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# Interpretation of Inotropic Effect Exhibited by *Desmodium gangeticum* Chloroform Root Extract Through GSMS and Atomic Mass Spectroscopy: Evaluation of its Anti Ischemia Reperfusion Property in Isolated Rat Heart

[1]G.A. Kurian, [3]R.S.S. Srivats, [3]R. Gomathi, [4]M.M. Shabi and [2]J. Paddikkala
[1]Department of Medical Biochemistry, School of Chemical and Biotechnology,
Sastra University, Thirumalaisamudram, Thanjavur, Tamil Nadu, India
[2]Department of Plant Biotechnology, Amala Cancer Research Center,
Malanagar, Trichur, Kerala, India
[3]Department of Biotechnology, Sastra University, India
[4]Division of Pharmacology and Toxicology,
Centre for Advanced Research in Indian System of Medicine,
Sastra University, India

**Abstract:** Cardiotonic effect mediated by *Desmodium gangeticum* (DG) chloroform root extract was determined in frog and assessed in isolated rat heart. Ischemia reperfusion injury was experimentally induced in rat by using Langendroff apparatus and was perfused with Kreb Hanseleit buffer through aorta. Ischemia-reperfusion injury was induced by 30 min of global ischemia and 45 min of reperfusion in isolated rat hearts. Heart rate, coronary flow and Left Ventricular Pressure (LVP) were recorded and the activity of lactate dehydrogenase in coronary effluent and creatine kinase and lactate dehydrogenase contents in myocardial tissues were measured. Administration of DG root chloroform extract (20 mg $kg^{-1}$ b.wt.) before global ischemia caused a significant improvement of cardiac function and a decrease in the release of lactate dehydrogenase in coronary effluent. Kymogram result in isolated frog heart showed that DG root extract possess positive inotropic effect. GSMS and atomic absorption analysis of DG root extract confirm the presence of bio-molecules that can stimulate the release of calcium in heart. From the above observations of present study, we can conclude that DG chloroform root extract can protect the myocardium against the damages induced by ischemia reperfusion in rats and the effect of the extract may be related to calcium releasing property.

**Key words:** *Desmodium gangeticum*, ischemic reperfusion, kymogram, gas spectroscopy/mass spectroscopy, atomic absorption

## INTRODUCTION

Myocardium calcium overload is one of the patho-physiological consequences of cardiac injury mediated by reperfusion of ischemic myocardium. In cardiac tissue, the most

**Corresponding Author:** G.A. Kurian, Department of Medical Biochemistry,
School of Chemical and Biotechnology, Sastra University,
Thirumalaisamudram, Thanjavur, Tamil Nadu, India

important regulator of $Ca^{2+}$ homeostasis is Sarcoplasmic Reticulum (SR), which serves as a sink for $Ca^{2+}$ ions during relaxation and as a $Ca^{2+}$ source during contraction (Lamb, 2009). It is well known that SR $Ca^{2+}$-ATPase transports $Ca^{2+}$ from the cytosol to the lumen of the SR at the cost of ATP and was depressed during ischemia reperfusion (Solaro and Arteaga, 2007).

Arrhythmogenesis, one of the clinical presentation of ischemia reperfusion injury (IRI), may be due to intracellular $Na^+$ and $Ca^{2+}$ loading (Huang *et al*., 2006). In fact, reperfusion of the ischemic myocardium results in an increase in intracellular $Na^+$ and $Ca^{2+}$ (Baczko *et al*., 2008). The exact mechanism for $Ca^{2+}$ influx into the myocardial cells remains unsolved; it is, however, generally thought that both $Ca^{2+}$ slow channels and $Na^+$-$Ca^{2+}$ exchangers play important roles in the $Ca^{2+}$ overload during the myocardial ischemia and reperfusion (Satoh *et al*., 2000). Even the inhibitors of $Na^+$-$Ca^{2+}$ exchange system such as nickel was also found to be beneficial for the functional recovery of myocardium from the insult of ischemia reperfusion (Han *et al*., 2002). Indeed, divalent and trivalent cations have been shown to inhibit $Ca^{2+}$ transport by $Na^+/Ca^{2+}$ exchanger in a concentration-dependent manner (Iwamoto and Shigekawa, 1998). Moreover, $Mg^{2+}$ inhibits $Na^+$ dependent $Ca^{2+}$ uptake by competing with $Ca^{2+}$ for the external exchanger site in cardiomyocytes and smooth muscle cells (Smith *et al*., 2001).

Despite of having ample experimental proof to reduce calcium overload in the myocardium, none of the agents were found to be effective in ameliorating IRI. Thus the search for new therapeutic agents continues.

A very few comparable studies investigated the possible role of herbal extract that modulate the $Na^+$- and $Ca^{2+}$-loading in ischemia induced reperfusion. We, therefore, decided to expand the above observations, using isolated rat model, to examine the possible role of herbal extract that can induce favorable cardiotonic effect and thereby act as possible agent against IRI. *Desmodium gangeticum* (DG) (Leguminosae) is absolutely found in India and is one of the important plants used in indigenous system of Indian medicine. Our laboratory previously reported the cardio protective effect of *Desmodium gangeticum* root methanol extract (muscarnic receptor agonist) in isolated rat heart as post conditioning agent (Kurian *et al*., 2008). But the negative inotropic response of the extract limited its use as a precondition mimetic drug. However our preliminary study with chloroform extract of DG root suggested using the extract as preconditioning mimetic drug. In this study, we explore the cardiotonic effect of DG root chloroform extract and its subsequent use as therapeutic agent against myocardial ischemia reperfusion.

## MATERIALS AND METHODS

### Preparation of Chloroform Root Extracts of *Desmodium gangeticum*

The plant, after collection from the herbal garden (September 2008) was washed, cleaned and maintained in the Biochemistry department at SASTRA University, Tamil Nadu, India. The voucher specimen A/C No. 3908 was retained in our laboratory for future reference. The dried roots were then milled to fine powder (10 kg) and extracted with chloroform in Soxhlet's apparatus for 24 h and the extract was evaporated to dryness under vacuum and dried in vacuum desiccators (815.8 g). The residue thus obtained was used for the experiments.

### Chemicals

DL isocitrate and N-Phenyl-P-Phenylenediamine were purchased from Acros organics, New Jersy 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 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 commercial pelleted rat chow (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.

**Animals for Experiment I and II**

Male Wistar albino rats (150 to 200 g) and frogs of *Rana hexadactyla* species were housed in cages and were maintained in controlled temperature at 23±2°C with 12 h light/dark cycle. 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.

**Protocol: Experiment 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. Experiments were done at room temperature (18-21°C). The hearts were 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 2.0 g in g $L^{-1}$. The force of contraction was recorded and the rate of contraction was counted and tabulated.

**Protocol: Experiment II**

The rats were divided into four groups (n = 6 in each group): group I, control; group 2, ischemic control; group 3, reperfusion and group 4, drug.

**Group 1: Normal Control**

In normal control group hearts were perfused for 90 min with KH buffer and used for the biochemical analysis.

**Group 2: Reperfusion**

In reperfusion control group, after 20 min of equilibration, rat hearts were subjected to 30 min global ischemia followed by 45 min reperfusion.

**Group 3: Pre Treatment of Nac Before Global Ischemia**

Rat hearts (n = 6) in this group, after the equilibration were pretreated with DG before global ischemia at a dose of 20 mg $kg^{-1}$ body weight for around 15 min. Hearts were then subjected to 30 min of global ischemia, followed by 45 min of reperfusion.

**Group 4: Pre Treatment of DG Chloroform Root Extract Before Global Ischemia**

Rat hearts (n = 6) in this group, after the equilibration were pretreated with DG before global ischemia at a dose of 100 mg $kg^{-1}$ b.wt. for around 15 min. Hearts were then subjected to 30 min of global ischemia, followed by 45 min of reperfusion.

**Heart Preparation**

Wistar male rats weighing 250-280 g were anesthetized with 40 mg $kg^{-1}$ sodium thiopentenone. After an intravenous injection of 300 U heparin, the heart was rapidly excised

via a mid-sternal thoracotomy and arrested in the ice cold Krebs-Henseleit buffer (KH) containing (mM $L^{-1}$) 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 an aorta for retrograde perfusion with KH buffer maintained at 37EC and pH = 7.4 and saturated with a gas mixture of 95% $O_2$, 5% $CO_2$. 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 Kreb solution was recorded with a 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 counting 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 0.1 M Tris-HCl (pH 7.4) buffer solution. The homogenate was centrifuged at 3000 rpm for 5 min. 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 and Schenkman and Ciniti, respectively. Assay of isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), succinate dehydrogenase (SDH), α-ketoglutarate dehydrogenase (α-KGDH), NADH dehydrogenase (NADH dH) and cytochrome c oxides were carried out in a UV-1601 Shimadzu spectrophotometer. Protein concentration was measured with Folin phenol reagent, following the procedure described by Lowry. Assay of creatine kinase, lactate dehydrogenase and aspartate transaminase were also estimated.

**Phytochemical Screening**

Phytochemical screening for secondary metabolites in the chloroform extract was carried out by Mayer = s and Dragendorff = s tests (alkaloids), Shinoda = s test (flavonoids), ethanolic KOH test (coumarins), Libermann-Burchard test (terpenoid/steroids) and froth formation test (saponins). A small quantity of the chloroform extract of DG was dissolved in chloroform and applied as a band (6 mm width) onto the HPTLC plate (silica gel 60 F 254, E. Merck, Germany, 10×10 cm) with an automatic Linomat V applicator (Camag, Switzerland). The HPTLC plate was developed to a height of 80 mm in hexane-chloroform-methanol (1.5 : 7.5 : 1) with pre-saturation for 15 min in a Camag twin trough glass tank. After development, the plate was derivatized using anisaldehyde-sulfuric acid reagent and dried. The spots were scanned at 580 nm (visible, tungston lamp) using Camag TLC Scanner 3 equipped with Wincat software at slit width 5×0.45 mm.

**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 poly siloxane). Helium was used as the carrier gas with a flow rate of 1 mL $min^{-1}$. The 1 µL chloroform root extract of DG was injected into the GSBMS in split less mode at

250°C. The column oven temperature was held at 110°C for 2 min, then programmed at 75°C $min^{-1}$ to 200°C for 1 min, 5°C $min^{-1}$ to 280°C and held for 9 min.

**Statistical Analysis**

All data were reported as Mean±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 AND DISCUSSION

The present study indicates inotropic action of *Desmodium gangeticum* chloroform root extract on isolated frog heart. The resultant effect of DG chloroform root extract was reassessed *in vivo* by subjecting it as anti ischemic reperfusion agent in isolated rat heart. The positive inotropic effect of the extract appeared to be linked to the release of calcium to myocardium. The calcium releasing effect of DG was utilized for cardio protection against ischemia reperfusion injury.

**Phytochemical Screening**

The chloroform extract of DG was tested positive for terpenoids and alkaloids in preliminary phytochemical tests. The HPTLC fingerprint of the chloroform extract is shown in Fig. 1. In the HPTLC fingerprinting of chloroform extract, ten spots at the following Rf values: 0.05 (20.37%), 0.14 (12.72%), 0.25 (16.03%), 0.29 (10.96%), 0.33 (10.70%), 0.40 (7.54%), 0.51 (6.06%), 0.57 (5.36%), 0.64 (7.38%), 0.71 (2.88%) were observed.

Oleic acid, N hexadecanoic acid, 9,9 Dimethoxybicyclo (3,3,1) nona 2,4dione, 9 Dodecanoic acid methyl ester, Didodecyl phthalate. It represents around 89% (Fig. 2). Minor compounds such as 4 dodecanol, 10 undecenal, 1, 2 benzenedicarboxylic acid bis

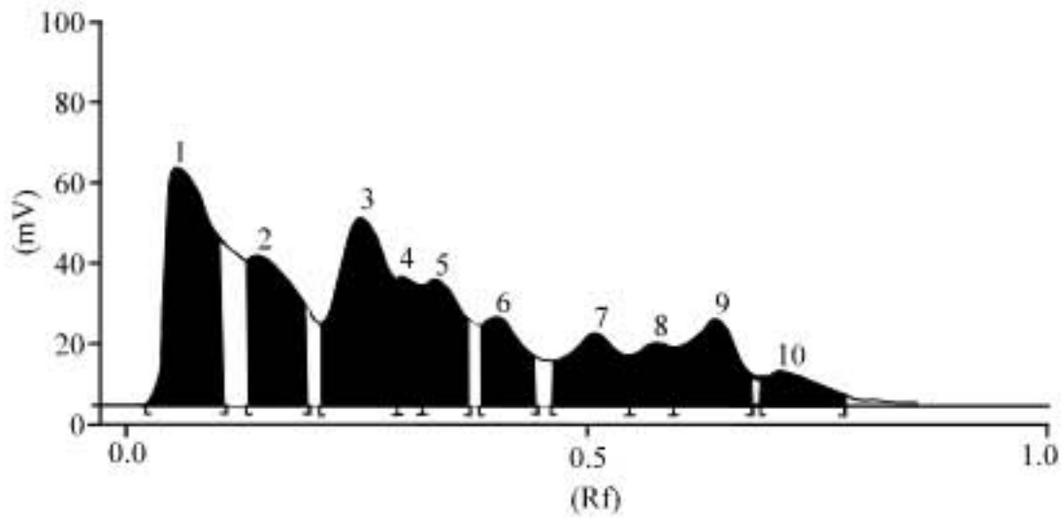


Fig. 1: HPTLC profile of the chloroform extract of DG in 1.5 : 7.5 : 1 hexane-chloroform-methanol, derivatized in anisaldehyde-sulfuric acid and scanned at 580 nm

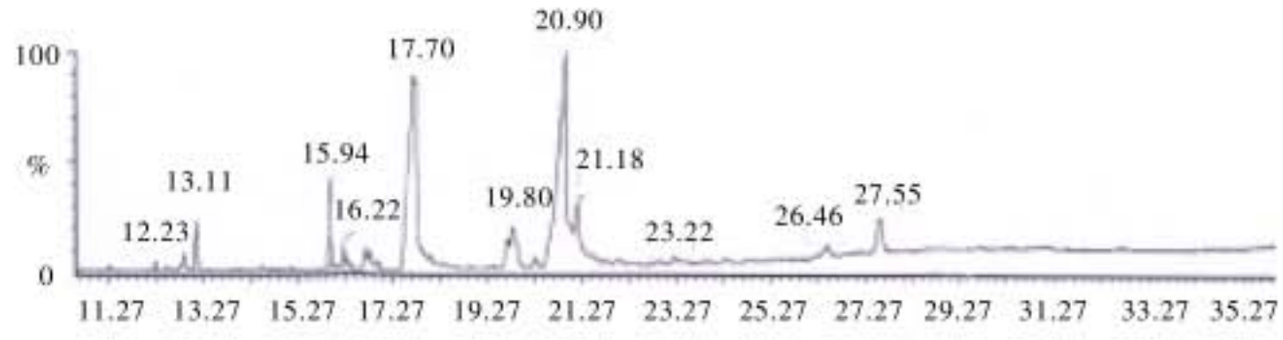


Fig. 2: GSMS of chloroform extract of *Desmodium gangeticum* root extract

(2 methylpropyl) ester, 1, 14 Tetradecanediol and 2 methyl pentanal were identified (Table 1) through GSMS analysis. In fact olive oil reported to be an effective agent against oxidative stress associated diseases. Moreover, olive oil contains minor components with antioxidant properties.

The presence of iron, magnesium, sodium, potassium, calcium and nickel (Table 2) were confirmed in the extract through atomic absorption spectrophotometer. An early report confers protection against the incidence of diabetes, metabolic syndrome, hypertension and cardiovascular disease by increased dietary magnesium intake. It ameliorates insulin resistance, serum lipid profiles, lowers inflammation, endothelial dysfunction, oxidative stress and platelet aggregability (Bo and Pisu, 2008). Few other studies explain the efficacy of dietary sodium supplementation to cardiac patients (Mervaala *et al*., 1997).

**Cardiotonic Assay**

*In situ* frog heart study indicated no change in the amplitude and force of contraction of frog myocardium when DG extract was given up to a dose of 2 mg (Table 3). However, the positive inotropic effect mediated by DG extract, initiates after a slight recession in the amplitude and force of contraction, when calcium channel blocker was administered along with it. On the other hand, 0.5 mg of calcium chloride along with calcium channel blocker did not show any delay in the increased amplitude and force of contraction (Fig. 3A, B). This suggested a delayed release of calcium into the myocardium by alternate calcium release mechanism. This may be due to sodium pump inhibition and elevated

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

| Drug and extract | Heart rate (Beats $min^{-1}$) | Flow volume (mL $min^{-1}$) | Amplitude (mm) |
|---|---|---|---|
| Baseline | 87 | 12 | 15 |
| Extract 500 µg | 72 | 11 | 10 |
| Extract 1000 µg | 70 | 11 | 08 |
| Extract 2000 µg | 75 | 19 | 09 |
| 5 µg of Ca blocker+2 mg extract | 63 | 12 | 08 |
| 500 µg of $CaCl_2$ | 67 | 11 | 17 |
| 5 µg of Ca blocker+ 500 g of $CaCl_2$ | 63 | 19 | 07 |
| 10 µg of Ca blocker+500 g of $CaCl_2$ | 65 | 20 | 09 |

Table 2: Hemodynamic characteristic

| Group | N | LVDP (mmHg) | CF (mL $min^{-1}$) | HR (bp $min^{-1}$) | RPP × $10^3$ (mm Hg bt $min^{-1}$) | MAP (mm Hg) |
|---|---|---|---|---|---|---|
| 1 | 6 | 99.64±4.0 | 9.1±1.01 | 340±19.1 | 33.88±5.3 | 120±7 |
| 2 | 6 | 40.29±4.3 | 9.1±1.09 | 237±30.1 | 9.55±7.4 | 113±8 |
| 3 | 6 | 75.45±4.2 | 9.1±0.95 | 321±30.2 | 24.22±5.6 | 104±5 |
| 4 | 6 | 84.18±4.6 | 9.3±1.05 | 320±30.5 | 26.94±7.4 | 103±6 |

Values are Mean±SD for 6 rats in each group. n,no. 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: Level of creatine kinase and lactate dehydrogenase (LDH) in the myocardium of isolated rat heart and the activity of LDH in the myocardial perfusate

| | | | LDH (units/mL/perfusate) | |
|---|---|---|---|---|
| Group | CK (µmol phosphorous liberated/min/mg protein) | LDH (nmol pyruvate liberated/min/mg protein) | 5 min | 10 min |
| 1 | 16.5±0.88$^a$ | 110.60±5.11$^a$ | 1.5±0.65$^a$ | 1.2±0.34$^a$ |
| 2 | 112.9±0.98$^c$ | 285.61±6.32$^d$ | 58.8±1.87$^b$ | 57.6±1.05$^b$ |
| 3 | 24.7±0.77$^a$ | 167.46±5.67$^b$ | 22.1±1.11$^d$ | 24.4±1.65$^c$ |
| 4 | 29.1±0.98$^b$ | 165.93±4.38$^b$ | 24.6±1.08$^d$ | 26.8±1.23$^c$ |

Results are Mean±SD (n = 6). Activity is expressed as µmoles of phosphorus liberated per sec per gram protein for $Na^+$ $K^+$ ATPase, $Ca^{2+}$ ATPase and $Mg^{2+}$ ATPase; mmoles of phosphorus released per mg protein per hour for 5'-nucleotidase. Values not sharing a common superscript differ significantly at $p<0.05$) when compared between the groups

Table 4: Metal composition of *Desmodium gangeticum* root chloroform extract by atomic absorption

| Element | Quantity (μg mL$^{-1}$) |
|---|---|
| Iron | 15.00±1.10 |
| Magnesium | 34.75±3.30 |
| Sodium | 227.28±10.3 |
| Potassium | 50.58±5.50 |
| Calcium | 284.52±14.7 |
| Nickel | 0.9292±0.1 |

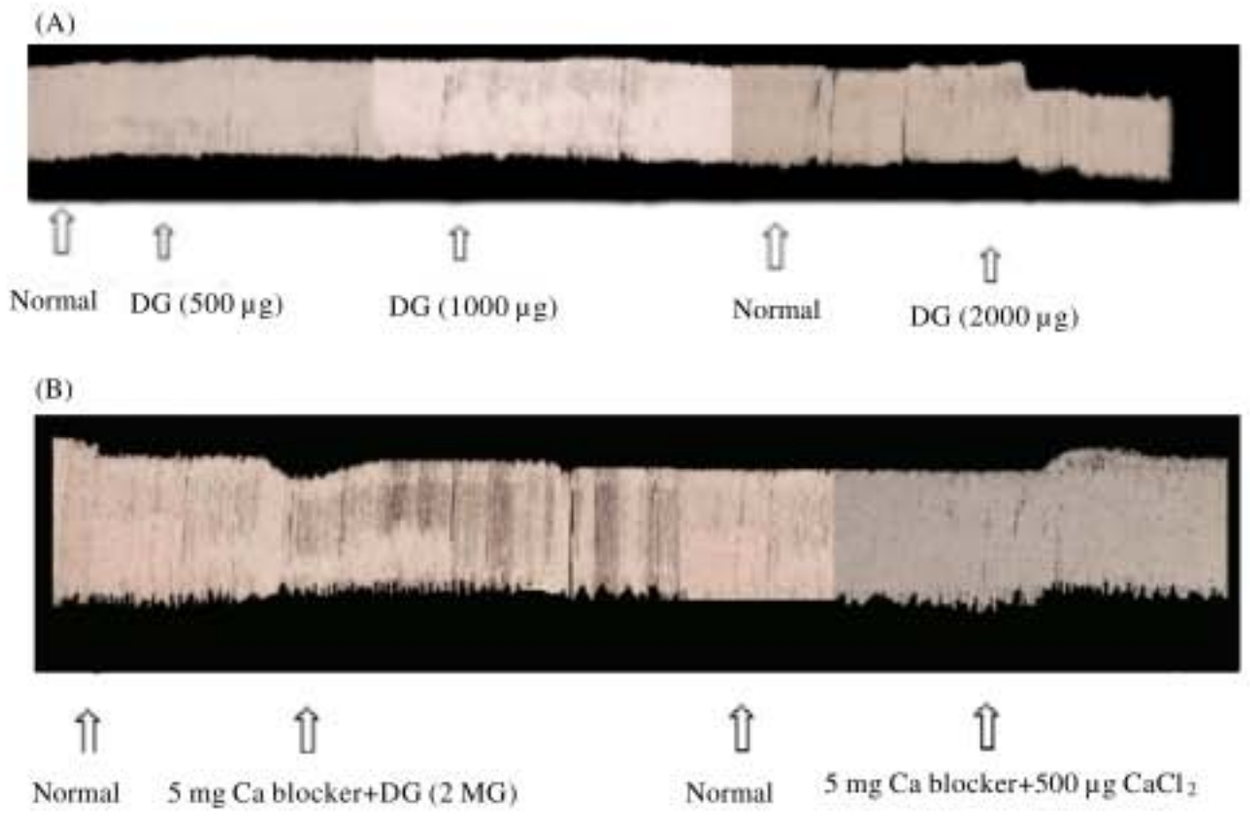


Fig. 3: The positive inotropic effect of *Desmodium gangeticum* root chloroform extract on isolated frog heart preparation. (A) with different concentrations of the drug and (B) insensitive to calcium channel blocker

sodium (Sheu and Fozzard, 1982). Indeed, slow calcium releases are known to show better cardiotonic activity in hypodynamic hearts than in normal hearts (Garaliene *et al*., 1998).

The positive inotropic effect of DG chloroform root extract was reassessed in isolated rat heart. The improved hemodynamic parameters in isolated rat heart are associated with DG root chloroform extract infusion (Table 4). Further, increased heart rate and force of contraction may be due to calcium influx, as predicted by above kymogram study. However, in isolated frog heart study, effect of DG root extract was observed to be insensitive to calcium channel blocker (Fig. 3), suggest that active ingredient of DG root extract may be acting indirectly through different sym or anti port system to release calcium. Further research is needed to establish the exact mechanism of action. But elevated calcium flux that may increased coronary flow rate observed in the present study suggest voltage independent calcium flux. Interestingly, our element analysis of DG root extract showed a significant level of nickel (Table 2) in the extract triggering a $Ca^{2+}$ influx sensitive to nickel, an inhibitor of voltage-dependent calcium channels (Komai and Rusy, 1993).

**Myocardial Ischemia Reperfusion Study**

Cardiac biomarkers are usually measured to assess myocardial damage which leads to the disintegration of heart cell membrane (Shell *et al*., 1971; Irvine *et al*., 1980). Hence the presence of cardiac enzymes like CK, LDH and Troponin in the coronary perfusate of the present study indicates the cardiac damage which was observed significantly in control

Table 5: Chemical composition of Desmodium gangeticum root chloroform extract by GS-MS analysis

| RT | Name of the compound | Molecular formula | Molecular weight | Peak area (%) |
|---|---|---|---|---|
| 12.84 | 2 methyl pentanal | $C_6H_{12}O$ | 100 | 0.89 |
| 13.11 | 1,14 Tetradecanediol | $C_{14}H_{30}O$ | 230 | 2.29 |
| 15.94 | 1,2 Benzenedicarboxylic acid bis (2 methylpropyl) ester | $C_{16}H_{22}O_4$ | 278 | 3.45 |
| 16.22 | 10 Undecenal | $C_{11}H_{20}O$ | 168 | 0.92 |
| 17.70 | N hexadecanoic acid | $C_{16}H_{32}O_2$ | 256 | 36.42 |
| 19.80 | 9 Dodecenoic acid methyl ester | $C_{13}H_{24}O_2$ | 212 | 5.06 |
| 20.90 | Oleic acid | $C_{18}H_{34}O_2$ | 282 | 36.63 |
| 21.18 | 9,9 Dimethoxybicyclo[3,3,1]nona 2,4 dione | $C_{11}H_{16}O_4$ | 212 | 7.32 |
| 23.22 | 4 dodecanol | $C_{12}H_{26}O$ | 186 | 0.75 |
| 26.46 | 1,2 Bis (trimethysilyl) benzene | $C_{12}H_{22}Si_2$ | 222 | 1.95 |
| 27.55 | Didodecyl phthalate | $C_{32}H_{54}O_4$ | 502 | 4.31 |

animal. However, administration of the DG root extract reduces the level of these enzymes in perfusate suggesting myocardial protection (Table 5). The myocardial depression is caused by the combined effect of a reduction in (1) trans sarcolemmal L type $Ca^{2+}$ current, (2) sarcoplasmic reticulum $Ca^{2+}$ content and (3) sensitivity of myofilaments to calcium (Pask *et al*., 1981). A high conductance $Ca^{2+}$ activated potassium (KCa) channel, located in the inner membrane of the mitochondrion, has been shown to mediate cardio protection against ischemia/reperfusion (Xu *et al*., 2002), as does the mitochondrial ATP-sensitive potassium (mitoKATP) channel (Garlid *et al*., 1997). Thus a decline in cardiac biomarkers in the perfusate predicts the intact cardiac membrane architecture.

Metal content of DG extract analyzed by atomic absorption spectroscopy also substantiate calcium like action of DG root extract (Table 2). Macro and microelements play a vital role in the medicinal value of plant therapy in health and diseases (Simon *et al*., 1990). It was previously reported that plants containing rich amount of calcium may support the medicinal uses of plants (Siddhuraju and Beckar, 2001). The high concentrations of Ca are very significant because Ca is known to enhance the qualities of bones and teeth and also of neuromuscular systemic and cardiac functions (Martin, 1984). Sodium and potassium content of the extract attribute to diuretic action (Sica *et al*., 2008) that can indirectly influence the cardiac function.

## CONCLUSIONS

From the above observation we can infer that chloroform root extract of *Desmodium gangeticum* posses positive inotropic effect that mediate cardio protection against injury due to ischemia/reperfusion. This effect may be related to the delayed increase of $Ca^{2+}$ in the myocardium due to the inhibition of sodium-potassium pump. The increased influx of $Ca^{2+}$ could also be associated to the inhibition of voltage dependent calcium channels by $Ni^{2+}$ as significant quantity is present in the extract. $Ca^{2+}$-activated potassium (KCa) channel present in the mitochondrion could have been activated either due to the presence of trace elements like $Mg^{2+}$ or by some principle component present in the drug for which further studies can help in confirming the mechanism involved in this drug mediated cardio protection.

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