

## Dietary UKMR-1 Roselle Supplementation Prevents Nicotine-Induced Cardiac Injury by Inhibiting Myocardial Oxidative Stress

(Suplemen Rosel Diet UKMR-1 Mencegah Kecelakaan Jantung Aruhan Nikotin dengan Menghalang Tekanan Oksidatif Miokardium)

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### ABSTRACT

*UKMR-1, a local variant of mutant Roselle strain (Hibiscus sabdariffa) is enriched with free radical scavenging polyphenols such as anthocyanin, vitamin C and hydroxycitric acid. However, pharmacological actions of UKMR-1 are not fully known. This study was conducted to determine whether supplementation of aqueous UKMR-1 calyx extract was able to protect against nicotine-induced cardiac injury in rats. In this experimental study, healthy male albino rats were randomly allotted into three groups (n=7 per group): control, nicotine and UKMR-1+Nicotine groups. Nicotine (0.6 mg/kg, i.p.) was administered to both nicotine and UKMR-1+Nicotine groups for 28 consecutive days. UKMR-1+Nicotine group also received 100 mg/kg UKMR-1 extract orally via gavage 30 min prior to nicotine injection, daily. UKMR-1+Nicotine group had significantly (p<0.05) higher lactate dehydrogenase (LDH) activity, as well as lower malondialdehyde content in heart tissue homogenate than nicotine group, suggesting its cardio protective activity by inhibition of lipid peroxidation. UKMR-1 also lowered (p<0.05) the blood pressure in nicotine-administered rats. In addition, UKMR-1 significantly (p<0.05) restored activities of cytosolic superoxide dismutase, glutathione peroxidase and glutathione-S-transferase as well as redox balance ratio (GSH:GSSG). In conclusion, UKMR-1 was able to protect against myocardial injury in rat model of nicotine administration possibly by inhibiting oxidative stress.*

*Keywords: Antioxidant; antihypertensive; cardioprotective; Hibiscus sabdariffa; nicotine*

### ABSTRAK

*UKMR-1 yang merupakan salah satu varian mutan Rosel tempatan (Hibiscus sabdariffa) yang diperkaya dengan polifenol perangkap radikal bebas seperti antosianin, vitamin C dan asid hidrositrik. Walau bagaimanapun, tindakan farmakologi UKMR-1 belum diketahui sepenuhnya. Kajian ini dijalankan untuk menentukan sama ada suplementasi ekstrak akues UKMR-1 dapat melindungi tikus daripada kecederaan jantung aruhan nikotin. Dalam kajian eksperimental ini, tikus jantan Albino yang sihat dibahagikan secara rawak kepada tiga kumpulan (n=7 setiap kumpulan): kawalan, nikotin dan UKMR-1 + nikotin. Nikotin (0.6 mg/kg, ip) telah diberi kepada kedua-dua kumpulan nikotin dan UKMR-1 + nikotin selama 28 hari berturut-turut. Kumpulan UKMR-1 + nikotin turut menerima ekstrak 100 mg/kg UKMR-1 secara oral melalui gavage 30 minit sebelum suntikan nikotin pada setiap hari. Kumpulan UKMR-1+nikotin mempunyai aktiviti laktat dehidrogenase (LDH) yang lebih tinggi secara signifikan (p<0.05) serta kandungan malondialdehid yang lebih rendah dalam homogenat tisu jantung daripada kumpulan nikotin, mencadangkan bahawa aktiviti perlindungan jantung dengan mencegah peroksidaan lipid. UKMR-1 juga menurunkan (p<0.05) tekanan darah dalam tikus diadministrasi nikotin. Di samping itu, UKMR-1 memulihkan aktiviti superoksida dismutase sitosolik, glutathione peroksida dan glutathione-S-transferase serta nisbah keseimbangan redoks (GSH: GSSG) dengan signifikan (p<0.05). Secara kesimpulannya, UKMR-1 dapat memberi perlindungan terhadap kecederaan jantung dalam model tikus diadministrasi nikotin, berkemungkinan melalui perencatan tekanan oksidatif.*

*Kata kunci: Antioksidan; antihipertensi; Hibiscus sabdariffa; nikotin; perlindungan jantung*

### INTRODUCTION

Heart diseases remain a major cause of death in developing and developed countries globally (Mensah & Brown 2007). One of the major pathophysiological contributors to heart diseases is oxidative stress, whereby cytotoxic reactive oxygen species (ROS) promotes lipid peroxidation and reversible oxidation of contractile proteins which compromise cardiac function (Sener et al. 2005b; Qin et al. 2013). Cardiomyocytes are equipped with endogenous

enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) which serve as first line of defence against cytotoxic ROS in the heart. Overexpression of enzymatic antioxidants was previously shown to protect against myocardial injury in animal models of myocardial infarction and aging, demonstrating crucial role of these enzymes in preserving cardiac function (van Deel et al. 2008; Wu et al. 2007).

Roselle (*Hibiscus sabdariffa*) is enriched with powerful antioxidants such as hydroxycitric acid, anthocyanins and vitamin C (Obouayeba et al. 2014) which contribute to its antihypertensive (Onyenekwe et al. 1999), antidiabetic (Pannangpetch et al. 2013) and antilipidemic (Ochani & D'Mello 2009) activities *in vivo*. Local researchers from Universiti Kebangsaan Malaysia (UKM) have successfully bred three mutant Roselle variants (UKMR-1, UKMR-2 and UKMR-3) by irradiating seeds of parental Arabic line of Roselle in Malaysia with gamma rays to improve quality of Roselle (Osman et al. 2011). UKMR-1 and UKMR-2 had significantly higher content of hydroxycitric acid, anthocyanin and vitamin C, as compared to parental line (Osman et al. 2011). Antioxidant effects of UKMR-2 were extensively studied in our laboratory, from which it was shown that aqueous UKMR-2 extract was able to protect red blood cells from haemolytic injury (Mohamed et al. 2014, 2013;) and alleviate sperm damage in streptozotocin-induced type I diabetic rats (Idris et al. 2012). However, the pharmacological effects of UKMR-1 are not fully known.

Nicotine is an environmentally abundant chemical substance which contributes to the initiation and progression of cardiovascular diseases (Balakumar et al. 2008). It has been shown to induce oxidative stress in major organs such as aorta, heart, kidney and testes of animal models (Gumustekin et al. 2010; Iranloye & Oludare 2011). Intraperitoneal injection of nicotine to rats subacutely, in doses corresponding to this study was able to induce lipid peroxidation and inhibit activities of SOD, CAT and GPX in rat hearts (Sener et al. 2005a). In addition, we have recently shown intraperitoneal injection of nicotine to rats also induces vascular endothelial dysfunction with characteristics of impaired vasoconstriction and vasorelaxation, increased intimal media thickness and proliferation elastic lamellae which translates to increased blood pressure (Zainalabidin et al. 2014). Owing to these, administration of nicotine would produce a reproducible rat model with myocardial injury driven by oxidative stress.

This study was undertaken to determine whether supplementation of aqueous calyx extract of UKMR-1 was able to protect against myocardial injury driven by oxidative stress in a rat model with nicotine administration.

## MATERIALS AND METHODS

### PREPARATION OF EXTRACT

Dried UKMR-1 calyces (Specimen Voucher No. UKM 40308), were blended in distilled water for 10 min, boiled and allowed to cool. The filtrate of this cold mixture was added with 10% maltodextrin and freeze-dried to obtain powdered extract. Aqueous UKMR-1 extract was prepared fresh each day by dissolving 1 g of powder in 10 mL of distilled water.

## ANIMALS

Healthy male Sprague-Dawley rats (6 weeks old, 180-220 g) were used in this study, fed with standard pellet diet and given free access to drinking water. All procedures were conducted in accordance with guidelines approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (FSK/BIOMED/2013/BALKIS/25-SEPT./539-OCT.-2013-FEB.-2014).

## STUDY DESIGN

Experimental rats were randomly allotted into three groups ( $n=7/\text{group}$ ); control, nicotine and UKMR-1+Nicotine group. UKMR-1 extract (100 mg/kg) was given orally via gavage to UKMR-1+Nicotine group alone. Both control and nicotine groups were fed saline orally via gavage. After 30 min, nicotine (0.6 mg/kg) was administered via intraperitoneal injection to nicotine and UKMR-1+Nicotine group (Sener et al. 2005b). Control group received saline injection alone. All regimens were given for 28 consecutive days.

## BLOOD PRESSURE, HEART RATE AND HEART WORK

Arterial blood pressure indices (systolic, diastolic and mean) and heart rate were measured with non-invasive tail cuff method using CODA II system (Kent Scientific, USA). Rate-pressure product (RPP) was calculated as described earlier, by multiplying systolic arterial pressure and heart rate as indicator of myocardial workload.

## SAMPLE COLLECTION AND PROCESSING

On 29th day, rats were sacrificed under urethane (1 g/kg) anaesthesia after which the hearts were excised and kept frozen for further analysis. Heart samples were homogenized in Tris-HCl buffer and centrifuged at 20000 g for 20 min upon thawing. Supernatants were kept frozen for biochemical studies.

## BIOCHEMICAL STUDIES

Intracellular LDH activity was measured in heart homogenate as index of cellular injury, according to method described by Wroblewski and Ladue (1955). Injury by lipid peroxidation was quantified by measuring malondialdehyde (MDA) in tissue homogenate through colorimetric assay (Stocks & Dormandy 1971). Activities of Cu/Zn-SOD and Mn-SOD were measured using colorimetric assay described by Beyer and Fridovich (1987), in which one unit of enzyme inhibits formation of purple formazan by 50%. To obtain activity of Mn-SOD, homogenate was treated with potassium cyanide before assay to inhibit Cu/Zn-SOD. Activity of Cu/Zn-SOD was calculated by deducting Mn-SOD activity from total SOD activity (Khan & Black 2003). CAT activity was measured using molybdate assay for hydrogen peroxide content (Goth 1991). GPx activity in tissue was assayed as described by Lawrence and Burk (1976) using NADPH-coupled colorimetric reaction.

Glutathione content and its recycling by glutathione reductase (GRx) were measured as indicator for tissue redox balance. Tissue content of glutathione (GSH) and glutathione disulphide (GSSG) was measured using enzymatic recycling colorimetric assay as described by Rahman et al. (2007). Whereas, GRx activity was assayed using spectrophotometer as described (Mannervik 2001). In addition, activity of phase II enzyme, GST was measured to study detoxifying activity using 1-chloro-2,4-dinitrobenzene conjugation reaction (Mannervik 1985). O-diasidine method was employed to measure MPO activity in tissue using spectrophotometer (Hillefuss et al. 1990). MPO activity is an index of neutrophil-mediated inflammation (Scumpia et al. 2004).

#### STATISTICAL ANALYSIS

All values were expressed as mean  $\pm$  standard error of mean (SEM). Statistical significance was determined using one-way analysis of variances (ANOVA) in Statistical Package for Social Sciences (SPSS) version 19. A  $p$ -value  $< 0.05$  was considered statistically significant.

#### RESULTS

In this study, the administration of nicotine for 28 days increased indices of arterial blood pressure (Table 1). UKMR-1 supplementation remarkably lowered the increase in blood pressure. In contrast, the heart rate of UKMR-1+Nicotine group was higher than control group. UKMR-1 also able to

reduce rate-pressure product (RPP) ( $p=0.035$ , with 95% CI: 12.79 to 405.2) in nicotine-administered rats as compared to nicotine group (Figure 1).

Apart from this, nicotine group had reduced intracellular lactate dehydrogenase (LDH) activity in heart tissue (Figure 2) and high content of lipid peroxidation marker, malondialdehyde (MDA) (Figure 3). Interestingly, UKMR-1 was able to attenuate these effects by preserving intracellular LDH activity ( $p=0.023$ , with 95% CI: -38.01 to -2.6) and lowering MDA formation ( $p<0.01$ , with 95% CI: 8.28 to 46.38).

The administration of nicotine lowered activities of Cu/Zn-SOD, Mn-SOD, catalase (CAT) and glutathione peroxidase (GPX) enzymes as compared to the control group (Figure 4). Supplementation with UKMR-1 had significantly restored activities of Cu/Zn-SOD ( $p<0.01$ , with 95% CI: -6.26 to -0.93) and GPX ( $p=0.043$ , with 95% CI: -41.76 to -0.63). However, no significant difference was noted for Mn-SOD ( $p=0.96$ ) and CAT ( $p=0.08$ ) between nicotine and UKMR-1+Nicotine groups.

Besides, nicotine also depleted myocardial glutathione (GSH) content, reducing the redox ratio (GSH:GSSG) as compared to control group. It was also shown that glutathione reductase (GRx) activity was lower in the heart of nicotine group. However, UKMR-1 was able to restore GSH content in heart, increasing GSH:GSSG ratio significantly ( $p<0.01$ , with 95% CI: -43.11 to -14.49). UKMR-1+Nicotine group exhibited significantly higher GRx activity ( $p=0.016$ , with 95% CI: -0.31 to -0.03) than nicotine group (Table 2).

TABLE 1. Blood pressure indices and heart rate of experimental rats by group

Parameter	Control	Nicotine	UKMR-1+Nicotine
Systolic BP (mmHg)	103.7 $\pm$ 1.5	127.4 $\pm$ 4.0*	95.3 $\pm$ 3.9 <sup>#</sup>
Diastolic BP (mmHg)	73.6 $\pm$ 5.7	82.9 $\pm$ 4.2*	59.1 $\pm$ 4.5 <sup>#</sup>
Mean BP (mmHg)	83.6 $\pm$ 3.0	97.7 $\pm$ 1.8*	71.2 $\pm$ 2.8 <sup>#</sup>
Heart rate (beats/min)	406.0 $\pm$ 15.6	453.6 $\pm$ 21.6*	461.2 $\pm$ 18.2*

BP = blood pressure

Values are given as mean  $\pm$  SEM. \*significant relative to control group at  $p<0.05$ ,

<sup>#</sup>significant relative to nicotine group at  $p<0.05$

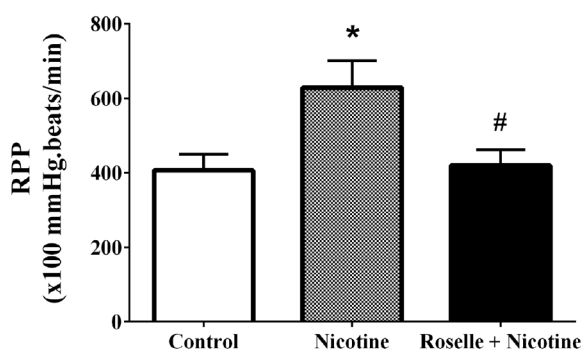


FIGURE 1. RPP ( $\times 100$  mmHg.beats/min) of experimental rats by group. Values given as mean  $\pm$  SEM. \*significant relative to control group at  $p<0.05$ , <sup>#</sup>significant relative to nicotine group at  $p<0.05$

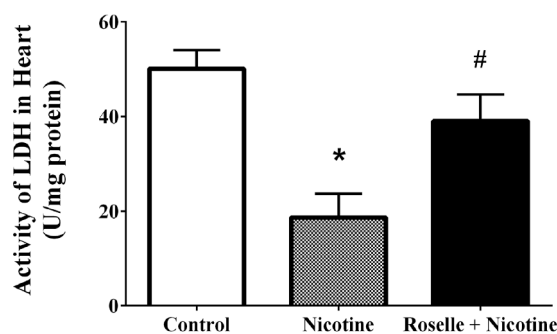


FIGURE 2. Intracellular LDH activity (U/mg protein) in heart of experimental rats by group. Values given as mean  $\pm$  SEM. \*significant relative to control group at  $p<0.05$ , <sup>#</sup>significant relative to nicotine group at  $p<0.05$

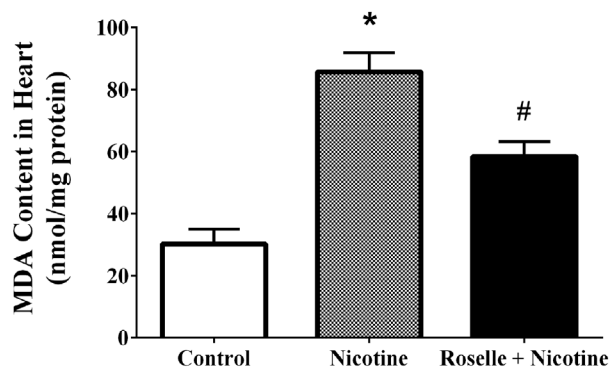


FIGURE 3. MDA content (nmol/mg protein) in heart of experimental rats by group. Values given as mean  $\pm$  SEM. \*significant relative to control group at  $p < 0.05$ , #significant relative to nicotine group at  $p < 0.05$

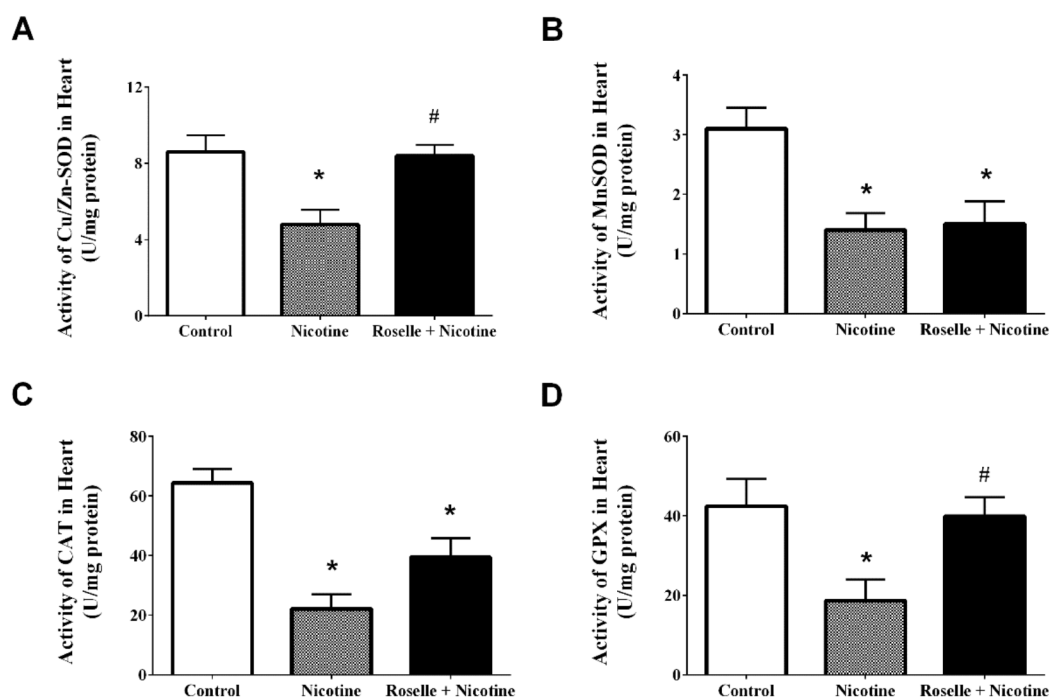


FIGURE 4. Activities of Cu/Zn-SOD, Mn-SOD, CAT and GPx enzymes (U/mg protein) in heart of experimental rats by group. Values given as mean  $\pm$  SEM. \*significant relative to control group at  $p < 0.05$ , #significant relative to nicotine group at  $p < 0.05$

TABLE 2. Glutathione balance and recycling in heart of experimental rats by group

Parameter	Control	Nicotine	UKMR-1+Nicotine
GSH ( $\mu\text{mol/mg protein}$ )	6.33 $\pm$ 0.34	4.76 $\pm$ 0.38*	5.90 $\pm$ 0.73#
GSSG ( $\mu\text{mol/mg protein}$ )	0.08 $\pm$ 0.01	0.12 $\pm$ 0.01*	0.09 $\pm$ 0.03#
GSH:GSSG	79.12 $\pm$ 3.40	39.67 $\pm$ 3.80*	68.42 $\pm$ 4.65#
GRx (U/mg protein)	0.72 $\pm$ 0.05	0.41 $\pm$ 0.02*	0.58 $\pm$ 0.04#

Values are given as mean  $\pm$  SEM. \*significant relative to control group at  $p < 0.05$ , #significant relative to nicotine group at  $p < 0.05$

Chronic administration of nicotine to rats also lowered activity of phase II enzyme, GST, which was significantly ( $p < 0.01$ , with 95% CI: -46.38 to -7.42) restored by UKMR-

(Figure 5). Nevertheless, UKMR-1 did not significantly ( $p = 0.94$ ) affect cardiac MPO activity (Figure 6).

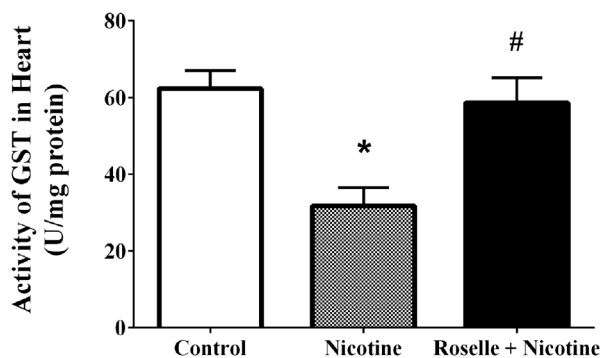


FIGURE 5. Activity of GST (U/mg protein) in heart of experimental rats by group. Values given as mean  $\pm$  SEM. \*significant relative to control group at  $p < 0.05$ , #significant relative to nicotine group at  $p < 0.05$

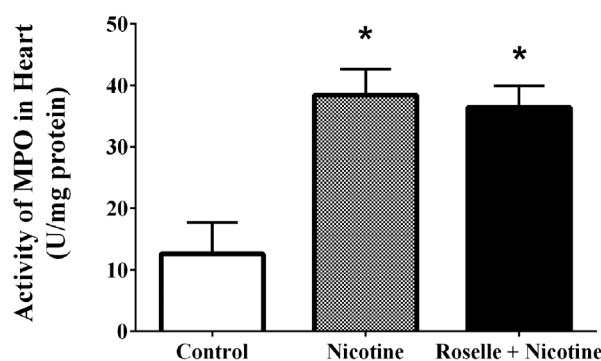


FIGURE 6. Activity of MPO (U/mg protein) in heart of experimental rats by group. Values given as mean  $\pm$  SEM. \*significant relative to control group at  $p < 0.05$ , #significant relative to nicotine group at  $p < 0.05$

## DISCUSSION

UKMR-1 is a mutant Roselle variant with high antioxidant capacity, however, its pharmacological actions are not fully known. In this study, we have shown for the first time that UKMR-1 protects against cardiac injury in a rat model of nicotine administration, by lowering blood pressure, preserving LDH activity in the heart, lowering MDA level in the heart, restoring activities of Cu/ZnSOD, GPX, GRX and GST as well as increasing GSH:GSSG ratio in the heart.

The administration of nicotine for 28 days resulted in extensive cardiomyocyte injury, as evidenced by depletion of intracellular LDH content. In addition, these injuries are likely to result from lipid peroxidation because rat heart from nicotine group has high content of lipid peroxidation product, MDA similar to previous studies (Gumustekin et al. 2010; Sener et al. 2005a). Interestingly, UKMR-1 supplementation to rat model of nicotine exposure exhibited cardioprotective effect via inhibition of lipid peroxidation, as prominently evidenced by preserved intracellular LDH content and lower level of MDA in heart than nicotine group. These effects are similar to actions of UKMR-2 shown in our previous studies (Mohamed et al. 2014, 2013) which suggested mutant Roselle variants are capable of inhibiting lipid peroxidation which helps to prevent damage to phospholipids bilayer in plasma membrane. UKMR-1 is likely to preserve structural

integrity of plasma membrane in cardiomyocytes which prevents oxidative stress driven necrosis.

Apart from that, nicotine administration also increases blood pressure and myocardial workload, which were both successfully attenuated by UKMR-1 Roselle. Oxygen consumption in heart increases as a result of elevated workload which forces overwhelming mitochondria respiration and generates superoxide anions through electron flux in mitochondria respiratory chain (Vincent et al. 2001). UKMR-1 likely to mimic parental Roselle which produces vasodilation through endothelium-derived nitric oxide-cGMP-relaxant pathway and inhibition of calcium influx into vascular smooth muscle cells (Ajay et al. 2007), thereby lowering blood pressure. In this study, UKMR-1 was also shown to increase heart rate in nicotine-administered rats. In a previous study, Roselle extract was shown to increase heart rate in diabetic rats to level comparable to control group, contradicting to current findings in which heart rate appears to be higher than control group (Mantrud et al. 2010). Though the mechanism for this observation is unknown, it is likely to be a compensatory process to drop in blood pressure such that heart work can be restored.

Mammalian cells are equipped with enzymatic antioxidants such as SOD, CAT and GPx to scavenge on free radicals. SOD is a key player which scavenges on superoxide anions in various compartments of cells. To date, there are

three major forms of SOD, namely cytosolic SOD (Cu/Zn-SOD) found in cytoplasm, mitochondrial SOD (Mn-SOD) found in mitochondria and extracellular SOD (EC-SOD) found in plasma and extracellular matrix (Choung et al. 2004). CAT and GPX scavenge on hydrogen peroxide mainly in cytoplasm (Sullivan-Gunn & Lewandowski 2013). In the present study, nicotine administration has compromised activities of Cu/Zn-SOD, Mn-SOD, CAT and GPX in rat heart probably due to exhaustion (Iranloye & Oludare 2011). UKMR-1 was shown to restore activities Cu/Zn-SOD and GPX. There was also trend to show UKMR-1 restored CAT activity ( $p < 0.1$ ). UKMR has high content mixture of natural antioxidants such as ascorbic acid, anthocyanin and hydroxycitric acid (Ajiboye et al. 2011). These natural antioxidants are likely to scavenge on ROS generated by nicotine, thereby being able to improve endogenous antioxidant status in nicotine-administered rat hearts.

UKMR-1 also improved redox balance in the rat heart by increasing GSH content and stimulating GRX activity in nicotine-administered rat hearts. GSH is an endogenous substrate which acts as an electron donor to reduce cytotoxic ROS into non-reactive species by forming GSSG (Golbidi et al. 2014). GRX helps to maintain redox balance in cells by catalysing conversion of GSSG to GSH. Inhibition of its activity further prevents restoration of redox balance in nicotine group (Erat et al. 2007). Lower GSH:GSSG in cells indicates a highly oxidized environment which oxidative post translational modifications of key cellular proteins (Chang et al. 2014), thus UKMR-1 prevents oxidation of cardiac proteins by improving GSH:GSSG ratio. It was also noted that rat heart of nicotine group had lower GST activity, which is a phase II enzyme in xenobiotic metabolism that detoxifies reactive metabolites in tissues through a GSH-dependent reaction. GST was inhibited in nicotine group due to alterations in gene expression (Hu et al. 2002) and depletion of GSH as substrate due to nicotine-induced oxidative stress. UKMR-1 increased GST activity of nicotine-administered rat hearts, which is likely to result from replenishment in GSH content.

In contrast, UKMR-1 failed to restore mitochondrial SOD (Mn-SOD) activity in nicotine-induced rats, although it was able to restore activity of cytosolic enzymes. It is not known why UKMR-1 failed to restore activity of Mn-SOD but it can only be speculated that UKMR-1 Roselle was unable to protect against mitochondria dysfunction which precedes cytosolic oxidative stress in nicotine-administered rats (Arany et al. 2013). Abnormalities to mitochondria respiratory chain may not be reversed due to which abundance of ROS generated within mitochondria may exhaust mitochondria antioxidants. Further studies, however, are warranted to specifically study action of UKMR-1 on mitochondria dysfunction *in vitro*. In addition to Mn-SOD, UKMR-1 also failed to restore MPO activity, which are likely due to lack of anti-inflammatory activity in UKMR-1.

We conclude that UKMR-1 protects against myocardial injury in rat model of nicotine administration by possibly inhibiting oxidative stress. Future studies are recommended to further investigate pharmacological effects of UKMR-1 using molecular approaches.

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