Hibiscus sabdariffa Linn. (Roselle) Protects Against Nicotine-Induced Heart Damage in Rats
(*Hibiscus sabdariffa* Linn. (Rosel) Melindungi Kerosakan Jantung pada Tikus yang Diaruh Nikotin)

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ABSTRACT

Nicotine has been identified as one of the causal factor for oxidative stress, hypertension and hyperlipidemia. Roselle has been widely studied for its potential as an antioxidant, antihyperlipidemic and antihypertensive. However, no studies have been done to investigate if roselle could diminish the oxidative stress caused by nicotine which could further lead to cardiac damages. Thus, this study was aimed to investigate the effect of roselle extract (HSE) on blood pressure, serum lipid profile, oxidative stress marker levels and histological changes to the heart in nicotine-treated rats. A total of 21 Sprague-Dawley rats were randomly divided into 3 groups (n=7 per group): Control group received normal saline (0.5 mL/day, i.p); nicotine group received 0.6 mg/kg/BW nicotine (i.p); and treated group received 100 mg/kg/b.w HSE through oral force feeding followed with 0.6 mg/kg/b.w nicotine (i.p) for 21 consecutive days. The results showed that HSE significantly (p>0.05) reduced the heart rate but no effect to the blood pressure. For lipid profile study, HSE increased the high-density lipoprotein (HDL) concentration significantly (p<0.05) in rats given with nicotine, without any significant changes in total cholesterol, triglyceride and low-density ipoprotein (LDL) concentration. Besides, HSE treatment was also found to reverse malondialdehyde (MDA) level, superoxide dimustase (SOD) enzyme activity and protein concentration significantly (p<0.05) in nicotine-treated rats. In summary, these results indicated that HSE is an effective antioxidant against oxidative damage in heart caused by nicotine, but not as antihyperlipidemic and antihypertensive agent in this rat model.

Keywords: Blood pressure; oxidative stress; nicotine; Roselle

ABSTRAK

Nikotin telah dikenal pasti sebagai salah satu faktor penyebab untuk tekanan oksida, hipertensi dan hiperlipidemia. Rosel telah dikaji secara meluas kerana potensinya sebagai antioksidan, antihiperlipidemik dan antihipertensi. Namun begitu, tiada lagi kajian setakat ini yang mengkaji sama ada rosel mampu merendahkan tekanan oksida yang disebabkan oleh nikotin yang kemudiannya boleh membawa kepada kerosakan jantung. Dengan itu, kajian ini telah dijalankan untuk menentukan kesan ekstrak rosel (HSE) pada tekanan darah, profil lipid serum, aras penanda tekanan oksida dan perubahan histologi jantung tikus terawat-nikotin. Sebanyak 21 tikus Sprague-Dawley telah dibahagikan secara rawak kepada 3 kumpulan (n=7 setiap kumpulan): Kumpulan kawalan yang menerima salina normal (0.5 mL/hari, i.p); kumpulan nikotin yang menerima 0.6 mg/kg/BW nikotin (i.p.); dan kumpulan rawatan yang menerima 100 mg/kg/BW HSE secara paksaan oral diikuti dengan 0.6 mg/kg/BW nikotin (i.p) selama 21 hari berturut-turut. Hasil kajian menunjukkan bahawa HSE menurunkan kadar denyutan jantung secara signifikan (p>0.05) tetapi tiada kesan pada tekanan darah. Bagi profil lipid pula, HSE mampu meningkatkan aras lipoprotein berketumpatan tinggi dengan signifikan (p<0.05) pada tikus diberi nikotin, namun tanpa sebarang perubahan signifikan pada aras kolesterol jumlah, trigliserida dan lipoprotein berketumpatan rendah. Selain itu, rawatan HSE juga didapati mampu untuk memperbaiki aras malondialdehid (MDA), aktiviti enzim superoksida dismutase (SOD) dan kepekatan protein secara signifikan (p<0.05) pada tikus terawat-nikotin. Secara kesimpulannya, hasil kajian ini telah menunjukkan bahawa HSE adalah antioksidan yang berkesan terhadap kerosakan oksida oleh nikotin pada jantung, namun bukan sebagai agen antihipertensi dan antihipersif di dalam model tikus ini.

Kata kunci: Nikotin; Rosel; tekanan darah; tekanan oksida

INTRODUCTION

Nicotine, an alkaloid isolated from the tobacco leaves, is usually found in cigars and cigarettes (Efraim et al. 2000). According to Joukar et al. (2012), smoking is one of the contributing factors for hypertension and atherosclerosis that lead to cardiovascular diseases. Nicotine enhances the process of lipolysis that releases plasma free fatty acids and further accelerates the synthesis of cholesterol, triglyceride, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) (Helen et al. 2000). In addition, high concentration of LDL will lead to development of atherosclerosis by stimulating LDL oxidation in the arterial

wall (Sreekala & Indira 2008). Activation of sympathetic activity triggered by nicotine could also lead to increased heart rate and vasoconstriction, thus elevates the blood pressure (Talukder et al. 2011; William 2013). This could be caused by the decreased production of nitric oxide (NO) due to excessive free radicals due to nicotine intake, leading to systemic vasoconstriction and hypertension (Zainalabidin et al. 2014). Subsequently, hypertension could lead to other complications such as left ventricular hypertrophy and heart failure (Lip et al. 2000).

Nicotine has been shown to cause oxidative stress which increases reactive oxygen species (ROS) and lipid peroxidation (Suleyman et al. 2002). Oxidative stress could cause heart failure through impairment of mitochondrial respiration that reduces ATP production and necrosis (Kunwar & Priyadarshini 2011; Nojiri et al. 2006). Lipid peroxidation in the heart causes Ca^{2+} overload that triggers development of atherosclerosis, hypertension, cardiac hyperthrophy and heart failure (Jamaludin 2008). This Ca^{2+} release from sarcoplasmic reticulum is by the reaction of superoxide, hydroxyl (OH) and NO with sulfhydryl groups in the heart (Valko et al. 2007). The increase of lipid peroxidation in nicotine-induced rats showed abolished antioxidant enzyme activities such as catalase (CAT) and superoxide dismutase (SOD) (Helen et al. 2000; Joukar et al. 2012). ROS produced in the cells were physiologically removed by endogenous antioxidant, a primary means to prevent cellular damage (Leiris et al. 2006). Thus, exogenus source of antioxidant has been considered as one approach to balance this oxidative redox status.

Roselle (*Hibiscus sabdariffa*) is a local tropical plant which offers a wide range of nutraceutical benefits. It is characterized by red calyces which is rich in antioxidant components, mainly anthocyanin, that counteracts with oxidative damages by scavenging the hydroxyl radicals, donating electron to free radicals and terminating radical chain reaction (Cissouma et al. 2013). Besides that, unique sour taste in roselle extract indicates that roselle contains phenolic acid components, such as hibiscus acid that has the ability to prevent carbohydrate metabolism and absorption, as well as inhibit lipolysis (El-Shafey et al. 2013). In a clinical study on hypertensive subjects, it was found that roselle could reduce mean arterial pressure (MAP), comparable to the effect of captopril, an antihypertensive drug (Harerra-Arellano et al. 2004). The antihypertensive activity of roselle could be possibly via the inhibition of angiotensin-converting enzyme activity (ACE) (Ojeda et al. 2010), enhancement of vascular activity by Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase (Olatunji et al. 2006) or by increment of NO production, an endothelium-derived relaxing factor (EDRF) (Ajay et al. 2006).

In spite of all these evidences, little is known about the effects of roselle on nicotine-induced heart damage. We hypothesized that roselle has protective potential towards heart damages caused by nicotine. Therefore, this study was aimed to investigate the effects of roselle extract (HSE) on blood pressure, serum lipid profile, oxidative stress marker level and histological changes to the heart in nicotine-treated rats.

MATERIALS AND METHODS

PREPARATION OF ROSELLE AQUEOUS EXTRACT

Dried roselle (*H. sabdariffa* L. UKMR-1) calyces were collected from Kuala Berang, Terengganu and authenticated by a plant taxonomist at Herbarium Unit, Universiti Kebangsaan Malaysia (UKM) and were deposited as voucher specimen UKMB 40308. Roselle aqueous extract was prepared based on the method by Jamaludin et al. (2013). First, dried calyces were added to distilled water in a 1:2 ratio and ground with blender (Cornell, Malaysia) for 10 min. After that, the roselle extract was heated until it boiled and allow to cool. Cooled extract solution was filtered and then the filtrate was kept frozen in an aluminium-wrapped round bottle flask overnight at -20°C before freeze-dried (Heto-Holten A/S, Denmark) and stored in a dark bottle at 4°C until use. From a 250 g dried calyx, a total yield 42.8 g of extract powder was obtained.

ANIMALS AND EXPERIMENTAL DETAILS

Sprague-Dawley male rats (270-300 g) were obtained from Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia (UKM). The animal ethic was approved by UKM Animal Ethic Committee (FSK/Biomed/2012/Satirah/12-Dec./488/-Dec.-2012-Dec-2014). The animals were kept in cages of 2 rats per cage and maintained under standard conditions at 12 h light and 12 h dark cycle. They were fed on standard rat chow and water *ad libitum*. Twenty-one rats were randomly divided into 3 groups: Group I (Control): Rats were given 0.5 mL/day normal saline, (i.p); Group II (Nicotine): Rats were given 0.6 mg/kg BW nicotine, (i.p) (Helen et al. 2000); and Group III (Nicotine + HSE): Rats were given 100 mg/kg BW HSE (oral gavage) (Idris et al. 2012) + 0.6 mg/kg BW nicotine, (i.p).

The regimens were continued for 21 consecutive days (Helen et al. 2000). Blood pressure was taken on day-0 and day-21 by using CODA II TM Non-Invasive Blood Pressure (NIBP) System (Kent Scientific Corporation, USA). At the end of the experiment, the rats were deprived of food overnight. On day-22, under the light anesthesia by diethyl ether, blood sample was taken via orbital sinus and centrifuged for serum separation. Serum was then stored at -40ºC for lipid profile analysis. The hearts were excised, washed with cold 1.15% KCl and then weighed. The heart tissue was homogenized in 10 mL of 1.15% KCl (ice-cold) and centrifuged at 6000 g, 15 min at 4ºC. After centrifuge, the clear supernatant solution was stored at -40ºC for biochemical analysis.

LIPID PROFILE

Serum total cholesterol (TC), high-density lipoprotein (HDL) and triglyceride (TG) levels were measured by using

semi-automated biochemical analyzer Biosystem BTS 350 (BioSystem Reagent & Instrument, USA). LDL is calculated according to Friedewald et al. (1972). Besides, atherogenic index (TC/HDL) and predictive indicator of cardiovascular disease (CVD) (LDL/HDL) were calculated according to Leimieux et al. (2001).

The production of MDA was measured for lipid peroxidation based on the Stocks and Dormandy (1971) methods. Briefly, heart sample homegenate was added to a trichloroacetic acid/hydrochloric acid (TCA/HCl) solution, vortexed and incubated at room temperature for 15 min. The mixture was added to thiobarbituric acid/sodium hydroxide (TBA/NaOH), vortexed and heated in a boiling water bath (100°C) for 30 min. The MDA in the heart sample reacted with the TBA to form a pink chromogen containing thiobarbituric acid reactive substances (TBARS). The level of TBARS in the heart sample was measured using a spectrophotometer at 532 nm. The MDA concentration was expressed as nM/mg of protein.

Superoxide dismutase (SOD) enzyme activity was determined using the Beyer and Fridovich (1987). Briefly, heart sample homogenate was mixed with the substrate containing PBS (EDTA), L-methionine, nitroblue tetrazolium chloride (NBT.2HCl) and Triton-X and mixed together with riboflavin. The mixture was incubated in an aluminium box under 20 Watt lamp for 7 min. The SOD activity was measured spectrometrically by monitoring the inhibition of ferricytochrome reduction using a xanthine-xanthine oxidase as a source of peroxides. SOD unit is defined as one unit of enzyme, which inhibits 50% of the nitro blue tetrazolium (NBT) reduction. SOD activity was calculated and expressed in U/mg protein.

Reduced glutathion (GSH) content was measured based on the Ellman (1959) method with some modification where 415 nm was replaced with 412 nm. Heart sample homogenate was mixed with a reaction buffer at pH8.0 and 5,5'-dithiobase-2-nitrobenzene (DTNB) for 15 min and measured at 412 nm by using a microplate reader. The GSH content was expressed as mmol/mg protein.

Protein concentrations in the plasma were measured by the method of Lowry et al. (1951). Briefly, plasma was added to Lowry stock reagent and incubated for 30 min at 27°C. Then, Follin-Phenol 2N was added, vortexed and incubated again for 30 min at 27°C. The level of protein was measured by using spectrophotometer at 595 nm. The protein level was expressed as mg/mL.

Histological observation of the heart was performed using Hematoxylin and Eosin (H&E) staining method.

The results were presented as mean ± SEM. Biochemical data were analyzed by One-Way ANOVA while blood pressure data were analyzed using Mixed-ANOVA. All data were tested for normality using the Shapiro-Wilk test ($p>0.05$) and Levene's test was used to assess homogeneity ($p>0.05$). P-value ≤ 0.05 was considered as statistically significant.

At the end of experiment, there was no significant difference in the rats body weight among the three groups ($p>0.05$) (Table 1).

Table 2 shows the blood pressure monitoring at day-0 and day-21. The basic parameters at the initial experiment were consistent between all groups (day-0). At day-21, there was no significant difference in the blood pressure parameters; namely systolic, diastolic and mean arterial pressure. However, the heart rate in nicotine + HSE decreased significantly compared to nicotine group ($p<0.05$).

TABLE 1. Body weight parameter before and after treatment

Group	Body weight (g)	
	Day-0	Day-21
Control	274.86±5.06	310.00±5.00
Nicotine	261.86±4.82	297.86±5.87
Nicotine + HSE	269.14±6.58	295.00±8.77

TABLE 2. Blood pressure and heart rate monitoring before and after treatment

Group	SBP (mmHg)		DBP (mmHg)		MAP (mmHg)		HR (bpm)	
	Day-0	Day-21	Day-0	Day-21	Day-0	Day-21	Day-0	Day-21
Control	115.00±2.90	120.00±4.90	87.33±3.30	86.17±2.12	96.44±3.03	97.33±2.90	360.00±8.47	380.00±10.81
Nicotine	118.00±0.87	124.00±0.99	88.58±2.30	88.76±2.11	98.16±1.88	100.00±1.28	363.00±3.80	400.00±6.36
Nicotine + HSE	121.00±3.21	110.00±3.50	84.00±5.80	80.50±2.36	95.83±4.69	88.83±1.36	358.00±7.10	336.00±0.60[b]

[b] $p<0.05$ vs. nicotine group

The serum lipid profile showed no significant changes in TC, TG and LDL level. However, the HDL level was decreased in nicotine group, and elevated in nicotine + HSE group ($p<0.05$) (Table 3). The atherogenic index (Figure 1) and predictive indicator for CVD (Figure 2) were significantly increased in nicotine group vs. control group and decreased in nicotine + HSE group vs. nicotine group ($p<0.05$).

MDA concentration was significantly increased in nicotine group vs. control and nicotine + HSE (Figure 3). Nicotine + HSE group showed an increase in SOD enzyme activity significantly ($p<0.05$) as compared to control and nicotine group (Figure 4). GSH concentration showed no significant difference ($p>0.05$) in all groups (Figure 5). Meanwhile, protein concentration was significantly higher in nicotine + HSE group as compared to nicotine group (Figure 6).

HISTOLOGY

Histological observation of the left ventricular tissue appeared normal in all groups. The nicotine induction failed to cause any morphological changes (Figure 7).

DISCUSSION

Our study has shown that *Hibiscus sabdariffa* extract (HSE) was able to protect the heart damage from nicotine by elevating antioxidant enzymes and reducing oxidative stress levels. Nicotine, which is the main psychoactive

TABLE 3. Serum lipid profile

Group	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Control	1.65±0.09	0.37±0.08	0.50±0.04	1.01±0.14
Nicotine	1.71±0.07	0.59±0.12	0.31±0.03[a]	1.13±0.10
Nicotine + HSE	1.66±0.08	0.35±0.05	0.74±0.05[a,b]	0.77±0.14

[a] $p<0.05$ vs. control group   [b] $p<0.05$ vs. nicotine group
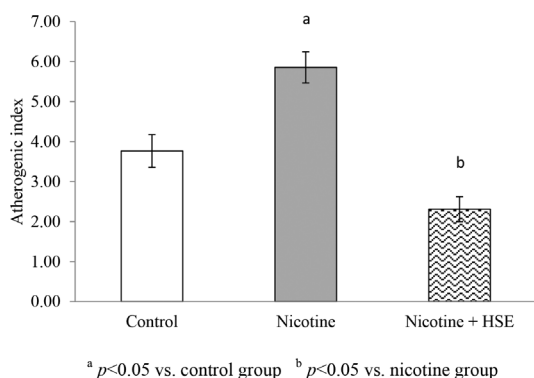

[a] $p<0.05$ vs. control group   [b] $p<0.05$ vs. nicotine group

FIGURE 1. Atherogenic index (TC/HDL) of heart tissue
Values are means ± SEM

[a] $p<0.05$ vs. control group   [b] $p<0.05$ vs. nicotine group
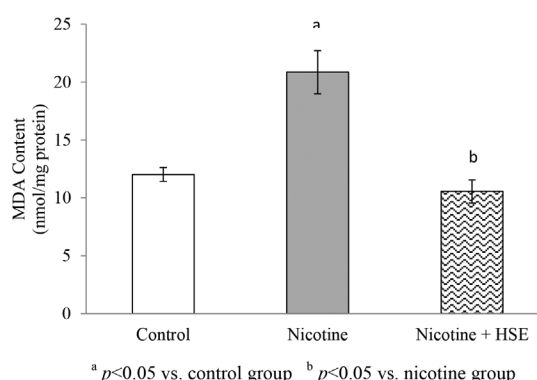
FIGURE 3. Malondialdehyde (MDA) content of heart tissue
Values are means ± SEM

[a] $p<0.05$ vs. control group   [b] $p<0.05$ vs. nicotine group
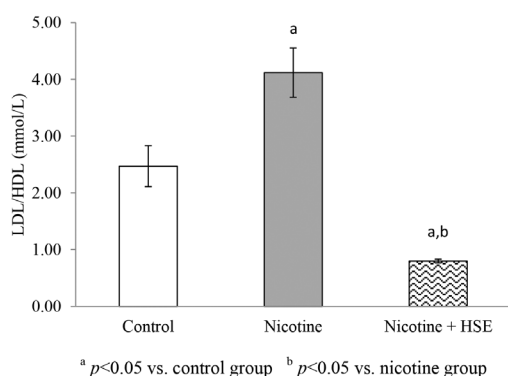
FIGURE 2. Predictive indicator for CVD (LDL/HDL) of heart tissue
Values are means ± SEM

[a] $p<0.05$ vs. control group   [b] $p<0.05$ vs. nicotine group

FIGURE 4. SOD enzyme activity of heart tissue
Values are means ± SEM

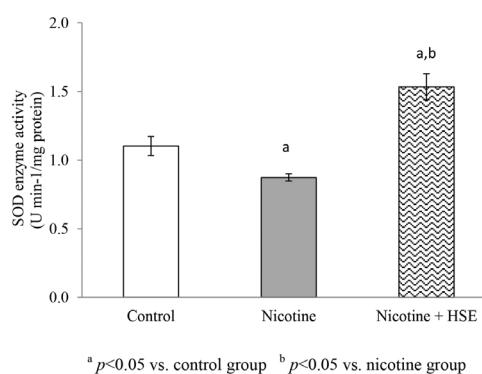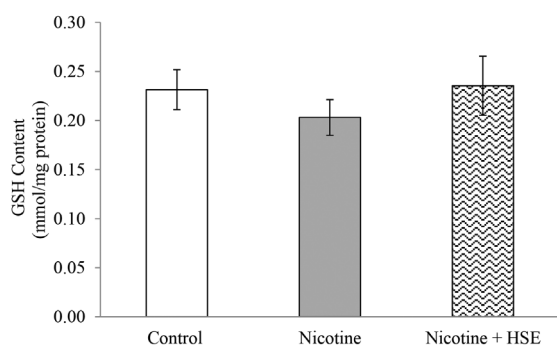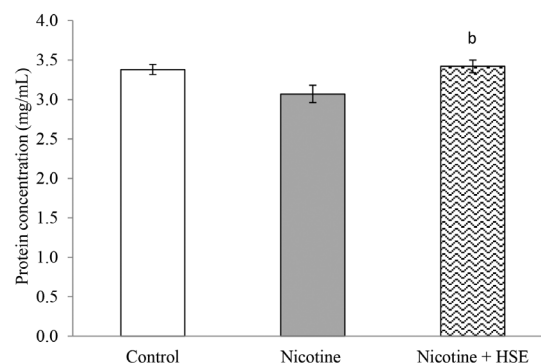FIGURE 5. GSH content of heart tissue
Values are means ± SEM. No significant difference was
observed in any of the groups

[b] $p<0.05$ vs. nicotine group

FIGURE 6. Protein concentration of heart tissue
Values are means ± SEM

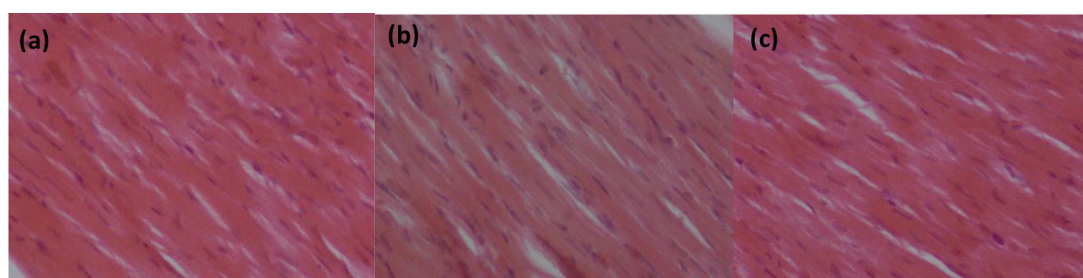
FIGURE 7. Photographs of the left ventricle (H&E) in (a) Control, (b) Nicotine and
(c) Nicotine + HSE under 40× magnification. No difference was seen among the groups

compound in cigarette, has been identified as the factor that causes cardiovascular diseases such as hypertension, atherosclerosis and myocardial ischemia (Yoshikawa & Naito 2002). According to Gomes et al. (2004), 0.6 mg/kg/day dose of nicotine in a rat is similar to the dose in light smoker (low dose), while a heavy smoker is equivalent to 1.2 mg/kg/day dose of nicotine (high dose). In rat, the LD_{50} of nicotine through intraperitoneum injection was found at 14.6 mg/kg of body weight (Karaconji 2005). Thus, this has proven that the nicotine dosage used in this study was considered as safe.

Smoking is often associated with weight loss as nicotine can increase metabolic rate and stimulate leptin which gives a feeling of fullness (McGovern & Benowitz 2011). However, in this present study, nicotine did not change the body weight increment among the groups, similarly found in other studies (Iranloye & Bolarinwa 2009; Olatunji et al. 2006). This was probably due to low dose of nicotine (0.6 mg/kg) that did not affect the amount of food intake (Iranloye & Bolarinwa 2009).

Heart rate can increase up to 30% during the first 10 min of smoking. This is because nicotine activates sympathetic nervous system by binding to nicotinic acetylcholine receptor (nAChR) and stimulates adrenaline release that increases the heart rate, blood pressure and respiratory rate (William 2013). In this study, HSE was found to decrease the heart rate significantly in nicotine-exposed rats. The

mechanism involved is not fully understood yet, but it might work like conventional medicine (beta blocker) by the blocking the effect of catecholamine. This action could prevent the coupling of beta-receptor (β-receptor) with G-proteins, thus block the Ca^{2+} entry into cardiac myocytes and subsequently reducing the heart contraction and heart rate (Opie 1997). We did not find any significant changes in systolic and diastolic blood pressure in all groups. Probably, the nicotine dose was inadequate to increase the blood pressure (Benowitz et al. 2002; Shahrokhi et al. 2006), thus hinder the antihypertensive activity of HSE. Supposedly, Roselle is able to lower blood pressure through enhancement of vascular Na^+-K^+-ATPase activity that inhibit Ca^{2+} entry and storage intracellularly (Olatunji et al. 2006). Our lipid profile has found there were no significant changes in the levels for TG, TC and LDL in all of the groups and that is similar with the previous studies (Joukar et al. 2012; Sun et al. 2001). A clinical study by Mohagheghi et al. (2011) on hypertensive patients, whereby 500 mg/kg of HSE was given for 30 days did not show any significant reduction in lipid profile reading. In contrary, another study which used the same dosage but with longer duration of 10 weeks was found to significantly lower the TG, TC and LDL level in hyperlipidemic rabbits (Chen et al. (2003). Roselle contains protocatechuic acid that is supposed to inhibit lipase and HMG-CoA enzyme on lipolysis activities (Hopkins et al. 2013; Sari et al. 2013). This probably caused

the significant decrease in serum HDL concentration in HSE treatment compared to control and nicotine groups. HDL is important to uptake excess cholesterol from plasma to the liver, thus reducing the risk of atherosclerosis (Cho 2009). Supportively, the atherogenic index and predictive indicator for CVD in our study were found significantly decreased with HSE treatment in nicotine-exposed rats.

Nicotine could also aggravate oxidative stress and generate free radicals which attack the lipid membrane of cells, resulting in the formation of malondialdehyde (MDA) (Balakrishnan & Menon 2007; Chattopadhyay & Chattopadhyay 2008). Our study has shown that the high MDA concentration in nicotine rats was significantly reversed by HSE treatment, similarly found by Hirunpanich et al. (2006) and Al-Kennany & Al-Khafaf (2010). Lipid peroxidation could cause depletion of antioxidant SOD activity which in longer period could then lead to heart failure (Nojiri et al. 2006). When nicotine rats were treated with HSE, SOD activity increased significantly which was similarly observed by Suleyman et al. (2002). This result might be caused by the antioxidant components of Roselle, mainly anthocyanin, which removes free radicals, donating electron to free radicals and terminating the radical chain reaction (Dai & Mumper 2010; El-Shafey et al. 2013). Jain and Flora (2012) stated that, aggravated lipid peroxidation can cause a decrease in GSH, as it is also involved in reducing lipid peroxidation. However, we have found that there was no significant difference in GSH concentration for all groups. This could be caused by other antioxidant enzymes such as SOD which quickly reacts with free radicals, as it is the first antioxidant produced in radical chain reaction. Lipid peroxidation does not only damage lipid component but also protein level (Noeman et al. 2011). In the present study, HSE treatment was found to significantly increase the protein level compared to nicotine group. This is consistent with previous study that stated reduced protein concentration was due to liver damage induced by peroxidation, which interferes with protein synthesis (Olorunnisola et al. 2012).

In summary, this study demonstrates that HSE treatment could reduce the heart rate, the risk of atherosclerosis and prevent oxidative damage to the heart tissue, suggesting its potential as functional food to prevent heart damage induced by nicotine. On a separate note, the molecular mechanisms underlying protective action of roselle against heart damage requires further investigation.

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