Biomedical & Pharmacology Journal, March 2019. Vol. **12**(1), p. 363-373

The Effects of Citrus Leaf Extract on Renal Oxidative Stress, Renal Function and Histological Changes in Rats Fed with Heated Palm Oil

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http://dx.doi.org/10.13005/bpj/1649

(Received: 14 October 2018; accepted: 12 January 2019)

Prolonged consumption of heated palm oil causes detrimental effects on cardiovascular system, liver and kidneys. The detrimental effects of heated oils are associated with oxidative stress; hence the role of antioxidants in attenuating heated oil-induced effects has been widely studied. The aim of the present study was to determine the effects of polyphenol-rich Citrus leaf extract (CLE) on renal oxidative stress parameters, renal function and kidney histological changes in rats fed with heated palm oil. Fifty-six male *Sprague-Dawley* rats *(n=56)* were divided into seven groups. The control group was given normal rat chow, while other groups were fed with 15% weight/weight (w/w) palm oil-enriched diet of either fresh palm oil (FPO), five-time-heated palm oil (5HPO) or ten-time-heated palm oil (10HPO); with or without the addition of CLE (0.15%, w/w) supplementation. After 16 weeks, the rats were sacrificed and the kidneys were harvested for analysis. CLE supplementation improved heated palm oil-induced oxidative stress parameters in the kidneys, shown by reduced levels of renal thiobarbituric acid (TBARS) and renal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in 5HPO and 10HPO groups. Renal haem oxygenase levels were restored with CLE supplementation in the heated oil groups. Decreased serum creatinine was observed in 5HPO group with CLE supplementation, but not in 10HPO group. Heated oil caused mild interstitial inflammation with vascular congestion in 5HPO and 5HPO+CLE groups, while 10HPO group had moderate inflammation and vascular congestion. CLE supplementation in 10HPO group was able to reduce these changes as only mild interstitial inflammation and congestion was observed. CLE has the potential to reduce renal oxidative stress parameters, improve renal function and reduce renal inflammation possibly mediated via its antioxidant properties.

Keywords: Citrus leaf;Oxidative stress; *Rutaceae*;Heated oil; Renal function; Polyphenols.

Palm oil is commonly used asthe major source of cooking oil in many households and food industries in this part of the world. In addition, the oils are commonly heated repeatedly as this practice helps to save cost[1]. However, it is established that the components of the oils tend to degenerate after repeatedly heating, leading to increased viscosity and darkening in color due to increased fatty acid composition during hydrolysis[2].

Heated palm oil undergoes hydrolysis, oxidation and polymerization which generates reactive oxygen species such as hydroperoxides and low molecular volatile compound[2] leading to increased oxidative stress. Subsequently, during frying process, these reactive oxygen species will be absorbed into food and therefore poses health threats when consumed[3].

Oxidative stress imposes multisystem detrimental effects on the body[4]. It has shown to cause cardiac damage and remodeling[5], endothelial dysfunction[6] and increased blood pressure leading to atherosclerosis[7]. In addition, studies have shown that consumption of heated palm oil (HPO) results in increased inflammatory liver markers and liver damage in experimental rats[8-9].Chronic consumption of heated oils was also reported to cause increased oxidative stress measured as renal thiobarbituric acid reactive substances (TBARS)[10-11], increased NADPH oxidase[12] and reduced renal haem oxygenase activity[13], eventually leading to impairment in renal function[14]; which was manifested as increased serum creatinine as well as chronic inflammation of the renal parenchyma in rats[13, 15].

The citrus leaf extract (CLE) studied in this research was derived from citrus leaves ofthe Rutaceae plant family. A previous study using similar leaf extract has reported that CLE contains polyphenols such as diosmin, lutein, isoquercitrin, obacunone, hesperidin and didymin[16-17]. It was originally developed and patented (patent number: US8425969B2) as an oil additive during frying process; targeted mainly to reduce oil absorption into food and at the same time provide anti-oxidative effects that render the frying oil to be more resistant against thermal oxidation[16]. CLE, which was also known as ADD-X in previous studies, was reported to possess antihypertensive effects by reducing vascular damage in hypertensive rats fed with heated palm oil diet[17]. An earlier study has also reiterated that CLE possessed antihypertensive properties as well as able to attenuate oxidative stress biomarkers when incorporated into the diet of ovariectomised rats[18]. CLE supplementation has also been shown to display anti-inflammatory and cardio-protective properties by preventing heated oil-induced necrotic changes in cardiac tissues of experimental rats [19]. Another study which reported the effects of CLE supplementation on aortic vascular reactivity in rats fed with heated palm oil has outlined that CLE supplementation managed to restore plasma nitric oxide levels, thereby reducing vasoconstriction-mediated vascular reactivity in the heated palm oil group [20]. Although CLE supplementation has been shown to have multiple beneficial effects; particularly on blood pressure, inflammatory markers and oxidative stress parameters, its effects on the kidneys has not been explored. Therefore, this study was undertaken to evaluate the effects of CLE specifically on the kidneys, by measuring its effects on renal oxidative stress, renal function and kidney histological changes in rats fed with heated palm oil.

MATERIALS AND METHOD

Materials

CLE was obtained from University Putra Malaysia (UPM), Malaysia. Palm oil used for this study was purchased from a local manufacturer (Lam Soon Edible Oils) in Selangor, Malaysia. Creatinine (serum) Colorimetric assay kit was purchased from Cayman Chemical, Ann Arbor, MI, USA. Other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Loius, MO, USA).

Animals and experimental design

This study and animal handling was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee. For this study, a total of fifty-six adult male Sprague-Dawley rats (weight between 180 to 200g) were obtained from the Laboratory Animal Research Unit, Universiti Kebangsaan Malaysia. All rats were housed in stainless steel cages in temperature maintained at 27ºC with 12-hours light-dark cycle. The animals had free access to water and food (rat chow) throughout the study. After one week of acclimatization, treatment was commenced according to the assigned groups. The animals were randomly divided equally into seven groups

with eight rats in each group; namely control, fresh palm oil (FPO)**,** fresh palm oil with CLE (FPO+CLE), five-times-heated palm oil (5HPO), five times-heated palm oil with CLE (5HPO+CLE), ten times-heated palm oil (10HPO) and ten times-heated palm oil with CLE (10HPO+CLE) groups. The control group were fed with normal rat chow without oil. The other groups were fed with diet consisting of rat chow fortified with 15% weight of total oil composition / total weight of basal diet (15% w/w). The total oil composition may be with or without CLE according to the assigned groups. After16 weeks, the rats were sacrificed and subsequently the kidneys were weighed and harvested for histological analysis, renal thiobarbituric acid reactive substance (TBARS) measurement, serum creatinine, heme oxygenase and NADPH levels.

Heated oil and animal diet preparation

For diet preparation, palm oil was used as either fresh palm oil (FPO), five-times heated palm oil (5HPO) or ten-times heated palm oil (10HPO) which was prepared according to the methods described earlier by Leong *et al*. [7]. 2.5 litres of the oil were used to fry 1 kg of peeled sweet potato slices in a stainless-steel wok at 180ºC for 20 minutes. The hot oil was allowed to cool down for at least 5 hours before the frying process was repeated to produce 5HPO and 10HPO. The frying process was repeated with a fresh batch of sweet potatoes (without addition of fresh oil) for another four times to obtain five-times-heated palm oil (5HPO) and another nine times to obtained ten-times-heated palm oil (10HPO). For FPO+CLE, 5HPO+CLE and 10HPO+CLE groups, the respective oils used were then mixed with 10% diluted CLE, with ratio of CLE to oil equals to 1:10 before the diet preparation. Standard rat chow (obtained from Gold Coin, Selangor, Malaysia) were grounded and mixed with 15% w/w of the respective oils. The rats were fed with the respective diets for 16 weeks.

Measurement of serum creatinine

Serum creatinine was measured using a commercial kit available in the market (Cayman's Creatinine (serum) Colorimetric Assay Kit. The developed color intensity was measured at 490 nm using ELISA microplate reader. The results for creatinine in serum were expressed as ìmol/l.

Measurement of renal thiobarbituric acid reactive substances (TBARS)

TBARS content in the kidney was determined by using previously described method by Ledwozyw *et al*.[21] with a few modifications. Firstly, kidney homogenate (0.5 mL) was added to 2.5 mL trichloroacetic acid (1.22mol/L) in 37% hydrochloric acid (HCl). Then, it was incubated for 15 min at room temperature. The mixture was mixed with 1.5 mL 0.67% of thiobarbituric acid in 0.05mol/L sodium hydroxide (NaOH) and incubated at 100 °C in a water bath for 30 min. The mixture was cooled down then added into 4 mL n-butanol. Subsequently, the mixture was vortexed vigorously and centrifuged at 3000 r/min for 10 minutes. The absorbance upper layer was read using a spectrophotometer at 532 nm against 1, 1, 3, 3-tetraethoxypropane standard curve. The results were expressed as nmol/g wet weight.

Measurement of renal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

NADPH oxidase was measured based on its capacity to reduce ferricytochrome c in ferrocytochrome at pH of 7.8[22]. Kidney homogenate (50 µg protein/experiment), cytochrome c (250 µg/l final concentration), and NADPH (100 µM) was incubated either in presence or absence of diphenyleneiodonium (DPI, 100 µM). The absorbance of reduction of cytochrome c was measured at 550 nm. The difference between the absorbance of samples between 0 to 120 min was measured in nmol/mg protein and the extinction coefficient 21 mmol/l/cm.

Measurement of heme oxygenase (HO-1) activity

The method described by Vera *et al*.[23] was used to determine heme oxygenase activity. Heme oxygenase activity was measured based on total bilirubin measurement. Initially, kidney homogenate in phosphate buffer (0.25mmol/L, pH 7.7) was centrifuged at 13 000 rpm at 4°C for a total of 15 minutes.5mg protein of sample was added into a reaction mixture containing 2mmol/L glucose-6-phosphate, 0.2 unit glucose-6-phosphate dehydrogenase, 0.8 mmol/L nicotinamide adenine dinucleotide phosphate, and 20 ìmol/L hemin. The mixture was subsequently incubated in the dark for 1 hour at 37°C. 1mL of chloroform was added to extract formed bilirubin. The samples were

then centrifuged at 7000r/min for 10 minutes. The optical density of the chloroform later was read at 466-530nm, Finally, the heme oxygenase activity was calculated based on extinction coefficient of 40 $mM^{-1}cm^{-1}$.

Kidney histology

Kidney samples were initially fixed in phosphate formalin buffer solution (10%) before being blocked in paraffin, sectioned at 5ìmol/L and stained with haematoxylin-eosin. Kidney histology was assessed by light microscopic examination and slides were read under 200x and 400x magnifications. All the slides were examined by an experienced pathologist in a blinded fashion and graded for the presence of inflammation (mononuclear cell infiltration), red blood cell (RBC) congestion and necrosis. The extent of the above mentioned histological changes in the kidney was graded using the following pre-defined scale:

Normal : 0 % of area is affected

Mild changes : <25% of the area is affected

Moderate changes : 25-50% of the is affected

Severe changes : >50% of the area is affected

Statistical analysis

All results for this study were expressed as mean ± SEM (standard error of mean). Shapiro-

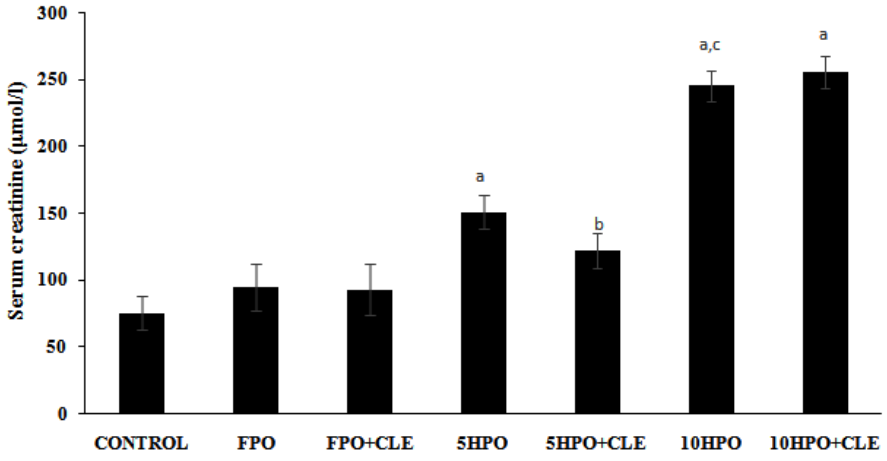

Fig. 1. Serum creatinine levels in rats fed with fresh palm oil (FPO), five-time-(5HPO) or ten-time-heated palm oil (10HPO), with and without CLE supplementation. Data are means ± SEM (n=8). [a]significant differencevs control and FPO ($p<0.05$); [b]significant difference vs 5HPO ($p<0.05$); [c]significant differencevs 5HPO ($p<0.05$)

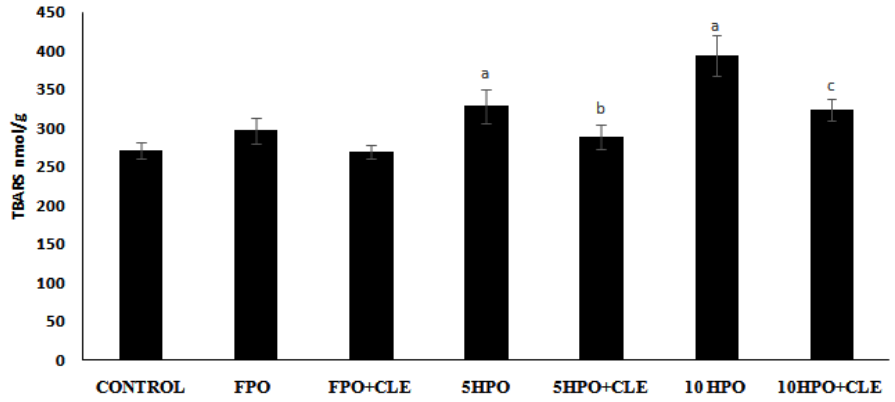

Fig. 2. Renal TBARS activity in rats fed with fresh palm oil (FPO), five-time-heated (5HPO) or ten-time-heated (10HPO), with and without CLE supplementation. Data are means ± SEM (n=8). [a] significant difference vs control and FPO ($p<0.05$); [b] significant differencevs 5HPO ($p<0.05$); [c] significant differencevs 10HPO ($p<0.05$)

Wilk test was applied to check for normality of data. Normally distributed data were analysed using ANOVA and followed by Tukey's Honestly Significant Differences (HSD) post-hoc test. A value of $p<0.05$ wasconsidered as significant. All statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) version 23 software (SPSS Inc, Chicago, IL, USA).

RESULTS

Serum creatinine

Fig. 1 shows the serum creatinine level of all experimental groups at the end of the study (week 16). There was no significant difference in the levels of serum creatinine of FPO and FPO + CLE groups when compared to control. Serum creatinine was significantly increased in 5HPO and 10HPO groups as compared to control and FPO groups. Serum creatinine level in 5HPO + CLE group was significantly lower compared to 5HPO ($p < 0.05$). However, serum creatinine level in 10HPO + CLE group was not significantly different compared with 10HPO group.

Renal thiobarbituric acid reactive substances (TBARS)

Figure 2shows renal TBARS level for all experimental groups.There was no significant

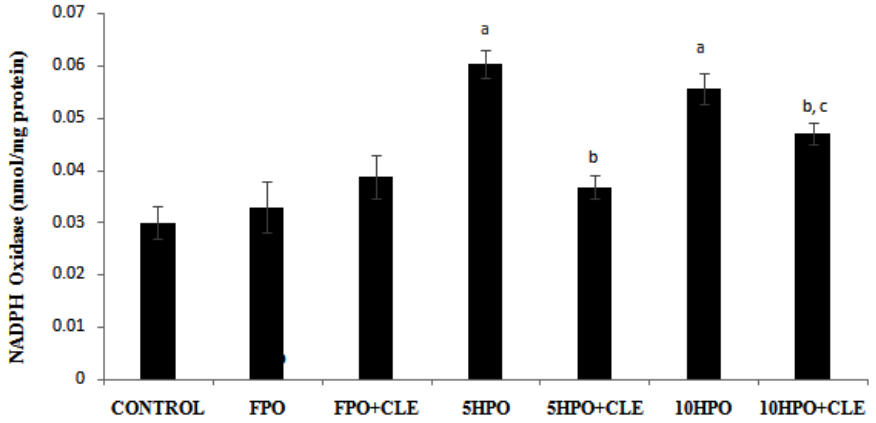

Fig. 3. Renal NADPH oxidase activity in rats fed with fresh palm oil (FPO), five-time-(5HPO) or ten-time-heated palm oil (10HPO), with and without CLE supplementation. Data are means ± SEM (n=8). [a]significant differencevs control and FPO ($p<0.05$); [b]significant differencevs 5HPO ($p<0.05$);[c]significant differencevs 10HPO ($p<0.05$)

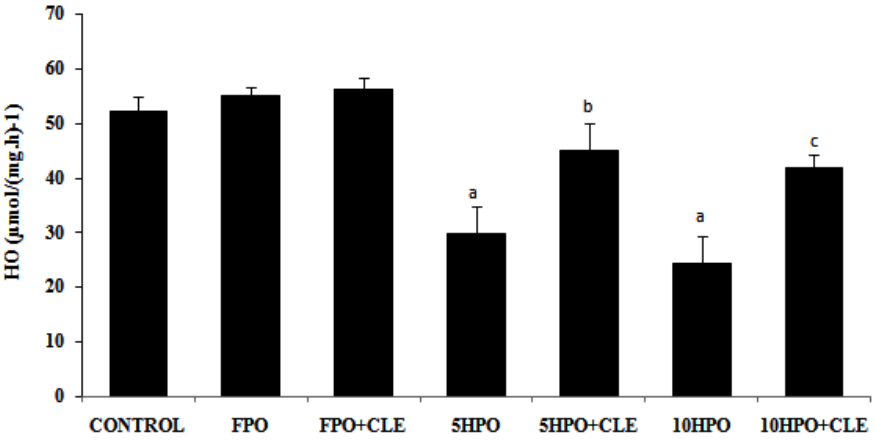

Fig. 4. shows HO levels in rats fed with FPO, 5HPO and 10HPO with or without CLE. Data are means ± SEM (n = 8). [a]significant difference vs control and FPO ($p < 0.05$); [b]significant difference vs 5HPO ($p < 0.05$); [c]significant difference vs 10HPO ($p < 0.05$)

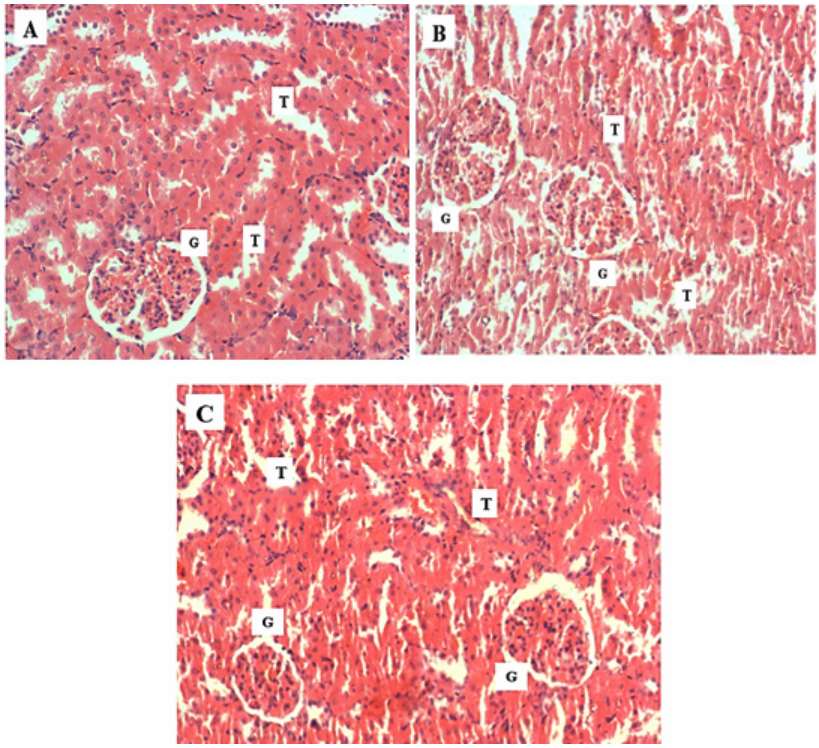

Fig. 5. Photomicrographs showing cross section of the rats' kidneys stained using Hematoxylin and Eosin (H&E), magnification x 200. All three groups namely **A)** Control, **B)** Fresh Palm Oil (FPO) and **C)**FPO+CLEgroups showed no congestion and inflammation. G: Glomerulus ; T: Tubule

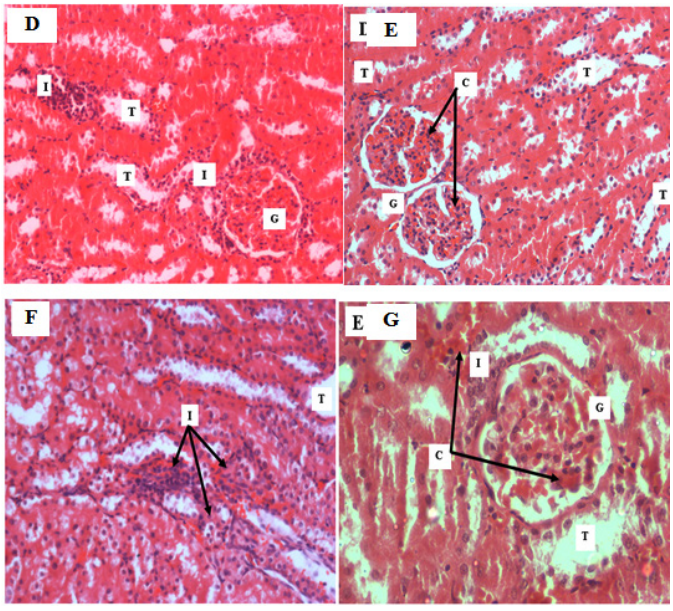

Fig. 6. Photomicrographs showing cross section of the rats' kidneys stained using H&E.
D) 5 times heated palm oil (5HPO) group showed mild inflammation in the interstitium (X200). **E)** 5HPO group showed mild congestion at the glomerulus (X200). **F)** 5HPO + CLE group showed mild inflammation at the interstitium (X200). **G)** 5HPO + CLE group showed mild inflammation at the interstitium and mild congestion at the glomerulus (x400). C: Congestion; G: Glomerulus I: inflammation; T: Tubule

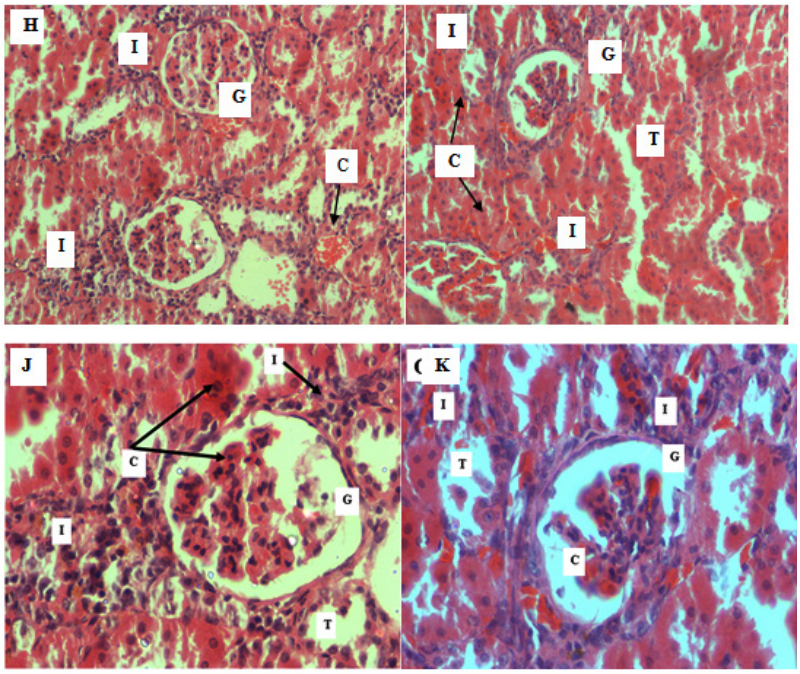

Fig. 7. Photomicrographs showing cross section of the rats' kidneys stained using H&E.
H) 10HPO group showed moderate congestion in the glomerulus and tubular sections and moderate inflammation at the interstitium. **I)**10HPO + CLE group showed mild inflammation at the interstitium and mild interstitial congestion. **J)** 10HPO group showed moderate congestion in the glomerulus and moderate inflammation in the interstitium. **K)** 10HPO+ CLE showed mild inflammation at the interstitium and mild congestion in the glomerulus.C: Congestion; G: Glomerulus; I: inflammation; T: Tubule

difference between renal TBARS in FPO and FPO+CLE compared to control group. There was a significant increase in renal TBARS in 5HPO and 10HPO groups compared to control ($p < 0.05$). Renal TBARS level is significantly reduced in 5HPO+CLE and 10HPO+CLE compared to 5HPO and 10HPO groups respectively.

Renal Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

Figure 3 shows renal NADPH oxidase activity of all experimental groups at the end of the study. There was no significant difference in renal NADPH oxidase activity in FPO and FPO +CLE compared to control. NADPH oxidase activity in 5HPO and 10HPO groups were significantly increased compared to control ($p < 0.05$). NADPH oxidase activities in 5HPO + CLE and 10HPO + CLE were significantly reduced compared to 5HPO and 10HPO groups respectively ($p< 0.05$).

Haem oxygenase (HO) activity

Figure 4 shows HO levelsfor rats fed with heated palm oil with or without CLE supplementation. FPO and FPO+CLEgroups were comparable to the control group. 5HPO and 10HPO groups showed significant decrease in HO activity compared to control and FPO groups ($p < 0.05$). With CLE supplementation, HO activity were significantly restored in both 5HPO and 10HPO groups ($p < 0.05$).

Kidney histology

Figure 5 shows photomicrographs (A - C) of the control, FPO and FPO+CLE groups. There was no congestion, inflammation or necrosis noted in the tubules, glomerulus and interstitium of control, FPO and FPO+CLE groups. Figure 6 shows photomicrograph (D - G), whereby there were mild congestion in the glomerulus and mild inflammation in the interstitium of the 5HPO and 5HPO+CLE groups. Figure 7 shows photomicrographs (H – K), which shows moderate congestion and inflammation in 10HPO group. In contrast, the 10HPO + CLE group showed mild congestion and inflammation in the interstitium

and glomerular areas. No necrosis was observed in any of the experimental groups.

DISCUSSION

Long-term consumption of repeatedly heated palm oil is known to cause detrimental health effects, including to the kidneys. In this study, intake of 5HPO and 10HPO diet for 16 weeks was observed to significantly increase serum creatinine and oxidative stress parameters such as renal TBARS and NAPDH oxidase, as well as reduced HO activity in the experimental groups. A significant increase in serum creatinine levels in/†5HPO and 10HPO groups/†was observed when compared to control group./†This finding is in accordance with other studies which reported a significant increase in serum creatinine concentration in thermoxidised palm oil group and repeatedly heated cooking oil groups respectively [14, 24]. One of the mechanisms proposed for causing deterioration of renal function is due to oxidative stress, whereby excessive reactive oxygen species (ROS) such as hydroperoxides produced as a result of repeated heating of oil[2], led to cellular damage by reacting with various biomolecules such as proteins, nucleic acids or lipids[25]. However, our research finding was in contrary with the findings of Jaarin *et al*.[15], which reported that there was no significant difference in serum creatinine among the groups at the end of study period. The reason for the discrepancy in this finding was unclear, although it could possibly be attributable to the shorter frying duration in the previously mentioned study. In our study, supplementation of CLE had significantly reduced the serum creatinine level in 5HPO but not in 10HPO group. This finding shows that CLE supplementation, through its ROS-scavenging activitywas able to reduce the extent of renal damage only in the 5HPO group due to less oxidative damage induced by less number of oil heating in this group. Therefore, the antioxidant properties of CLE might no longer be effective in 10HPO group when the oxidative insult is greater[17], as caused by the increased number of oil heating.

Renal TBARS levels were significantly increased in the 5HPO and 10HPO groups compared to control. † This finding is in line with previous studies indicating that repeated heating increases oil oxidation, which subsequently increases lipid peroxidation[10, 11, 18, 26]. CLE supplementation was able to significantly reduce the renal TBARS in 5HPO and 10HPO groups. This finding was in agreement with previous studies which reported that citrus leaf extract supplementation reduced plasma TBARS in heated oil groups [10, 18]. Similarly, as highlighted by a previous report, oxidative stress has the capability to promote renal injury by inducing cytotoxicity, as shown by accumulation of renal malonyldialdehyde levels which represents renal TBARS levels in our present study[25].

Chronic consumption of 5HPO and 10HPO were shown to increase NADPH oxidase levels in our present study. Similarly, a study by Perez-Herrera *et al*.[12] has demonstrated that consumption of repeatedly heated oil increased NADPH oxidase gene expression. Jaarin *et al*. also showed the association between oxidative stress and cardiac NADPH oxidase activity in hypertensive rats[27]. As reported previously, the reduction in NADPH oxidase activity comes hand in hand with decreased ROS production[28-29], therefore as proven in our study, CLE supplementation was able to attenuate the ROS-associated increase in NADPH levels in the heated oil groups. In favour of this, several studies have also showed the efficacy of polyphenols and dietary flavonoids in reducing cellular NADPH oxidase activity[30-31],which may be attributed to reduced ROS production that subsequently attenuated high levels of NADPH oxidase activity [28-29].

There is an inverse relationship between heme oxygenase-1 (HO-1) levels and oxidative stress. Higher levels of oxidative stress induced by chronic consumption of repeatedly heated palm oil have been shown to reduce renal heme oxygenase[13, 32-33], which is supposed to have anti-oxidative effects. In contrast, when the activity of the enzyme is increased, bilirubin formation will rise, which subsequently will function as an antioxidant with anti-inflammatory properties[34]. In this present study, the heated oil groups (5HPO and 10HPO groups) had reduced levels of heme oxygenase activity, indicating that oxidative stress were markedly increased due to repeated heating in these experimental groups. However, with CLE supplementation, heme oxygenase activity was restored in 5HPO and 10HPO groups which are in accordance with the study reported by Siti *et al*.[17].

Histological analysis showed that there was evidence of inflammation in the heated oil groups after 16 weeks of treatment. Mild interstitial inflammation and mild congestion were observed at the glomeruli area of the rats' kidneys for both 5HPO groups, with and without CLE supplementation. This further reiterates the fact that oxidative process which occurs as the oil is repeatedly heated is damaging to the renal cells[25, 35] as the findings in our study has also highlighted that there was a similar increasing trend of serum creatinine levels for both heated oil groups. However, 10HPO group showed more severe histological changes, as moderate inflammation and congestion were observed surrounding the glomeruli and interstitium. This was in accordance with Jaarin et.al[15] which highlighted that the degree of congestion and inflammation in the kidneys was more severe in 10HPO compared to 5HPO experimental groups. However, in our study, the addition of CLE into 10HPO group was able to reduce the severity of congestion and interstitial inflammation observed, possibly due to its antioxidant properties. This finding is in accordance with the findings highlighted by Jones and Shoskes[36], which reported that the addition of polyphenols such as curcumin and quercetin were able to ameliorate ischaemic perfusion injury that is associated with irreversible lipid peroxidation in animal model. Other previous studies have also shown a strong correlation between nephroprotective effects of flavonoid-rich plant extracts against nephrotoxicity induced in experimental rat model, possibly mediated via their high antioxidant content and free-radical scavenging properties[37-38]. Although the method of inducing nephrotoxicity in the experimental animals might differ with our study (drug-induced as opposed to nephrotoxicity induced via heated oil diet supplementation), it is possible that similar nephroprotective mechanisms offered by the polyphenol contents in the CLE has managed to restore the oxidative stress parameters in this study and reversed the histological changes in the heated palm oil groups.

CONCLUSION

Based on the findings of this study, CLE supplementation after 16 weeks of treatment was capable to improve renal oxidative stress parameters, reduce renal injury and preserve renal function in heated oil group. These findings suggest that CLE has renoprotective effects against heated oil-induced oxidation and renal injury, possibly mediated by its antioxidant properties that is rich in polyphenols.

ACKNOWLEDGEMENT

This research was funded by grant from Universiti Kebangsaan Malaysia Medical Centre (grant number FF-2017-077).The researchers would like to acknowledge Mr. Fadhlullah Zuhair Japar Sidik, Mrs. Juliana Abd Hamid and Ms Nur Hani Suhaimi for providing technical assistance for the research.

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