Journal of Medicinal Plants Studies

www.PlantsJournal.com

ISSN 2320-3862
JMPS 2014; 2(6): 01-08

Received: 16-09-2014
Accepted: 11-10-2014

Abi Beaulah G
Department of Chemistry, Auxilium College, Vellore, Tamil Nadu, India.

Mohamed Sadiq A
Department of Biochemistry, APCAS, Kalavai, Tamil Nadu, India.

Sivakumar V
Department of Biochemistry, APCAS, Kalavai, Tamil Nadu, India.

Jaya Santhi R
Department of Chemistry, Auxilium College, Vellore, Tamil Nadu, India

Correspondence:
R. Jaya Santhi
PG and Research Department of Chemistry, Auxilium College (Autonomous), Vellore, Tamil Nadu, India.

Cardioprotective activity of methanolic extract of *Croton sparciflorus* on isoproterenol induced myocardial infarcted wistar albino rats

Abi Beaulah G, Mohamed Sadiq A, Sivakumar V and Jaya Santhi R

Abstract
The methanolic extract of *Croton sparciflorus* was studied for the cardioprotective activity using Isoproterenol induced myocardial infarction in wistar albino rats. Methanolic extract of this plant showed significant cardioprotective effect by lowering the serum levels of various biochemical parameters like CPK, LDH and transaminases in the selected model. The results obtained in the present work are in turn with histopathological examinations of heart tissue sections and are comparable with the standard cardioprotective drug. The results also suggests that the biologically active phytoconstituents such as flavonoids, glycosides, alkaloids present in the methanolic extract of plant which is confirmed from the qualitative analysis may be responsible for the significant cardioprotective activity.

Keywords: *Croton sparciflorus*, Cardioprotective activity, Isoproterenol, Myocardial infarction, CPK.

1. Introduction
Myocardial infarction (MI) is a complex phenomenon affecting the mechanical, electrical, structural and biochemical properties of the heart [1]. MI and the resultant complication in cardiac function is one of the leading causes of death for both men and women [2]. Due to changing lifestyles in developing countries, particularly in urban areas, MI is making an increasingly important contribution to mortality statistics [3]. Antioxidant compounds, highly present in plants have shown protective effects against diseases without reducing their therapeutic efficacy. Moreover, there is a growing interest in the usage of natural antioxidants as a protective strategy against cardiovascular related problems such as ischemia reperfusion [4]. The chemo-therapeutic agents, which inhibit the free radical formation and can reduce the risk of heart diseases, have gained imperative value in the modern medicines [5, 6]. Herbal medicines having antioxidant properties, may therefore, have a protective role in cardiovascular diseases [7].
Isoproterenol (ISPH), a synthetic catecholamine and β-adrenergic agonist that causes severe stress in myocardium and infarct-like necrosis of the heart muscles [8]. ISPH induced myocardial injury involves membrane permeability alterations, which brings about the loss of functions and integrity of myocardial membranes [9]. ISPH induced myocardial necrosis is a wellknown standard model to study the beneficial effect of many drugs on cardiac dysfunction. Although modern drugs are effective in preventing cardiovascular disorders, their use is often limited because of their side effects [10]. Herbal medicine is increasingly gaining acceptance from the public and medical professionals due to advances in the understanding of the mechanisms by which herbs positively influence health and quality of life [11]. The use of herbal medicines has been steadily increasing over the past decade because of the ill effects of the chemical medicines. Many medicinal plants have been found to possess beneficial effects in pathological conditions like cancer, liver diseases, cataract and myocardial ischemia [12, 13]. A considerable number of these plants and plant based products have been widely used [14]. Therefore, interest in the examination of plants as potential sources of new drugs is increasing. The prophylactic and therapeutic effect of many plant foods and extracts in reducing cardiovascular disease has been reviewed as they are inexpensive, efficacious and safe [15].
Croton sparciflorus Morong (*synonym* = *Croton bonplandianum* Bail.) belongs to the family Euphorbiaceae is a small annual herb, growing up to 1-2 feet tall found in southern parts of Indian subcontinent. The latex of this plant is being used in folk medicines by tribal people of Tamil Nadu to treat wasp sting [16].

The powdered leaves are useful in controlling high blood pressure and for the treatment of skin diseases, cuts and wounds [17-18]. The hexane, chloroform, methanol and ethyl acetate extract of the different parts of the plants exhibit antifungal activity [19]. Exhaustive literature survey shows that very few research work has been carry out in the plant *Croton sparciflorus*. So an attempt has been made for the first time to investigate the cardioprotective activity of the plant through curative mode of treatment. This study is an effort to evaluate the cardioprotective effects of the methanolic extract of whole plant of *Croton sparciflorus* in IDPH induced myocardial infarction in Wistar *albino* rats.

2. Materials and Methods

2.1 Experimental animals

Male Wistar albino rats numbering 30 of 8 weeks old, weighing 160–210 g, were obtained from the Laboratory Animal centre and were housed in solid bottom polycarbonate cages under controlled environmental conditions (22 ± 2 °C with 55 ± 5% humidity and a 12/12 hour light/dark cycle). The rats were acclimatized to the environment for 1 week prior to the initiation of the experiment. The rats were offered standard diet supplied by Hindustan Lever Ltd, Mumbai and water *ad libitum*. Ethical clearance was obtained from the Institutional Animal Ethical Committee [No:1282/ac/09/CPCSEA]. All procedures were in strict compliance with relevant laws, the Animal Welfare Act, Public Health Services Policy and guidelines established by the Institutional Animal Care and Use Committee of the University.

2.2 Plant collection

Croton sparciflorus is an herb that grows throughout south India in the fields. The plant was collected in the month of August from Auxilium College campus, Vellore, Tamil Nadu, India and was taxonomically identified by the Department of Botany, Auxilium College, Vellore, Tamil Nadu, India and a voucher specimen [CS-ACK-CHEM-001] is retained as an herbarium in the department for future reference.

2.3 Extraction

The freshly cut plants were dried in the shade with active ventilation at ambient temperature and pulverized to a coarse powder using mechanical grinder and sieved with the help of 40 mesh size. The powder was percolated in methanol for about 20 days. The miscella was then concentrated using a vacuum rotary evaporator under reduced pressure. The dark brownish semisolid extract was preserved in tightly closed container and used for the analysis.

2.4 Experimental Design

The experimental rats were divided into 5 groups, each consisting of 6 animals.

Group I, the control group, served as negative control received a control diet and a single intra peritoneal (i.p.) injection of normal saline (2.5 ml/kg).

Group II, the ISPH induced group, served as a positive control was given a single i.p. injection of ISPH, 2 mg/kg of bodyweight on 14th day and a control diet.

Group III, the Rats were administered with methanolic extract of *Croton sparciflorus* 100 mg/bw, given orally once daily up to 30 days, followed by ISPH administration on 14th day .

Group IV, the Rats were administered with methanolic extract of *Croton sparciflorus* 200 mg/bw, given orally once daily up to 30 days, followed by ISPH administration on 14th day .

Group V, the Isoproterenol induced group, after the development of myocardial infarction, was treated with the standard drug Verapamil (5µmol/kg body weight) [Sigma-SO292, USA].

The duration of the treatment was 30 days. At the end of the treatment, blood was collected from all the groups by puncturing the retro-orbital plexus and was allowed to clot at room temperature and serum was separated by centrifugation at 2500 rpm for 10 min. The animals were sacrificed to remove the heart for histopathological studies.

2.5 Acute toxicity studies

The dose limits were selected on the basis of oral acute toxicity studies in rats, in accordance with the Organization for Economic Co-operation and Development **(**OECD) guidelines [20]. The acute toxicity test was carried out in 5 rats by giving doses of 100, 200, 300, 400, 500 mg/kg body weight. All groups of test drug showed neither any toxic effect, nor any lethal effect in the dose range of 100 to 500 mg/kg body weight. So a minimum dose of 100 mg/kg and 200 mg/kg of body weight was taken for further studies.

2.6 Biochemical estimation from serum

The serum was analyzed for the presence of different enzymes related to myocardial infarction such as Lactate dehydrogenase (LDH) [21], Creatine kinase-MB fraction (CK-MB) [22], Aspartate transaminase (AST) [23, 24], Alanine transaminase (ALT), Total cholesterol, Total triglycerides (TGL), High density lipoproteins (HDL) and Low density lipoproteins (LDL) [25]. All analysis was performed with commercially available kits based on the references using analyzer.

2.7 Biochemical estimation from Tissue homogenate

After sacrificing the rats by cervical dislocation, the heart tissue was excised immediately and washed with chilled isotonic saline. The heart tissue homogenate was prepared in 0.05 M phosphate buffer, pH 7.4 and homogenated in tissue homogenizer at 2,000 rpm for 10 min. And used for analyzing antioxidant activities like Lipid peroxides (LPO) [26], Superoxide dismutase (SOD) [27] and Catalase (CAT) [28] activity.

2.8 Histological Studies

After sacrificing the rats by cervical dislocation, some portion of atria and ventricle was collected, washed in normal saline and was perfused with 10% formalin and stored in the same for histopathological studies. It was fixed by using 40% formaldehyde as fixative for 24 hours and dehydrated with alcohol. All tissues were cleaned and embedded by using xylene and molten paraffin wax (melting point 58-60 ^{0}C). Sections were cut at 5 µm thickness and were stained by double staining. To differentiate the nucleus and cytoplasm, the basic dye haematoxylin and the acid dye eosin were used [29, 30]. Electron micrographs were performed using transmission electron microscope and photographed by photomicrography. The sections were then viewed under the

Nikon microscope, ECLIPSE E400, model 115, Japan.

2.9 Statistical Analysis
The data were collected, calculated and tabulated as Mean ± SD and significant difference between the groups were analyzed by Student's 't' test using SPSS software version 20 and the 'p' values were used to judge the significant level.

3. Results and Discussion
Myocardial infarction remains a leading cause for death worldwide and prompt treatment for a heart attack is indispensable to save the life. In the traditional Indian medicinal system, a major role has been played by the plants, especially, in the aspect of cardio protection. Several herbs and herbal products have been recommended for prophylactic and therapeutic effects in reducing cardiovascular diseases (CVDs) and that have been reviewed [31]. These include *Allium sativum* (garlic) [32], Allium cepa (onion) [33], *Daucus carota* (wild carrot) [34], Ocimum sanctum (tulsi), *Withania somnifera* (ashwagandha) and *Zingiber officinalis* (ginger) [35]. In this context, there is a need to reveal the cardio protective activity of extract of the *Croton sparciflorus* plant. Myocardial ischaemia results from the reduction of coronary flow to such an extent that supply of oxygen to the myocardium does not meet the oxygen demand of myocardial tissue. When this ischaemia is prolonged and irreversible then myocardial cell death and necrosis occurs which is defined as myocardial infarction [36]. Recent studies suggest that increased free radical formation and subsequent oxidative stress associated with the occurrence of a relative deficit in the endogenous antioxidants, may be one of the mechanisms for the development of heart failure after myocardial infarction [37]. In our study, ISPH is used to induce myocardial damage and it has been found to cause a severe stress in the myocardium resulting in necrosis of the heart muscle. It is also well known to generate free radicals and stimulate lipid peroxidation, which may be a causative factor in irreversible damage to the myocardial membrane in experimental myocardial infarction [38]. The rat model of ISPH induced myocardial infarction offers a reliable non-invasive technique for studying the effects of various potential cardioprotective agents [39]. Amongst various mechanisms proposed to explain ISPH induced cardiac damage, generation of highly cytotoxic free radicals through auto oxidation of catecholamines has been implicated as one of the important causative factors [40]. The pathophysiological changes following ISPH administration are comparable to those taking place in human myocardial alterations. Hence ISPH induced myocardial infarction model was used in the present study [41, 42].

The oral acute toxicity study of methanolic extract of *Croton sparciflorus* showed no mortality up to 500 mg/kg body weight in rats and did not produce any toxic symptoms. Hence the extract was considered to be safe and non-toxic for further pharmacological screening. The maximum nonlethal dose was found to be 4000 mg/Kg body weight, orally was also reported in the previous studies [43]. This may be due to the broad, non-toxic range of the plant, where the plant extract showed a high safety index.

The results of cardioprotective activities of methanolic extract of *Croton sparciflorus* against ISPH induced myocardial infarction are shown in Table-1.

Table 1: Effect of Croton sparciflorus extract in Cardiac marker enzymes

	CPK (IU/L)		LDH (IU/L)	
	Serum	**Tissue homogenate**	**Serum**	**Tissue homogenate**
Group I	45.96 ± 2.43	49.96 ± 1.43	127.49 ± 1.87	123.39 ± 1.07
Group II	156.08 ± 6.50	30.45 ±0.55#	247.59 ± 3.01	99.45 ±0.33#
Group III	43.36 ± 1.61*	42.33 ±0.98	133.06 ± 3.14*	125.07 ±2.44
Group IV	49.15 ± 0.79*	46.15 ±0.22	136.79 ± 4.19*	130.45 ±1.47
Group V	49.03 ± 1.07*	48.06 ±2.01	136.22 ± 2.76*	133.00 ±2.54

Values are mean± SD;
* Significant reduction in Group III, IV and V compared to Group II ($p < 0.05$);
# Significant reduction in Group II compared to Group I ($p < 0.05$).

Heart contains an abundant concentration of diagnostic marker enzymes like CPK, LDH and transaminases (AST & ALT) and once the heart is metabolically damaged, it releases its content into the extra cellular fluid (ECF). In the present study, it was noted that in ISPH myocardial infracted rats, the increased activities of the serum marker enzymes accompanied by their concomitant reduction in the heart homogenate confirm the onset of myocardial necrosis. Hence the total concentration of the marker enzymes was found to be decreased in heart tissue of ISPH infracted rats as compared to control, which may be the reflection of consequences of cellular injury due to lipid peroxides. ISPH is well known to generate free radicals and to stimulate lipid peroxidation, which may be a causative factor for the irreversible damage to the myocardium [44]. CPK is a muscle specific enzyme mainly for heart and brain; therefore, its increase in serum is the result of myocarditis, cardiac insufficiency, arrhythmias and myocardial infarction [45]. The inhibition of glycolytic pathways of energetic in cardiac muscles of ISPH treated rats, lead to glycogen break down, loss of pyridine nucleotides and ATP, which resulted in an increase in intracellular calcium [46]. The calcium is an essential factor in phospholipase-associated degradation of membrane phospholipids, causing damage of mitochondrial membrane, which leads to impaired electron transport along with the leakage of lysosomal enzymes. Treatment with Calcium antagonist nifedipine, β-adrenergic blocker, propranolol and lipid lowering drug like guggulsterone partially reversed the changes in sarcolemma enzymes and stimulate the Calcium uptake in the damaged heart [47]. Decreased activities of these enzymes were due to the leakage from the damaged heart tissues into the blood stream as a result of necrosis induced by ISPH in rats [48]. It is observed that these cardio specific marker enzymes are released from the heart into the blood during myocardial damage due to myofibril degeneration and myocyte necrosis. Significant increase was noticed in the activities of cardiac markers like LDH and CPK in plasma of ISPH-treated rats, which is consistent with earlier reports [49, 50] might be due to enhanced

susceptibility of myocardial cell membrane to the ISPH mediated peroxidative damage, resulting in increased release of these diagnostic marker enzymes into the systemic circulation.

The levels of CPK was found to be 45.96 ± 2.43 in Group I normal rats which increased to 156.08 ± 6.50 when the rats were treated with ISPH. Then the rats on treatment continuously with the plant extract (100 mg and 200 mg) for 30 days, showed a decrease in the values, which were found to be 43.36 ± 1.61 and 49.15 ± 0.79 respectively, which were comparable with the standard drug Verapamil treated group whose values were found to be 49.03 ± 1.07. The levels of LDH was found to be 127.49 ± 1.87 in Group I normal rats which increased to 247.59 ± 3.01 when the rats are treated with ISPH. Then the rats on treatment continuously with the plant extract (100 mg and 200 mg) for 30 days, showed a decrease in the values, which were found to be 133.06 ± 3.14 and 136.79 ± 4.19 respectively which were comparable with the Verapamil treated group whose values were found to be 136.22 ± 2.76. But the levels of CPK and LDH reduced in the heart muscles, when the rats were induced with ISPH. But on treatment with the plant extract, the levels of CPK and LDH increased because of the cardioprotective activity of the plant which is comparable with the standard drug treatment.

Transaminase (AST and ALT) levels are a sensitive indicator of liver cell injury and are also helpful in recognizing other diseases also. AST is found in decreasing order of concentration in the liver, cardiac muscle, skeletal muscle, kidney, brain, pancreas, lungs, leucocytes and erythrocytes. ALT is maximally concentrated in liver and is relatively less specific for the other muscle injury. Both enzymes are released into the body in increasing amounts when the tissues are damaged. Despite being a sensitive indicator of liver cell injury, the transaminase correlates poorly with severity of cardiac disease also. The variations in the levels of the transaminases in ISPH induced wistar albino rats for myocardial infarction were shown in **Table-2**.

Table 2: Effect of Croton sparciflorus extract in Transaminases enzymes

Parameters	Group I	Group II	Group III	Group IV	Group V
AST (IU/L)	96.40 ± 2.86	210.40 ± 6.79	93.35 ± 1.59	99.15 ± 0.79	99.16 ± 0.88
ALT (IU/L)	64.58 ± 1.57	173.20 ± 5.91	67.51 ± 2.10	65.93 ± 1.93	66.76 ± 1.45

Values are mean± SD; Significance p< 0.05, versus control.

The levels of AST and ALT were found to be 96.40 ± 2.86 and 64.58 ± 1.57 in Group I normal rats which increased to 210.40 ± 6.79 and 173.20 ± 5.91when the rats were treated with ISPH respectively (Table.2). Then the rats on treatment for 30 days continuously with the plant extract showed a decrease in the values, which was found to be 93.35 ± 1.59 and 67.51 ± 2.10 for Group III and 99.15 ± 0.79 and 65.93 ± 1.93 for Group IV which is comparable with the standard drug Verapamil treated group whose values were found to be 99.16 ± 0.88 and 66.76 ± 1.45. The prevention of the leakage of AST and ALT from the heart tissues in Group III and Group IV suggested a potential protective effect of the plant extract against ISPH induced heart damage [51]. The effect could be due to the stabilization of heart membrane by the plant extract with a consequent decrease in the leakage of these marker proteins. The tendency of these enzymes to return to near normal in extract administered group is a clear manifestation of the cardioprotective activity of the extract.

The lipid profile is a group of tests comprising total cholesterol, triglycerides, HDL and LDL cholesterol. The lipid profile is used, together with other risk factors, to assess a person's risk of cardiovascular disease (CVD). The results of the levels of lipid profile for the treatment period in wistar albino rats are given in **Table-3**.

Table 3: Effect of Croton sparciflorus extract in Lipid profile

	Cholesterol (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Group I	96.40 ± 2.86	64.58 ± 1.57	33.90 ± 1.91	64.58 ± 1.57
Group II	210.40 ± 6.79	173.20 ± 5.91	24.12 ± 1.30	173.20 ± 5.91
Group III	93.35 ± 1.59	67.51 ± 2.10	36.43 ± 2.00	67.51 ± 2.10
Group IV	99.15 ± 0.79	65.93 ± 1.93	32.68 ± 1.52	65.93 ± 1.93
Group V	99.16 ± 0.88	66.76 ± 1.45	32.95 ± 1.93	66.76 ± 1.45

Values are mean ±SD; Significance p< 0.05, versus control

The significant acute myocardial infarction was indicated by the elevated levels of total cholesterol, Triglycerides (TGL) and low density lipo proteins (LDL) in Group II. In the treated groups III and IV, the levels were reduced nearest to the normal values, because of the action of plant extract. The results were significantly comparable with the standard group V. The levels of HDL reduced to 24.12 ± 1.30 in DEN induced group, reflecting the reduction of Good cholesterol. But in the treated groups III (36.43 ± 2.00) and IV (32.68 ± 1.52) the HDL level increased significantly which is comparable with the Group V (32.95 ± 1.93).

Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals [52]. Formation of free radicals in a biological system includes xanthine oxidase, activated neutrophils, direct donation of electron from myocardial electron transport chain, catecholamine oxidation, cyclo-oxygenase and lipoxygenase enzymes [53]. The treatment with free radical scavenger reduces infarct size after regional myocardial ischemia and reperfusion in a canine preparation has been reported. These reports provide strong support for the hypothesis that oxygen radical formation is a major contributor to reperfusion injury. Naturally occurring antioxidants might be useful in oxidative stress. Lipids are the most susceptible macromolecules to oxidative stress. Free radicals peroxidase polyunsaturated membrane lipids and damage their structure and functions [54]. The results of CAT, SOD and MDA are

given in **Table 4.**

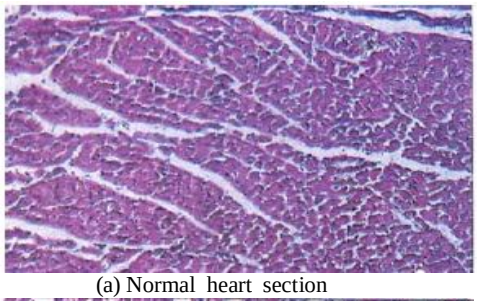
Table 4: Effect of Croton sparciflorus extract on the level of antioxidant enzymes

Parameters	CAT units/ml	SOD units/ml	MDA nmol/dl
Serum			
Group I	1.07± 0.08	4.32± 0.45	109.50± 12.08
Group II	0.55± 0.23	1.77 ±0.06	230.60 ±4.82
Group III	1.25 ±0.11	2.27± 0.38	121.80 ±1.52
Group IV	0.82 ±0.13	3.05±0.75	101.83± 9.61
Group V	1.28± 0.10	4.22±0.56	92.66 ±1.00
Heart tissue homogenate			
Group I	1.67±1.05	4.11±2.01	99.50± 2.28
Group II	0.32±2.01	1.05±0.15	130.60 ±4.82
Group III	0.99±2.00	2.97±0.89	121.80 ±1.52
Group IV	1.46±0.45	4.01±0.22	100. 3± 9.61
Group V	1.89±0.55	4.27±1.03	92.66 ±1.17

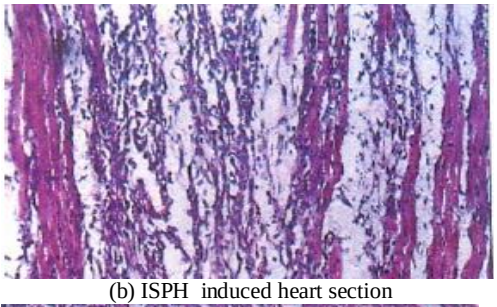
Values are mean ±SD; Significance p< 0.05, versus control

Malondialdehyde is one of the many products of lipid peroxidation. In the present investigation, we observed a significant elevation in MDA levels, 230.60 ±4.82 and 130.60±4.82 in the ISPH induced group II as compared to control group I which showed a value of 109.50± 12.08 and 99.50±2.28 in serum and heart tissue respectively. The results clearly are depicting the injured state of myocardium following ischemia-reperfusion injury. The treatment with methanolic extract of Croton sparciflorus for 30days orally, elevated the malondialdehyde level in serum and heart tissue significantly compared to control animals. The levels of CAT and SOD showed a significant decrease in the value of Group II compared to group I. But on treatment with the plant extract significantly elevates the CAT and SOD levels.

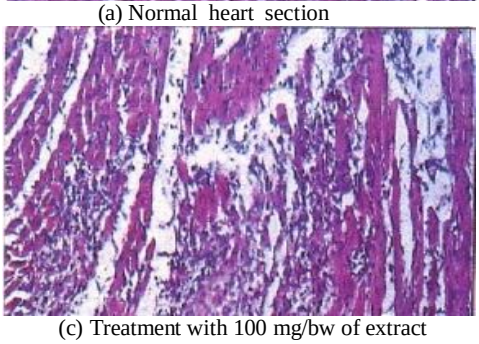
Results of the histopathological studies for the treatment period are presented in Figure. 1(a-e).



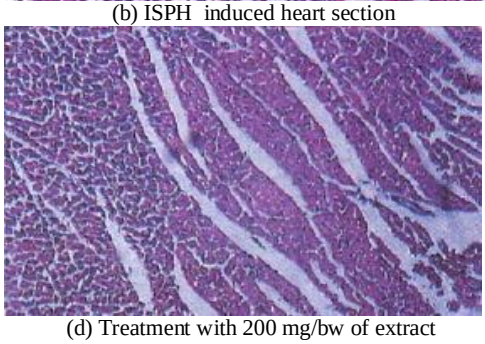
(a) Normal heart section



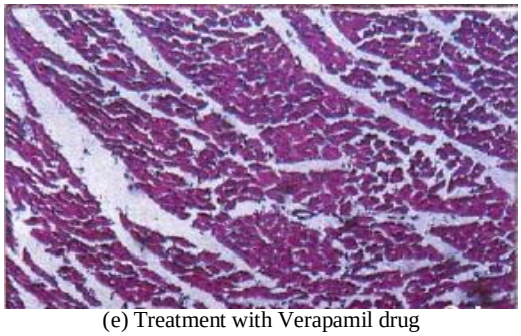
(b) ISPH induced heart section



(c) Treatment with 100 mg/bw of extract



(d) Treatment with 200 mg/bw of extract



(e) Treatment with Verapamil drug

Fig 1: Histopathological section of heart after a treatment period of 30 days.

In histopathological studies the control rats showed the regular arrangement with clear striations of myocardial fibers without any histological alterations because of degeneration or necrosis. Group II- ISPH induced rats (Fig.1-b) showed pathological changes in heart including several congestions, subendocardial necrosis and abundant hyperplasia along with increased edematous intramuscular space. The heart was having near normal appearance with mild changes in congestions and necrosis in rats treated with standard drug verapamil (Fig.1-e). Same pattern was obtained in rats treated with methanolic extract of *Croton sparciflorus* 100 mg/kg and 200 mg/kg (Fig. 1-c, d). So this research proved that the methanolic extract of the C*roton sparciflorus* plant can protect the heart from the myocardial infarction condition.

The phytochemical analysis carried out with conventional methods [55-57] showed the presence of phytoconstituents such as Alkaloids, Flavonoids, Phenols, Tannins, Saponin and Terpenoids in the methanolic extract of *Croton sparciflorus* and this may be responsible for the significant cadioprotective activity. The presence of above phytoconstituents was analyzed by various qualitative tests [58-61].

4. Conclusion

The plant extract of *Croton sparciflorus* has a potential to inhibit the cardio toxic effects induced by ISPH and possesses a significant medicinal value in the prophylactic treatment of MI. Efforts are in progress to isolate and characterize the active principle, which is responsible for the cardioprotective efficacy of this valuable medicinal plant. The search for new pharmacological-active compounds for drug development is an important issue, as the trend toward using standardized plant extracts of high quality, safety and efficacy will continue. Therefore, all efforts have to be targeted to reveal the chemical-pharmacological profiles of extracts and fixed combinations and to rationalize their therapeutic application.

5. Acknowledgement

Authors are thankful to the management of APCAS, Kalavai, for providing necessary facilities to carry out the animal studies.

6. References

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