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Methanol extract of *Desmodium gangeticum* DC root mimetic post-conditioning effect in isolated perfused rat heart by stimulating muscarinic receptors

Gino A Kurian[1*], Jose Paddikkala[2]

[1]*Medical Biochemistry, School of Chemical and Biotechnology, SASTRA University, Thirumalaisamudram,Thanjavur,Tamil Nadu, India, Pin: 613402*
[2]*Department of Plant Biotechnology, Amala Cancer Research Center, Amalanagar, Trichur, Kerala, India*

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ABSTRACT

Objective: To evaluate pharmacological mimetic action of herbal extract *Desmodium gangeticum* (DG) roots on ischemia reperfusion injury. **Methods:** With the help of Langendroff perfusion technique, ischemic post condition (POC) mimetic action of DG methanol root extract was evaluated and compared by using standard drugs that acts as muscarinic receptor agonist and antagonist, namely acetylcholine (Ach) and atropine (Atr) respectively in an isolated rat heart. **Results:** The physiological parameters like left ventricular developed pressure, end diastolic pressure and working index of isolated rat heart showed significant recovery in DG root extract administrated rat heart, similar to the recovery by POC. Kymogram results showed muscarinic receptor agonist like action for DG methanol root extract, confirmed in rat heart by muscarnic receptor agonist (acetylcholine) and anatoginst (atropine). Administration of DG root extract prior to reperfusion showed better antioxidant status in myocardial tissue homogenate and mitochondrial, complemented by the levels of cardiac specific marker proteins in myocardial tissue and perfusate. Even though DG methanol root extract mimics its action similar to that of Ach, the myocardial protection mediated by the extract was superior to Ach, due to the presence of antioxidants in the crude extract. **Conclusions:** DG methanol root extract provides myocardial protection towards IRI by stimulating muscarinic receptors.

1. Introduction

Myocardial ischemia reperfusion injury is a complex process involving several cell types (endothelial cells, neutrophils, and cardiomyocytes), soluble proinflammatory mediators, oxidants, ionic and metabolic dyshomeostasis, and cellular and molecular signals[1] are not mutually exclusive in the pathobiology of reperfusion injury. Some of these events take place during the very early moments of reperfusion, while others, seemingly triggered in part by the early events, are activated within a later timeframe.

The cardio-protection was associated with a reduction in: endothelial cell activation and dysfunction, tissue superoxide anion generation, neutrophil activation and accumulation in reperfused myocardium, microvascular injury, tissue edema, intracellular and mitochondrial calcium accumulation[2]. It has been shown that repeated brief coronary occlusions increase myocardial resistance towards prolonged episodes of ischemia, renders the heart more tolerant to ischemia with subsequent limitation of infarct size, has been termed ischemic preconditioning (IP). The ability of IP to protect the myocardium against prolonged ischemia may derive from improved energy balance[3]. Unfortunately, ischemic preconditioning is not feasible in the clinical practice because the coronary artery is already occluded at the time of hospital admission of the AMI patient.

Recently, in the dog model, a phenomenon called "ischemic post-conditioning (POC)", a brief episode of ischemia-reperfusion performed after prolonged ischemia [4], can reduce ischemic myocardial injury. POC can terminate

*Corresponding author: Gino A Kurian, Assistant Professor Medical Biochemistry, School of Chemical and Biotechnology, SASTRA University, Thirumalaisamudram,Thanjavur,Tamil Nadu, India, Pin: 613402.
E-mail: ginokurian@hotmail.com

reperfusion arrhythmias with no reduction of cardiac function, and may be useful for correcting stunned myocardium. Moreover, it may protect myocardium against ischemia/reperfusion injury via activating kappa-opioid receptors, muscarinic receptors, adenosine receptors and mitochondrial K_{ATP}[5]. However, practical clinical difficulty in the above procedure compels to search new therapeutic agents that can mimetic the mediators of post condition mechanism.

Our lab had already reported an anti ischemic reperfusion herbal agent *Desmodium gangeticum* (*D. gangeticum*, DG) roots methanol extract[6]. The herb plays an important role in traditional Indian and Chinese medication[7]. DG is common on the lower hills and plains throughout India; on the Himalayas it ascends to 5 000 feet. It is spread east to Pegu and Ceylon, the Malay Peninsula and Archipelago, and is distributed to China, the Philippine Islands and tropical Africa. The phytochemical screening of DG indicates the presence of cardio-stimulatory molecules in the extract. But its competency to act as pharmacological post conditioning agent was not being studied. Thus the present study aimed to find the possible mode of action of DG as post conditioning agent against ischemic reperfusion injury.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals

Adult male albino rats of the Wistar strain, weighing approximately 250-280 g were obtained from King Institute of Preventive Medicine, Chennai, India. They were acclimatized to animal - house conditions and were fed with commercial rat pellet (Hindustan Lever Ltd., Bangalore, India) and had free access to water (ethically approved by Ministry of Social Justices and Empowerment Government of India). The experimental protocol was approved by the institutional animal ethical committee (817/ac/CPCSEA, dated 6/8/04).

2.3. Preparation of methanol extract of roots of DG

After collection from the herbal garden, the plant maintained in the department was washed and cleaned. The plant material was taxonomically identified at 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.

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 2 L methanol for 72 h. 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 ℃ until further use (yield of the extract was 6.1% w/w).

The phytochemical screening of DG methanol extract revealed the presence of alkaloids, flavanoids, carbohydrates, saponins, phytosterols, phenols, terpenoids, tannins and phlobatannins. Anti ischemia reperfusion action of volatile compounds are reported earlier and no such study was conducted in DG. GS/MS analysis of DG methanol root extract resulted in the identification of 64 compounds (ure 1). Major compounds comprises of 4-[2-(dimethylamino)ethyl] phenol -(Cactine) (R.T 15.41), glycerine, 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- anthracene dione (R.T 29.10) and Piperine (R.T 43.56). It represents around 33%. Minor compounds such as conhydrin, oxirane, 2,5-dihydro-1-H-pyrrole, thymol, Eugenol, apiol, eicosane, 3- methyl-2-(2-oxo propyl) furan and1-methoxy-10-H-phenothiazine were identified.

2.4. Perfusion protocols

Hearts were removed and mounted on the Langendorff apparatus as previously described[8].

2.5. Experimental groups

Rats were randomly divided into 4 main groups; control, reperfusion (I/R), ischemic post conditioning (POC) and pharmacological post conditioning groups. Isolated hearts from control group was subjected to continuous perfusion of Krebs Henseleit (KH) buffer for 90 minutes. In the other groups, isolated rat hearts were stabilized for 25 minutes to establish its baseline parameters as a reference to the effects caused by subsequent manipulations. In I/R group, 30 minutes global ischemia was induced followed by 45 minutes reperfusion. In Ischemic post conditioning group, isolated rat hearts was subjected to 30 minutes global ischemia followed by five cycles of 2 minutes ischemia and 2 minutes reperfusion, then subjected to 45 minutes reperfusion. Pharmacological post conditioning group was subdivided into five subgroups namely, post treated acetylcholine (Ach), post treated atropine (Atr), post treated DG, post treated actylcholine + atropine, post treated DG + atropine. In post treated acetyl

choline group, after stabilization and 30 minutes global ischemia, isolated rat hearts were administered with acetylcholine (80 μg/mL) for 15minutes, followed by 45 minutes reperfusion. In post treated atropine and DG groups, same procedure mentioned for post treated acetylcholine was followed except the doses as 1 μg/mL and 1 mg/mL respectively. In post treated acetylcholine + atropine group, after 20 minutes of equilibration and 30 minutes global ischemia, acetylcholine (80 μg/mL) and atropine (1 μg/mL) were administered for 15 minutes at a flow rate of 7-8 mL per minutes, followed by 45 minutes of reperfusion. Similarly in post treated DG +atropine group, DG (1 mg/mL) and atropine (1 μg/mL) were administered for 15 minutes after 30 minutes of global ischemia, followed by 45 minutes of reperfusion. The hemodynamic parameters were monitored throughout the entire duration of each experiment. The biochemical parameters were measured in heart tissue samples taken at pre-determined time points along the protocol. At these time points, the heart was quickly frozen in liquid nitrogen and kept at - 80 ℃ until analyzed.

The following hemodynamic parameters were evaluated: left ventricle peak systolic pressure (PSP), end diastolic pressure (EDP), developed pressure (DP = PSP- E DP), heart rate (HR), work index (WI = DP × HR), + $(dp/dt)_{max}$ (denoted + dp/dt) and - $(dp/dt)_{max}$ (denoted - dp/dt).

2.6. 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 3 000 rpm for 5 minutes. The supernatant was used for the estimation of various biochemical parameters.

2.7. Biochemical assays

Mitochondria[9] fractions from the myocardium were isolated. The cardiac marker enzymes like creatine kinase[10], lactate dehydrogenase[11] and aspartate transaminase[12] were estimated in the cardiac tissue. The LDH release in the perfusate was also analyzed.

Thiobarbituric acid reactive substances (TBARS)[13] was measured as a marker of lipid per-oxidation and endogenous antioxidants, e.g., superoxide dismutase (SOD): Cu, Zn, SOD and Mn SOD[14], catalase[15], and glutathione peroxidase (GPx)[15] were carried out in a UV-1601 Shimadzu spectrophotometer. Protein concentration was measured with Folin phenol reagent, following the procedure described by Lowry[16]. Assay of isocitrate dehydrogenase (ICDH)[17], malate dehydrogenase (MDH)[18], succinate dehydrogenase (SDH)[17], -ketoglutarate dehydrogenase (-KGDH)[17], NADH dehydrogenase (NADH dH)[19] and cytochrome c oxides [20] were also estimated.

2.8. Frog heart in situ preparation

Frog hearts were isolated from specimens of *Rana hexadactyla* [weighing (22.015± 1.200)g, (mean ± SE)] and connected to a perfusion apparatus. Experiments were done at room temperature (18-21℃). The heart was perfused with frog-Ringer solution containing NaCl 6.5 g, KCl 0.14 g, CaCl 0.12 g, and $NaHCO_2$ 0.2 g, NaH_2PO_4 0.01 g, Glucose 2.0g in g. per liter. The force of contraction was recorded and the rate of contraction was counted and tabulated. Frog hearts were treated with different doses of methanol extract of DG and were compared with acetyl choline effect. Atropine, antagonist of acetyl choline was used to confirm the effect of acetyl choline and DG root methanol extracts.

2.9. Statistical analysis

The comparison between values of the same group, at various time points along the experiment and differences in variables between groups for a specific time point were analyzed using one-way ANOVA.

3. Results

The hemodynamic parameters were recorded during the experiment. Cardiac functional damage caused by IR injury is shown in table 1. Following reperfusion, end diastolic pressure was increased, systolic pressure decreased and thus developed pressure (systolic minus diastolic) was depressed. POC improved the developed pressure and there by increased the working index. Generally, rat hearts undergone pharmacological preconditioning showed an improved working index (Table 1). In addition post treatment with DG extract showed similar working index as that of Ach post treated rat hearts.

Table 1

Hemodynamic parameters during the experiment (n=6).

Groups	EDP	LVDP	WI
Normal control	3.3±1.9	96.8±3.9	93.5±1.9
IRC	45.5±7.2*	42.7±4.2*	33.1±2.2*
IPC	19.4±3.1*	93.2±3.6	83.2±2.1*
Post(Ach)	17.2±3.7*	92.2±2.5	84.2±2.8*
Post(Atr)	19.4±3.8*	90.7±3.6	83.7±4.2*
Post(DG)	12.8±2.6*	93.1±2.8	86.5±3.3
Post(Ach+Atr)	19.9±3.3*	87.7±2.4*	82.6±2.9*
Post(DG+Atr)	18.2±2.2*	86.7±3.5*	82.9±3.1*

Values are mean ± SD for 6 rats in each group. n, number of hearts in each group; LVDP, left ventricular developed pressure; EDP, end diastolic pressure; WI, working index; *$P < 0.05$, compared with normal control.

In order to find the mode of action of DG methanol extract, inotropic and chronotropic effects of DG in isolated frog hearts were recorded and the results were shown in Figure 2. The baseline reading of cardiac flow rate, heart rate and force of contraction were found to be changed with extract, acetyl choline and atropine. The negative inotropic and chronotropic effect shown by the extract followed a dose dependent change and the maximum response was produced by 8 mg of the extract (Figure 2). However, the decreased heart rate and force of contractions were recovered partially when 50 micrograms of atropine was administered along with the extract (Figure 2). Similarly when Ach was treated after atropine, the heart rate and force of contraction were declined. In order to explore the possible biomolecule that can induce negative inotropic and chronotropic effect in the extracts we did GS/MS analysis of DG methanol root extract, resulted in the identification of 64 compounds (ure 1) that includes cardio-stimulatory molecules namely 4-[2-(dimethylamino)ethyl] phenol -(Cactine) and 2,5-bis (1,1-dimethyl ethyl) phenol.

The presence of cardiac marker enzymes in coronary perfusate in an isolated rat heart model is considered to be an index for myocardial injury, used in this study to assess the efficacies of cardio protective interventions like POC and pharmacological agents. ures 3 shows the results of cardiac marker enzymes in tissue homogenate and coronary perfusate of different experimental group rat hearts. A significant decrease in cardiac marker enzymes were found in tissue homogenate of rat hearts subjected to I/R (ure 3a), and were in accord to decreased marker enzymes in coronary perfusate (ure 3b), indicates myocardial injury. Post treatment of rat hearts with Ach+Atr and DG+Atr also showed elevated levels of marker enzymes in the perfusate showing the negation of Ach mediated action of heart. Pharmacological post conditioning of rat hearts with drugs namely Ach and DG reduced the level of CK and LDH in coronary perfusate as compared to I/R, indicate its cardio protective action. On the other hand, atropine, antagonist of acetyl choline showed increased presence of CK and LDH in coronary perfusate (Figure 3b).

Increased flux of reactive oxygen species and subsequent calcium overload mainly in mitochondria are the cardinal features of myocardial ischemia reperfusion injury. Myocardial oxidative stress was determined by the level of TBARS and antioxidant enzyme levels in tissue and mitochondria. Tissue homogenate showed significant elevation of TBARS in I/R control rat hearts, indicate lipid peroxidation (Figure 4a). Pharmacological post treated groups except atropine post treated, showed decreased TBARS level. Similarly I/R and Atr groups showed declined CuZn SOD activity in tissue homogenate (Figure 4a). However in mitochondrial samples, antioxidant enzyme actives were found to be low in I/R group and atropine post treated groups (Figure 4b).

Thiol levels in the tissue are another indicator of oxidative stress. Total thiol, protein thiol and non-protein thiol levels in tissue homogenate were found to be decreased in I/R groups (Figure 5a).

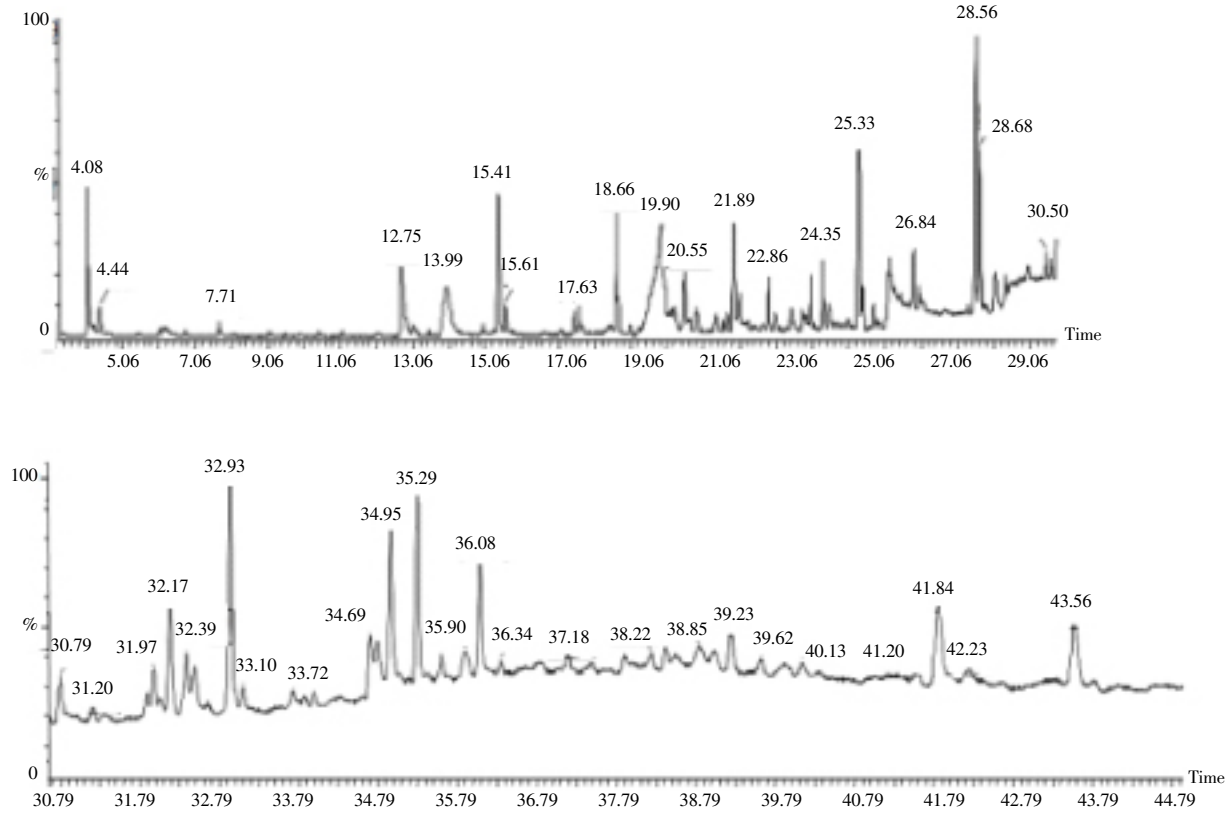
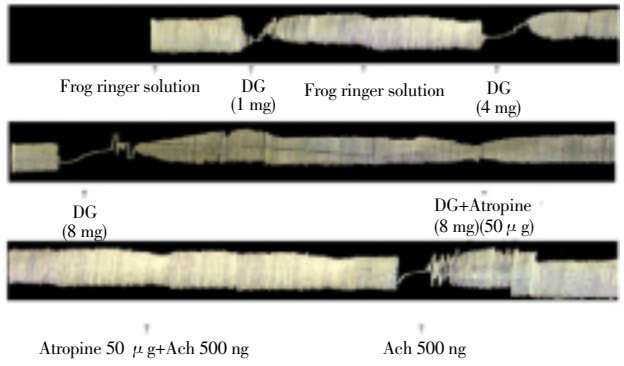


Figure 1. GC/MS of methanol extract of DG root.

In fact, a 25% decrease in total thiol concentration was observed in atropine and DG post treated rat hearts (Figure 5a). Similarly in Ach + Atr and Atr +DG groups, a significant decline of non-protein thiol was observed (46% and 69% respectively). In mitochondrial sample also total, protein and non-protein thiols were decreased significantly (P>0.05) in I/R rat hearts and in all pharmacologically post treated rat hearts (Figure 5 b).



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 8 mg	4.5	58	14
Atropine 50 μ g+Ach 500 ng	4.8	60	17
Ach 500 ng	3.0	15	07

Figure 2. Kymograph of the treated frog hearts.

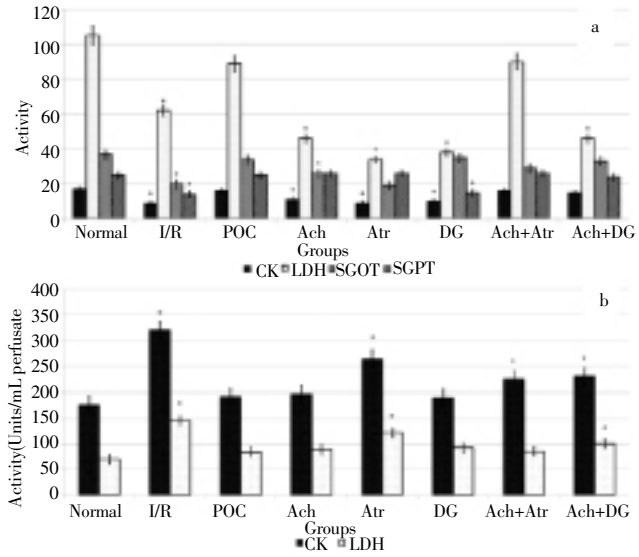


Figure 3. Cardiac markers: a) in the heart tissue b) in the coronary perfusate.

CK: Creatinine Kinase, expressed as μ M ofphosphorous generated/minute/mg protein in tissue homogenate. LDH: Lactate dehydrogenase, expressed as nM of pyruvate generated /minute/mg protein in tissue homogenate. Both SGOT and SGPT were expressed as nanomol pyruvate generated /minute/mg protein in tissue homogenate. Results are expressed as mean ± SD of n=6 independent assays: (*) P<0.05, statistically different from normal controls.

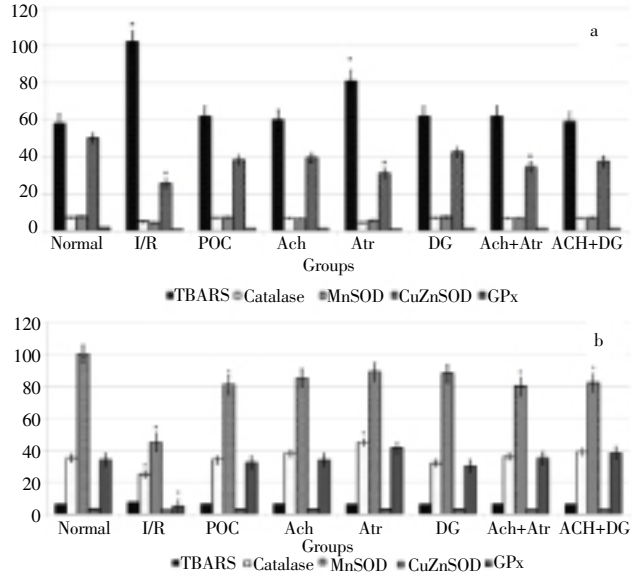


Figure 4. Lipid peroxidation and antioxidant enzyme activities: a) in tissue homogenate b) in mitochondria

TBARS was expressed as mM/g wet tissue in tissue homogenate and in mitochondrial samples. Catalase was expressed as U/mg protein in tissue homogenate and as mU/mg protein in mitochondrial samples. Both MnSOD and CuZN SOD were expressed as U/mg proteins in both tissue homogenate and mitochondrial samples. Similarly glutathione peroxidase (GPx) was expressed as μ g of GSH consumed /min/mg proteins in both tissue homogenate and mitochondrial samples.

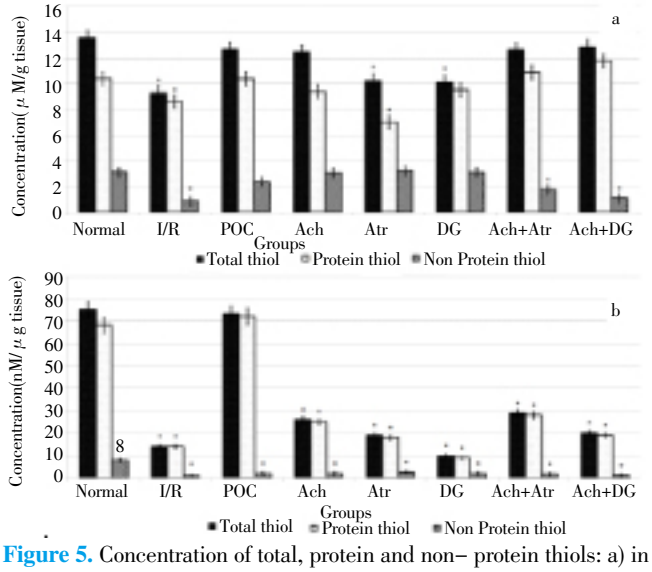


Figure 5. Concentration of total, protein and non- protein thiols: a) in tissue homogenate b) in the mitochondrial samples.

4. Discussion

The major findings of this study are as follows: DG methanol root extract can mediate cardio protection when administered after global ischemia (post conditioning) by stimulating muscarinic receptor. Myocardial protection induced by Ach, known as muscarinic receptor agonist, involves activation of mitochondrial KATP channels and increased free radical signals from mitochondria[21]. The binding of acetylcholine to receptors in the intact heart causes a decrease in the frequency (chronotropic

effect) and force (ionotropic effect) of contraction[22]. Agreement to these findings, our results also showed Ach mediated negative chronotropic and ionotropic effect, thereby probably mediate protection to I/R injury by reducing calcium overload and free radical release. Interestingly, a similar effect (negative inotropic and chronotropic) was shown by rat hearts post treated with DG root extract, mimetic Ach action. To confirm the Ach linked muscarinic receptor action of DG, atropine, muscarinic receptor antagonist was used for the study. Importantly, the above physiological change mediated by Ach/DG in kymogram was reversed by the addition of Ach receptor/muscarinic receptor antagonist Atr, suggesting the involvement of DG extracts on Ach receptor/ muscarinic receptor. Kymogram results reveal that physiological response mediated by 50 μg atropine in frog heart can be reversed by the addition of DG at the concentration of 160 μg.

On the other hand, Ach, muscarinic receptor agonist needs only 500 ng to do the same function. This difference may be due to the nature and purity of the agents. But reversion of physiological response of atropine by DG extract re-confirms the muscarinic receptor mediated action of the herbal extract. Furthermore, with Langendroff technique, effect of DG root extract was compared with Ach and Atr, the agonist and antagonist of muscarinic receptors respectively, results are discussed as follows. Post ischemic treatment of DG did not affect the basic hemodynamic parameters as compared to control.

Increased cardiac marker enzymes in tissue homogenate and perfusate in ischemia reperfusion control rats indicate severity of damage to myocardial membrane. However, a myocardial protection was observed by administrating Ach and DG before reperfusion, agree with the finding of Richard and his co workers, who reported that acetylcholine limited infarct size and protected coronary endothelial cells in a rat model during ischemia or reperfusion and these effects were prevented by atropine[23]. On comparison, DG extract may provide a better preservation of myocardial cell architecture during revascularization than acetylcholine. This may be due to the presence of phytochemical molecules in DG extract possessing antioxidant property apart from the presence of alkaloid, that stimulate the cardiac receptors[24].

Generation of oxygen free radicals and lipid peroxidation has been suggested to play an important role in the pathogenesis of post-ischemic myocardial dysfunction. Administration of Ach and DG root extract before myocardial ischemia reperfusion showed significant improvement in tissue lipid peroxidation and antioxidant enzymes like catalase, GPx and SOD as compared to ischemic reperfused control hearts in both tissue homogenate and mitochondria. The antioxidant potential possessed by Ach reported to be mediated by protein kinase C [25]and thereby prevents the further release of free radicals. However, in case of DG root extract, apart from muscarinic stimulation action, the presence of phytochemical molecules possessing free radical scavenging action[6], may provides the cardio-protection. Thus DG root extract when administered prior to reperfusion prevented the oxidative stress significantly than acetylcholine. The above results were complemented with the level of total thiol, protein thiol and non protein thiol in rat heart. Protein thiols are frequent sites for posttranslational modification[26] mediated by oxidative stress in a wide variety of disorders including aging, ischemia-reperfusion, and alcohol hepatotoxicity. The increased protein thiol content of the rat heart post treated with DG suggested a better preservation of antioxidant enzymes.

In the mitochondrial samples, pharmacological preconditioning of isolated rat heart with Ach, Atr and DG root extract showed similar pattern of changes in the activity of catalase, GPx , SOD and the level of TBARS. Myocardial non protein thiol to protein thiol ratio was significantly higher in rats postconditioning with DG root extract, indicate better antioxidant status than post conditioning with Ach/Atr This finding emphasis the importance of bio-molecules that can targets the antioxidants in mitochondria. Generally mitochondria do not synthesize non protein thiol namely, GSH but are able to transport and accumulate up to ~15% of the total cellular GSH. From the above results, we can conclude that DG extract mediate its cardio protection by stimulating muscarinic receptors similar to that of acetylcholine. In addition, DG extract suppressed the free radical accumulation which enhanced the cardio protection even better than POC. The major short coming of the study is that, we used the crude methanol extract of DG, rather pure compound. Similarly the study was conducted in an isolated rat heart model, where the hormonal interaction was negated; similar study has to be done in *in vivo* animal model to confirm the results. On the same time, it is worth to mention here that, to our knowledge, no single drug entity has completely cured the abnormalities related to ischemia reperfusion injury, indicate the relevance of multiple drug entity especially for ischemia reperfusion injury.

Conflict of interest statement

We declare that we have no conflict of interest.

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