Ismail Nurul-Ilmi[1], Abdullahisham Mohamad Husnal Al-Haqeem[1], Hj Mohd Saad Qodriyah[1], Norliana Masbah[1], Kamsiah Jaarin[2], Suhaila Mohamed[3] and Yusof Kamisah[1]*

[1]Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia
[2]Department of Pharmacology, Faculty of Medicine and Defence Health, Universiti Pertahanan Nasional Malaysia, Kuala Lumpur, Malaysia
[3]Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Abstract: Addition of citrus leaf extract (CLE) into frying oil was found to be renoprotective in rats that consumed heated palm oil diet. This study examined the effects of dietary CLE supplementation on renal vasoactive substances in rats given heated palm oil diet. Forty-two male *Sprague-Dawley* rats were randomly split and fed with (i) control, (ii) fresh palm oil (FPO), (iii) FPO + CLE, (iv) five-time-heated palm oil (5HPO), (v) 5HPO+CLE, (vii) ten-time-heated palm oil (10HPO) and (vii) 10HPO+CLE diets for 16 weeks. CLE was added into diet at 0.15% (w/w). CLE decreased renal angiotensin-converting enzyme, inducible nitric oxide synthase and angiotensin II expressions in rats given heated oil diets, but only decreased renal NADPH oxidase activity in the 5HPO group. Supplementation of citrus leaf extract has shown beneficial effects in regulating renal vasoactive substances in rats consumed heated palm oil diet.

Keywords: Angiotensin II, heme oxygenase, kidney, nitric oxide, citrus leaf extract, heated palm oil, vasoactive substances.

INTRODUCTION

Cardiovascular disease is a major cause of morbidity and mortality globally (WHO, 2021a). Several modifiable risk factors have been identified in contributing to cardiovascular disease, which includes hypertension, obesity, dyslipidemia, smoking and diabetes. However, hypertension remains the most common risk factor in cardiovascular disease (WHO, 2021b). Over time, uncontrolled hypertension will cause damage to the kidney as the renal arteries become narrowed, thickened and hardened. Thus, oxygen deprivation to the kidney occurs, which leads to renal impairment (Ott and Schmieder, 2022; Pruijm *et al*., 2013). The kidneys also play a role in blood pressure regulation (Patel *et al*., 2017).

One of the factors that lead to hypertension is a dietary habit, including consumption of reused heated cooking oil. The habit of utilizing reheated palm oil is common to reduce cost without considering the potentially harmful effects on health (Abdullah *et al*., 2010). On repeated heating, there will be a more significant generation of free radicals in the oil, which in turn, enhancing oxidative stress. Consumption of the reused heated oil, therefore, causes damage to the arterial wall, which finally promote the development of hypertension (Siti *et al*., 2019). In rats fed with heated oil, increases in angiotensin-converting enzyme (ACE), renal nitric oxide (NO) and a decrease in heme oxygenase (HO) were noted (Kamisah *et al*., 2016; Siti *et al*., 2017). ACE is involved in the conversion of angiotensin I to angiotensin II (Ang II) (Bellomo et al., 2020), which causes vasoconstriction (Řezáčová *et al*., 2019). Binding of the Ang II to its receptor will activate NADPH oxidase, a significant generator of superoxide anion (Siti *et al*., 2021).

Due to elevated oxidative stress, treatment with antioxidants showed protective effects on heated-oil-induced renal damage (Masbah *et al*., 2017). Citrus leaf extract (CLE), which is rich in polyphenol, especially flavonoids, was shown to reduce oxidative stress triggered by intake of heated palm oil diet in rats (Sukalingam *et al*., 2016). Flavonoids being an antioxidant inhibit NADPH oxidase (Siti *et al*., 2021) and ACE activities which eventually would decrease Ang II levels (Korystova *et al*., 2018). Furthermore, flavonoids which include quercetin and luteolin, can upregulate the expression of HO-1 protein (Croft *et al*., 2017).

Previous studies (Siti *et al*., 2017; Sukalingam *et al*., 2016) showed that CLE can reduce blood pressure, reduce oxidative stress biomarkers and vascular damage in hypertension induced by heated palm oil intake. Li *et al*. (2019) also reported that in rats fed CLE-added frying oil, renal oxidative stress and inflammation were significantly reduced. Hence, the purpose of the present study was to ascertain the effects of CLE supplementation on the

**Corresponding author:* e-mail: kamisah_y@yahoo.com

modulation of renal vasoactive substance in rats given repeatedly heated palm oil after 16 weeks.

MATERIALS AND METHODS

Diet preparation

CLE obtained from leaves of *Citrus hystrix* DC. was supplied by the Institute of Bioscience, Universiti Putra Malaysia, Malaysia, while the palm oil was purchased from a local market. The diet was formulated following a technique described by Siti *et al*. (2017). The oil was utilized either fresh (FPO), heated five times (5HPO) or heated ten times (10HPO). Two and half liter palm oil were utilized to fry a kilogram of sweet potatoes in stainless-steel wok at 180ºC for 15 min. To generate 5HPO, the process was repeated for another four times, while for 10HPO, an additional nine times frying process was conducted. The heated oil was cooled down at least for five hours between frying. For CLE-added frying oil, 1.5 g CLE was put into 148.5g oil before frying. The diets were then formulated to contain 15% oil. The final content of CLE in the selected diets was 0.15%. Then, the diet was left overnight at 70ºC in an oven.

Experimental design

Male Sprague Dawley rats (*n*=42) with initial body weight of 200-250g (Universiti Kebangsaan Malaysia Laboratory Animal Resource Unit) were randomly assigned into seven groups; (i) control, (ii) FPO, (iii) FPO+CLE, (iv) 5HPO, (v) 5HPO+CLE, (vi) 10HPO and (vii) 10HPO+CLE. The rats were maintained with the respective diets for 16 weeks. The rats in the control group consumed standard rat chow only. Subsequently, the kidneys were harvested after the rats were humanely sacrificed. The animal handling procedure was endorsed by the institutional animal ethical committee (Approval No.: PP/Far/2014/Kamsiah/22-Jan./571-Mar.-2014-Feb.-2016).

Immunohistochemical study

Preparation of kidney histological section was done following a previously described method (Bai *et al*., 2011). The tissues were embedded in paraffin after being fixed with formalin (10%) for a day. The sections were deparaffinated in xylene and rehydrated in decreasing concentrations of ethanol. The ACE expression was determined using MaxPoly-One™ Polymer Horseradish Peroxidase (Axil Scientific, Singapore) detection kit, while Ang II expression using Two-Step IHC Detection Reagent (PV-9001, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), and inducible nitric oxide synthase (iNOS) using Vector® Red Substrate Alkaline Phosphatase Kit (SK-5100, Vector Laboratories, Burlingame, CA, USA). The sections were incubated for 30 min with 1% bovine serum albumin or 10% goat serum to avoid non-specific binding of immunoglobulins, before being incubated with an anti-mouse anti-ACE monoclonal antibody (1:50, sc-23908, Santa Cruz Biotechnology, Santa Cruz, Dallas, TX, USA), anti-rabbit anti-Ang II polyclonal antibody (1:100, ab124505, Abcam, Cambridge, MA, USA), or anti-nitric oxide synthase 2 (anti-NOS2) monoclonal antibody (1:100, sc7271, Santa Cruz Biotechnology, Santa Cruz, CA, USA). 3,3'-Diaminobenzidine (DAB) substrate kit (Abcam, Cambridge, MA, USA) was employed for the color development. The expressions of ACE, Ang II and iNOS were quantified using an image analyzer.

Biochemical analysis

Renal NADPH oxidase activity was measured according to an established method (Mustapha *et al*., 2010), using an extinction coefficient of 21 mmol/L/cm and expressed in nmol/mg protein. Kidney homogenates for NO content measurement were prepared according to a described method (Mansour *et al*., 2011). The NO content in the kidneys was then calculated by measuring its metabolite, nitrite level (Miranda *et al*., 2001). Renal HO activity was analyzed accordingly as previously described by Vera *et al*. (2008) based on total bilirubin production. An extinction coefficient (40 mmol/L/cm) was adopted in the calculation.

STATISTICAL ANALYSIS

The results were expressed as the mean ± standard error of the mean (SEM). Shapiro-Wilk test was used to test for the data distribution. One-way analysis of variance (ANOVA) followed by Tukey post hoc test was used for normally distributed data, while Kruskal-Wallis test followed by Mann-Whitney U test was applied for not normally distributed data. A value of $P<0.05$ was considered statistically significant. The statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) version 20 software.

RESULTS

Renal ACE and Ang II expressions

Renal ACE expression was significantly higher in rats fed 5HPO and 10HPO diets than the control and FPO groups (fig. 1A). Addition of CLE into the diet of 5HPO and 10HPO groups had significantly decreased the ACE expression. Similar level of expressions was seen between the 10HPO and 5HPO groups. The expression was also similarly lower among the FPO, FPO+CLE and control groups.

Heating frying oil repeatedly for five- and ten-times increased Ang II expression in the kidneys of rats fed these diets compared to control and FPO (fig. 1B). The expression in the 10HPO group was significantly higher than that of the 5HPO. The Ang II expression was lower in 5HPO and 10HPO groups which were added with CLE, than their respective heated oil group ($P<0.05$).

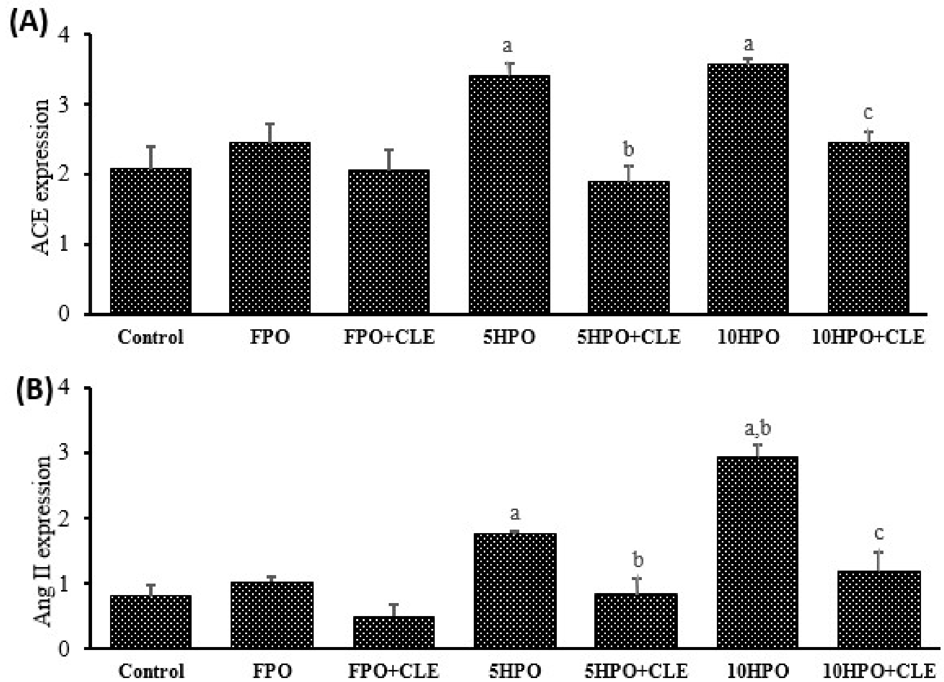

Fig. 1: Renal angiotensin-converting enzyme (ACE) (A) and angiotensin II (Ang II) (B) expressions in rats fed with fresh palm oil (FPO), five-time (5HPO) or ten-times-heated palm oil (10HPO), with or without CLE. Data are means ± SEM (n=6). [a]Significantly different compared with control (p<0.05); [b]significantly different compared with 5HPO (p<0.05); [c]significantly different compared with 10HPO (p<0.05).

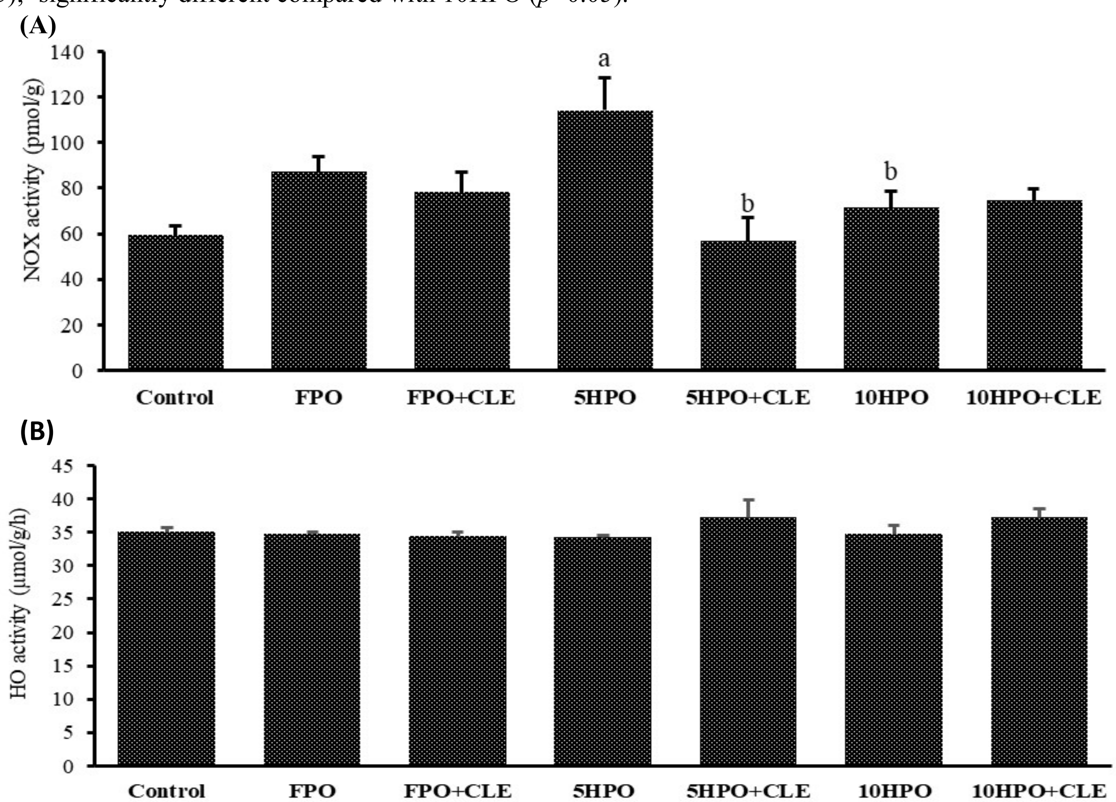

Fig. 2: Renal NADPH oxidase (NOX) (A) and heme oxygenase (HO) (B) activities in rats fed with fresh palm oil (FPO), five-time (5HPO) or ten-times-heated palm oil (10HPO), with or without CLE. Data are means ± SEM (n=6). [a]Significantly different compared from control (p < 0.05); [b]significantly different compared from 5HPO (p < 0.05).

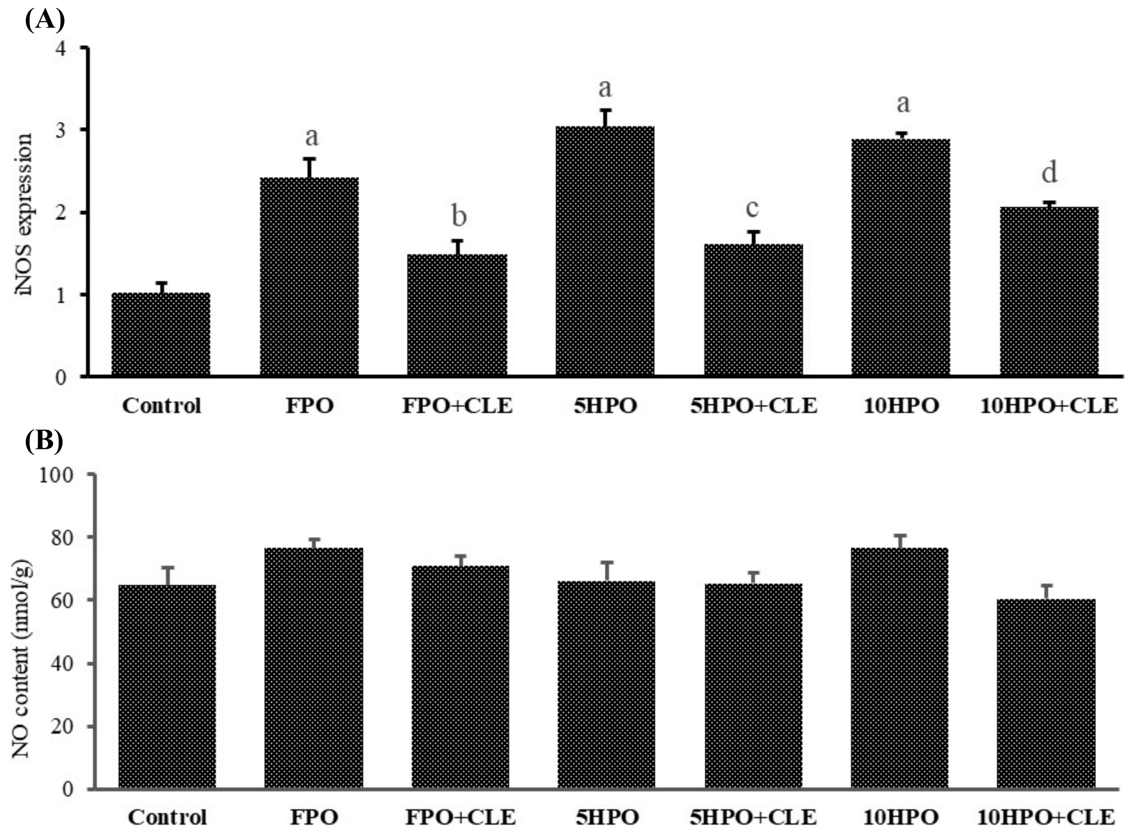

Fig. 3: Renal inducible nitric oxide synthase (iNOS) expression (A) and nitric oxide (NO) content (B) in rats fed with fresh palm oil (FPO), five-time (5HPO) or ten-times-heated palm oil (10HPO), with or without CLE. Data are means ± SEM (n=6). [a]Significantly different compared from control ($p < 0.05$); [b]significantly different compared from FPO ($p < 0.05$); [c]significantly different compared from 5HPO ($p < 0.05$); [d]significantly different compared from 10HPO ($p < 0.05$).

No significant difference in the expression was seen between the FPO and control groups. Addition of CLE into the FPO group did not affect the expression.

Renal NADPH oxidase

Renal NADPH oxidase activity was significantly augmented in the rats fed 5HPO, but not in the 10HPO group compared with the control (fig. 2A). The enzyme activity was significantly lower in the 10HPO group than that of the 5HPO. A decline in the enzyme activity was seen in the 5HPO group supplemented with CLE. The enzyme activity was similar in the control and FPO groups. Renal HO activity (fig. 2B) in rats fed fresh and heated palm oils in the presence or absence of CLE was not affected by the treatment.

Renal iNOS expression and NO content

Renal iNOS expression was similarly elevated in the groups fed FPO and heated oils (5HPO and 10HPO) compared with the control (fig. 3A). Addition of CLE into the diet had significantly brought down the iNOS expression in these groups. The renal NO content (fig. 3B) in rats fed fresh and heated palm oils in the presence or absence of CLE was not affected by the treatment.

DISCUSSION

Renal ACE and Ang II expressions

Consumption of reheated edible oils has been claimed to cause hypertension arising from endothelial damage (Siti *et al*., 2019; Ng *et al*., 2012). Elevation of systemic blood pressure would affect the kidneys because the organ is also involved in the regulation of blood pressure through the renin-angiotensin-aldosterone system (RAAS) (Patel *et al*., 2017). In our study, dietary repeatedly heated palm oils increased ACE and Ang II expressions. Both ACE and Ang II are involved in the RAAS (Patel *et al*., 2017). Ang II is known to cause vasoconstriction via its binding to Ang II type 1 receptors, leading to increased blood pressure (Rezacova *et al*., 2019). Our previous study had demonstrated the development of systemic hypertension in the same set of animals fed 5HPO and 10HPO (Siti *et al*., 2017).

The presence of oxidative stress in the heated oils might induce the activation of ACE expression, leading to increased expression of the Ang II in this study. Previous studies had indicated that increased oxidative stress was associated with the increases in ACE and Ang II expressions (Cui *et al*., 2019; Zhou *et al*., 2016). The inhibitory effects of CLE supplementation on both expressions could be due to the reduced oxidative stress in the oils, attributable to the antioxidant properties of its minor components. The phytochemical profile of CLE in the present study was previously reported, which showed the presence of various flavonoids, namely, diosmin, lutein, obacunone, isoquercitrin, didymin, and hesperidin (Siti *et al*., 2017). Hesperidin, lutein and isoquercitrin are known antioxidants (Huang *et al*., 2019; Cao *et al*., 2018; Li *et al*., 2016). The inhibitory effects of flavonoids on the ACE expression have been consistently demonstrated in many studies (Khan *et al*., 2018; Xing *et al*., 2014).

Renal NADPH oxidase and HO

Even though oxidative stress induces Ang II expression, its over expression also promotes oxidative stress (Xiong *et al*., 2018) via NADPH oxidase activation, as seen in the 5HPO group. The enzyme majorly produces superoxide anion (Gui *et al*., 2019a). However, no similar effect was seen in the 10HPO group. A possible explanation could be that the overwhelming oxidative stress in the 10HPO group had partially destroyed the enzyme. The CLE supplementation blocked the effect of the heated oil consumption on the NADPH oxidase activity. Li *et al*. (2019) had similarly reported the inhibitory effect of CLE, which was added into frying oils. Pretreatment with a flavonoid-rich extract was shown to prevent H_2O_2-induced NADPH oxidase elevation in cardiomyocytes (Gui *et al*., 2019a).

Renal HO activity was not affected by the heated oil diets, with or without CLE supplementation. This finding was again dissimilar to previous studies which showed a reduction in renal HO in rats fed heated oil (Kamisah *et al*., 2016; Li *et al*., 2019). The discrepancy found could not be explained. The enzyme has vasodilating and antioxidant properties (Imai *et al*., 2001) and is inducible by oxidative stress (Forstermann, 2010).

Renal iNOS expression and NO content

In the groups fed heated palm oil, raised oxidative stress level present in the oils augmented iNOS expression. Its increased expression in the FPO group was not understood. iNOS is an enzyme that is inducible by oxidative stress, producing NO (Gui *et al*., 2019b). However, the renal NO content was unaltered by the diet. This finding was different from a previous study (Kamisah *et al*., 2016). It is possible that the NO produced reacted with the super oxide anion, generating peroxynitrite radicals, and thereby reducing the NO bioavailability (Radi, 2018). However, the production rate of the super oxide anion or peroxynitrite was not measured in this study to confirm this postulation. The decrease in iNOS expression by the CLE supplementation indicated that the extract was able to inhibit the upregulation of the protein triggered by free radicals. This finding suggests that the antioxidants in the extract had normalized the detrimental effects of free radicals in the heated oil, resulting in reduced oxidative stress level.

CONCLUSION

Hence, our findings suggest that CLE supplementation could afford renoprotective effects against the detrimental effects of repeated heated oil consumption in rats. It is possibly by modulating the expressions of renal vasoactive substances like angiotensin II, ACE2 and iNOS, as well as NADPH oxidase activity. However, its effects on the histological features of the kidneys were not confirmed in this study. Further study needs to be conducted to elucidate its other potential renoprotective effects.

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