

Cardiotonic and Anti Ischemic Reperfusion Injury Effect of *Desmodium Gangeticum* Root Methanol Extract

[*Desmodium gangeticum* kök metanol ekstresinin kardiyotonik ve anti-iskemik reperfüzyon hasarına etkisi]

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ABSTRACT

Objectives: Anti ischemic reperfusion effect of crude methanol extract of *Desmodium gangeticum* root had been reported earlier but how is modulating its cardiotonic effect to render cardio-protection is largely unknown.

Methods: The inotropic effect of methanol extract of *Desmodium gangeticum* root was investigated on isolated frog heart and further analyzed the physiological and biochemical effects in isolated rat hearts. Global ischemia (30min) and reperfusion (45min) were modeled in-vitro condition by using Langendorff perfusion apparatus.

Results: Our results indicate that methanol extract of *Desmodium gangeticum* root showed a dose dependent negative inotropic and chronotropic effect on isolated frog heart and mimetic acetylcholine like action. In the isolated rat heart model for ischemia reperfusion injury, the results from different biochemical and physiological effect of *Desmodium gangeticum* root suggested the cardio-protective nature of the herbal extract that acts similar to acetylcholine (G protein agonist). Moreover, GS/MS result of *Desmodium gangeticum* root shows the presence of 4-[2-(dimethylamino) ethyl] phenol, a G protein agonist and α -asarone, a potent antioxidant.

Conclusions: The above results suggest that *Desmodium gangeticum* root extract mediates the cardio protection in ischemic reperfusion injury model in rat heart through negative inotropic and chronotropic effect by stimulating the G coupled receptors similar to the action of acetylcholine.

Keywords: *Desmodium gangeticum*, myocardial ischemia reperfusion, inotropes, GS/MS, isolated rat heart

ÖZET

Amaç: *Desmodium gangeticum* kök ham metanol ekstresinin anti iskemik reperfüzyon etkisi daha önce bildirilmiş olmasına rağmen kalbi korumak için kardiyotonik etkiyi nasıl düzenlediği büyük ölçüde bilinmemektedir.

Yöntemler: İzole edilmiş kurbağa kalbinde *Desmodium gangeticum* kök ham metanol ekstresinin inotropik etkisi incelendi ve ileri fizyolojik ve biyokimyasal çalışmalar ise izole edilmiş rat kalbinde analiz edildi. Langendorff perfüzyon cihazı kullanılarak genel iskemi (30 dk) ve reperfüzyon (45 dk) *in vitro* koşullarda oluşturuldu.

Bulgular: Sonuçlarımız izole kurbağa kalbinde *Desmodium gangeticum* kök ham metanol ekstresinin doz bağımlı negatif inotropik, kronotropik ve asetilkolin benzeri mimetik etki gösterdiğini işaret etmektedir. *Desmodium gangeticum* köklerinin, iskemi reperfüzyon hasar modelinden izole edilen rat kalbinde farklı biyokimyasal ve fizyolojik etkiler oluşturması sonucunda, bu bitkisel ekstrenin asetilkoline (G protein agonisti) benzer şekilde kalp koruyucu özelliği gözlemlendi. Ayrıca *Desmodium gangeticum* köklerinin GS/MS sonuçları, bu köklerde G protein agonisti olan 4-[2-(dimetilamino)etil] fenol ve etkili antioksidan olan α -asarone varlığını göstermektedir.

Sonuç: Yukarıda bahsedilen sonuçların da gösterdiği gibi *Desmodium gangeticum* kök ekstresi iskemi reperfüzyon hasarı modelinde rat kalp dokusundaki koruyucu etkisine, negatif inotrop ve kronotropik etkileri ile asetilkolinin etkilerine benzer şekilde G bağımlı reseptörleri aktive ederek aracılık etmektedir.

Anahtar Kelimeler: *Desmodium gangeticum*, miyokardial iskemi reperfüzyon, inotropolar, GS/MS, izole rat kalbi

Introduction

Several endogenous mediators are released during ischemic reperfusion that could potentially modulate intracellular signaling by acting on surface membrane receptors or on intracellular components of signaling pathways. The endogenous protective mechanism against ischemic reperfusion mediated by ischemic precondition is receptor mediated and initiated by stimulation of several Gi protein-coupled receptors including adenosine A₁/A₃, bradykinin B₂, and opioid δ receptors. [1] On the other hand there are some Gi-coupled receptors on myocardial cells that are capable of triggering a preconditioned state, but agonists for them are either simply not released or are released in insufficient quantity to impart protection during preconditioning ischemia. One such receptor is the muscarinic M₂ receptor. Previous report on muscarinic receptor stimulation by acetylcholine predict the reduction of myocardial infarct size in dog and rabbit models of ischemic reperfusion. [2]

The root of *Desmodium gangeticum* (DG), has been reported in the ancient Indian medicinal literature with beneficial effects in heart diseases. [3] In recent times, experimental studies have shown that the root of DG has significant protective effects in ischemic heart disease. [4] Moreover, few recent studies from our laboratories suggest the protective nature of DG against ischemic reperfusion injury (IRI). [5] However, the exact mechanism of its cardio-protective effects, in respect to the present knowledge of the patho-physiology of IRI, is not well investigated. The present study was designed to investigate the cardio tonic effect of methanol extract of DG root in isolated frog heart and to evaluate how inotropic changes can modulates the mitochondrial and sarcoplasmic ATPase function in an isolated rat heart. An understanding of the mechanisms involved in these responses not only informs us of how the heart reacts to injurious stimuli, but may provide avenues for developing novel protective strategies applicable in the setting of ischemia-reperfusion.

Methods and materials

Collection of Plant

The plant, after collection from the herbal garden was washed, cleaned and maintained in the department. *Desmodium gangeticum* (DC) (Leguminosae) was taxonomically identified by Prof. James Joseph, Head of the Department, Department of Botany, Saint Berchman's College, Mahatma Gandhi University, Kerala. The voucher specimen A/C no. 3908 was retained in our laboratory for future reference.

Preparation of methanol extract of roots of *Desmodium gangeticum*

One kilogram (1 kg) of fresh secondary roots of DG were sliced and air-dried at room temperature. The sliced, air-dried roots of the plant were milled into fine powder in a

warring commercial blender. The powdered plant material was soaked in 2L methanol for 72 hrs and the extract was filtered and distilled on 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 of powdered methanol extract.

Drug preparations

i) Methanol extract

1 g portions of the lyophilized extract was treated with 0.2 ml of dimethyl sulphoxide (DMSO) and then the mixture was topped up with 0.8ml of Frog Ringer's solution to give a stock solution of 1g/ ml, from which the following serial dilutions were made: 100mg/ml, 20mg/ml, 2mg/ml, 1mg/ml, 0.2mg/ml, and 0.02mg/ml.

ii) Acetylcholine

A stock solution of 10mg/ml was made with Frog Ringer's solution. The following working concentrations were prepared from the stock: 1mg/ml and 0.1mg/ml.

iii) Atropine

A stock solution of 10mg/ml was made with Frog Ringer's solution. The following working concentrations were prepared from the stock: 1mg/ml and 0.1mg/ml. 0.5 ml of this concentration was added to the bath and used as an antagonism of muscarinic receptor.

Chemicals

DL isocitrate and N-Phenyl-P-Phenylenediamine were purchased from Acros organics, New Jersey USA. Acetylcholine, Atropine, Cytochrome C & ATP were purchased from sigma chemical Co., St. Louis, MO USA. All other chemicals used were of analytical grade.

Animals

Frogs of *Rana hexadactyla* species maintained in the animal house and male Wistar albino rats (150 to 200 g) housed in cages and were maintained in controlled temperature at 23±2°C with 12hr. light / dark cycle were used for the studies. The animals were fed with food and water *ad libitum*. The animals were maintained as per the norms of CPCSEA and the experiments were cleared by CPCSEA and the local ethics committee (Reg No. and date 48/SASTRA/IAEC/RDP, dated 16/6/2009).

Experimental Protocol I

Frog heart in situ preparation

Frog hearts were isolated from specimens of *Rana hexadactyla* [weighing 22.015± 1.2 g (mean ± SE)] and connected to a perfusion apparatus as previously described [6]. Experiments were done at room temperature (18–21°C). The hearts were perfused with frog-Ringer solution containing NaCl 6.5g, KCl 0.14g, CaCl₂ 0.12g, and NaHCO₂ 0.2g, NaH₂PO₄ 0.01g, Glucose 2.0g in g. per liter. The force of contraction was recorded and the rate of contraction was counted and tabulated.

Experimental protocol II

The rats were divided into three groups, as follows: Group 1, the control group; group 2, reperfusion; and group 3, drug.

Group 1: Normal control

In normal control group, hearts (n=6) were perfused for 90 minutes with KH buffer and used for the biochemical analysis.

Group 2: Reperfusion

In reperfusion group, after the proper stabilization period, the 30 minutes ischemic hearts (n=6 in each sub groups) were subjected to 30 minutes reperfusion (2.1) and 45 minutes reperfusion (2.2) respectively.

Group 3: Drug

The animals in the drug group were subdivided into 5 groups.

Group 3.1: Rats (n=6) in this groups were pretreated orally (through ball tipped classic steel 15-16 gauge hypodermic needle) with DG at a dose of 100mg /kg b. wt. for thirty days. Hearts were perfused for 90 minutes with KH buffer and used for the biochemical analysis.

In group 3.2 and 3.3, rats (n=6) were pretreated orally (through ball tipped classic steel 15-16 gauge hypodermic needle) with DG at a dose of 100mg /kg b. wt. for thirty days. After equilibration and proper stabilization period, hearts were subjected to 30 minutes of global ischemia followed by 30 minutes of reperfusion and 30 minutes of global ischemia followed by 45 minutes of reperfusion respectively.

In group 3.4 and 3.5, a similar procedure was followed as above except, instead of DG a standard drug verapamil (0.2 mg/kg b.wt.) was used.

Heart Preparation

Wistar male rats weighing 250-280 g were anesthetized with 40 mg/kg sodium thiopentone. After an intravenous injection of 300U heparin, the heart was rapidly excised via a mid-sternal thoracotomy and arrested in the ice cold Krebs-Henseleit buffer (KH) containing (mM/L) NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.8, NaHCO₃ 25 and C₆H₁₂O₆ 11. The heart was attached to a Langendorff apparatus via an aorta for retrograde perfusion with KH buffer maintained at 37°C and pH = 7.4 and saturated with a gas mixture of 95% O₂ 5% CO₂. The coronary perfusion pressure was maintained at 80 mm Hg. the left ventricular pressure developed with the ventricle filled with Krebs solution. The left ventricular pressure developed with ventricle filled with Krebs solution was recorded with a pressure transducer, which in turn was connected to a device amplifier and chart recorder. This left ventricular pressure gave an indication of the mechanical performance of the heart. Coronary flow was measured simply by collecting the perfusate draining from the heart in a graduated cylinder for a defined time. The heart rate was measured by coun-

ting the number of contractions (obtained from the left ventricular pressure record) per minute.

Tissue Preparation

The heart was excised, rinsed in ice cold isotonic saline, blotted with filter paper, weighed, homogenized in 0.1M Tris – HCl (pH 7.4) buffer solution. The homogenate was centrifuged at 300*g for 5 minutes. The supernatant was used for the estimation of various biochemical parameters.

Biochemical assays

Mitochondria and microsomal fractions from the myocardium was isolated by the method of Johnson and Lardy [7], and Schenkman and Ciniti [8], respectively. Assay of isocitrate dehydrogenase (ICDH) [9], malate dehydrogenase (MDH) [10], succinate dehydrogenase (SDH) [11], α -ketoglutarate dehydrogenase (α -KGDH) [12], NADH dehydrogenase (NADH dH) [13] and cytochrome c oxides [14] were carried out in a UV-1601 Shimadzu spectrophotometer. Protein concentration was measured with Folin phenol reagent, following the procedure described by Lowry.[15] Assay of creatine kinase,[16] lactate dehydrogenase [17] and aspartate transaminase [18] were also estimated.

GS/MS Analysis

All analysis was conducted with a Perkin Elmer Clarus 500 GC equipped with mass spectrometry. The chromatographic conditions were as follows: Column: Elite -1 (100% dimethyl polysiloxane). Helium was used as the carrier gas with a flow rate of 1ml/minute. The 1 μ L methanol root extract of DG was injected into the GS–MS in split less mode at 250°C. The column oven temperature was held at 110°C for 2 minutes, then programmed at 75°C/min to 200°C for 1 min, 5°C / min to 280°C and held for 9 minutes.

Statistical analysis:

All data were reported as mean \pm SD. Results were statistically analyzed by a one-way analysis of variance (ANOVA) by SPSS software 12.00, followed by Duncan's multiple range test (DMRT). P < 0.05 was considered to be significant.

Results

Recording of inotropic and chronotropic effects

The baseline reading of cardiac flow rate, heart rate and force of contraction were found to be changed with extract, acetylcholine and atropine (Table 1). The negative inotropic and chronotropic effect shown by the extract followed a dose dependent change (1mg, 4mg, 8mg) and the maximum response was produced by 8mg of the extract. However, the decreased heart rate and force of contractions were recovered partially when 50micrograms of atropine was administered along with the extract (Fig 1). Similarly when Ach was treated after atropine, the heart rate and force of contraction again declined.

Table 1. Effect of DG on flow rate, heart rate and force of contraction in frog heart

Drug & Extract	Flow Volume (ml/min)	Heart Rate (Beats/min)	Amplitude (mm)
Baseline	9.0	62	18
Extract 1mg	5.0	28	10
Extract 4mg	3.7	16	08
Extract 8mg	3.1	14	05
Atropine 50 μ g + Extract 8mg	4.5	58	14
Atropine 50 μ g + Ach 500ng	4.8	60	17
Ach 500ng	3.0	15	07

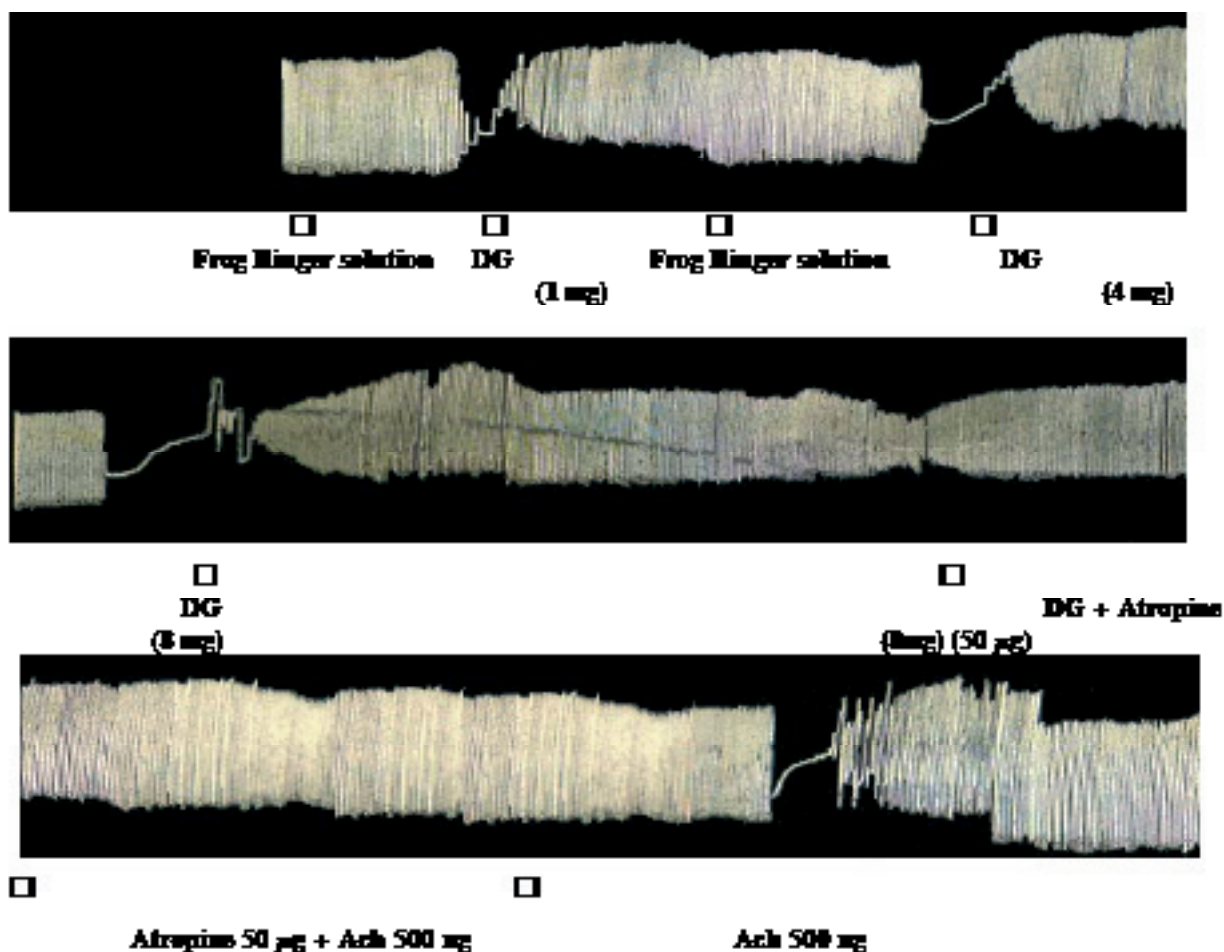


Figure 1. Effect of DG on isolated frog heart

GS/MS analysis

GS/MS analysis resulted in the identification of 64 compounds (figure 2). Major compounds comprises of 4-[2-(dimethylamino)ethyl] phenol -(Cactine) (Retention time (R.T) 15.41), glycerin, sucrose, asarone (R.T 18.66), trans -Z- α - bisabolene epoxide (R.T 20.55), 2,5-bis (1,1-dimethyl ethyl) phenol (R.T 21.89), trans-2-methyl-4-n-pentylthiane S,S-dioxide (R.T 22.86), decahydro-1,1-dimethylnaphthalene (R.T 25.33), 4,5 dihydro-2-(phenyl methyl) 1-H-imidazole (R.T 32.17), (-)-nortrachelogenin (R.T 39.23), 2-methyl-9,10- anthra-

cene dione (R.T 29.10) and Piperine (R.T 43.56). It represents around 33%.

Physiological and biochemical changes in myocardial ischemia reperfusion in isolated rat heart

Table 2 shows the hemodynamic changes in the rat heart subjected to ischemia and reperfusion. A significant fall in the mean arterial pressure, heart rate, coronary flow rate, left ventricular developed pressure and rate pressure products were observed in myocardial ischemia re-

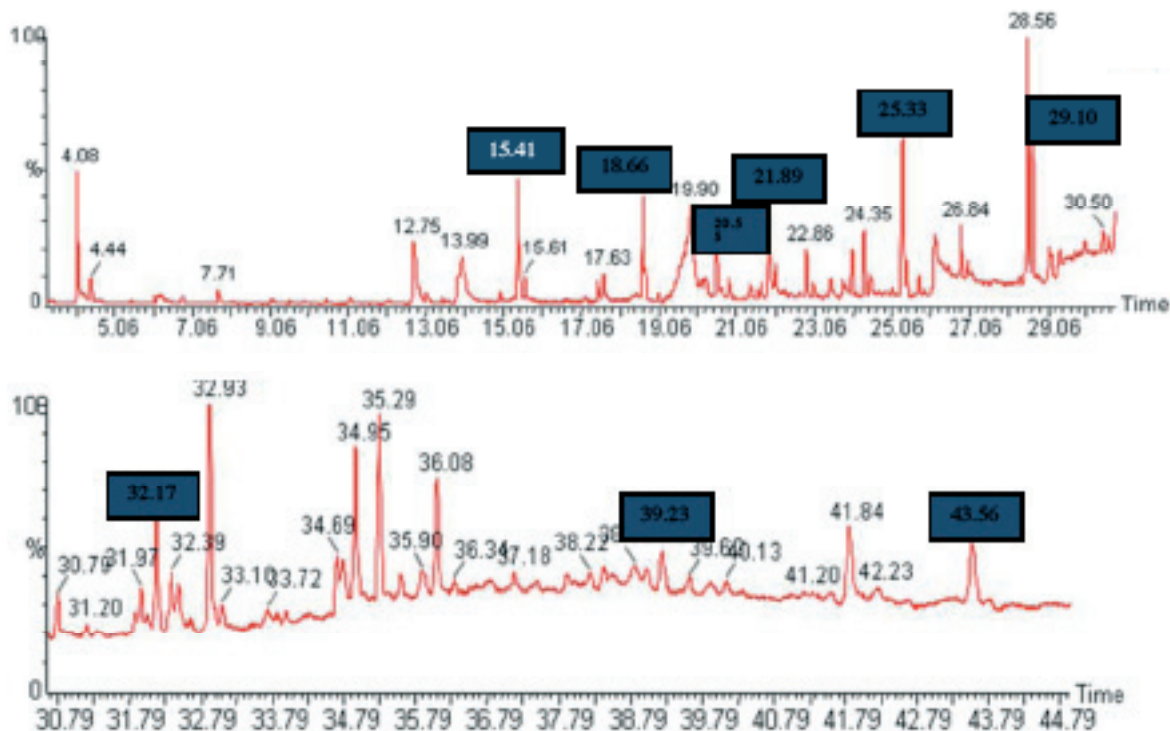


Figure 2. GC Mass spectrum for methanol extract of *Desmodium gangeticum* root

perfused myocardium. Administration of DG and verapamil, calcium channel blocker, had improved the hematological parameters to a near normal value showing cardio protective effect. Similarly mitochondrial enzymes and microsomal enzymes like ICDH, SDH, MDH, α KGDH, NADH dH cytochrome c oxidase, Na^+K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase were observed to be depressed in their activity (Table 3 and 4) during ischemia reperfusion. However, supplementation of Table V shows the level of cardiac marker enzymes in tissue homogenate and perfusate. In ischemia reperfusion control rats, increased level of these enzymes in tissue homogenate and perfusate indicate severity of damage to myocardial membrane. However, administration of DG before reperfusion preserved cardiac marker enzymes in both tissue homogenate and perfusate.

Discussions

Our results from this study indicates that methanol extract of DG stimulate cardiac muscarinic receptors thereby mediates its protection against ischemia reperfusion injury in rat heart. The kymogram results (Table 1, Fig 1) indicate negative inotropic and chronotropic effect of DG extract similar to the response mediated by acetylcholine (ACh) in frog heart. This physiological response was reversed by the addition of acetylcholine receptor /muscarinic receptor antagonist namely atropine, suggesting methanol extract of DG mediate its action through ACh receptor/ muscarinic receptor, a G protein receptor activator. However the concentration required by DG (160 μg) was more as compared to ACh (500ng)

to negate the atropine (50 μg) action. This difference in concentration for DG and ACh may be due to the nature its purity. In fact, reversion of physiological response of atropine by DG extract re-confirms the muscarinic receptor mediated action of the herbal extract. In fact our GS/MS results reveals the presence of 4-[2-(dimethylamino)ethyl] phenol (Fig 2) a known G protein coupled receptor agonist. [19] Acetylcholine has for long been known as a transmitter that has the property of lowering the blood pressure and also bringing about bradycardia through its muscarinic receptor. [20] Importantly, suppressed parasympathetic nervous system (PSNS) function has been found in a variety of cardiovascular diseases, such as hypertension, heart failure, and diabetes. Cardiac regulation by the PSNS is primarily mediated by the M_2 muscarinic acetylcholine receptor. [21] Pretreatment of the animal with atropine significantly reduced hypotensive and negative chronotropic activities of the extract. On the contrary, some other studies on perfused heart have reported coronary vasoconstriction [22] and increase myocardial contractility by acetylcholine. The positive inotropic and coronary vaso-constriction effect of ACh on isolated rat heart are modulated by the release of prostaglandins and NO, and both actions are muscarinic receptor mediated. [23]

Isolated rat heart model was used to evaluate the cardio protection stimulated by cardiotoxic effect of the extract. Hemodynamic variables like left ventricular developed pressure, mean arterial pressure and rate pressure product were depressed in ischemia reperfusion control. But administration of DG remarkable recovery of

hemodynamic variables (Table 2). This result indicate a possible similarity in their mode of action towards the physiological response on heart as observed in frog heart.

Table 3 shows the effect of DG root methanol extract on mitochondrial enzymes. Mitochondrial enzyme (ICDH, MDH, SDH, NADH dH, cytochrome c oxidase) showed a significant decrease ($P < 0.05$) in their activity. Experimental evidences suggested that when the myocardium becomes ischemic, the resulting hypoxia prevents oxidative phosphorylation, and consequently resulted in decreased tissue ATP concentrations and increased AMP and phosphate concentrations. However these enzymes recovered their activities by the administration of DG. Previous report suggested acetylcholine (muscarinic agonist) mediated protective action against infarction [24] and is dependent on mitochondrial $K_{(ATP)}$ channel opening. Significantly improved activity of mitochondrial enzymes in DG pretreated rat heart indicates the possibility of mitochondria mediated action by DG root extract. In addition, α -asarone present in the extract (Fig 2) exhibits characteristic antioxidant properties, [25] as well as acetylcholinesterase inhibitory activity.

[26] Even though administration of the standard drug, verapamil improves mitochondrial enzyme function; its effect was not as significant as DG extract. This may be due to the presence of free radical scavenging molecules along with cardio-stimulant biomolecules in DG extract (Fig 2).

Physiological dysfunction of rat heart during ischemic reperfusion is predominantly due to the disturbances in ionic balance. Microsomal ATPase analyzed in the present study (Table 4) showed an improved activities in DG extract treated rat heart as compared to ischemia reperfusion control. Trautwein and Dudel, [27] were among the first to suggested that the action of acetylcholine on cardiac muscle involves a specific increase of membrane permeability of K^+ . Further, atropine antagonism of Ach produced arrest observed in kymogram result may be linked to the inhibition of both Ach induced sodium influx and K^+ out flux from atrial tissue. [28] Hence the improved Na^+K^+ ATPase (Table 4) by the DG extract in the present study reemphasizes the acetylcholine like action by DG extract. Similarly, the improved Ca^{2+} ATPase (Table 4) activity and reduced cardiac marker enzymes in myocardial perfusate (Table 5) indicates an improved

Table 2. Hemodynamic characteristic of isolated rat heart

Group	n	LVDP(mmHg)	CF(ml/min)	HR(b.p/min.)	RPP 10^3 (mmHg.bt min ⁻¹)	MAP (mmHg)
1	6	105.22±4.3	9.2±1.00	342 ± 20.1	35.98 ± 7.1	122± 7
2.1	6	90.41±4.1	9.1±0.98	262 ± 18.2 ^c	23.68 ± 6.6 ^c	99± 6 ^c
2.2	6	94.2±4.4	9.1±1.02	240 ± 19.3 ^c	22.60 ± 6.3 ^c	98 ± 7 ^c
3.1	6	107.2±4.2	9.2±1.08	339 ± 34.1	36.34 ± 8.2	115± 8
3.2	6	107.3±4.5	9.3±1.10	338 ± 31.3	36.26 ± 5.1	114± 7
3.3	6	106.4±4.6	9.2±0.94	323 ± 33.2	32.94 ± 6.8	104± 5
3.4	6	106.2±4.0	9.4±1.05	329 ± 33.5	34.94 ± 7.4	103± 6
3.5	6	106.1±4.1	9.3±1.00	319 ± 32.8	33.84 ± 5.9	107± 7

Values are mean ± SD for 6 rats in each group. n, number of hearts in each group; LVDP, left ventricular developed pressure; CF, coronary flow; HR, heart rate; RPP, rate pressure product; MAP, mean arterial pressure. * $P < 0.05$, compared with control.

Table 3. Effect of methanol extract of DG root on mitochondrial enzymes in isolated rat heart

Group	ICDH	SDH	MDH	α KGDH	NADH dH	Cyt.c. Oxidase
1	^a 746.3 ± 24.3	^a 249.1± 8.7	^a 354.4± 12.1	^a 74.3 ± 3.3	^a 143.2 ± 3.5	^a 31.1 ± 0.8
2.1	^c 583.2 ± 19.3	^c 115.4 ± 4.2	^c 219.9 ± 12.5	^c 29.0 ± 0.8	^c 89.0 ± 2.4	^c 15.0 ± 0.3
2.2	^c 599.5 ± 18.6	^c 118.5 ± 4.3	^c 228.5 ± 10.3	^c 31.0 ± 0.9	^c 92.5 ± 2.5	^c 15.6 ± 0.3
3.1	^a 740.3± 23.2	^a 241.6± 9.3	^a 342.6± 14.2	^a 70.1± 4.1	^a 140.3± 2.3	^b 25.3± 0.6
3.2	^a 731.0± 11.6	^b 216.0± 6.3	^b 326.0± 15.7	^b 59.6± 1.8	^b 125.0± 3.3	^b 23.3± 0.6
3.3	^a 741.2± 12.4	^b 222.2± 8.2	^b 328.4± 23.4	^a 65.7 ± 1.9	^a 140.1± 3.7	^a 29.2± 0.7
3.4	^b 728.3± 20.1	^b 210.7± 10.2	^b 308.6± 18.2	^b 48.7± 2.2	^b 123.7± 8.2	^b 22.8± 0.7
3.5	^a 737.4 ± 19.3	^b 218.9 ± 11.3	^b 320.3± 16.5	^b 57.8± 3.4	^a 139.9 ± 6.2	^a 28.8± 0.9

Results are mean ± sd (n=6). Activity is expressed as nmol of NADP reduced per min per mg protein for ICDH, nmol of succinate oxidized per min per mg protein for SDH; nmol of NADH oxidized per min per mg proteins for MDH; nmol of α -keto glutarate formed per hour per mg proteins for α KGDH and nmol of NADH oxidized per min per mg protein for NADH dH; change in optical density per minutes per mg protein for cytochrome c oxidase.

Values not sharing a common superscript (a,b,c,d,e,f) differ significantly at $P < 0.05$ when compared between the groups

Table 4. Effect of methanol extract DG root on microsomal ATPase in isolated rat heart

Group	Na ⁺ K ⁺ ATPase	Ca ²⁺ ATPase	Mg ²⁺ ATPase	5' nucleotidase
1	^a 0.565± 0.01	^a 0.269 ± 0.01	^a 0.474± 0.01	^a 4.24± 0.12
2.1	^c 0.509± 0.01	^b 0.229± 0.03	^c 0.369± 0.02	^b 3.99± 0.11
2.2	^b 0.533 ± 0.01	^b 0.241± 0.02	^c 0.384 ± 0.01	^b 4.04 ± 0.12
3.1	^a 0.563± 0.01	^a 0.264± 0.01	^a 0.470± 0.01	^a 4.22± 0.01
3.2	^a 0.549± 0.01	^a 0.271 ± 0.01	^b 0.452± 0.01	^a 4.21± 0.10
3.3	^a 0.559 ± 0.01	^a 0.273 ± 0.01	^b 0.457± 0.02	^a 4.23± 0.10
3.4	^b 0.547± 0.02	^a 0.270± 0.01	^a 0.448± 0.01	^a 4.19± 0.01
3.5	^a 0.555± 0.01	^a 0.270± 0.02	^b 0.454± 0.02	^a 4.21± 0.02

Results are mean ± s.d (n=6). Activity is expressed as μmoles of phosphorus liberated per sec per gram protein for Na⁺ K⁺ ATPase, Ca²⁺ ATPase and Mg²⁺ ATPase; mmoles of phosphorus released per mg protein per hour for 5'-nucleotidase

Values not sharing a common superscript (a,b,c,d,e,f) differ significantly at P<0.05) when compared between the groups

Table 5. Level of creatine kinase and lactate dehydrogenase (LDH) in the myocardium of isolated rat heart and the activity of LDH in the myocardial perfusate.

Group	CK (μmol phosphorous liberated/min/mg protein)	LDH (nmol pyruvate liberated/min/mg protein)	LDH (units/ml/perfusate)	
			5minutes	10minutes
1	^a 16.5 ± 0.88	^a 110.60 ± 5.11	^a 1.5± 0.65	^a 1.2± 0.34
2.1	^c 110.4± 0.83	^d 280.35± 7.13	^b 65.5± 2.11	^b 58.2± 1.54
2.2	^c 112.9± 0.98	^d 285.61± 6.32	^b 58.8± 1.87	^b 57.6± 1.05
3.1	^a 15.2± 0.89	^a 99.76± 5,21	^c 9.2± 0.62	^a 7.4± 0.45
3.2	^a 24.7± 0.77	^b 167.46± 5.67	^d 22.1± 1.11	^c 24.4± 1.65
3.3	^b 29.1± 0.98	^b 165.93± 4.38	^d 24.6± 1.08	^c 26.8± 1.23
3.4	^b 33.2± 0.95	^b 181.12± 7.67	^e 33.6± 1.03	^c 27.4± 1.22
3.5	^b 33.6± 0.87	^c 192.65± 5.05	^e 29.4± 1.43	^c 26.9± 0.65

Results are mean ± s.d (n=6). Activity is expressed as μmoles of phosphorus liberated per sec per gram protein for Na⁺ K⁺ ATPase, Ca²⁺ ATPase and Mg²⁺ ATPase; mmoles of phosphorus released per mg protein per hour for 5'-nucleotidase.

Values not sharing a common superscript (a,b,c,d,e,f) differ significantly at P<0.05) when compared between the groups

Ca²⁺ sensitivity of contractile protein that is essential to cardiac contractility. Myocardial injury was assessed by estimating the activity of cardiac markers in both heart tissue and perfusate. Higher cardiac marker enzyme activity in the tissue perfusate of ischemia reperfused rat heart indicates the myocardial injury. On the other hand low activity of the above enzyme in perfusate as well as tissue homogenate of rat heart pretreated with drug predicts the cardio-protective nature of drug. The decreased level of above enzymes in the perfusate and tissue (Table 5) substantiate the cardio-protection of the extract.

We conclude from the above observation that DG methanol extract mediates its cardio protection in isolated rat heart during ischemia reperfusion by eliciting the action of muscarinic receptor.

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