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Cardioprotective effects of *Gynura procumbens* extract on oxidative status and myocardial injury in rats with isoproterenol-induced myocardial infarction

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Abstract

Gynura procumbens (GP) grows abundantly in Southeast Asia. The present work was conducted to investigate the cardioprotective potential of ethanol extract of GP on cardiac markers, antioxidant levels, and histopathology of isoproterenol-induced myocardial infarction (MI). A total of 36 adult Sprague-Dawley rats were randomly divided into six groups. Treatments were given via oral gavage for 28 days: two groups were given normal saline 0.9%; two groups were given GP250 mg/kg/day- and two groups were given GP500 mg/kg/day. On day 27 and 28, MI was induced with a subcutaneous injection of 85 mg/kg isoproterenol. The rats were sacrificed 48 h after the 1st injection. Cardiac markers, lipid peroxidation, oxidative status, and histopathological analyses were evaluated. Isoproterenol significantly increased the levels of troponin T, creatine kinase MB isoenzyme (CKMB), lactate dehydrogenase (LDH), and malondialdehyde (MDA), whereas the level of superoxide dismutase (SOD), catalase, and glutathione peroxidase were significantly decreased. In addition, the histopathological findings showed a necrosis of the myocardium as evidenced by neutrophil infiltration, and interstitial oedema with acceleration of apoptosis in MI. Interestingly, treatment with GP restored the levels of troponin T, LDH, MDA, SOD, and catalase significantly. Moreover, GP preserved the myocardial architecture while decreasing both necrosis and apoptosis. GP has the potential to limit myocardial injury after MI, and this is most likely achieved through its modulation of antioxidant enzyme activities.

Introduction

Myocardial infarction (MI) is the most dreaded consequence of cardiovascular disease (CVD). In 2016, approximately 17.9 million people died from CVD, of which about 85% of deaths were caused by MI and strokes (WHO, 2017). Mortality is expected to increase each year, with an estimated 23.6 million deaths are attributed to CVD by 2030 (Aniza *et al.,* 2016). As a result, various health agencies have made tremendous efforts to overcome this problem. Numerous measures and financial support have been invested in preventing and treating CVD and its risk factors (Switaj *et al.,* 2017). Currently, the treatment of MI requires intervention through the use of multiple drugs such as antiplatelet drugs, fibrinolytic agents, angiotensin converting enzyme inhibitors, and beta-blockers. Despite their efficacy, these drugs are known to have side effects, and have to be taken cautiously with safety considerations (Dai and Ge, 2011). Therefore, research on plant-based medications has been conducted extensively over the years in search for safer and more natural alternatives.

Generally, MI is characterised by necrosis or the death of cardiomyocytes in a segment of the heart muscle. Following deprivation of blood supply to the heart, the cardiac markers become elevated (Switaj *et al.,* 2017). Coronary insufficiency and ischaemia induce metabolic and cellular changes in myocardium as the constant contractile function of the myocardium relies mostly on aerobic metabolism for energy. Therefore, prolonged deprivation of oxygen supply results in detrimental consequences to myocardial cells due to damages caused by the

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generation of free radicals, increased lactate accumulation, reduced pH, and increased intracellular Ca^{2+}, which eventually lead to the inhibition of cellular functions (de Zwaan *et al.,* 2001).

Reactive oxygen species (ROS) play an important role in the pathogenesis of MI. The ROS at suboptimal level work as a mediator to regulate normal cardiovascular function. However, excessive production of ROS is hazardous as it leads to oxidative stress, which is marked by an increase in tissue lipid peroxidation as indicated by high level of malondialdehyde (MDA), and decreased level of antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). As a consequence, cellular damage, necrosis, and apoptosis of cardiomyocytes occur due to the direct oxidising impact on proteins, lipids, and DNAs (Moris *et al.,* 2017). There is a positive correlation between antioxidant enzymes and MI. It was reported that during the MI events in transgenic mice with overexpressed SOD, the size of the infarcted region in the heart was notably reduced (Hori and Nishida, 2009).

Isoproterenol (ISO)-induced MI is an established method for inducing MI in animal models (Syed Abd Halim *et al.,* 2018). It has been widely used to study the cardioprotective effects of natural and synthetic products (Upaganlawa *et al.,* 2011). This method is simple, easy to execute, and causes less complications in creating myocardial injury that mimics the necrotic myocardium in human (Syed Abd Halim *et al.,* 2018). The ISO is a synthetic sympathomimetic with potent chronotropic and inotropic effects that cause severe stress on myocardium. High level of blood catecholamine augments the contraction force of the heart, and increases ATP utilisation (de Snchez *et al.,* 2012). Upon administration of ISO, the heart rate is elevated, and myocardial oxygen demand increases with drops in systolic and diastolic blood pressure. This condition leads to functional hypoxia that results in MI (de Snchez *et al.,* 2012). Subsequent oxidative stress further deteriorates the myocardial condition in ISO-induced MI due to hypoxia, Ca^{2+} overload, and mitochondrial alteration (de Snchez *et al.,* 2012).

Gynura procumbens (GP) grows abundantly in Southeast Asian countries such as Malaysia, China, Thailand, Indonesia, and Vietnam (Rahman and Asad, 2013). The species belongs to the family Asteraceae, and is described as small green shrub with lanceolate-shaped leaves and fleshy stems (Rahman and Asad, 2013). Among the Malays, GP is known as *"sambung nyawa"* which translates to extension of life, whereas in Chinese it is known as *"bai bing chao"* (grass for a hundred ailments) or *"feng wei jian"* (pointed phoenix tail) (Wiart, 2002; Tan *et al.,* 2016). In Malaysia, the leaves of GP are consumed raw as salad, while in Thailand the leaves are used in cooking. The plant is rich in bioactive compounds such as phenolic acids, flavonoids, alkaloids, tannins, triterpenes, and sterol glycosides (Puangpronp *et al.,* 2010). Previous studies have reported multiple therapeutic properties of GP such as antihypertensive (Kim *et al.,* 2006), antihyperglycaemic (Akowuah *et al.,* 2002), antioxidant (Rosidah *et al.,* 2008), and antimicrobial (Rahman and Asad, 2013). The present work thus aimed to investigate the cardioprotective effects of the GP extract on oxidative status and cardiac injury in rats with isoproterenol-induced MI.

Materials and methods

Plant material

Fresh GP was purchased from a nursery in Semenyih, Selangor, Malaysia. It was then validated and deposited in the Herbarium of the Faculty of Science and Technology, Universiti Kebangsaan Malaysia (voucher no.: UKMB40411). All parts of GP were cleaned and dried at room temperature for 3 d. The dried parts were then ground and extracted in 80% ethanol at room temperature for 72 h. This cycle was repeated three times. The solvent was filtered and dried using a rotary evaporator at 55°C. The extraction produced 9.2% yield. Liquid chromatography-tandem mass spectrometry profiling of GP was performed by Ahmad Nazri *et al.* (2019).

Animals

A total of 36 male Sprague-Dawley rats (200 - 250 g) were obtained from the Laboratory Animal Resource Unit (LARU) - Universiti Kebangsaan Malaysia. The sample size was determined using the resource equation approach for animal studies, which is based on the degree of freedom of analysis of variance (ANOVA). However, the present work considered an attrition rate of 30% for each group, thus giving a total sample size of 36 animals (six animals per group) (Charan and Kantharia, 2013; Arifin and Zahiruddin, 2017).

The animals were housed in individual cages at constant room temperature (22 ± 2°C) with 12-h light-dark cycles. The diet consisted of chow pellets (Gold Coin, Malaysia) and tap water *ad libitum*. The present work was conducted following the approval by Universiti Kebangsaan Malaysia Animal Ethics

Committee (ANAT/PP/2017/NORZANA/27 SEPT/869-OKT 2017-SEPT 2018).

Experimental protocol

The animals were randomly divided into six groups (n = 6). The control and MI groups were forced-fed orally with distilled water daily for 28 d. The rats in groups GP250, MI+GP250, GP500, and MI+GP500 were forced-fed orally with ethanol extracts of GP250 and GP500 mg/kg daily also for 28 d. The doses of GP were chosen based on the study by Kim *et al.* (2006).

On days 27 and 28, MI was induced in group MI, GP250+MI, and GP500+MI with 85 mg/kg ISO injection. The non-MI group (Control, GP250, and GP500) received subcutaneous normal saline injection. On day 29, the rats were anesthetised with KTX (zoletil-50, ketamine, xylazine) cocktail intravenously; the blood samples were collected from the retro-orbital sinus, and the serum was separated to determine the level of cardiac markers such troponin T, CKMB, and LDH. Then, the rats were sacrificed via decapitation. The abdomen was incised, and the heart dissected. The dissected heart was then washed with phosphate buffered saline, and the left ventricle was isolated and horizontally cut into two — the upper and lower parts. The lower part of left ventricle was kept in 4% formalin for histopathological analysis, whereas the upper part was homogenised for antioxidant enzyme analysis (Zhang *et al.,* 2008; Wu *et al.,* 2018).

Determination of cardiac markers in serum

The blood samples were centrifuged to isolate the serum for estimation of the level of troponin T, CKMB, and LDH, measured using a commercial ELISA kit (Elabscience, USA).

Determination of malondialdehyde level

The level of malondialdehyde (MDA) was also determined by using a commercial ELISA kit (Elabscience, USA). Prior to the procedure, the heart tissues were homogenised according to the method specified by the manufacturer.

Determination of antioxidant enzymes activity

Superoxide dismutase, catalase, and GPx were assayed by using a commercial kit (Cayman, USA). Prior to the procedure, the heart tissues were homogenised according to the method specified by the manufacturer, and the supernatants were collected for subsequent assay using the commercial kit.

Histopathological analysis

The left ventricle was immersed in a 10% formalin solution prior to tissue processing. The processed tissue was embedded in the paraffin block, sectioned at 5 μm thickness, and mounted on a glass slide. Then, the slide was stained with haematoxylin-eosin (H&E) and Masson's trichrome. The detection of apoptosis was performed by using CardioTAC *in situ* apoptosis detection kit (Trevigen, USA).

Statistical analysis

Data were expressed as mean ± SEM, and analysed with SPSS version 23. The differences between the experimental groups were analysed using one-way ANOVA. Values of $p < 0.05$ were considered to be statistically significant.

Results

The effect of GP extract on cardiac markers

As shown in Figure 1, MI increased the serum concentration of cardiac markers, *i.e.* troponin T, CKMB, and LDH in the MI group as compared to the control group. It was observed that the pre-treatment with GP significantly decreased the concentration of troponin T and LDH as compared to the MI group.

The effect of GP extract on oxidative status

The level of MDA significantly increased in the cardiac tissue homogenate of the MI group as compared to the control (Figure 2). GP extract of 250 mg/kg significantly decreased the level of cardiac MDA in MI group. The activities of SOD, catalase, and GPx decreased in cardiac tissue homogenate of MI group as compared to the control group (Figure 2). Pre-treatment with GP prior to MI significantly increased the activities of SOD and catalase.

The effect of GP extract on histopathological section of cardiac tissue

Photomicrographs of the rat's heart from the non-MI group (Figures 3A, 3C, 3E and 4A, 4C, 4E) show cardiomyocytes with normal architecture. Normal cardiomyocytes are cylindrical, highly branched, and have a striated banding pattern. The ISO administration led to structural changes such as fragmentation of cardiomyocytes, necrotic tissue, infiltration of leukocytes, and intramuscular oedema as observed in the MI group, shown in Figures 3B and 4B. However, minimal alteration in the structure of cardiomyocytes with less infiltration of leukocytes was observed in the rats receiving GP

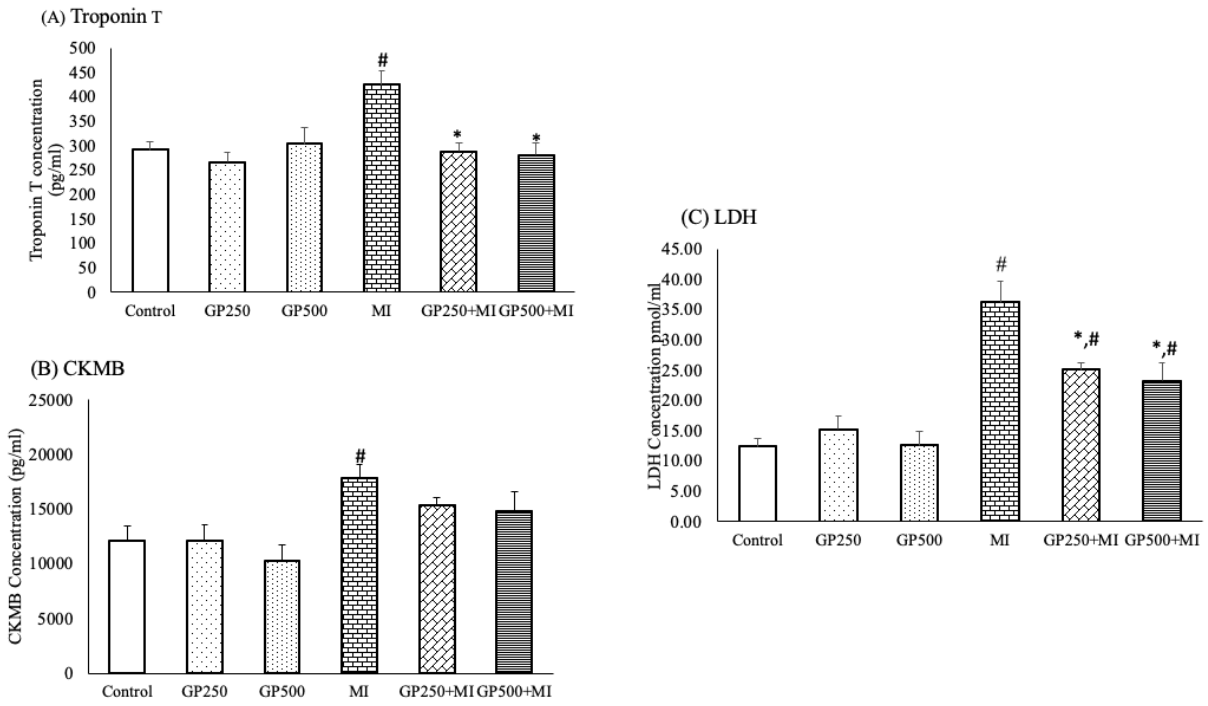

Figure 1. The effect of GP extract on the concentration of serum cardiac enzyme markers. (A) cardiac troponin T, (B) CKMB, and (C) LDH. Error bars indicate SEM (n = 6). #indicates significant differences ($p < 0.05$) as compared to control. *indicates significant differences ($p < 0.05$) as compared to MI.

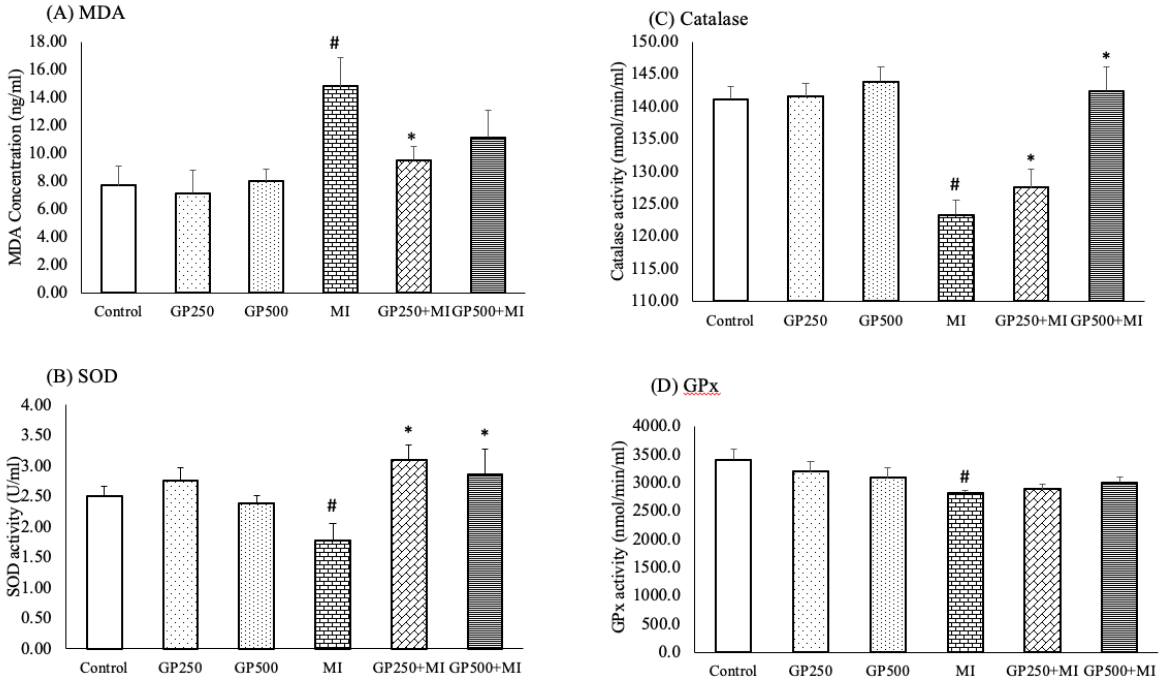

Figure 2. The effect of GP extract on cardiac oxidative status. (A) MDA, (B) SOD, (C) CAT, and (D) GPx. Error bars indicate SEM (n = 6). #indicates significant differences ($p < 0.05$) as compared to control. *indicates significant differences ($p < 0.05$) as compared to MI.

supplementation (Figures 3D, 3F and 4D).

The effect of GP extract on cardiac apoptosis

The dark blue precipitate indicated the presence of apoptotic cells. The histopathological sections of rat's heart from non-MI groups (Figures 5A, 5C, and 5E) were devoid of dark-blue precipitate, whereas in the MI group (Figure 5B),

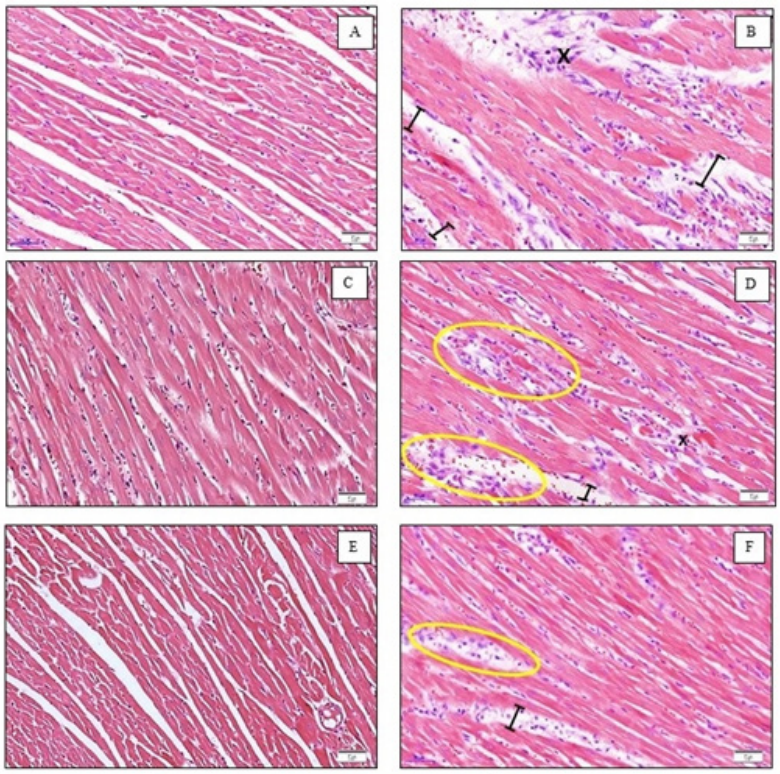

Figure 3. Longitudinal section of myocardium with H&E staining at 200× magnification with a 50-µm bar scale. (A) Control group, (B) MI, (C) GP250, (D) GP250+MI, (E) GP500, and (F) GP500+MI. Fragmentation of cardiomyocytes is marked with X, with leucocytes infiltration circled in yellow which indicates intramuscular oedema.

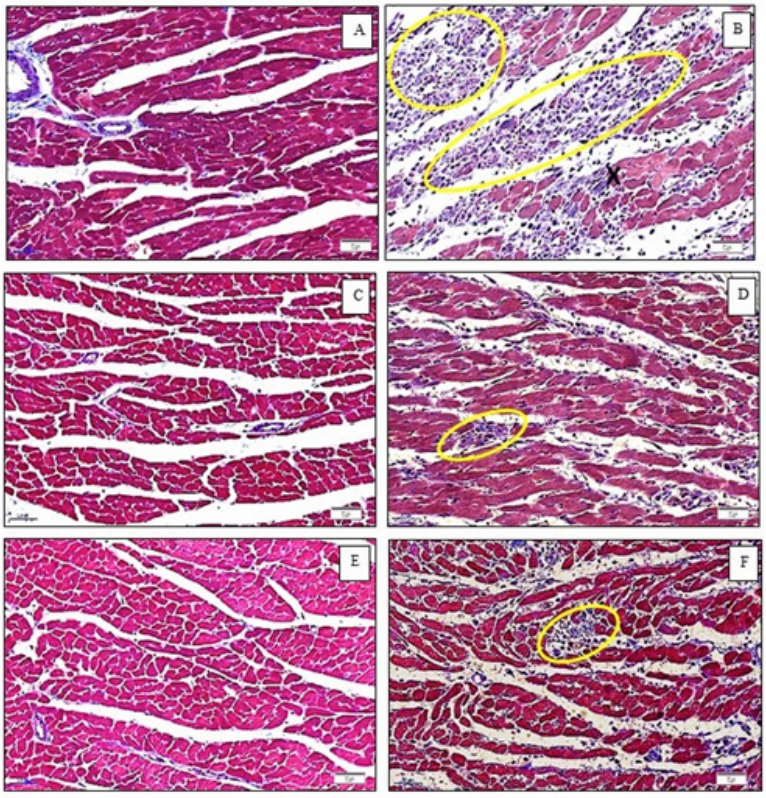

Figure 4. Cross section of myocardium with Masson's trichrome staining at 200× magnification with 50-µm bar scale. (A) control group, (B) MI, (C) GP250, (D) GP250+MI, (E) GP500, and (F) GP500+MI. Fragmentation of cardiomyocytes is marked with X, with leucocytes infiltration circled in yellow.

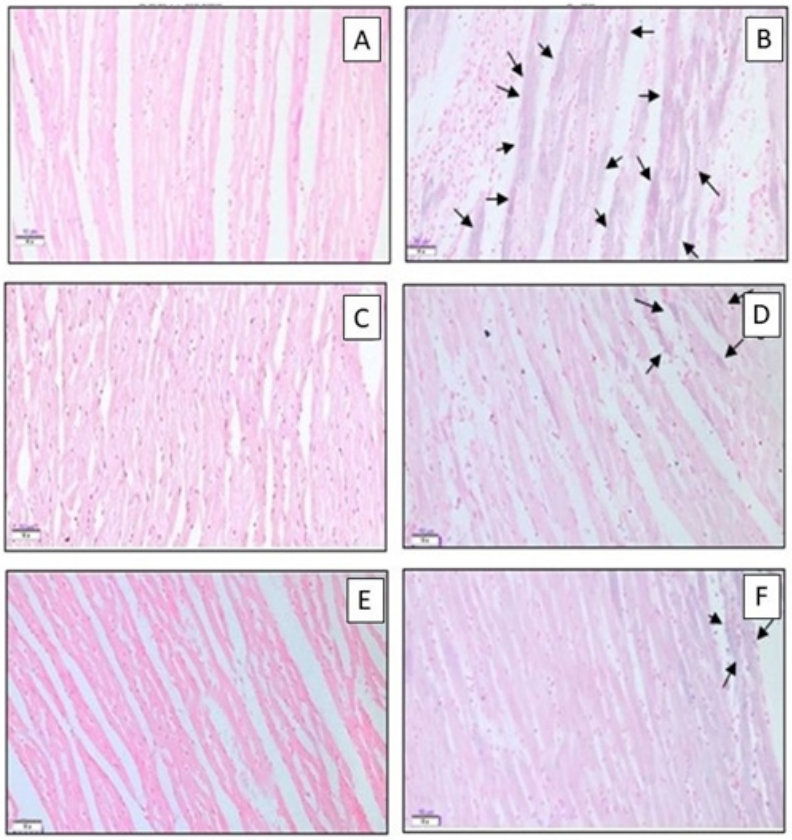

Figure 5. Immunohistochemical staining at 100× magnification with a 100-µm bar scale. The arrows point out the apoptotic bodies.

abundant dark blue precipitates were observed in cardiomyocytes. However, the groups receiving GP supplementation (Figures 5D, 5F) exhibited a condition of preserved cardiomyocytes with minimal cell apoptosis.

Discussion

The present work gives important insights on the possible cardioprotective action of GP, a natural product rich in antioxidants, against ISO-induced MI in rats. Various studies have been conducted to explore the therapeutic components of GP to evaluate their potential application in disease prevention and treatment (Akila and Vennila, 2016).

Isoproterenol, a synthetic sympathomimetic β adrenergic agonist, inflicts myocardial damage through several mechanisms that include coronary insufficiency, oxidative stress, depletion of high phosphate energy, intracellular Ca^{2+} overload, and alteration of metabolism (Upaganlawa *et al.,* 2011). The damage results in the elevation of serum cardiac enzyme markers such as troponin T, LDH, and CKMB in the blood, as demonstrated in the present work. The findings are consistent with those of Patel *et al.* (2010) and Khalil *et al.* (2015) who reported that ISO causes damage to the membrane sarcolemma of the myocardium and leakage of cardiac markers into the circulation. Troponin measurement is the gold standard in diagnosing MI, particularly the cardiac troponin T and I (Patel *et al.,* 2010; Khalil *et al.,* 2015). This is because troponin T and I in the cardiac muscle have different structures of amino acid than the troponin present in skeletal muscle (Mythili and Malathi, 2015).

The leakage of cardiac markers from the myocardium occurs due to the rupture of cellular membrane caused by oxidative damage (Patel *et al.,* 2010). The accumulation of lipid peroxide triggers the impairment in the structure of membrane and its function. As a result, changes such as necrosis, intramuscular oedema, and leukocyte infiltration will manifest, as observed in the histopathological findings. Furthermore, neutrophils release enzymes that dissolve the necrotic myocardium before phagocytosis by monocytes and macrophages. After clearance of the debris, fibrosis occurs at the site of infarction. Oedema can be found in between the muscle fibres due to excessive water accumulation that results from the activation of sodium-calcium channels (Bhandari *et al.,* 2008). The alteration in the membrane permeability is another factor contributing to the accumulation of water (Othman

et al., 2017). The addition of 1% myocardial water content due to interstitial oedema has been associated with a reduction in myocardial function by 10% (Toutounchi *et al.,* 2017). During pathological events in myocardium, apoptosis plays a role to accelerate the process of cell death (Zhang *et al.,* 2008; Wang *et al.,* 2017). Although cardiomyocyte death may be due to either apoptosis or necrosis, it is difficult to differentiate them via basic histological staining. Therefore, the TUNEL assay was used in the present work to identify the apoptotic bodies, which were mostly present in the necrotic area.

Auto-oxidation reaction in ISO generates abundant free radicals which may attack any cells in the body. However, the primary attack involves the formation of peroxyl radicals from polyunsaturated fatty acids (PUFA), which can be found in the cell membrane. The radicals attack the nearby fatty acids present in the membrane, and trigger the lipid peroxidation reaction (Patel *et al.,* 2010). The oxidised product of ISO also increases the amount of ROS by interacting with sulfhydryl group to form superoxide anions, and subsequently, hydrogen peroxide (H_2O_2) (Akila and Vennila, 2016). As a result, oxidative stress occurs and impairs the structure and function of myocardium. The present work found that ISO causes oxidative stress, as evidenced by the increase in MDA level as well as the significant decrease in antioxidant enzyme activity. These results are consistent with the findings obtained in previous research (Griendling *et al.,* 2016; Shikalgar and Naikwade, 2018). Since direct measurement of free ROS is difficult due to its instability, the end product of lipid peroxidation, MDA, is used as an indicator of ROS production instead (Goyal *et al.,* 2015). Therefore, the increase in MDA activity indicates the excessive production of ROS. Once the overproduction of MDA outweighs the antioxidant enzymes, oxidative stress occurs (Upaganlawar *et al.,* 2009; Long *et al.,* 2012).

Antioxidant enzymes such as SOD and catalase are important reducing agents that reduce and detoxify ROS in order to maintain the integrity of cellular membranes (Ighodaro and Akinloye, 2017). The harmful presence of superoxide and H_2O_2 are reduced by SOD and catalase (Upaganlawar *et al.,* 2009; Long *et al.,* 2012). The conversion of H_2O_2 is not only done by catalase, but also by GPx. However, the conversion of H_2O_2 by GPx requires glutathione (GSH) as a substrate (Shikalgar and Naikwade, 2018). GSH is a non-enzyme antioxidant in the body. It reacts with superoxide radicals and singlet oxygen to form oxidised glutathione. The oxidation and reduction cycle of glutathione is a reversible reaction, catalysed by glutathione reductase and GPx. The decrease in the activity of GPx is due to the lack of GSH which is used to neutralise the excessive production of ROS (Patel *et al.,* 2010; Long *et al.,* 2012).

The present work demonstrated that supplementation of GP before MI induction restored MDA activity, and increased the level of antioxidant enzymes. The findings suggest the ability of GP to inhibit lipid peroxidation and prevent the accumulation of MDA in the heart. These results are consistent with a previous study which discovered that GP could inhibit lipid peroxidation (Akowuah *et al.,* 2012). A study conducted by Akowuah *et al.* (2012) demonstrated that the administration of methanol extract of GP before the event of oxidative stress reversed the process of lipid peroxidation. This is probably due to the antioxidant property of GP rendered by its high phenolic and flavonoid contents. Chlorogenic acid is one of the bioactive compounds present in GP (Ahmad Nazri *et al.,* 2019) which acts as a free radical scavenger, thus inhibiting the initiation and progression of lipid peroxidation (Akila and Vennila, 2016). In addition, chlorogenic acid enables the protection of mitochondrial respiratory chain enzymes from the inhibitory effect of ROS (Wei *et al.,* 2017). Therefore, chlorogenic acid is beneficial to prevent mitochondrial dysfunction and further mitochondrial release of ROS.

Certain components in GP might also increase the level of antioxidant enzymes such as SOD and catalase. The results obtained in the present work agree with those of previous studies where the antioxidant properties of fulvic acid and malvidin rendered a protective effect in ISO-induced MI animal models (Wei *et al.,* 2017; Shikalgar and Naikwade, 2018). Endogenous antioxidant stimulation in myocardium is a treatment strategy used for diseases associated with increased oxidative stress. Therefore, the enhancement in the activity of endogenous SOD and catalase is significant to counter ROS production. The present work demonstrated that GP supplementation provided reliable protection for animals suffering from ISO-induced oxidative stress by maintaining antioxidant activities in the heart. However, the level of GPx remained unchanged even after the pre-treatment with GP. These results contradict those of the previous research which showed that the supplements containing antioxidant properties could restore GPx level. Previous studies have proven that antioxidants from animal or plant sources were able to restore the GPx activity as they have the capacity

to remove free radicals (Ganesan *et al.,* 2010; Ighodaro and Akinloye, 2017). Although GP has antioxidant and radical scavenging properties, the GPx activity was not restored. This may be due to the deactivation of ROS activity by catalase.

The protection rendered by antioxidant enzymes is indicated by the preservation of cardiomyocyte and its contractile apparatus function. Pre-treatment with GP has been found to reduce necrotic tissue, leukocyte infiltration, and minimal intramuscular oedema based on the microscopic analysis. Moreover, the levels of troponin T, CKMB, and LDH leakage have also decreased with the treatment, thus reflecting the extent of the myocardial necrosis and infarct size (Tungmunnithum *et al.,* 2018). The positive result obtained in the present work may also be due to the presence of secondary metabolites such as flavonoids and phenolics that serve as effective antioxidants (Kasote *et al.,* 2015). Flavonoid is an excellent antioxidant due to its high reactivity to donate hydrogen and electron. Flavonoid does not only act as a free radical scavenger but also serves to strengthen the antioxidant defence system in the body (Kaewseejan *et al.,* 2015). There is a strong positive relationship between the total amount of flavonoid and antioxidant activities, as observed in the tests with DPPH (2,2-diphenyl-1picrylhydrazyl) and FRAP (ferric reducing antioxidant power) (Rosidah *et al.,* 2008). There is also a significant interconnection between total phenolic content and antioxidant capacity (Rosidah *et al.,* 2008).

Based on the results, both doses of GP, 250 and 500 mg/kg, produced positive effects on the studied parameters. However, GP500 mg/kg was more capable to protect cardiomyocytes from leakage of troponin, LDH, and CKMB into the serum. This is because the higher dose of GP inhibited ROS generation, and strengthened the endogenous antioxidant enzyme activity via elevation of SOD and catalase. In summary, the animal model receiving GP supplementation of 500 mg/kg orally for 28 days exhibited a condition that potentially indicated cardioprotective effect, particularly in the ISO-induced MI group.

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