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In-vitro and *In-vivo* Contractile Effect of *Desmodium gangeticum* L. Extract on Isolated Rat Ileum and Electrocardiogram Recording.

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ABSTRACT

The present study was to evaluate the effect of petroleum ether extract of *Desmodium gangeticum* (L) on calcium channels in in-vitro and in-vivo model. In in-vitro study, the dose response curve was recorded with different concentration (250-4000 µg/ml) of *Desmodium gangeticum* (L) extract using rat ileum preparation. Followed by, antagonist effect of *Desmodium gangeticum* (L) extract on pre- contracted rat ileum using calcium chloride. Further to confirm the calcium channel blocking property of *Desmodium gangeticum* (L) extract, synergistic contractile dose response curve was recoded with different dose of *Desmodium gangeticum* (L) extract and fixed dose of verapamil 10^{-12} g/ml) as a standard calcium channel blocker. In in-vivo study, the effect of *Desmodium gangeticum* (L) extract on electrocardiogram (ECG) was investigated using Wistar rats. Two groups of twelve rats were divided into normal and test group. They were fed with normal saline and *Desmodium gangeticum* extract (500 mg/kg) per oral route. The in-vitro study results showed that the different concentration of *Desmodium gangeticum* extract significantly affect the calcium channels of the rat ileum preparation as evidenced by significant ($p<0.01$) inhibition of calcium chloride induced contraction and also significant ($p<0.01$) potentiation with verapamil mediated relaxation in rat ileum preparation. Further the in-vivo study results also demonstrated that per oral administration of *Desmodium gangeticum* extract significantly affect the rat heart electrophysiological properties as evidenced by significant ($p<0.01$) increase in P wave, Q wave and QRS wave but not T waves as compared with normal control group. It can be concluded that the extract of *Desmodium gangeticum* affects electrophysiological property of the heart in in-vivo and calcium channel blocking properties in in vitro. The study provides a scientific base regarding the potential of *Desmodium gangeticum* extract against myocardial ischemia-reperfusion injury and the possibilities for exploring its therapeutic benefits.

Keywords: *Desmodium gangeticum* L, ileum, electrocardiogram, synergistic effect

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INTRODUCTION

The World Health organization (WHO) estimates that 80% of the people in developing countries rely on Herbal medicines, mostly plant derived drugs, for their primary health needs [1]. Medicinal plants are commonly used in treating and preventing specific ailment and are considered to play a significant role in health care. *Desmodium gangeticum* (L) (DG) an herb 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 in the Indian System of Medicines (ISM) [2] for bitter tonic, febrifuge, digestive, antiemetic, anti- inflammatory agent for chest and other inflammatory conditions [3]. This plant has been used in Ayurveda for the treatment of various diseases like typhoid fever, urinary discharges, piles, asthma, bronchitis, vomiting, dysentery and hemicranias [4]. Roots of *Desmodium gangeticum* (L.) are 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 [5].

Three pterocarpenoids, gangetin, gangetinin and desmodin, are the major chemical constituents of the roots [6, 7]. Alkaloids such as indol-3-alkyl-amines and β-carbolines were isolated from aerial part and shown to possess anticholine sterase, smooth muscle stimulant, CNS stimulant and depressor response [8]. Gangetin, a pterocarpan, shows anti-fertility activity by affecting alkaline phosphatase activity in uterine fluids [9].

The aqueous extract of this species has been reported to show severe anti-writhing activity, moderate central nervous system (CNS) depressant activity and anti-leishmanial activity [10, 11]. Gangetin, a pterocarpnoid from D. gangeticum has been shown to possess anti-inflammatory and analgesic activities [12-14].

However, there was no scientific statement available on traditional claims of the petroleum ether extract of root of *Desmodium gangeticum* (L.). Therefore, keeping above facts in view the present study was planned to evaluate the effects of petroleum ether extract of *Desmodium gangeticum* (L.) on the functional evidence for voltage gated ion channels in isolated rat ileum preparation and its agonist effect of *Desmodium gangeticum* (L.) In - vitro and in vivo correlations.

MATERIALS AND METHODS

Chemicals

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

Collection, Identification and Authentication of Plant

The plant material used in this study was root parts of DG collected from Kerala state, India, during October 2013**.** The plant was identified and authenticated taxonomically by Botanist, Department of Environmental & Herbal Science, Tamil University, Tamil Nadu, India. A voucher specimen of the collected sample was also deposited in the same department for future reference

Preparation of Petroleum ether extract

The root was shade dried for 15 days and they were coarsely powdered. The powdered material was passed through 10-mesh sieve. They were soaked in petroleum ether in the ratio of 1:4 (w/v). The solvent was removed under reduced pressure and temperature using rotary vacuum evaporator. The yield of extract was found to be 2.1% w/w. A semi solid extract was obtained after complete elimination of petroleum ether and it was stored in refrigerator for experimental evaluation.

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Stock solution of DG

Ten 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 [15]. The following working concentrations were used from the stock: 1mg/0.1mL and 1mg/0.2mL of this concentration were added.

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

Wistar strain male rats, weighing 300-350 g were used for the present study. The animals were housed individually in polypropylene cages under hygienic and standard environmental conditions (22 ± 2ºC, humidity 60-70%, 12 h light/dark cycle). The animals were provided standard pellet diet (Tetragon Chemie Pvt. Ltd., Pet Care Division, Bangalore, Tamilnadu, India) and water ad libitum. All the animals were allowed to acclimatize for 10 days prior to the experiment. The study protocol was carried out as per the rules and regulation of the Institutional Animal's Ethics Committee (IAEC) of SASTRA University (IAEC no.48/ SASTRA/ IAEC/ RPP)

In - vitro study

Experimental Protocol I

Study design for Depolarization and its antagonist effect on rat ileum preparation

The experimental plan consisted of two steps

Step I: Tissues (n=6), DG

Stabilization	**DG (250 µg)**	**Drug Washout Period**	**DG (500 µg)**	**Drug Washout Period**

DG (1000 µg)	**Drug Washout Period**	**DG (2000 µg)**	**Drug Washout Period**	**DG (4000 µg)**

Step II: Tissues (n=6), DG Vs CB

CB (10^{-12} g/ml) + DG (500 µg)	**Drug Washout Period**	**CB (10^{-12} g/ml) + DG (1000 µg)**	**Drug Washout Period**	**CB (10^{-12} g/ml) + DG (2000 µg)**

CB= Calcium Channel Blocker

Step1: At the end of a 15 min stabilization period, tissues (n=6) received continuous superfusion along with DG at the dose of 250 µg, 500µg, 1000 µg, 2000 µg & 4000 µg. **Step 2:** In this period, tissues (n=6) were administered with CB (10^{-12} g/ml) Followed by DG at the dose of 500 µg, 1000 µg. & 2000 µg.

Experimental Protocol II

Study design for synergistic effect and its potency on isolated smooth muscle

Tissues (n=6), DG

Stabilization	**Ca^{++} (1ml)**	**Drug Washout Period**	**Ca^{++} (1ml) + DG (500 µg)**	**Drug Washout Period**

Tissues (n=6), DG Vs CB

Drug Washout Period	**Ca^{++} (1ml)**	**Drug Washout Period**	**Ca^{++} (1ml) + DG (1000 µg)**	**Drug Washout Period**	**Ca^{++} (1ml)**

CB= Calcium Channel Blocker

Experimental Procedure of rat ileum preparation and DRC Recording

Tissue preparation

Male rats were sacrificed by stunning and cervical dislocation (The experiments were performed in accordance with the regulations of the local Institutional Animal Ethical Committee regulations on Animal Care, 2009) and 2-cm pieces of the ileum were dissected from the ileum segment 10 to 20 cm proximal to the ileocecal valve. Tissues were mounted for tension recording and allowed to equilibrate for 1h in 20-mL chambers containing Tyrode's solution (136.0 mM NaCl, 5.0 mM KCl, 0.98 mM MgCl2, 2.0 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 5.5 mM glucose, pH 7.4, at 37°C) and bubbled with air. 3-6 fold magnification was adjusted with the resting load of 1g. The tissue was washed every 10 min up to 1 hr.

Measurement of contractile activity

The mechanical responses in ileum were recorded with a frontal lever and a kymograph. Dose Response Curve (DRC) was recorded using the standard drugs their antagonist effect was also recorded in dose dependent manner. Effects of drugs were recorded on smoked kymograph by means of an isotonic frontal writing lever. The contact time for baseline was 30 second and the contact time for drug action was 60 second. The tissue was superfused with three to four times by using physiological salt solution between every dose of drugs added.

In- vivo Method

Effect of *Desmodium gangeticum* (L.) in electrocardiogram analysis in wistar rats

The male Wistar rats (300 to 350 gm) were used for electrocardiogram analysis. ECG Lead were fixed to right arm, left arm and left leg and a bipolar transthoracic ECG is obtained on a Biopac MP100 Data Acquisition Unit (Biopac Systems, Inc., USA). During ECG recording, the rats were anesthetised by Ketamine at the dose of 100 mg/kg body weight. The normal ECG of animals (n=6) was performed followed by the oral administration of *Desmodium gangeticum* extract at the dose of 500 mg/kg, body weight. Bipolar transthoracic ECGs were obtained before dosing, and every 30 minutes post dosing for 2 hours. ECG of the animal performed using Acknowledge 3.9.0 software.

Hardware Setup

Snap the ECG100C module to the UIM100
Select channel 1 on the ECG100C amplifier
Place the ECG100C amplifier gain to 500, and the module switch settings to:
35 Hz LPN Filter and 0.05 Hz HP Filter

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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, (Abytech Sdn. Bhd, A-5-18, One South Streetmall, Jalan OS, Taman Serdang Perdana,43300 Seri Kembangan, Selangor Darul Ehsan, Malaysia) followed by Duncan's Multiple Range Test (DMRT), $p<0.05$ was considered to be significant.

RESULTS

Contractile effect of *Desmodium gangeticum* (L.) and antagonist effect verapamil on Desmodium mediated contractile effect in rat ileum

The effects of DG on rat ileum contractility were shown in Table 1 and Figure (1 & 2). When ileum was treated with different dose of DG (250-4000 µg) there was an increase in amplitude of contraction from 2 mm to 16 mm from base line and the effect was dose dependent. The lower concentration of DG (250 µg) was produced the initial contraction of muscle. The maximum contraction caused by DG at the concentration of 4000 µg. The contractile effect of DG at the concentration of 500 µg was partially antagonized by verapamil (10^{-12} g/ml). At the same concentration of verapamil was completely antagonize the 1000 and 2000 µg of DG induced rat ileum contraction.

Table 1: Dose response effect of Desmodium gagenticum (L.) on rat ileum preparation in the presence and absence of verapamil.

Treatment	Dose	Response (mm)
DG	250 µg	2 .2± 0.11
DG	500 µg	4.83± 0.29**
DG	1000 µg	10.83± 0.76**
DG	2000 µg	13.33± 0.58**
DG	4000 µg	16.24± 0.61**
CB + DG	10^{-12} g/ml + 500 µg	3.33± 0.58 **
CB + DG	10^{-12} g/ml + 1000 µg	2.50± 0.50
CB + DG	10^{-12} g/ml + 2000 µg	1.33± 0.58**

DG = *Desmodium gangeticum*; CB = Calcium channel blocker (Verapamil)

Activity is expressed as mm for tissue contraction. Values are expressed as mean ± S.D. ** Significant difference ($P<0.01$) between base line vs DG 250,500, 1000, 2000 &4000 µg; base line vs 10^{-12} g/ml CB& 500, 1000 & 2000µg ; $p<0.01$Duncan's Multiple Range Test (DMRT).

Fig 1: Dose response curve *Desmodium gangeticum* (L.) on rat ileum preparation in the presence and absence of verapamil

Baseline contact time : 30 sec
Action of DG contact time : 30 sec
Action of verapamil (CB) contact time : 60 sec
Rotation speed : 0.25mm/ sec

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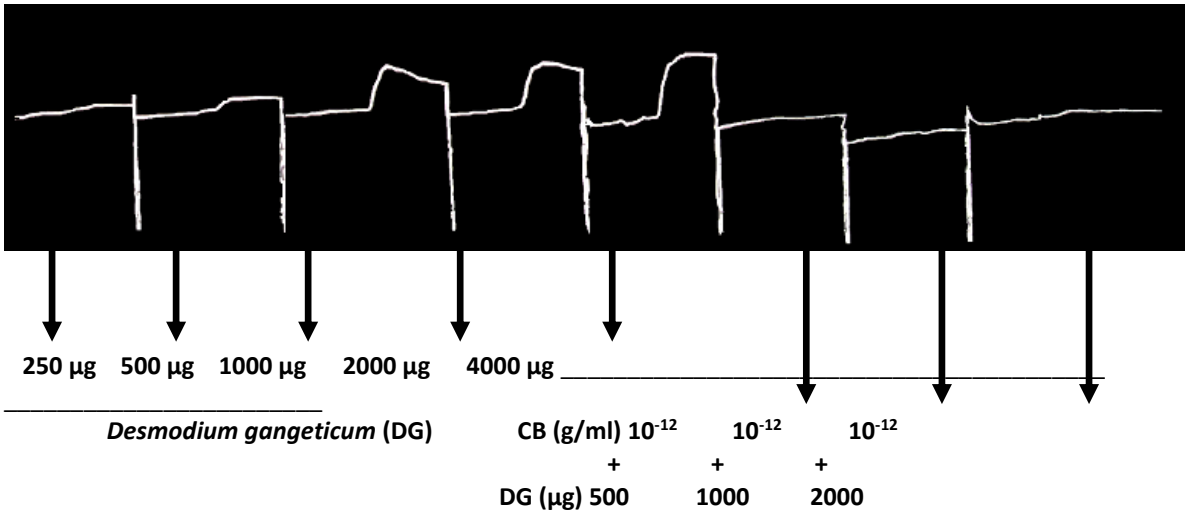
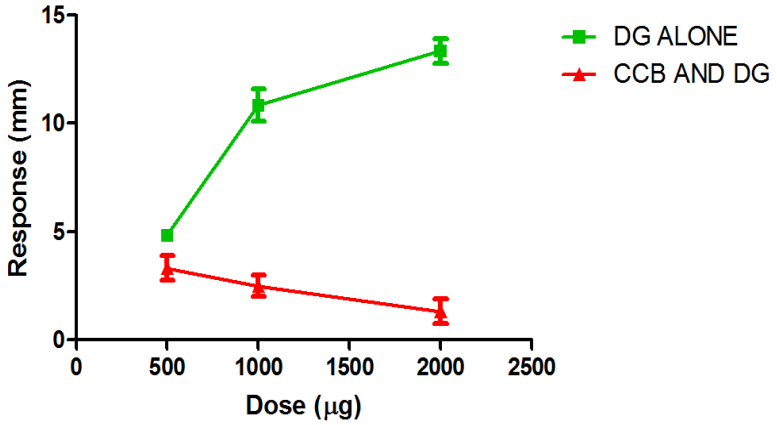


Figure 2: Graphical representation of *Desmodium gangeticum* (L.) on isolated ileum preparation in the presence and absence of antagonism



Additive and synergistic effect of *Desmodium gangeticum* (L.) and its potency effect in isolated smooth muscle preparation

The additive contractile effect of DG and calcium chloride was shown in Figure 3. The contractile amplitude of 3.5 to 4.0 mm was recorded when calcium chloride added into bath fluid at the concentration of 0.5 ml of 1%. Interestingly, additive contractile amplitude was noted with 500 µg and 1000 µg DG with calcium chloride at the concentration of 0.5 ml of 1%.

Both the concentration of DG was significantly ($p<0.001$) increased the contraction of the rat ileum with the presence of calcium chloride as compared with only calcium chloride mediated contraction.

Figure 3: Kymographical representation of *Desmodium gangeticum* (L.) and its potency effect on isolated smooth muscle preparation

Basic Contact Time: 30 sec : 30 Sec
Action of DG along with $Cacl_2$ Contact Time : 60 Sec
Actions of $Cacl_2$ contact Time : 60 Sec
Rotation Speed : 0.25 mm/ Sec
Calcium Chloride Concentration : 1% Soln.

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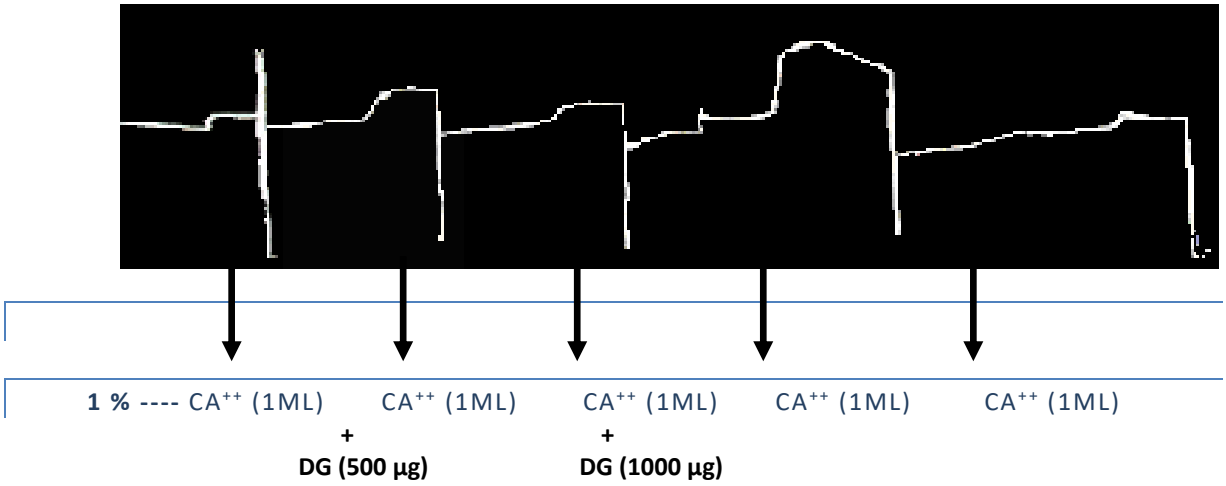


Effect of *Desmodium gangeticum* (L.) in electro cardiogram (EGC) of the anesthetized rat

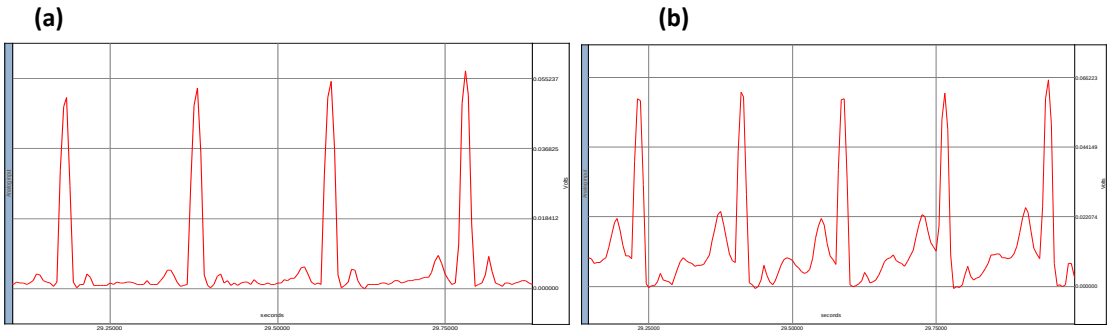
Electrocardiogram patterns of normal and experimental animals and changes are shown in **Table 2 & Fig. 4**. Normal control and DG at the dose of 500mg/kg treated rats showed a significant ($P < 0.05$) increase in ST-segment, QT interval along with a significant ($P<0.05$) increase in the P wave, QRS complex and RR interval as compared to the normal control group.

Table 2: Conformational study of electrophysiological activity of *Desmodium gangeticum* (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 (500mg/kg)	0.049± 0.009**	0.005± 0.001**	0.037± 0.008**	0.004± 0.000	372.333± 7.506**

Values are expressed as mean ± S.D. ** Significant difference ($P<0.01$) between Normal vs DG treated rats. $p<0.01$Duncan's Multiple Range Test (DMRT).

Figure 4: Pictroial Electrocardiogram representation of Wistar Albino rats showing: a normal (a) & DG treated ECG pattern was changed (b)



DISCUSSION

The present study results showed that the contractile effect of petroleum ether extract in rat ileum preparation by affecting its Ca^{2+} ion channel. The in-vitro results showed that DG increases the contraction of ileum in a concentration-dependent manner as evidenced by significant increase in amplitude of contraction. The excitatory amplitude effect of DG on the contractile frequency of ileum was blocked by pre-treatment with verapamil. The potential contractile action of DG was might be dependent on extracellular Ca^{2+}. In

smooth muscle, there are several factors that increase the frequency of contraction, such as increased pacemaker activity and shortened action potential[16]. The extracellular Ca^{2+} and Ca^{2+} influx is essential for excitability and contractility in smooth muscle cells. Intracellular Ca^{2+} elevation is the main regulating factor of smooth muscle tension [17]. DG stimulatory effect of Ca^{2+} was increased, indicating that potentiated Ca^{2+} mediated spasmogenic effect.

It is well recognized that voltage- gated Ca^{2+} channels are important for regulating entry of extracellular Ca^{2+} in smooth muscle cells. The influx of Ca^{2+} is essential for the development of muscle tension. The contractile effects of the smooth muscles of the intestines are due to the cytosolic free calcium levels [18]. There is an exchange of calcium between extracellular and intracellular calcium stores. The voltage dependent calcium channels (VDCs) are responsible for the influx of calcium into the sarcoplamic reticulum [19, 20]. This leads to periodic depolarization and repolarization of the intestinal tissues that accounts for its spontaneous responses. The effect of different concentration of calcium chloride on L-type calcium channel on smooth muscle strip has been demonstrated. The contractile effect stimulated by calcium chloride. Calcium chloride treated isolated tissues that opens the voltage–operated calcium channels and allows the extra-cellular calcium into the cytosol resulting in the depolarization of the tissue [21, 22]. The present study confirmed that DG may increase the extracellular calcium, elicits marked stimulation of intestinal smooth muscle. The calcium channel blocker has completely antagonist effect against increase level of intracellular calcium ion. These findings indicate that the stimulating effect of DG mediated through channel based pathway. Calcium channel blockers such as verapamil, diltiazem, nifidipine interfere with smooth muscle contraction by binding to voltage sensitive calcium channels in a way that prevents depolarization induced Ca2+ influx [23]. Here it was used verapamil as a standard drug for comparison between DG and verapamil. The additive effect of *Desmodium gangeticum* study was may be Ca2+ influx and potentiate the action of extract.

The electrophysiological activity of *Desmodium gangeticum* (L.) 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. No 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[24]. In current study, there was no elevation of ST segments as well as abnormal heart rhythm. The QT interval correlates with measurements of cardiac 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 prolongation of QT interval is considered as a hallmark parameter to analysis the cardiac toxicity. 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 ion channel pathway conformational study has been demonstrated by the effects of ***Desmodium gangeticum* (L.)** on intestinal smooth muscle contractility were investigated on isolated ileum preparation of rats. These results showed that DG increases the frequency of ileal contraction in a concentration-dependent manner. The excitatory amplitude effect of DG on the contractile frequency of ileum was blocked by pre-treatment with calcium channel blocker. The action of ***Desmodium gangeticum* (L.)** was might be dependent on extracellular $Ca2^{+}$. DG stimulatory effect of Ca2+ was increased, indicating that potentiated Ca2+ spasmogenic effect.

The electro pharmacological activity of *Desmodium gangeticum* (L.) 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. 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 ***Desmodium gangeticum* (L.)** against myocardial ischemia-reperfusion injury and the possibilities for exploring its therapeutic benefits. Considering the safety, efficacy and acceptability of ***Desmodium gangeticum* (L.)** further researches are needed to establish its therapeutic and preventive role in myocardial ischemic reperfusion injury. These observations highlight that ***Desmodium gangeticum* (L.)** is one of the challenging herbal drug for improving defense

mechanisms in the physiological systems against oxidative stress and tissue injury caused by ischemia-reperfusion injury.

In future, the potential and mechanism of action of ***Desmodium gangeticum* (L.)** at the molecular and cellular level have to discover in different pathway through receptor and ion channel based mechanism on smooth muscle and cardiac muscle in various events by both in-situ, in- vivo and ex-vivo techniques. The active constituent of ***Desmodium gangeticum* (L.)** have to isolated and evaluated for tissue protective activity using modern methods of drug discovery.

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