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Methanol extract of *Desmodium gangeticum* roots preserves mitochondrial respiratory enzymes, protecting rat heart against oxidative stress induced by reperfusion injury

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Abstract

Ischaemia and reperfusion result in mitochondrial dysfunction, with decreased oxidative capacity, loss of cytochrome c and generation of reactive oxygen species. The aim of this study was to evaluate the effect of a methanol extract of *Desmodium gangeticum* (L) DC (Fabaceae) (DG) on lipid peroxidation and antioxidants in mitochondria and tissue homogenates of normal, ischaemic and ischaemia-reperfused rats. Myocardial lipid peroxidation products (thiobarbituric acid reactive substances; TBARS) in cardiac tissue homogenates and mitochondrial fractions were significantly increased during ischaemia reperfusion. Antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase (GPx) and glutathione reductase) in the myocardial tissue homogenate and mitochondria decreased significantly during ischaemia reperfusion, accompanied by a decreased activity of mitochondrial respiratory enzymes. Daily pretreatment of rats with DG (50 or 100 $mg\,kg^{-1}$) orally for 30 days had a significant effect on the activity of mitochondrial and antioxidant enzymes. In-vitro studies showed that DG inhibited lipid peroxidation, and also scavenged hydroxyl and superoxide radicals. The concentrations required to scavenge 50% of the superoxide and hydroxyl radicals were 21 and 50.5 $\mu g\,mL^{-1}$, respectively. Administration of DG to normal rats did not have any significant effect on any of the parameters studied. The results of our study showed that DG possesses the ability to scavenge the free radicals generated during ischaemia and ischaemia reperfusion and thereby preserves the mitochondrial respiratory enzymes that eventually lead to cardioprotection.

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Introduction

Ischaemia reperfusion of the heart leads to many biochemical alterations that may contribute to post-ischaemic contractile and electric dysfunction and lead to potentially fatal arrhythmias (Carmeliet 1999). But reperfusion of the ischaemic myocardium is the only logical approach to the management of patients with acute obstruction of the coronary arteries. However, myocardial ischaemia reperfusion may result in various metabolic stresses that can lead to calcium overload and an increase in the production of reactive oxygen species (ROS), which favour the degradation of mitochondrial integrity, leading to necrotic and apoptotic cell death (Gogvadze & Galitovsky 2001). Sources of ROS include activated neutrophils, stressed cardiomyocytes, the mitochondrial electron transport chain, activated vascular endothelium and, to a minor extent, perivascular tissue (Kloner & Jennings 2001). Moreover, mitochondria are susceptible to injury from increased fluxes of superoxide ions and hydrogen peroxide (Malis & Bonventre 1998).To minimize the toxicity of ROS, mitochondrial antioxidant systems maintain concentrations at low steady-state levels.

Many pharmacological interventions are used to provide cardioprotection against oxidative stress by ischaemia reperfusion injury (Bandyopadhyay et al 2004). Herbal agents such as garlic (Banerjee et al 2001), *Emblica officinalis* (Rajak et al 2004) and *Terminalia arjuna* (Gauthaman et al 2001) have been found to be effective in the management of ischaemia reperfusion of rat hearts. Nevertheless, the search for pharmacological agents that will render the myocardium resistant to ischaemia and delay the development of irreversible cell injury continues.

Desmodium gangeticum (L.) DC (Fabaceae) (DG) is a perennial non-climbing herb or shrub and is widely used as a medicinal herb in the treatment of ischaemic heart diseases (Kirthikar & Basu 1975). Phytochemical analysis of DG has described flavonoids, glycosides, pterocarpanoids, lipids, glycolipids and alkaloids (Mishra et al 2005).

Experimental findings suggest that controlled management of mitochondrial calcium and/or ROS levels before reperfusion may be effective in providing cardioprotection.

The aim of the present study was to determine the cardioprotective effect of DG against ischaemia reperfusion injury, by assessing parameters relating to oxidative stress and mitochondrial respiratory enzymes.

Materials and Methods

Preparation of methanol extract of roots of *D. gangeticum*

After collection from the herb garden maintained in the department, the plant was washed and cleaned. The plant material was taxonomically identified by Professor James Joseph (Head of the Department of Botany, Saint Berchman's College, Mahatma Gandhi University, Kerala, India). The voucher specimen A/C no. 3908 is retained in our laboratory for future reference.

One kilogram of fresh secondary roots of DG were sliced and air-dried at room temperature and then milled into fine powder in a commercial blender. The powdered plant material was soaked in 2 L of methanol for 72 h and the extract filtered and distilled in a water bath. The last traces of the solvent were removed under vacuum drier and the solid brown mass obtained was stored at −4°C until further use. The yield of the extract was 6.1% w/w. The extract was lyophilized. For the in-vitro antioxidant assay, the methanol extract was dissolved in 0.9% saline. For in-vivo experiments DG was suspended in water and administered orally.

Dose determination

A pilot study was conducted to determine the effect of DG at four different doses (25, 50, 100 and 150 $mg\,kg^{-1}$) for a period of 30 days in ischaemia-reperfused (IR) rats. Since 50 and 100 $mg\,kg^{-1}$ showed significant effects ($P<0.05$), we used these doses for further studies.

Chemicals

DL-Isocitrate and N-phenyl-p-phenylenediamine were purchased from Acros Organics (Morris Plains, NJ, USA). Cytochrome c and ATP were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were of analytical grade.

Animals

Adult male Wistar rats, weighing 250–280 g, were obtained from King Institute of Preventive Medicine (Chennai, India). They were acclimatized to conditions in the animal house and were fed commercial pelleted rat chow (Hindustan Lever Ltd, Bangalore, India) and had free access to water.

Ethical approval was obtained from the Ministry of Social Justices and Empowerment, Government of India. The experimental protocol was approved by the institutional animal ethical committee.

Heart preparation

Rats were anaesthetized with sodium thiopentenone (40 $mg\,kg^{-1}$). After an intravenous injection of 300 IU heparin, the heart was excised rapidly via a mid-sternal thoracotomy and arrested in ice-cold Krebs–Henseleit buffer (KH) containing (in $mmol\,L^{-1}$): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25 and $C_6H_{12}O_6$ 11. The heart was attached to a Lagendorff apparatus via the aorta for retrograde perfusion with KH buffer maintained at 37°C and pH 7.4 and saturated with a mixture of 95% O_2/5% CO_2. The coronary perfusion pressure was maintained at 80 mmHg. The left ventricular pressure (LVP) that developed when the ventricle filled with KH was recorded with a pressure transducer connected to an amplifier and chart recorder. LVP gives an indication of the mechanical performance of the heart. Coronary flow was measured by collecting the perfusate draining from the heart in a graduated cylinder for a defined time. The heart rate (HR) was measured by counting the number of contractions (obtained from the LVP record) per min.

Experimental protocol

The rats were divided into three groups (n = 6 in each group): group 1 were controls; group 2 were the IR group; group 3 underwent IR and also received drug treatment. In the control group, hearts were perfused for 90 min with KH buffer and used for the biochemical analysis. Animals in the IR group (group 2) were divided into two subgroups of six rats. After 20 min equilibration, the rat hearts were subjected to 30 min' global ischaemia, followed by 30 min reperfusion in one group and 45 min reperfusion in the other (groups 2.1 and 2.2, respectively).

Animals in the drug group were divided into eight subgroups of six rats. DG was administered by gavage. Rats in group 3.1 and 3.2 were pretreated with 50 or 100 $mg\,kg^{-1}$ DG, respectively, for 30 days. Hearts were perfused for 90 min with KH buffer, followed by biochemical analysis. Rats in groups 3.3 and 3.4 were pretreated with 50 $mg\,kg^{-1}$ DG for 30 days. Hearts were then subject to 30 min' global ischaemia after equilibration, followed by 30 or 45 min reperfusion, respectively. Rats in groups 3.5 and 3.6 were pretreated orally with 100 $mg\,kg^{-1}$ DG for 30 days. Hearts were then subject to 30 min' global ischaemia after equilibration, followed by 30 or 45 min' reperfusion, respectively.

Hearts from rats in groups 3.7 and 3.8 were perfused with KH buffer for 20 min, followed by infusion of the reference drug verapamil (0.2 $mg\,kg^{-1}$) for 30 or 15 min, respectively. They were then subject to 30 min' global ischaemia followed by 30 or 45 min' reperfusion, respectively.

Tissue preparation

The heart was excised, rinsed in ice-cold isotonic saline, blotted with filter paper, weighed, and homogenized in 0.1 M Tris-HCl (pH 7.4) buffer. The homogenate was centrifuged at 3000 rev min^{-1} for 5 min. The supernatant was used for the estimation of various biochemical parameters.

Preparation of mitochondria

The heart was excised, rinsed in ice-cold isotonic saline, blotted with filter paper, weighed and then homogenized in 0.25 M sucrose for 5 s using a polytron homogenizer at 4°C and maximum power (20000 rev min^{-1}). The homogenate was centrifuged for 10 min at 600 *g* and the nuclear and cytoskeleton fractions were discarded. The supernatant was centrifuged for 20 min at 15 000 *g* to pellet the mitochondria (Johnson & Lardy 1967). The mitochondrial fraction was suspended in 0.25 M sucrose containing 10 mM Tris-HCl and 1 mM EDTA in a total volume of 3 mL.

Acute toxicity studies

These studies were performed according to the OECD 425 guidelines. Wistar rats weighing 150–250 g were maintained under standard laboratory conditions. Five rats each received a single dose of DG of 2000 mg kg^{-1}. Animals were fasted overnight before drug administration, and food was withheld for 3–4 h after administration of DG. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for 14 days. Once-daily cage-side observations included changes in skin and fur, eyes and nasal mucous membrane. Changes in respiratory rate, circulatory (heart and blood pressure), autonomic activity (salvation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) were also recorded. No acute toxicity was noted.

Biochemical assays

Thiobarbituric acid reactive substances (TBARS) (Ohkawa et al 1979) were measured as a marker of lipid peroxidation and endogenous antioxidants. Cu-Zn-superoxide dismutase (SOD), Mn-SOD (Geller & Winge 1983; Marklund 1985), catalase (Aebi 1984) and GPx (Wendel 1981) were measured by UV spectrophotometry. The protein concentration was measured using the Folin phenol reagent according to the method described by Lowry et al (1951). Levels of isocitrate dehydrogenase (ICDH) (Bell & Baron 1968), malate dehydrogenase (MDH) (Mehler et al 1948), succinate dehydrogenase (SDH) (Slater & Bonner 1952), α-ketoglutarate dehydrogenase (α-KGDH) (Reed & Savage 1995), NADH dehydrogenase (NADH dH) (Minakami et al 1962) and cytochrome c oxides (Pearl et al 1963) were also estimated using previously reported methods.

In-vitro antioxidant activity

Superoxide radical scavenging activity

Superoxide scavenging was determined by the nitroblue tetrazolium (NBT) reduction method (McCord & Fridovich, 1969). The reaction mixture contained EDTA (6 μM), NaCN (3 μg), riboflavin (2 μM), NBT (50 μM), various concentrations of DG extract (5–50 μg mL^{-1}) and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 mL. The tubes were uniformly illuminated with incandescent light for 15 min and the optical density was measured at 530 nm before and after the illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes.

Hydroxyl-radical scavenging activity

Hydroxyl-radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The hydroxyl radical attacks deoxyribose, which eventually results in formation of TBARS (Elizabeth & Rao 1990). The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), KH_2PO_4 KOH buffer (20 mM, pH 7.4) and various concentrations of DG extract (25–400 μg mL^{-1}) in a final volume of 1 mL. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated.

Lipid peroxide scavenging activity

Reaction mixture (0.5 mL) containing rat liver homogenate (0.1 mL, 25% w/v) in Tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous iron (0.16 mM) and ascorbic acid (0.06 mM) was incubated for 1 h at 37°C in the presence and absence of DG extract (20–180 μg mL^{-1}). Lipid peroxidation was measured by TBARS formation (Ohkawa et al 1979). For this, incubation mixture (0.4 mL) was treated with sodium dodecyl sulfate (8.1%, 0.2 mL), thiobarbituric acid (0.8%, 1.5 mL) and acetic acid (20%, 1.5 mL, pH 3.5). The total volume was then made up to 4 mL with distilled water and kept in a water bath at 100°C for 1 h. After cooling, 1 mL distilled water and 5 mL of a mixture of n-butanol and pyridine (15:1 v/v) were added and the samples shaken vigorously. After centrifugation, the absorbance of the organic layer was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing results obtained with the test compounds with those of controls not treated with the extracts. Tocopherol (10 mmol/L) was used as standard.

GC–MS analysis

Analysis was conducted with a Perkin Elmer Clarus 500 gas chromatogram equipped with mass spectrometry (MS). For gas chromatography (GC) an Elite-1 column (100% dimethyl polysiloxane (Perkin Elmer, Waltham, MA, USA) was used. The carrier gas was helium, delivered at a flow rate of 1 mL min^{-1}. An aliquot (1 μL) of the methanol root extract of DG was injected into the GC–MS in splitless mode at 250°C. The column oven temperature was held at 110°C for 2 min, then programmed to increase at 75°C min^{-1} to 200°C, held for 1 min, then increased at 5°C min^{-1} to 280°C and held for 9 min.

Statistical analysis

All data are given as mean ± s.d. Results were analysed by a one-way analysis of variance using SPSS software 12.00

(Chicago, IL, USA), followed by Duncan's multiple range test. $P < 0.05$ was considered significant.

Results

Initial value for mean arterial pressure (MAP) and HR in the control IR groups (groups 2.1 and 2.2) were 99 ± 6 mmHg and 262 ± 18.2 beats min^{-1}, respectively. Both haemodynamic variables in the control IR group remained depressed throughout the IR duration compared with control baseline values. Because HR and the developed LVP may recover to different degrees, rate pressure product (RPP) was calculated by multiplying HR by LVP, which provides a reliable parameter for left ventricular function in the isolated heart. There were no differences between the experimental groups for RPP at the end of the 20 min equilibration period before starting treatments and global ischaemia (Table 1). During the 30 min' global ischaemia RPP decreased to 0 but then recovered gradually as the reperfusion progressed. Pretreatment with DG improved the recovery of the RPP in the drug group (69% of the baseline value) compared with the IR group (38% of the baseline value, $P < 0.05$) (Figure 1). There was no significant difference in RPP between the DG-treated group and the control group (90% of baseline value).

Table 1 Haemodynamic characteristics. Treatment of the different groups of rats is described in the text

Group	LVP	CF	HR	RPP	MAP
1	105.22 ± 4.3	9.2 ± 1.00	342 ± 20.1	35.98 ± 7.1	122 ± 7
2.1	90.41 ± 4.1	9.1 ± 0.98	262 ± 18.2*	23.68 ± 6.6*	99 ± 6*
2.2	94.2 ± 4.4	9.1 ± 1.02	240 ± 19.3*	22.60 ± 6.3*	98 ± 7*
3.1	107.2 ± 4.2	9.2 ± 1.08	339 ± 34.1	36.34 ± 8.2	115 ± 8
3.2	107.3 ± 4.5	9.3 ± 1.10	338 ± 31.3	36.26 ± 5.1	114 ± 7
3.3	106.4 ± 4.6	9.2 ± 0.94	323 ± 33.2	32.94 ± 6.8	104 ± 5
3.4	106.2 ± 4.0	9.4 ± 1.05	329 ± 33.5	34.94 ± 7.4	103 ± 6
3.5	106.1 ± 4.1	9.3 ± 1.00	319 ± 32.8	33.84 ± 5.9	107 ± 7
3.6	105.6 ± 4.2	9.2 ± 1.02	321 ± 33.5	33.89 ± 7.3	105 ± 6

Values are mean ± s.d. for six rats/hearts in each group. LVP, developed left ventricular pressure, in mmHg; CF, coronary flow, in mL min^{-1}; HR, heart rate, in beats min^{-1}; RPP, rate pressure product (HR × LVP, in mmHg beat^{-1} min^{-1} × 10^3; MAP, mean arterial pressure in mmHg. *$P < 0.05$ vs control.

The free-radical scavenging activity of DG was evaluated by comparing it with the activity of standard substances such as tocopherol and ascorbic acid, which possess some antioxidant potential. DG inhibited lipid peroxidation and production of hydroxyl and superoxide radicals. The concentrations of DG that scavenged 50% of superoxide and hydroxyl radicals were 21 and 50.5 μg mL^{-1}, respectively (Figure 2).

Lipid peroxidation was measured in-vivo by assaying TBARS. Tables 2 and 3 show the effects of various doses of DG on lipid peroxidation and antioxidant activities in myocardial tissue and mitochondria from normal, ischaemic and IR hearts. A significant ($P < 0.05$) increase in myocardial TBARS was observed in the ischaemia and IR groups compared with the controls. However, rats pretreated with various doses of DG (50 or 100 mg kg^{-1}) for 30 days showed a significant decrease in the level of TBARS in both tissue homogenates and mitochondrial extracts. Furthermore, levels in rats pretreated with DG (groups 3.5 and 3.6) showed considerable improvements compared with rats treated with the standard drug verapamil (groups 3.7 and 3.8).

The activity of mitochondrial antioxidant enzymes declined significantly compared with whole-tissue homogenate, except for the activity of Cu-Zn-SOD. Both catalase and GPx had significantly lower activities in the mitochondrial sample, while GPx activity was found to be significantly decreased in tissue homogenates. As with the standard drug verapamil, pretreatment of rats with DG (50 or 100 mg kg^{-1} for 30 days) significantly improved the activities of these enzymes.

Activities of the mitochondrial respiratory enzymes ICDH, MDH, SDH, NADH dH and cytochrome c oxidase were decreased in IR hearts (Table 4) but recovered significantly ($P < 0.05$) in hearts from rats pretreated with DG. The 50 mg kg^{-1} dose had a more pronounced effect than 100 mg kg for all parameters studied. Treatment with either dose of DG had no significant effects on normal rats.

GC–MS analysis identified 64 compounds (Figure 3). Major compounds were 4-[2-(dimethylamino)ethyl]phenol (cactine) (retention time (RT) 15.41 min), glycerine, sucrose, asarone (RT

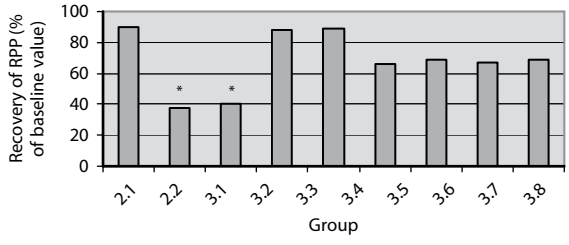

Figure 1 Recovery of the rate pressure product (RPP), an index of ventricular function (calculated by multiplying heart rate by left ventricular pressure). Treatment of groups is described in the text. Data are mean ± s.d. *$P < 0.01$ vs control group.

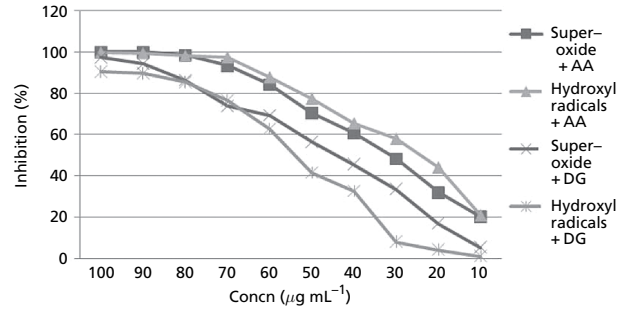

Figure 2 Superoxide and hydroxyl radical scavenging activity by DG root extract and ascorbic acid (AA; reference compound). Data are mean ± s.d. The concentrations of DG that resulted in 50% decrease in free radical concentration were 21 ± 1.16 μg mL^{-1} for superoxide radicals and 50.5 ± 3.41 μg mL^{-1} for hydroxyl radicals.

Table 2 Effect of the methanol extract of DG root on TBARS (mmol per 100 g wet tissue), catalase (μmol H_2O_2 consumed min^{-1} per mg protein), superoxide dismutase (SOD; U† per mg protein) and glutathione (GSH) peroxidase (GPx; μg GSH consumed min^{-1} per mg protein) in the tissue homogenate of isolated rat hearts. Treatment of the different groups of rats is described in the text

Group	TBARS	Catalase	Mn-SOD	Cu-Zn-SOD	GPx
1	$^{a}0.61 \pm 0.04$	$^{a}7.60 \pm 0.52$	$^{a}8.1 \pm 0.71$	$^{a}52.5 \pm 4.3$	$^{a}1.83 \pm 0.17$
2.1	$^{a}0.74 \pm 0.05$*	$^{b}5.17 \pm 0.47$*	$^{d}6.0 \pm 0.55$*	$^{b}36.1 \pm 3.3$*	$^{c}1.11 \pm 0.11$*
2.2	$^{a}0.70 \pm 0.05$*	$^{b}5.20 \pm 0.41$*	$^{b}5.7 \pm 0.58$*	$^{b}35.2 \pm 3.4$*	$^{c}1.21 \pm 0.11$*
3.1	$^{a}0.59 \pm 0.04$	$^{a}7.85 \pm 0.54$*	$^{a}8.1 \pm 0.73$	$^{a}51.6 \pm 4.1$	$^{a}1.85 \pm 0.17$
3.2	$^{a}0.58 \pm 0.04$	$^{a}7.57 \pm 0.53$	$^{a}8.1 \pm 0.71$	$^{a}51.0 \pm 4.1$	$^{a}1.80 \pm 0.18$
3.3	$^{b}0.47 \pm 0.03$*	$^{c}6.17 \pm 0.55$*	$^{a}7.2 \pm 0.65$*	$^{a}47.3 \pm 4.0$	$^{b}1.57 \pm 0.17$*
3.4	$^{b}0.44 \pm 0.03$*	$^{b}5.86 \pm 0.47$*	$^{a}7.4 \pm 0.62$	$^{b}44.5 \pm 3.8$	$^{b}1.51 \pm 0.16$*
3.5	$^{a}0.58 \pm 0.04$	$^{b}5.51 \pm 0.45$*	$^{b}6.9 \pm 0.62$*	$^{b}41.1 \pm 3.6$*	$^{b}1.43 \pm 0.16$*
3.6	$^{a}0.55 \pm 0.05$	$^{b}5.23 \pm 0.50$*	$^{b}6.8 \pm 0.68$*	$^{b}35.6 \pm 3.3$*	$^{b}1.39 \pm 0.16$*
3.7	$^{a}0.59 \pm 0.05$	$^{b}5.11 \pm 0.48$*	$^{b}6.5 \pm 0.62$*	$^{b}39.7 \pm 3.1$*	$^{b}1.48 \pm 0.15$*
3.8	$^{a}0.64 \pm 0.06$	$^{b}5.08 \pm 0.43$*	$^{b}6.1 \pm 0.71$*	$^{b}34.1 \pm 3.2$*	$^{b}1.40 \pm 0.16$*

Values are mean ± s.d. for six rats in each group. †One unit is defined as the enzyme concentration required to inhibit 50% of optical density produced by the chromogen in 1 min, measured at 560 nm. *$P < 0.05$ vs normal control group. Values not sharing a common superscript (a, b, c, d, e, f) differ significantly ($P < 0.05$) between the groups.

Table 3 Effect of methanol extract of DG root on TBARS (pmol per total protein), catalase (μmol H_2O_2 consumed min^{-1} per mg protein), superoxide dismutase (SOD; U† per mg protein) and glutathione (GSH) peroxidase (GPx; μg GSH consumed min^{-1} per mg protein) in the mitochondria of isolated rat heart. Treatment of the different groups of rats is described in the text

Group	TBARS	Catalase	Mn-SOD	Cu-Zn-SOD	GPx
1	$^{a}600.5 \pm 22.4$	$^{a}0.031 \pm 0.004$	$^{a}99.1 \pm 5.2$	$^{a}3.02 \pm 0.21$	$^{a}33.8 \pm 2.3$
2.1	$^{b}769.7 \pm 22.7$*	$^{b}0.006 \pm 0.001$*	$^{b}40.8 \pm 4.1$*	$^{b}2.41 \pm 0.26$*	$^{b}3.3 \pm 0.8$*
2.2	$^{c}710.0 \pm 23.8$*	$^{c}0.018 \pm 0.002$*	$^{b}42.7 \pm 4.0$*	$^{a}2.55 \pm 0.24$*	$^{b}5.5 \pm 0.9$*
3.1	$^{a}597.6 \pm 23.8$	$^{a}0.030 \pm 0.004$	$^{a}95.5 \pm 5.1$	$^{a}3.01 \pm 0.32$	$^{a}32.1 \pm 2.2$
3.2	$^{a}594.1 \pm 21.1$	$^{a}0.029 \pm 0.004$	$^{a}93.9 \pm 5.7$	$^{a}2.97 \pm 0.29$	$^{a}30.6 \pm 2.1$
3.3	$^{d}366.6 \pm 20.4$*	$^{d}0.038 \pm 0.004$*	$^{a}90.2 \pm 5.1$	$^{a}2.61 \pm 0.26$	$^{c}23.9 \pm 1.6$
3.4	$^{d}395.8 \pm 20.2$*	$^{a}0.030 \pm 0.004$	$^{c}87.0 \pm 4.8$	$^{a}2.59 \pm 0.31$*	$^{c}21.8 \pm 1.7$*
3.5	$^{e}453.0 \pm 22.7$*	$^{c}0.022 \pm 0.004$*	$^{a}91.3 \pm 4.9$	$^{a}2.69 \pm 0.25$*	$^{c}22.3 \pm 1.9$*
3.6	$^{e}488.7 \pm 23.9$*	$^{b}0.011 \pm 0.003$*	$^{c}83.7 \pm 4.2$*	$^{b}2.45 \pm 0.21$*	$^{c}19.9 \pm 1.7$*
3.7	$^{e}423.9 \pm 22.5$*	$^{c}0.023 \pm 0.004$*	$^{c}86.9 \pm 4.1$*	$^{a}2.68 \pm 0.29$*	$^{c}20.3 \pm 1.8$*
3.8	$^{e}444.7 \pm 22.4$*	$^{c}0.018 \pm 0.003$*	$^{d}79.2 \pm 3.9$*	$^{a}2.58 \pm 0.25$*	$^{c}18.8 \pm 1.6$*

Values are mean ± s.d. for six rats in each group. †One unit is defined as the enzyme concentration required to inhibit 50% of optical density produced by the chromogen in 1 min, measured at 560 nm. *$P < 0.05$ vs normal control group. Values not sharing a common superscript (a, b, c, d, e, f) differ significantly ($P < 0.05$) between the groups.

18.66 min), trans-Z-α–bisabolene epoxide (RT 20.55 min), 2,5-bis (1,1-dimethylethyl)phenol (RT 21.89 min), trans-2-methyl-4-n-pentylthiane S,S-dioxide (RT 22.86 min), decahydro-1,1-dimethylnaphthalene (RT 25.33 min), 4,5 dihydro-2-(phenylmethyl) 1-H-imidazole (R.T 32.17), (–)-nortrachelogenin (RT 39.23 min), 2-methyl-9,10-anthracene dione (RT 29.10 min) and piperine (RT 43.56 min), representing about 33%. Minor compounds such as conhydrin, oxirane, 2,5-dihydro-1-H-pyrrole, thymol, eugenol, apiol, eicosane, 3-methyl-2-(2-oxo propyl) furan and 1-methoxy-10-H-phenothiazine were also identified.

Discussion

Previous phytochemical screening of DG had suggested the presence of 5-methoxy-N,N-dimethyltryptamine, N,N-dimethyltryptamine and their N-oxides, N-methyl-tetrahydroharman and 6-methoxy-β-carbolinium cation from aerial parts, and ter-β-phenylethylamines and candicine in the roots (Banerjee & Ghosal 1969). Some pterocarpanoids such as gangetin, gangetinin and desmodin (Mishra et al 2005), the isoflavanoid phytoalexin desmocarpin (Singh et al 2005) and the flavone glycosides 4′,5,7-trihydroxy-8-prenyl-flavone, 4′O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside have been isolated from the stems of DG. However, there has been little study of the volatile components of the roots. Many studies have shown the significance of volatile compounds like isoflurane, enflurane and halothane in cardioprotection against IR injury (Zaugg et al 2003).

The present study was performed with a crude extract of DG root. Probable phytochemical constituents have been explored by others; we focused our work on the volatile components identified through GC–MS (Figure 3).

Table 4 Effect of methanol extract of DG root on mitochondrial enzymes in isolated rat heart. Treatment of the different groups of rats is described in the text.

Group	ICDH	SDH	MDH	α–KGDH	NADH dH	Cytochrome c oxidase
1	$^{a}741.5 \pm 21.3$	$^{a}250.6 \pm 7.7$	$^{a}352.1 \pm 11.2$	$^{a}77.3 \pm 2.4$	$^{a}134.6 \pm 3.9$	$^{a}33.5 \pm 0.9$
2.1	$^{b}563.1 \pm 16.3$*	$^{b}112.2 \pm 3.4$*	$^{b}216.4 \pm 2.4$*	$^{b}31.6 \pm 0.9$*	$^{b}91.2 \pm 2.1$*	$^{b}16.2 \pm 0.3$*
2.2	$^{b}575.2 \pm 16.8$*	$^{b}115.3 \pm 3.7$*	$^{b}225.6 \pm 5.6$*	$^{b}33.4 \pm 0.7$*	$^{b}94.3 \pm 2.3$*	$^{f}19.3 \pm 0.5$*
3.1	$^{a}743.6 \pm 22.3$	$^{a}252.1 \pm 6.7$	$^{a}354.6 \pm 10.6$	$^{a}78.3 \pm 2.3$	$^{a}136.3 \pm 3.4$	$^{a}33.6 \pm 0.9$
3.2	$^{a}745.3 \pm 23.4$	$^{a}254.8 \pm 6.1$	$^{a}356.3 \pm 11.4$	$^{a}79.4 \pm 2.6$	$^{a}137.4 \pm 3.8$	$^{a}34.2 \pm 0.8$
3.3	$^{c}702.3 \pm 18.7$*	$^{c}163.2 \pm 4.8$*	$^{c}300.3 \pm 9.2$*	$^{c}48.1 \pm 1.4$*	$^{c}110.3 \pm 3.3$	$^{c}25.5 \pm 0.7$
3.4	$^{a}729.3 \pm 19.4$	$^{c}169.1 \pm 5.1$*	$^{c}301.9 \pm 9.5$*	$^{c}52.2 \pm 1.4$*	$^{c}114.2 \pm 3.4$	$^{c}26.3 \pm 0.7$
3.5	$^{a}719.0 \pm 9.5$	$^{d}210.0 \pm 6.3$	$^{c}316.0 \pm 18.7$	$^{d}57.6 \pm 1.7$	$^{c}115.0 \pm 3.4$	$^{e}22.3 \pm 0.6$
3.6	$^{a}731.2 \pm 10.4$	$^{d}217.2 \pm 7.2$	$^{c}320.4 \pm 21.4$	$^{e}65.7 \pm 1.9$	$^{a}130.1 \pm 3.9$	$^{d}28.2 \pm 0.8$
3.7	$^{c}700.3 \pm 21.2$	$^{e}238.5 \pm 7.1$	$^{c}326.4 \pm 19.7$	$^{e}69.7 \pm 2.0$	$^{d}121.1 \pm 3.6$	$^{c}27.3 \pm 0.8$
3.8	$^{c}690.2 \pm 20.2$	$^{c}171.2 \pm 5.1$	$^{d}259.1 \pm 17.7$	$^{f}41.2 \pm 1.2$	$^{c}107.2 \pm 2.2$	$^{b}15.2 \pm 0.4$

Results are mean ± s.d. (n = 6). ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; SDH, succinate dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; NADH dH, NADH dehydrogenase. Activities are expressed as nmol NADP reduced min^{-1} per mg protein for ICDH, nmol succinate oxidized min^{-1} per mg protein for SDH; nmol NADH oxidized min^{-1} per mg protein for MDH and NADH dH; nmol α-keto glutarate formed h^{-1} per mg protein for α-KGDH, and change in optical density min^{-1} per mg protein for cytochrome c oxidase. *$P < 0.05$ vs normal control group. Values not sharing a common superscript (a, b, c, d, e, f) differ significantly ($P < 0.05$) between the groups.

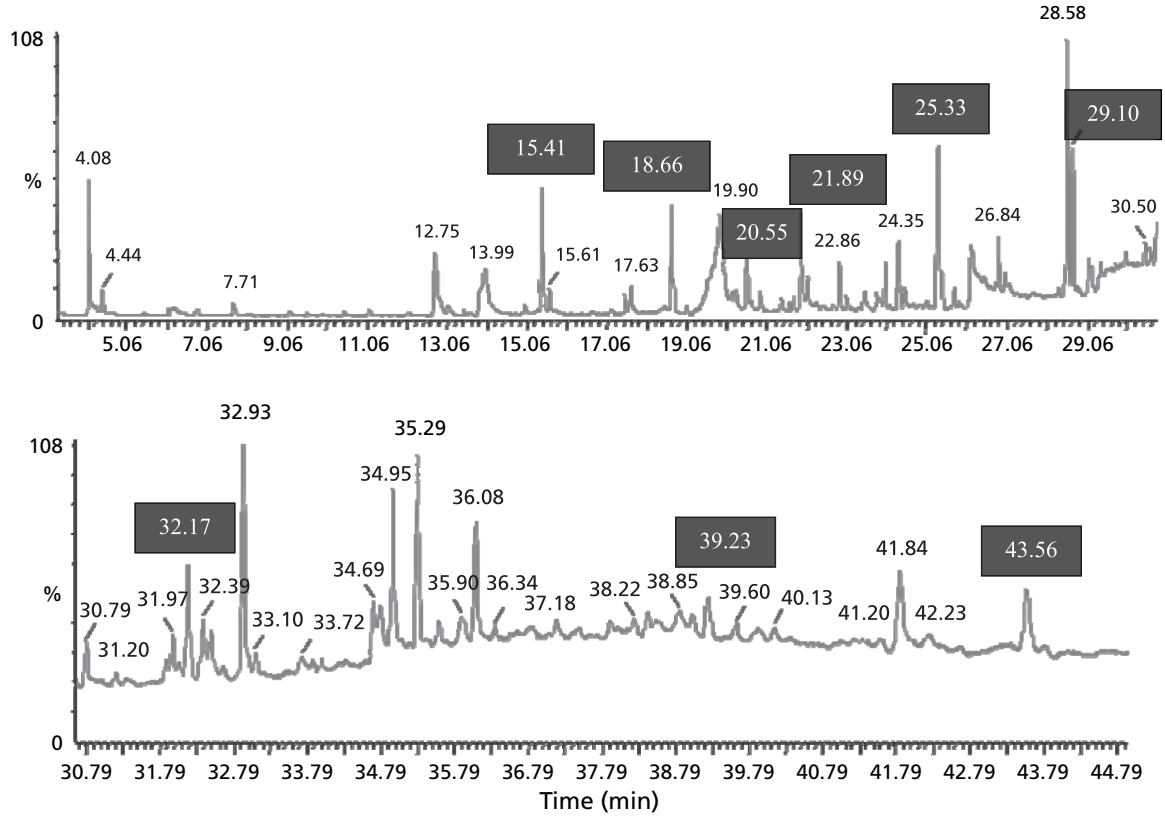

Figure 3 GC–MS for methanol extract of DG root.

Interestingly, we found the presence of two specific compounds (around 5% of volatile composition) namely (E)-2,4,5-trimethoxypropenylbenzene (asaron) and p-[2-(dimethylamino)ethyl]phenol, both of which have been reported to have action on cardiac tissue (Percot et al 2007).

Our experimental work shows that DG root extract has differing potential to scavenge the various types of radicals encountered. The effect of the DG root methanol extract on superoxide radicals (measured by the TBARS assay) is shown in Figure 4. DG probably inhibits the generation of superoxide radicals in-vitro.

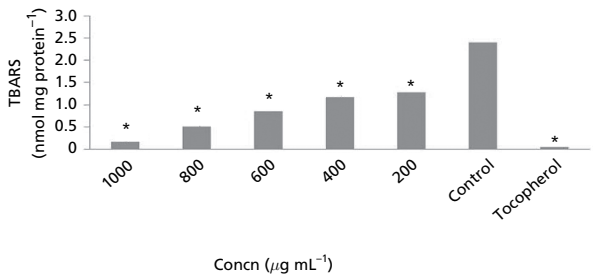

Figure 4 Inhibition of lipid peroxidation by DG root extract. Tocopherol (10 mmol L^{-1}) was used as a reference compound. Data are mean ± s.d. *$P < 0.01$ vs control group.

The effect of the DG extract on production of hydroxyl radicals was assessed by the iron(II)-dependent deoxyribose damage assay (Figure 4). The significant scavenging activity of DG can be explained by the Fenton reaction (Halliwell 1981) that generates hydroxyl radicals. This radical may attack the sugar, which leads to sugar fragmentation. Addition of transition metal ions such as iron at low concentrations to deoxyribose causes degradation of sugar into malondialdehye and other related compounds, which form a chromogen with thiobarbituric acid.

IR injury was associated with increased oxidative stress, as evidenced by an increase in myocardial TBARS and depletion of endogenous myocardial antioxidants such as SOD, catalase, glutathione and GPx in tissue homogenate and mitochondrial fractions of myocardial tissue (Tables 2 and 3). Similar observations using similar models have been reported previously (Maulik 1999). It is interesting to note that different plants and plant extracts can also stimulate the synthesis of cellular antioxidants (Banerjee et al 2001; Gauthaman et al 2001). In our study, oral administration of DG for 30 days prevented the oxidative stress and improved the activity of mitochondrial enzymes (Tables 2–4). Protection against IR-induced oxidative stress in DG-treated rat hearts was evidenced by preservation of endogenous antioxidants and prevention of the increase in TBARS. Recent reports have suggested the involvement of mitochondrial-mediated antioxidant responses in cardioprotection, mainly delayed preconditioning of the heart (Hoshida 2002).

Free-radical scavenging enzymes such as SOD, catalase and GPx are the first line of cellular defence against oxidative injury. Under normal conditions, scavenging mechanisms operate swiftly to remove excess ROS. Superoxide ions are removed by SOD in cytosol (Cu-Zn-SOD) and in mitochondria (Mn-SOD) (Kinnula & Crapo 2004) and the resultant H_2O_2 is removed by catalase, GPx and peroxiredoxin. However, elevated levels of ROS can swamp the protective mechanisms of the cell, and are a significant cause of IR damage, as shown in Tables 2 and 3. The protective effect against ROS mediated by DG in IR rats (Tables 2 and 3) shows a significant therapeutic effect of the methanol root extract.

In general, ROS may arise from the activity of cytoplasmic xanthine oxidase. However, in the heart, mitochondria probably constitute the principal source of ROS, since the respiratory chain deals with most of the electrons potentially capable of reducing oxygen (Rao & Mueller 1983). Mn-SOD and GPx are the two main enzymes that act as primary defence against superoxide ions and H_2O_2 in eukaryotic cell mitochondria (Radi et al 1991).

Mn-SOD dismutes O_2^- at almost diffusion-controlled rates (Fridovlch 1976), while mitochondrial H_2O_2 is eliminated by GPx. In spite of these antioxidant enzymes, significant amounts of H_2O_2 can diffuse from mitochondria. Once in the cytosol, detoxification of H_2O_2 is mostly via cytosolic GPx or by diffusion to peroxisomes, where it is metabolized by catalase (Chance et al 1979). GPx detoxifies most mitochondrial and cytosolic H_2O_2 under normal conditions because it has a low Km value for H_2O_2 and a diffuse intracellular distribution, while peroxisomal catalase becomes a more significant scavenger of H_2O_2 at higher concentrations (Chance 1979). However, the catalase enzyme that occupies 0.025% of heart mitochondrial protein is important for detoxifying mitochondrial-derived H_2O_2 because it can 'site specifically' eliminate mitochondrial-derived H_2O_2 (Radi et al 1991).

Pretreatment of rats with DG significantly improved the activities of catalase and GPx in heart mitochondria and tissue homogenate. Cardiac ischaemia results in increased generation of ROS, and subsequent reperfusion can result in toxic overproduction of ROS, which may contribute to irreversible damage to mitochondrial function, consequently impairing recovery of physiological function.

Activities of the mitochondrial enzymes (ICDH, MDH, SDH, NADH dH and cytochrome c oxidase) in rat heart during IR were significantly decreased in our study (Table 4) ($P < 0.05$). Experimental evidence suggests that when the myocardium becomes ischaemic, the resulting hypoxia prevents oxidative phosphorylation, resulting in decreased tissue concentrations of ATP and increased concentrations of AMP and phosphate. Further degradation of AMP to adenosine, inosine and xanthine leads to depletion of tissue adenine nucleotides (Halestrap 1994). The much decreased tissue ATP concentration also inhibits Na^+/K^+ ATPase and leads to a progressive increase in the intracellular sodium ion concentration, which in turn causes a rise in intracellular calcium (Reimer & Jennings 1992) and ultimately leads to alterations in myocardial physiological activities and even cell death. Pretreatment of ischaemic myocardium with DG brought the activities of the mitochondrial respiratory enzymes to near-normal levels. The improvement of 5′-nucleotidase activity in the DG-pretreated group suggests an early release of adenosine, which is considered to be cardioprotective (Headrick et al 2003). Moreover, a significant recovery of α-KGDH activity (which is more susceptible to free-radical-mediated inactivation) (Humphries Szweda 1998) in DG-treated rats during IR emphasizes the cardioprotective nature of the extract.

Conclusions

In this study, we provide evidence that cardiac mitochondria exhibit an increased susceptibility to reperfusion-induced dysfunction due to oxidative damage. Our results have shown that DG pretreatment significantly improved the antioxidant activities of both myocardium and cardiac mitochondria against oxidative stress mediated by IR. Furthermore, improved activities of mitochondrial enzymes suggests that

DG has myocardial salvaging effects, which might contribute to the observed preservation in cardiac function and cardioprotective effects.

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