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DESMODIUMGANGETICUM (L) TRIGGERS INOTROPIC AND ELECTROPHYSIOLOGICAL CHANGES IN CARDIAC TISSUE THROUGH INACTIVATION OF THE NA+, K+-ATPASE SIGNALING CASCADE - AN *IN – VITRO* AND *IN- VIVO* CORRELATIONS

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ABSTRACT

Background: *Desmodiumgangeticum*(L) (DG) an herb which belongs to the family *Fabaceae*is a perennial shrub widely distributed in tropical and sub-tropical habitats and particularly abundant in India. DG is widely used for the treatment of cardiovascular disease in the Indian System of Medicines. The petroleum ether root extract of DG was evaluated for its cardio-protective activity using isolated frog´s heart perfusion method and to assess the electrocardiographic changes in rats. **Method:** The*in-vitro* positive inotropic and chronotropic activities in isolated frog heart were recorded using perfusion apparatus bykymographical techniques. The in-vivo method, rats were treated with *Desmodiumgangeticum* (500mg·kg^{-1}, p.o) for a period of 30 days. At the end of experiment, electrocardiographic changes were monitored from control and experimental groups. **Result:** The petroleum ether root extract of *Desmodiumgangeticum* showed a dose dependent positive inotropic and negative chronotropic action on isolated frog heart. The pharmacological effect may be due to theinactivation of the Na^+, K^+-ATPase signaling cascade.In addition, *Desmodiumgangeticum* administered rats showed a significant alteration in electrocardiograph pattern changes when compared to control groups. Moreover, GC- MS of DG root extract confirm the presence of bio-molecules that can stimulate the release of calcium in heart and protect from oxidative stress in heart.

Conclusion: In present study, we can conclude that root extract of *Desmodiumgangeticum* can protect the myocardium against the cardiac depression by blocking Na^+, K^+-ATPase signaling cascade similar to the action of digoxin. The effect of the *Desmodiumgangeticum*extract may be presence of glycosides.

KEYWORDS: DG, Positive inotropic, Electrocardiograph, GC- MS, heart.

BACKGROUND

The vast majority of isolated compounds have been shown a remarkably high correlation of structure and specificity to produce pharmacological effects. Experimental evidence indicates that drugs interact with receptor sites localized in macromolecules.[1] Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cardiovascular diseases. Although, drug discovery from medicinal plants continues to provide an important source of new drug leads, numerous challenges are encountered including the procurement of plant materials, the selection and implementation of appropriate high-throughput screening bioassays, and the scale-up of active compound.[2]

Desmodiumgangeticum(L) (DG) an herb belongs to the family *Fabaceae*is a perennial shrub distributed in tropical and sub-tropical habitats and particularly abundant in India. DG is widely used for the treatment of ischemic heart disease in the Indian System of Medicines (ISM).[3] It has been extensively used in the ISM as a bitter tonic, febrifuge, digestive, anticatarrhal, antiemetic, anti- inflammatory agent for chest and other inflammatory conditions.[4] This plant has been used in Ayurveda for the treatment of various diseases like typhoid fever, urinary discharges, piles, asthma, bronchitis, vomiting,dysentry and hemicranias.[5] Roots of DGare one of the components of ayurvedic preparations used frequently in the management of ischemic heart diseases and are reported to contain flavones and isoflavonoid glycosides.[6] However, there was no scientific statement available on traditional claims of the petroleum ether extract of root of DG.Therefore, keeping above facts in view the present study was planned to evaluate thefunctional evidence of inotropic and chronotropic effect of petroleum ether extract of DGthrough voltage gated ion channels in isolated frog heart preparationcorrelated with electrophysiological evidence in rats by*in – vitro* and *in- vivo* correlations involving different animals.

MATERIALS AND METHODS

Plant

Collection, Identification and Authentication of Plant

The plant material used in this study was root parts ofDG collected from Kerala state, India, during October 2013. The plant wasidentified and authenticated taxonomically by Botanist, Department of Environmental& Herbal Science, Tamil University, Tamilnadu, India. A voucherspecimen of the collected sample was also deposited in the same departmentfor future reference.

Preparation of Petroleum ether extract

The collected plant root was shade dried for 15 days and the root were coarselypowdered and passed through 10-mesh sieve. The coarsely powdered materialswere soaked in petroleum ether in the ratio of 1:4 (w/v). The solvent wasremoved under reduced pressure and temperature using rotary vacuum evaporator.The yield of extract was calculated as 2.1%. A semi solid extract wasobtained after complete elimination of petroleum ether and it was stored inrefrigerator for experimental evaluation.

Stock solution of DG

10 mg of DG was dissolved in 1.0 mL of Frog Ringer's solution. A stock solution of 10 mg/mL was made with of Frog Ringer's solution. The following working concentrations were used from the stock: 1mg/0.1mL and 1mg/0.2mL of this concentration were added.

Chemicals

Verapamil was purchased from Sigma ChemicalCo., St. Louis, MO, USA. All other chemicals and reagents used in this studywere of analytical grade with high purity and were purchased by M/S. KeerthiChemicals, Chennai, India.. Physiological salt solution compositions were purchased from NICE chemicals, Kerala, India. All the chemicals used in this experiment were of analytical grade.

Preparation of Verapamil

A stock solution of verapamil at the concentration of 1000 µg/mL was made with frog ringer solution. The following working concentration was prepared from the stock solution: 500µg/mL, 100 µg/mL and 10 µg/mL of this concentration was added to the inner organ bath and used as an antagonist effect against calcium channel.

Animals

Frogs of *Ranahexadactyla*species maintained in theanimal house and Wistar strain male albino rats, weighing 300-350 g were selected for thepresent study. The animals were housed individually in polypropylene cagesunder hygienic and standard environmental conditions (22 ± 2ºC, humidity60-70%, 12 h light/dark cycle). The animals were provided standard pellet diet(Tetragon ChemiePvt ltd., Pet Care Division, Bangalore, Tamilnadu, India)and water *ad libitum.* All the animals were allowed to acclimatize for 10 daysprior to the experiment. The study protocol was carried out as per the rulesand regulation of the Institutional Animal's Ethics Committee (IAEC) ofSASTRA University (IAEC no.48/ SASTRA/ IAEC/ RPP)

MATERIALS AND METHOD

Chemical charecterisation of DG using GC- MS

All analysis was conducted with a Perkin Elmer Clarus 500 GC equipped with mass spectrometry. 100 g of DGleaves was subjected to hydro-distillation using Clevenger apparatus and the separated oil was used for the GC-MS analysis. 1.0 mL of oil was dissolved in 1.0 mL of hexane and the sample was injected to the GC in the following conditions. The GC utilized for analysis was equipped with Elite – 1 column. The sample was run in the GC at 600°C for 0 minute, followed by 100°C increased at the rate of 10°C/min and then, the temperature was increased up to 260°C at the rate of 40°C /min. Totally the sample was run in the GC for 50 min. Helium at the rate of 1.0 mL/min was used as carrier gas. The conditions for the MS analysis were as follows. Inlet line temperature - 200°C, Source Temperature - 200°C, Electron energy - 70 eV, Mass scan - 25 – 400 and total MS time - 50 min.

Experimental Protocol I

Experimental Plan

The experimental plan consisted of three steps

Step I: Hearts (n=6) ,*DG*

Normal	**500 µg DG**	**Normal**

Normal	**1000 µg DG**	**Normal**

Step II: Hearts (n=6),Atenololvs*DG*

Normal	**50 µg Atenolol+ 500µg DG**	**Normal**
Normal	**50 µg Atenolol+ 1000 µg DG**	**Normal**

Step III: Hearts (n=6), Verapamilvs*DG*

Normal	**1 µg Verapamil**	**Normal**
Normal	**1 µg Verapamil+ 500 µg DG**	**Normal**

Step1: At the end of a 15 min stabilisation period, hearts (n=6) received continuous perfusion along with DG at the dose of 500 µg& 1000 µg. ***Step 2:***In this period, hearts (n=6) were administered with Atenolol(50 µg)Followed by DG at the dose of 500 µg& 1000 µg.***Step 3:***In this period, hearts (n=6) administered withverapamil(1 µg) and verapamil(1 µg) followed by DG at the dose of 500 µg.

Inotropic and Chronotropic effect of DG on isolated heart preparation

An average sized frog was pithed and destroyed by passing a stiletto through the occipito-atlantic junction without causing any injury to its heart and associated blood vessels.[7] The anterior chest wall was opened and a pericardiectomy was performed and opened to expose the heart. Tie one end of the aorta, inferior vena cava was identified. A small cut inferior vena cava and a syme's cannula was inserted towards the heart. A steady flow of the perfusion Frog-Ringer solution containing oxygenated, fluid of the following composition: NaCl 6.5, KCl 0.14, $CaCl_2$ 0.12, and $NaHCO_3$ 0.2, NaH_2PO_4 0.01, Glucose 2.0 in g/ litre.[8] pH was adjusted to 7.20 by adding Na_2HPO_4.[9] It was perfused through this cannula and there was an opening in the cannula through which drugs could be injected by pushing a capillary tube attached to a syringe through an injection needle. A very thin hook was attached to the apex of beating heart, which was tied with a cotton thread. The other end of the thread was attached with the Starling heart lever so that the movements of the beating of the heart could be recorded on a smoked paper of a kymographic drum. The force of contraction was recorded and the rate of contraction was counted.[10] A frog heart was perfused with DG, atenolol and verapamil in frog Ringer solution and the recording were noted and tabulated. All animal experimentations were carried out with the guidelines of Institutional Animal Ethics Committee (IAEC).

Experimental Protocol II

Rats were divided in two groups. Group I rats received of 2 mL of distilled water, p.o. for 30 days. Group II rats received DG (500 mg·kg^{-1}, p.o) for 30 days.

Measurements of ECG changes

On the 30th day of DG administration, the leads of ECG were attached to right arm, left arm and left leg of ratsand a bipolar transthoracic ECG is obtained on a Biopac MP100 Data Acquisition Unit (Biopac Systems, Inc., USA). During this process, the rats were under anesthesia by Ketamine at the dose of 100 mgkg^{-1} body weight. Bipolar transthoracic ECGs were obtained in every 30 min. The changes in QRS complex, P wave amplitude along with changes in heart rate were determined from ECG. ECG of the animals was performedby using Acqknowledge 3.9.0 software.

STATISTICAL ANALYSIS

All data were reported as Mean ± SD. Results were statically 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

GS/MS analysis

GS/MS analysis resulted in the identification of 16 compounds (**Figure 1**). Major compounds comprises of Cyclohexanol, 1-methyl-4-(1-methylethyl), - à-Curcumene, 2-Butanone, 4-(4-methoxyphenyl)-, à-Farnesene, Elemicin, à-Bisabolol, 2-Octenoic acid, 4-isopropylidene-7-methyl-6-methylene-, methyl ester, Spathulenol, Caryophyllene oxide, Asarone, trans-Z-à-Bisabolene epoxide, Cubenol, ë-Cadinol, (-)-,Lanceol, cis, Azulol, 2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro-, 2,4,5,5,8a-Pentamethyl-6,7,8,8a-tetrahydro-5H-chromene and Cubenol is represents around 11.9542 %. In fact above mentioned compound to be an effective agent against oxidative stress associated with cardiac diseases.

Inotropic and chronotropic effect

The inotropic and chronotropic effects of isolated heart were recorded from apex of beating heart connected to the isolated perfusion systems. The normal rate of contraction of isolated heart was recorded after stabilization for 15 minutes. In step I, The different doses 500 µg DG and 1000 µg of petroleum ether extract of DG were injected directly to the isolated heart along with perfusion. The graded doses of DG significantly ($P <0.01$) increase the cardiac

flow rate, and force of contraction. However, the maximum response was produced by 1000 µg of DG. In II step, beta receptor blocker such as atenolol was added in perfusateat the dose of 50 µg followed by DG at the doses of 500 µg and 1000 µg.The atenolol at the dose of 50 µg was not partially and fully blocked by 500 µg and 1000 µg of DG. In III step, before adding DG to the perfusate, calcium channel blocker such as verapamil was added at the dose of 1 µg followed by DG at the doses of 500 µg. The verapamil at the dose of 1µg was fully blocked the action of 500 µg of DG. The responses observed of the DG and after administration of the antagonists are shown in **Table 1& Figure 2.** The maximum contractility and heart rate were 21 mm and 22 beats /min at the dose of 1000 µg.

Study of electrophysiological activity of Desmodiumgangeticum (L) in wistar rats by electrocardiogram

The effect of DG on ECG pattern in normal and DG treated rats are shown in Table 2. Normal animals groups showed normal pattern of ECG. DG treated showed a significant (P<0.01, P<0.001) increase in p wave, Q wave, QRS complex and beat/min when compared to normal animals. Moreover T wave has no significant. **(Table 2, Figure 3)**

Table 1. Effect of *Desmodiumgangeticum*(L) on cardiac output, heart rate and amplitude in isolated rat hearts preparation

Steps	Groups	Drug and Extract	Cardiac output (mL min^{-1})	Heart Rate (Beats min^{-1})	Amplitude (mm)
Step I	I	Base line	16.83± 0.29	78± 2.00	13.67± 0.58
	II	DG 500 µg	17.83± 0.76^{ns}	76.33± 1.15^{ns}	17.33± 0.58**
	III	DG 1000 µg	22.50± 0.50**	79.33± 1.53^{ns}	22.33± 1.15**
Step II	IV	Atenolol 50 µg +DG 500 µg	17.63± 0.60^{ns}	75.33± 1.53^{ns}	17.33± 0.58**
	V	Atenolol 50 µg +DG 1000 µg	18.00± 0.30^{ns}	77.00± 2.65^{ns}	21.67± 1.15**
Step III	VI	1 µgverapamil	14.90± 0.36**	74.00± 1.73^{ns}	12.33± 0.58 ^{ns}
	VII	1 µgverapamil + DG 500 µg	17.20± 0.53^{ns}	76.33± 1.53^{ns}	13.67± 1.53 ^{ns}

Activity is expressed as mL/min for cardiac output; beats/ min for heart rate; mm for amplitude Values are expressed as mean ± S.D. ** Significant difference (P <0.01) between base line vs DG 1000 µg; base line vs 1 µg verapamil; base line vs DG 500 µg;base line vs DG 1000 µg; base line vs Atenolol 50 µg +DG 500 µg; base line vs Atenolol 50 µg +DG 1000 µg; p <0.01Duncan's Multiple Range Test (DMRT).

Table 2. Conformational study of electro pharmacological activity of *Desmodiumgangeticum*(L) in wistar rats by electrocardiogram

Treatment	P wave	Q wave	QRS complex	T wave	Beat/ min
Normal	0.023± 0.001	0.008± 0.001	0.032± 0.003	0.005± 0.001	336.000± 5.196
DG (500 $mg \cdot kg^{-1}$)	0.049± 0.009**	0.005± 0.001*	0.037± 0.008*	0.004± 0.000^{ns}	372.333± 7.506**

ns = not significant, *P<0.05, ** P<0.01 values compared to normal groups

Instrument Details

Make: PerkinElmer Clarus 500

Column Type: Capillary Column Elite-5ms (5%Phenyl 95% dimethylpolysiloxane)

Column length: 30m

Column id: 250µm

Oven program: 80ºC- 300ºC 2@ 10ºC/min

Injector temp. 280ºC

Carrier gas: He @1mL/min

Mass range: 40-450daltons

Scan Type: Full Scan

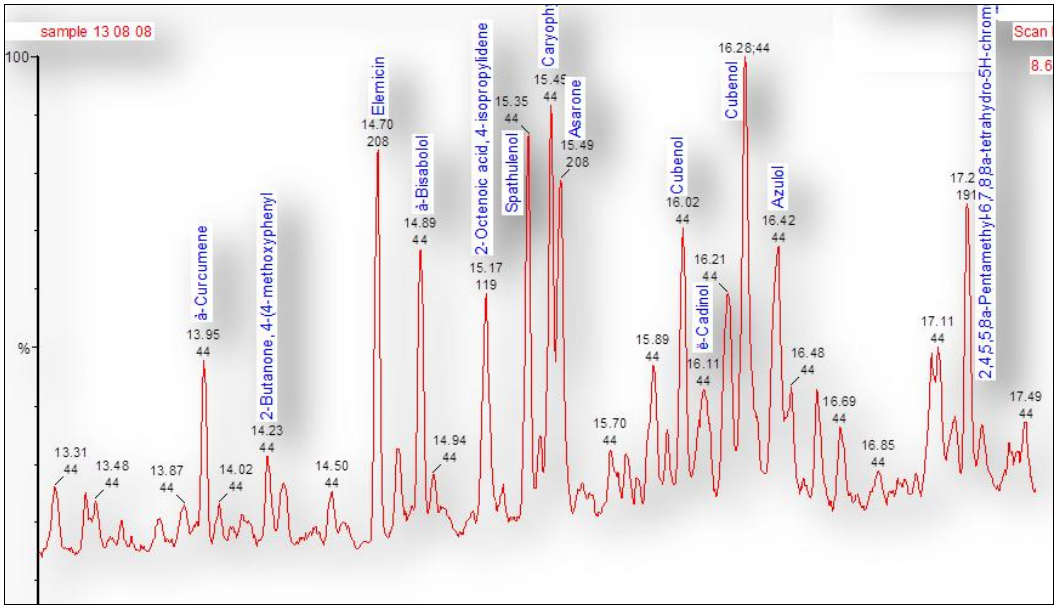


Figure 1. GC- MS of petroleum extract of *Desmodiumgangeticum* (L) root extract

Figure 2.Kymographical representation of *Desmodiumgangeticum*(L)in the presence and absence of antagonism in isolated rat hearts preparation; A- elevation of force of contraction of heart in the presence of DG; B- elevation of force of contraction of heart in the

presence of verapamil and DG; C- elevation of force of contraction of heart in the presence of atenolol and DG

Base line : 60 sec

Action of DG contact Time : 120 sec

Action of β Blocker + 500 µg DG contact time : 180 min

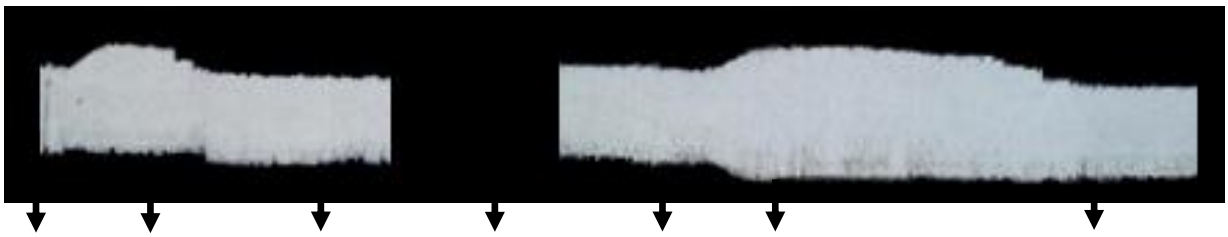
Action of Ca^{++} Blocker + 500 µg DG contact time: 180 min

Drum Rotation Speed : 0.25 mm/sec

A.Group I heart

Step

I

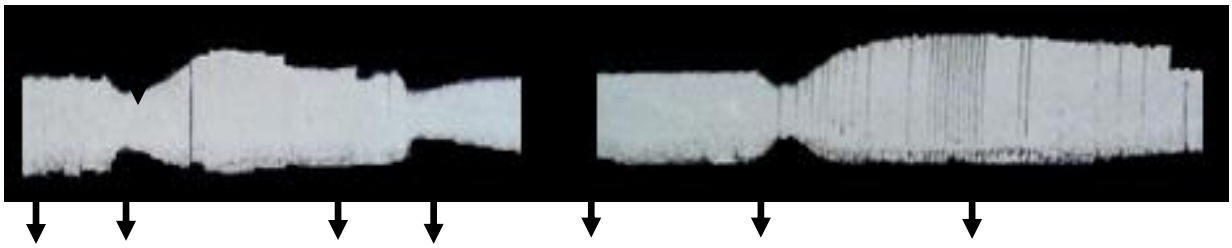


N 500 µg DGN N1000 µg DG N

B. Group II heart

Step

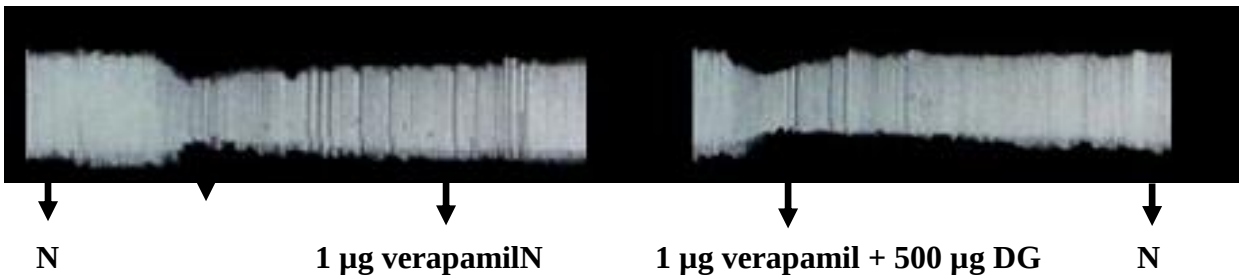
II



N50 µg atenolol + 500 µg DG N N 50 µg atenolol + 1000 µg DG

C. Group III

heartStepIII



N 1 µg verapamilN 1 µg verapamil + 500 µg DG N

N = Base line;DG = *Desmodiumgangenticum*

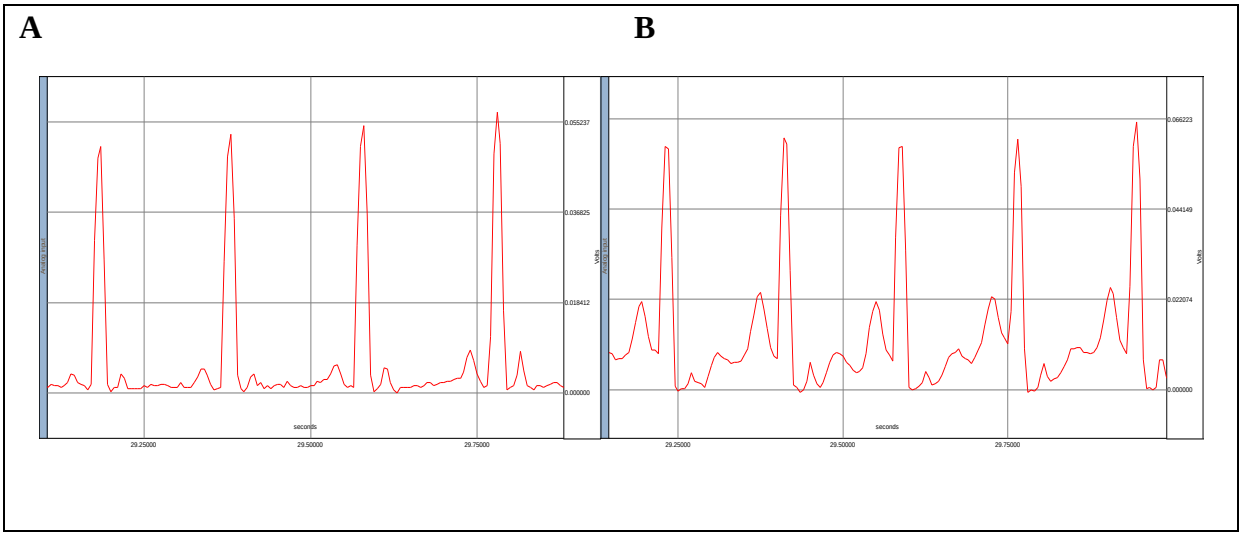


Figure 3. Pictorial Electrocardiogram leads showing A- normal ECG pattern of control group; B- slight elevation of 'P' wave and Q wave and marked elevation of QRS complex in DG treated group

DISCUSSION

The purpose of this study was to discover the possible mechanism of action of DG in targeted site. .In positive chronotropic activity, the extract elicited a potent cardiotonic effect, which was characterized by positive ionotropic and negative chronotropic actions. This effect was not significantly blocked by beta blocker .This action may have due to positive inotropic and chronotropic by Na^+K^+ATPaseinhibition by DG. It is cardiac glycosides like action leads ultimately to increase intracellular $Ca2^+$concentrations through Na^+/ Ca 2^+exchange and an associated increase in slow inward $Ca2^+$current as well as in transient $Ca2^+$current[11] $Ca2^+$induced $Ca2^+$release is a general mechanism that most cells use to amplify Ca2+ signals.[12] In heart cells, this mechanism is operated between voltage-gated L-type calcium channels (LCCs) in the plasma membrane and calcium release channel, commonly known as ryanodine receptors in the sarcoplasmic reticulum.[13] Cardiac glycosides inhibit the membrane bound Na^+ and K^+ activated adenosine triphosphatase, the receptor for cardiac glycosides. Hydrolysis of adenosine triphosphate by this enzyme provides the energy for sodium pump-the system in the sarcolemma of cardiac fibres that actively extrudes sodium and transports potassium into the fibres. It binds specifically to the Na^+ K^+ ATPase, inhibits its enzymatic activity and impairs the active transport of sodium and potassium. As a result, there is a gradual increase in intracellular sodium and decrease in potassium. Cardiac fibres possess the mechanism for exchange of intracellular sodium for extra cellular calcium. When inhibition of the pump of cardiac glycosides causes sodium to increase, there is an augmented

exchange of intracellular sodium for extra cellular calcium. This causes an increase in the net influx of calcium and presumably an increase in the concentration of calcium in the sarcoplasm. Calcium plays an important role in the genesis of oscillatory after-potentials in the His-Purkinje tissue.[14] The DG produced positive ionotropic effects similar to that of cardiac glycosides by stimulating the Na^+K^+ATPase inhibition. This effect appears to be due to a direct effect of digitalis like drugs on cardiac muscle probably involving an alteration in calcium flux.

Cardiac enzyme profile indicates that the extract of DG exhibited powerful cardio tonic like activity which manifested as a result of general decrease in the activity of Na^+K^+ATPase and $Mg2^+$ATPase and an increase in $Ca2^+$ATPase. Most important electrolytes like sodium, potassium, calcium, magnesium and bicarbonate provide inorganic chemicals for biochemical processes as well as act at the cell membrane to allow transmission of electrochemical impulse in nerve and muscle fibers.[15] The intracellular cation plays a significant role in the regulation of normal physiology and biochemistry of cardiac and smooth muscles. Dysregulation of these processes is an important factor in the genesis of various serious arrhythmias.[16] In heart cells, this mechanism is operated between voltage-gated Ltype calcium channels in the plasma membrane and calcium release channel in the sarcoplasmic reticulum.[13] Calcium Channel blocker is a LCC antagonist.[12] Since calcilum Channel blocker, blocks the cardio tonic action of the DG extract significantly, the extract might have produced its action by opening the voltage sensitive slow $Ca2^+$ channel. In connection with the cardiotonic effects observed one could see a relationship that exists between the inhibitory levels of the activities of $Mg2^+$ATPase and Na^+K^+ATPase.[17] The significant rise in the level of activity of $Ca2^+$ATPase might be due to the rise of cytosolic $Ca2^+$.[18]

In fact our GS/MS result reveals the presence of chemical compound in DG has effective agents against oxidative stress. It may give additional support for protecting cardiac muscle.The recent study shown that ouabain can trigger the Na^+/K^+-ATPase signaling cascade and then stimulate PKC-ε in the heart, resulting in the opening of the mitochondrial K_{ATP} channel and subsequent increases in the production of reactive oxygen species (ROS).[19,20 &21] Significantly, the positive inotropic effect of ouabain on myocardium can be attenuated by antioxidants.[22, 23& 24] These new findings suggest an important role of PKC-ε and ROS in the regulation of cardiac contraction.Binding of cardiac glycoside to this receptor

complex activates Src, leading to the recruitment and tyrosine phosphorylation of PLC-γ1 in pig kidney cells.[25]

The electropharmacological activity of DG in wistar rats by electrocardiogram showed that significant alteration of ECG patterns was observed in DG administered rats when compared to normal control rats. The characteristic findings were amplify in the P wave, QRS complex significant but there were no ST segment and abnormal elevation of heart rate when compare to normal animals. A slight change was observed in QRS complex when compared to normal control. The manifestation of ST segment elevation is some of the indicative signs of ischemia and consecutive loss of cellular may be characterized by ST segment elevation.[26& 27] In current study, there is no elevation of ST segments as well as abnormal heart rhythm. The QT interval correlates with measurements of cardia autonomic function, with cardiac vagal dysfunction resulting prolongation of the QT interval. QT interval represents both the dispersion and the lengthening of the action potential. The prologation of QT interval is considered as a hallmark parameter to analaysis the cardiac toxicity. From this result, DG administration showed that may altered ECG patterns and its protecting the cell membrane damage due to cardiac contractile disability.

SUMMARY AND CONCLUSIONS

According to our experimental data on infers that root extract of DGposses positive inotropic effect that mediate cardio protection through sodium potassium pump. This effect may be related to the delayed increase of Ca^{2+} in the myocardium due to the inhibition of sodium-potassium pump. The DG produced positive ionotropic effects similar to that of cardiac glycosides by stimulating the $Na^{+}K^{+}$ATPase inhibition. This effect appears to be due to a direct effect of digitalis like drugs on cardiac muscle probably involving an alteration in calcium flux.

The electrophysiological activity of DG in wistar rats by electrocardiogram showed that significant alteration of ECG patterns was observed in DG administered rats when compared to normal control rats. The characteristic findings were amplified in the P wave, QRS complex significant but there were no ST segment and abnormal elevation of heart rate when compared to normal animals. From this result, DG administration showed that may alter ECG patterns and it's protecting the cell membrane damage due to cardiac contractile disability. The study provides a scientific basis regarding the efficacy of DGagainst myocardial inability and the possibilities for exploring its therapeutic benefits of cardiovascular disease such as

congestive heart failure. The GC- MS observation highlights that DG is one of the challenging herbal drug for improving defense mechanisms in the physiological systems against oxidative stress and tissue injury caused by cardiac injury.

In future, the cardio-protective potential and mechanism of action ofDG at the molecular and cellular level have to discover in different pathway through receptor and ion channel based mechanism on cardiomyocytes in various events by both *in-situ*, *in- vivo* and *ex-vivo* techniques. The active constituents of DG have to be isolated and evaluated for cardio-protective activity using modern methods of drug discovery.

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