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## Tualang honey supplementation improves oxidative stress status among chronic smokers

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Free radicals induced by cigarette smoking have been linked to an increase in oxidative stress resulting in smoking-related cardiovascular diseases. However, the possible effect of honey that has antioxidant property in improving oxidative stress status among smokers has not yet been reported. Hence, this study was to determine the effects of 12-week Tualang honey supplementation on F<sub>2</sub>-isoprostanes, superoxide dismutase, glutathione peroxidase, catalase, and total antioxidant status among chronic smokers. A total of 32 non-smokers and 64 chronic smokers were recruited from Quit Smoking Clinic and Health Campus, Universiti Sains, Malaysia. Smokers were randomized into two groups ( $n = 32/\text{group}$ ) namely smokers without supplementation and smokers with honey supplementation (20 g/day) for 12 weeks. Blood was obtained from non-smokers and smokers at pre-intervention and from smokers at post-intervention. During pre-intervention, the levels/activity of F<sub>2</sub>-isoprostanes, total antioxidant status, and catalase were significantly higher while superoxide dismutase and glutathione peroxidase were lower in smokers than non-smokers. During post-intervention, in supplemented smokers, there were significant decrease in F<sub>2</sub>-isoprostanes and increase in total antioxidant status, glutathione peroxidase and catalase levels/activities compared with pre-intervention. This study indicates that honey supplementation improves oxidative stress status suggesting a beneficial role of honey in reducing the risk of cardiovascular diseases.

**Keywords:** cigarette smoking; oxidative stress; Tualang honey; antioxidant; smokers

### Introduction

Cigarette smoking is a major risk factor for the development of cardiovascular diseases such as atherosclerotic vascular disease, hypertension, coronary artery disease, myocardial infarct, and stroke (Ockene and Miller 1997). Endothelial dysfunction, inflammation, oxidant–antioxidant imbalance as well as an alteration of antithrombotic and prothrombotic factors are among the smoking-related major determinants of initiation and acceleration of the atherothrombotic process leading to these cardiovascular diseases. Exposure to cigarette smoke is associated with increased lipid peroxidation (Garg et al. 2006; Bloomer 2007; Pasupathi, Saravanan, and Farook 2009), alteration in erythrocyte antioxidant enzymes, (Pannuru et al. 2011; Tonguc et al. 2011; Aziz et al. 2012) and decreased total antioxidant status (TAS) (Bloomer 2007; Jha et al. 2007; Aziz et al. 2012) in human studies.

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Studies have shown that several antioxidants are effective in reducing oxidative stress among smokers (Lee et al. 2010; Leelarungrayub et al. 2010). Supplementation of a green algae namely *Chlorella vulgaris* for six weeks significantly increases the plasma antioxidant status such as vitamin C and  $\alpha$ -tocopherol in male smokers. The levels of erythrocyte antioxidant enzymes activities such as catalase (CAT) and superoxide dismutase (SOD) are also significantly increased (Lee et al. 2010). Meanwhile, another study reported that supplementation of another herb namely *Vernonia cinerea* Less for eight weeks significantly increases TAS and decreases malonaldehyde levels in active smokers (Leelarungrayub et al. 2010).

Honey is a natural product of bees formed from nectar. Interestingly, apart from sugars such as glucose and fructose, honey also has minerals, proteins, organic acids, and antioxidants such as vitamins A and E, glutathione reductase, CAT, and polyphenols (Gheldof, Wang, and Engeseth 2002b; Al-Waili 2003; Mato et al. 2003; Yao et al. 2004; Bloomer 2007; Michalkiewicz, Biesaga, and Pyrzynska 2008; Khalil et al. 2011). Honey has been shown to have antioxidant activity as measured by ferric reducing antioxidant power assay and antiradical activity as measured by 1,1-diphenyl-2-picrylhydrazyl assay (Mohamed et al. 2010). Honey supplementation significantly reduces oxidative stress and toxic effects of cigarette smoke on the testicular structures in rats (Mohamed et al. 2011). Furthermore, significant increases in seminal SOD, CAT, and TAS are found after honey supplementation for eight weeks in male road cyclist. (Tartibian, Hajizadeh Maleki, and Abbasi 2011)

To date, the possible effect of honey supplementation in improving oxidative stress status in chronic smokers has yet to be reported. Therefore, the aim of this study was to determine the effects of Tualang honey supplementation on plasma F<sub>2</sub>-isoprostanes, erythrocyte antioxidant enzymes, and plasma TAS among chronic smokers.

## Materials and methods

The study protocol was performed in compliance with the institutional guideline and approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USM) (approval code: JEPeM (243.3.(6))). This study involved 32 male non-smokers and 64 male chronic smokers aged between 20 and 50 years. The non-smokers were recruited from the Health Campus USM staff and the chronic smokers were volunteers or patients of the Quit Smoking Clinic, USM Hospital and Health Campus USM staff. Participants from the smoker group had smoked at least 10 cigarettes per day for more than five years. The exclusion criteria included a history of taking regularly consuming multivitamins and/or dietary supplements three months before participation in the study and any history of cardiovascular diseases. Parameters such as body mass index and blood pressure were taken and recorded for each participant. Carbon monoxide in the breath of smokers and non-smokers was determined (Sabzwari and Fatmi 2011).

Participants who had fulfilled the criteria were briefed on the nature of study and informed consent was obtained. Randomization was computer generated, and participants of the smoker's group were randomly divided into two groups, namely smokers without supplementation ( $n = 32$ ) and smokers with supplementation ( $n = 32$ ) of 20 g a local wild honey (Tualang honey, supplied by Federal Agricultural & Agro Based Industry, Malaysia) per day for 12 weeks. The honey was prepared in sachets to ensure correct dosage. Analysis of Tualang honey used in this study has been reported earlier whereby its phenolic and flavonoid contents were  $27.0 \pm 0.1$  mg gallic acid equivalents per kg and  $21.7 \pm 0.4$  mg catechin equivalents per kilogram, respectively. High-performance liquid

chromatography analysis showed that it contained phenolic acids such as benzoic, gallic, syringic, and trans-cinnamic acids as well as flavonoids such as catechin and kaempferol (Khalil et al. 2011). During the initial visit (pre-intervention, week 0), 10 mL of blood was taken for oxidative stress status assessment before supplementation with honey was started. Any possible side effects of supplementation and compliance with sachet counting were monitored at weeks 4, 8, and 12, after which post-intervention oxidative stress status was assessed. As for the non-smoker group, blood was collected only at pre-intervention.

Venous blood (10 mL) samples were collected (from all participants) into EDTA tubes (Shanghai Orsin Medical Technology Co. Ltd, Shanghai, China), plasma was immediately separated by centrifugation at  $1000 \times g$  for 10 minutes at  $4^\circ\text{C}$ , aliquoted (2 mL each) for biochemical analysis of TAS and  $\text{F}_2$ -isoprostanes, and kept frozen at  $-80^\circ\text{C}$  until assayed. For determination of the activities of SOD, CAT, and glutathione peroxidase (GPx), the remaining packed erythrocytes were lysed with four volumes deionized water, followed by centrifugation at  $10,000 \times g$  for 15 minutes at  $4^\circ\text{C}$ . The lysate samples were kept frozen at  $-80^\circ\text{C}$  until analysis.

CAT, GPx, and SOD were determined using commercially available kits (CAT and GPx assay kits, Bioassay Systems, Hayward, USA; SOD assay kit, Northwest Life Sciences, Vancouver, USA) and expressed as U/mg Hb. Hemoglobin was assayed using a non-cyanide method (Shah, Shah, and Puranik 2011). Plasma TAS was measured as the trolox equivalent antioxidant capacity using quantichrom antioxidant assay kit (Bioassay Systems,). For  $\text{F}_2$ -isoprostanes, plasma was hydrolyzed followed by solid-phase extraction purification according to manufacturer's instruction (STAT-8-Isoprostane EIA Kit, Cayman, Michigan, USA).

Statistical analyses were carried out using the Statistical Package for Social Science version 20 (International Business Machines Corporation, North Castle, USA). Boxplots were used to test normality of the data. Normally distributed data were analyzed using independent and paired *t*-tests while non-normally distributed data were analyzed using Wilcoxon signed-rank test. Independent *t*-test was used to analyze the differences between non-smokers and smokers group. The baseline and changes over a 12-week intervention period in each group of smokers were assessed by paired *t*-test or Wilcoxon signed-rank test accordingly. A value of  $p < 0.05$  was considered statistically significant. Data are presented as mean  $\pm$  standard error of the mean (SEM) or median (interquartile range).

## Results

A total of 32 male non-smokers and 64 male chronic smokers participated in this study. The characteristics of the participants are summarized in Table 1. Non-smokers and smokers did not differ ( $p > 0.05$ ) in their mean age. Smokers had significantly higher ( $p < 0.05$ ) levels of CO than to non-smokers.

In smokers, plasma  $\text{F}_2$ -isoprostanes, plasma TAS, and erythrocyte CAT were significantly higher while erythrocyte SOD and GPx activities were significantly lower than in non-smokers (Table 2).

In smokers with honey group, there was a significant decrease in plasma  $\text{F}_2$ -isoprostanes and a significant increase in the erythrocyte GPx and CAT activities following 12 weeks of intervention (Table 3). No significant difference was observed in the level of erythrocyte SOD activity while plasma TAS was significantly increased compared to pre-intervention.

Table 1. Characteristics of the non-smokers' and smokers' groups.

	Non-smokers ( <i>n</i> = 32)	Smokers ( <i>n</i> = 64)	<i>p</i> value <sup>#</sup>
Age (years)	35.2 (1.5)	38.4 (1.0)	0.066
CO Analysis (mg/m <sup>3</sup> )	0.4 (0.2)	7.5 (0.8) <sup>*</sup>	<0.001

Note: Data are presented as mean (SEM). CO: carbon monoxide.

<sup>#</sup>Independent *t*-test.

<sup>\*</sup>*p* < 0.05 compared to non-smokers.

Table 2. Pre-intervention oxidative stress status between non-smokers' and smokers' groups.

	Non-smokers ( <i>n</i> = 32)	Smokers ( <i>n</i> = 64)	<i>p</i> value <sup>#</sup>
F <sub>2</sub> -isoprostanes (pg/mL)	221 (13)	945 (56) <sup>*</sup>	<0.001
GPX (U/mg Hb)	0.58 (0.03)	0.47 (0.03) <sup>*</sup>	0.022
SOD (U/mg Hb)	2450 (200)	1914 (117) <sup>*</sup>	0.015
CAT (U/mg Hb)	0.143 (0.004)	0.171 (0.005) <sup>*</sup>	<0.001
TAS (μmol/L Trolox equivalent)	497 (16)	568 (16) <sup>*</sup>	0.006

Note: Data are presented as mean (SEM).

<sup>#</sup>Independent *t*-test.

<sup>\*</sup>*p* < 0.05 compared to non-smokers.

Table 3. Pre-intervention and post-intervention oxidative stress status in smokers with and without honey.

	Smokers with honey ( <i>n</i> = 32)			Smokers without honey ( <i>n</i> = 32)		
	Pre-intervention	Post-intervention	<i>p</i> value <sup>b</sup>	Pre-intervention	Post-intervention	<i>p</i> value <sup>b</sup>
F <sub>2</sub> -isoprostanes (pg/mL)	846 (75)	528 (56) <sup>*</sup>	0.001	1005 (87)	1146 (131)	0.413
GPX (U/mg Hb)	0.547 (0.045)	1.16 (0.08) <sup>*</sup>	<0.001	0.41 (0.21) <sup>a</sup>	0.61 (0.23) <sup>a</sup>	0.068 <sup>c</sup>
SOD (U/mg Hb)	1518 (154)	1590 (167)	0.760	2263 (162)	1501 (173) <sup>#</sup>	0.005
CAT (U/mg Hb)	0.169 (0.006)	0.201 (0.010) <sup>*</sup>	0.004	0.17 (0.01)	0.19 (0.01)	0.073
TAS (μmol/L Trolox equivalent)	550 (20)	568 (23) <sup>*</sup>	0.037	582 (122) <sup>a</sup>	623 (211) <sup>a</sup>	0.136 <sup>c</sup>

Note: Data are presented as mean (SEM),

<sup>a</sup>median (interquartile range).

<sup>b</sup>Paired *t*-test.

<sup>c</sup>Wilcoxon signed-rank test.

<sup>\*</sup>*p* < 0.05 compared to pre-intervention in smokers with honey group.

<sup>#</sup>*p* < 0.05 compared to pre-intervention in smokers without honey group.

In smokers without honey supplementation, after 12 weeks no significant differences were found for plasma F<sub>2</sub>-isoprostanes, TAS, as well as the activities of erythrocyte GPx and CAT, but the activity of erythrocyte SOD was significantly decreased.

## Discussion

During pre-intervention, the level of plasma F<sub>2</sub>-isoprostanes, the lipid peroxidation end products that indicate oxidant activity, was higher in smokers than in non-smokers. As

cigarette smoke has been demonstrated to have radical species such as quinone, semiquinone, hydroquinone, nitric oxide, and carbon-centered acyl- and alkylaminocarbonyl radicals (Church and Pryor 1985; Bartalis et al. 2008), the higher oxidant activity could be produced by cigarette smoke. Quinone/hydroquinone complex is capable of reducing oxygen to produce superoxide anion which may give rise to other reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical (Church and Pryor 1985). Lipid peroxidation of cellular membrane may eventually result in the damage of the cell structure and function. Thus, our finding provides compelling evidence that smoking causes oxidative modification of biologic components in humans (Morrow et al. 1995). The increased lipid peroxidation observed in smokers is in agreement with previous studies as reported earlier (Bloomer 2007; Pasupathi, Saravanan, and Farook 2009; Seet et al. 2011). However, several studies reported no significant difference in the level of plasma lipid peroxidation among both groups as the human body is able to compensate by removal of its adducts (Jha et al. 2007).

Reduced F<sub>2</sub>-isoprostanes suggests a reduced lipid peroxidation among smokers supplemented with honey. Similar findings have been reported on the beneficial effect of honey in reducing lipid peroxidation in human and animal studies (Kilicoglu et al. 2008; Erejuwa et al. 2010; Mohamed et al. 2011; Tartibian, Hajizadeh Maleki, and Abbasi 2011; Erejuwa et al. 2012). Kilicoglu and colleagues (2008) reported that supplementation of honey to the bile duct ligated rats significantly reduces the malonaldehyde (MDA) levels. Apart from that, in pancreas of diabetic rats, honey administration significantly reduces the elevated level of MDA (Erejuwa et al. 2010) and prevents the formation of MDA in the kidney of spontaneously hypertensive rats (Erejuwa et al. 2012). Honey supplementation has been found to reduce oxidative stress in the testis by reducing the level of lipid peroxidation in rats exposed to cigarette smoke for 13 weeks (Mohamed et al. 2011).

During the pre-intervention, the activity of erythrocyte SOD was significantly lower in smokers than to non-smokers which is similar with previous studies (Pasupathi, Saravanan, and Farook 2009; Tonguc et al. 2011). The imbalance between ROS generation and scavenging activities may lead to oxidative stress which in turn causes oxidative damage to the cellular component and alters many cellular functions including loss of enzymatic activity (Kohen and Nyska 2002). Therefore, it is suggested that low SOD activity found in the present study might be due to the inactivation of the enzyme by the ROS. As SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide, the low in SOD activity could also be attributed to its utilization in scavenging superoxide anion into hydrogen peroxide, which is then removed by GPx and CAT. On the contrary, other studies have reported a higher SOD activity among smokers (Tonguc et al. 2011; Aziz et al. 2012). Increased activity of gingival SOD in smokers may be the consequence of adaptive and protective mechanism against oxidative stress developing in the gingival tissue (Tonguc et al. 2011). Duthie and colleague reported that the activity of SOD between smokers and non-smokers is similar possibly due to potent antioxidant defense capacity of the erythrocytes (Duthie, Arthur, and James 1991). This difference could be attributed to the characteristics of the study subjects.

In this study, the activity of SOD remained unchanged among smokers with honey supplementation. This could possibly be attributed to the lower formation of radicals as a result of the radical scavenging effect of honey as shown by the reduced F<sub>2</sub>-isoprostanes. In contrast, Tartabian, Hajizadeh, and Abbasi (2011) observed that supplementation of honey for eight weeks to male cyclists significantly increased seminal SOD activity. This difference could be attributed to the duration of supplementation and the characteristics of the study subjects. The activity of SOD was significantly reduced in the group of

smoker without honey supplementation after 12 weeks which might be due to the inactivation of the enzyme by ROS.

At pre-intervention, the activity of GPx was significantly lower in smokers which is consistent with previous reports (Pasupathi, Saravanan, and Farook 2009; Aziz et al. 2012). The level of GPx activity was significantly increased with the supplementation of honey among smokers in the present study suggesting that honey might increase the bioavailability of GPx that scavenges hydrogen peroxide resulting in reduced level of oxidative stress damage of the cell. The high activity of GPx could also be a compensatory mechanism to scavenge high hydrogen peroxide formed by concomitant higher oxidant activity produced by cigarette smoke. This finding is consistent with previous report which observed that honey supplementation significantly increased GPx activity in kidneys of streptozotocin-induced diabetic rats (Omotayo et al. 2010).

At pre-intervention, the activity of CAT revealed to be significantly higher in smokers than non-smokers suggesting the compensatory mechanism of this enzyme to overcome the increased oxidative stress by continuously decomposing hydrogen peroxide into water and oxygen. Our result is in accordance with previous studies (Garg et al. 2006; Tonguc et al. 2011). The increased activity of CAT after honey supplementation among smokers may suggest the increased bioavailability of CAT to scavenge hydrogen peroxide by honey supplementation. Our finding is supported by previous reported studies (Erejuwa et al. 2010; Yao et al. 2011). Honey supplementation was shown to restore the activity of CAT in the erythrocytes of young and middle-aged rats (Yao et al. 2011) and in the pancreas of diabetic rats (Erejuwa et al. 2012).

In this study, the higher level of TAS, in smokers than non-smokers, was in accordance with the finding of a previous study in which increased levels of superoxide radical scavenging activity, compared to non-smokers, was reported (Durak et al. 2002). The increased TAS level could be a compensatory effect in reducing the increased oxidative stress and might partly contribute by the high CAT activity in smokers. In contrast, previous studies reported lower (Bloomer 2007; Aziz et al. 2012) and similar (Jha et al. 2007) level of TAS between smokers and non-smokers. These differences could be due to the characteristics of the study subjects. Following 12 weeks of honey supplementation, a significant increase in the plasma level of TAS may suggest the capability of honey to increase plasma antioxidant capacity and offset oxidative stress as shown by the significant increases in CAT and GPx activities in this study. This finding is supported by previous studies (Omotayo et al. 2010; Mohamed et al. 2011; Tartibian, Hajizadeh Maleki, and Abbasi 2011).

Tualang honey used in the present study has been reported to consist of antioxidants such as phenolic acids (benzoic, gallic, syringic, trans-cinnamic acids) and flavonoids (catechin, kaempferol), which have strong free radical-scavenging activities (Khalil et al. 2011). Therefore, it is suggested that the reduced oxidative stress status among smokers after honey supplementation could be partly attributed to the antioxidant property of honey.

In conclusion, chronic smokers had significantly higher levels of F<sub>2</sub>-isoprostanes, TAS and CAT activity as well as significantly lower levels of SOD and GPx activities than non-smokers at pre-intervention. Supplementation of honey for 12 weeks significantly reduced the level of F<sub>2</sub>-isoprostanes and increased the level of TAS as well as the activities of CAT and GPx among chronic smokers. Our findings may suggest that honey can be used as a supplement among those who are exposed to free radicals in cigarette smoke either as active or passive smokers in order to protect or reduce the risk of having cardiovascular diseases.

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## Disclosure statement

The authors have declared that there is no conflict of interest.

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