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Effect of cardioprotective polyherbal formulation on isoproterenol induced myocardial infarction in experimental animals

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Cardiovascular disease (CVD), with annual mortality of 17.5 million people, 31% of all deaths worldwide, is reported to be the World's leading cause of death. More than 75% of CVD deaths occur in low and middle income countries. In India, the prevalence of CVD is 7-13%.The increased risk is due to multiple risk factors with the lifestyle. Herbs are known to have potential benefits against CVD. In this study, we explored the cardioprotective effect of polyherbal formulation on isoproterenol induced myocardial infarction in male Wistar rats. The Polyherbal formulation was prepared using 6 different plant samples that includes *Allium sativum* (pulp), *Andrographis paniculata* (leaves), *Boerhavia diffusa* (leaves), *Moringa oleifera* (bark), *Piper betle* (leaves), *Piper longum* (seed). The quantitative analysis of phytochemicals revelead the presence of alkaloid, carbohydrates, triterpenoids and phytosterols in CPHF. 100, 250, 500 mg/kg of body wt of CPHF was administered orally to complete the acute toxicity studies. The activity of liver enzymes in serum sample was analyzed and found to be non-toxic with maximum dosage. The cardio protective study was carried out by administering 250 mg/kg of body wt. of the CPHF in the experimental rats for 30 days. After the study period, serum sample was collected for biochemical analysis like cholesterol, free fatty acids, and triglycerides. The activity of cardiac marker enzymes like AST, ALT, ALP, LDH, CK, and CK-MB were also analyzed. The level of cholesterol, LDL, Triglycerides and cardiac marker enzymes activity in treated groups were found to be decreased as compared with the control group rats. The histopathological observations of heart tissue have also confirmed the cardioprotective role of CPHF.

Keywords: *Allium sativum*, *Andrographis paniculata*, Ayurvedic, Betelvine, *Boerhavia diffusa*, Cardiac markers, Cardiovascular diseases (CVD), Drumstick tree, Garlic, Green chiretta, Heart attack, Indian long pepper, *Kalmegh*, King of Bitters, *Moringa oleifera*, Paan, *Piper betle*, *Piper longum*, Pipli, *Punarnava*, Red spiderling, Spreading hogweed, Tarvine

Cardiovascular diseases (CVD) globally, comprise a leading cause of mortality with annual mortality of 17.5 million people, 31% of all deaths worldwide. Of this, >75% occur in low and middle income countries[1]. Countries like India are also struggling to manage the impact of CVD along with the increasing yoke of obesity. By 2020, India has been reported to be holding the largest CVD burden among the world. The occurrence of these diseases is more in metropolitan than in countryside areas[42]. About 80% CVD deaths occur due to heart attacks and strokes[1].

Myocardial infarction (MI), the myocardial cell death owing to delayed ischemia, is obvious with the impaired systolic and diastolic function, ventricular dilatation eventually leading to congestive heart failure[3,4]. Apoptosis is also known to play a role in the process of tissue damage leading to myocardial infarction[5,6].

Herbal medicine is progressively gaining acceptance among people owing to better understanding of the mechanisms by which herbs influence health positively and the quality of life[7-9]. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging critical bio-molecules[6,8]. Several scientific reports have showed that polyherbal is also useful in the prevention of isoproterenol induced myocardial infarction in experimental animals[5,6,10]. In traditional medicine, more than 2000 plants have been listed and some of them provide wide range of relieves from cardiovascular diseases to various other ailments[11-16]. Herbal drugs have been used to treat patients with "hyperlipidemia"[14,17-19] and "ischemic heart disease"[6,11,20].

Here, we prepared a cardio tonic (polyherbal formulation) based on Ayurvedic pharmacopeia using

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six different plant sources *viz*. *Allium sativum* L. (pulp), *Andrographis paniculata* (Burm.f.) Wall. Nees. (leaves), *Boerhavia diffusa* L. (leaves), *Moringa oleifera* Lam. (bark), *Piper betle* L. (leaves), *Piper longum* L. (seeds) known for various properties apart from cardioprotective including antioxidant, antihyperlipidemic and antilipid peroxidative properties, etc. (Table 1)[9,21-30].

Materials and Methods

Materials

The dried plants samples were purchased from commercial market, Gandhigram Trust, Dindugal, Tamil Nadu (Regd. No. 18/16/5509). Isoproterenol was purchased from Hi-Media and the other chemicals for biochemical studies were purchased from Sigma Companies Group Pvt. Ltd.

Preparation of cardio tonic and qualitative analysis of phytochemicals

The polyherbal formulation was prepared using the 6 different plant samples according to Indian Ayurvedic Pharmacopeia standard. To one part of plant 16 parts of water was added and reduced to 1/8 of its initial volume finally ("*Sarangadhar samhita*" – Ayurvedic literature PHC Murthy, Chaukhambha publications). It was then filtered and stored in amber bottles for future use. To one litre of the prepared tonic 1 g of sodium benzoate was added to preserve.

The cardio protective polyherbal formulation (CPHF) obtained was subjected to preliminary phytochemical screening. Test for alkaloids (Mayer's test), flavonoids (Alkaline reagent test), carbohydrates (Molischs test), glycosides (Legals test), saponins, tannins, phytosterol (Salkowski test), triterpenoid (Libermann Burchard test), proteins and amino acids (Ninhydrin test), biuret test, anthraquinones, steroids, reducing sugars (Fehling's Test), acidic compounds were done to check the presence of phyto constituents[31-33].

High Performance Thin Layer Chromatography analysis of CPHF

The cardio tonic was centrifuged at 3000 rpm for 5 min and 2 µL of test sample (CPHF) solution and 2 µL of standard alkaloid solution were loaded as 5 mm band length in the 3 × 10 Silica gel 60 F 254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The plate was developed in the respective mobile phase up to 90 mm. The plate was kept in photo documentation chamber. Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanned at UV 254 nm using software Win CATS 1.3.4 version. Ethyl acetate-methanol-water (10:1.35:1) was used for mobile phase and the the Spray reagent was Dragendorff's reagent followed by 10 % Ethanolic sulphuric acid reagent.

Animals

Adult male Wistar rats (weighing 110±10 g) purchased from Kerala Veterinary College were used for this study, and the experiments were conducted as per the Institutional Animal Ethical Committee, India (IAEC) guidelines and necessary approval (IAEC no. IAEC/KU/BT/14/07). The animals were maintained

Table 1—Cardioprotective role of *Allium sativum*, *Andrographis paniculata*, *Boerhavia diffusa*, *Moringa oleifera*, *Piper betle*and *Piper longum*

Plants	Justification
Allium sativum (Garlic) Alliaceae	Protective effect on atherosclerosis is attributed to its capacity to reduce lipid content in arterial wall. Depresses the hepatic activities of lipogenic and cholesterogenic enzymes. Also reduces the mechanism of LDL oxidation. Used as an adjuvant with lipid-lowering drugs for control of lipids[9,21,22].
Andrographis paniculata (Green chirayta) Acanthaceae	Hydroalcoholic extract inhibits lipid peroxidation and prevention of leakage of myocytes injury marker enzymes from heart. Pretreatment reduces myocardial I-R injury in rats by reducing myonecrosis, oedema and inflammation along with improving cardiac function and tissue defense network[23,24].
Boerhavia diffusa (Punarnava) Nyctaginaceae	Ethanolic extract exhibits hyolipidemic and anti inflamatory effects in isoproterenol induced MI rats[25].
Moringa oleifera (Drumstick tree) Moringaceae	Polyphenols inhibit lipid peroxidation by acting as chain breaking peroxyl radical scavengers and can protect LDL from oxidation58 and also inhibit hepatic lipid synthesis. Prevents oxidative stress due to High fat diet[26-28].
Piper betle (Betle leaves) Piperaceae	Pretreatment shows beneficial effects on myocardial antioxidant status and modulation of perturbed hemodynamic[29].
Piper longum (Pepper) Piperaceae	Pretreatment helps in restoration of antioxidant enzymes and cellular damage. Methanolic extract protects against adrimycin induced myocardial oxidative stress induced injury in rats[30].

at 24±2°C, humidity 55-60% and photoperiod 12:12 h light:dark cycle. A commercially balanced diet and tap water were provided *ad libitum*. After a period of 14 days study the blood sample was collected from experimental animals for analyses such as Haematological parameters, Aspartate aminotransferase (AST), Alanine Transaminase (ALT), glucose, cholesterol and creatinine. Initial and final body weights, water and food intake, behavioural changes were monitored and mortality was noted.

Experimental design for acute toxicity studies and Myocardial Infarction studies

The toxicity studies were carried out with three different concentrations 100, 250 and 500 mg/kg body wt. Animals were divided into four groups, consisting of a minimum of six animals each. Group I had Normal diet; Groups II-IV received CPHF as mentioned above[34]. Myocardial infarction was induced by isoproterenol injection (150 mg/kg of body wt. i.p.). It was weighed and mixed with 0.5 mL and 0.9% saline. Animals were divided into four different groups, with a minimum of six animals in each group, as follows: Group I, normal diet; Group II, induced Myocardial infarction by administering isoproterenol i.p. on 29th and 30th day (150 mg/kg of body wt.); Group III, isoproterenol i.p. (150 mg/kg of body wt.) + pre-treated with Zocor (simvastatin) (10 mg/kg of body wt.); and Group IV, isoproterenol (150 mg/kg of body wt. i.p.) + pre-treated with CPHF (250 mg/kg of body wt.)[35].

Determination of serum biochemical parameters

Rats were administered with CPHF @ 250 mg/kg orally for 30 days and at the end of treatment myocardial infarction was induced by injecting isoproterenol @ 150 mg/kg i.p. twice at the interval of 24 h. At the end of experiment, rats were sacrificed under chloroform anesthesia, blood was collected through carotid artery, and serum was separated. The levels of cholesterol, triglycerides (TG), high density lipoproteins (HDL), low density lipoproteins (LDL) and urea were estimated using Span Diagnostics kits. The protective effect of formulation was evaluated by comparing the results with vehicle-treated rats injected with isoproterenol.

Collection of blood and tissue samples

Paired blood samples were collected into heparinized bottles for hematological studies; blood samples collected in clean non-heparinized bottles were allowed to clot. The serum was separated from the clot and centrifuged into clean bottles and was used for the assay of marker enzymes lactate dehydrogenase (LDH)[36], aspartate transaminase (AST)[36], alanine transaminase (ALT)[36] and creatine kinase (CK, CK-MB)[37]. The heart was dissected out, immediately washed in ice-cold saline, preserved for further use[38].

Histopathology examination

Histopathological evaluation was performed on the heart specimen fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4 µM in thickness, stained with haematoxylin and eosin and viewed under light microscopy for histological changes in the cardiomyocytes[6].

Statistical analysis

All the grouped data was statistically evaluated via the INSTAT software, version 3.0. Data were analyzed statistically by one-way ANOVA. Values are expressed as mean ± SD for six animals in each group. Unit comparison test was used for statistical comparison between the four different groups. Changes were considered statistically significant if the *P*-value was less than 0.05.

Results

Qualitative analysis of phytochemicals

The presence of alkaloids, carbohydrates, saponins, tannins, phytosterol, triterpenoid, steroids and reducing sugars were observed in the polyherbal formulation but anthraquinones, flavonoids and glycosides were absent (Table 2).

HPTLC analysis details

Yellow coloured zone at visible mode was present in the tracks, it was observed from the chromatogram after derivatization, which confirmed the presence of alkaloid/nitrogen containing compound in the given standard and may be in the samples. (Fig. 1 A and B)

Table 2—Qualitative analysis of phytochemicals in CPHF

Phytochemicals	Inference
Alkaloids	+
Flavonoids	-
Carbohydrates	+
Glycosides	-
Saponins	+
Tannins	+
Phytosterol	+
Triterpenoid	+
Anthraquinones	-
Steroids	+
Reducing Sugars	+
Acidic Compounds	-

'+' Present; '-' Absent

Acute toxicity studies

Table 3 shows the weight of animals observed during the study, there were no significant changes in the body weight of the animals during the period of acute toxicity studies and Table 4 shows the levels of RBC, WBC and Hemoglobin for all four groups. There was no mortality observed in any group of animals. The levels of biochemical parameters such as cholesterol, triglycerides, low density lipoprotein, high density lipoprotein, and urea are shown in Fig. 2A. The range of cholesterol, triglycerides, low density lipoprotein in group IV was found to be significantly lower as compared to the group II. Fig. 2B reveals the activity of cardiac marker enzymes. The activity of these enzymes were found decreased in group IV after the treatment with CPHF.

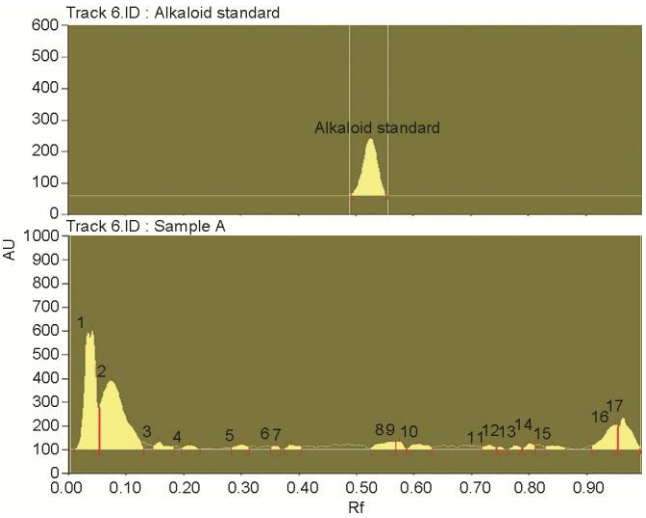

Fig. 1—Densitogram display at 254 nm. (A) Alkaloid standard peak; and (B) Sample peak

Histopathology

Fig. 3 depicts the histological observations revealing observable changes as detailed below. The section of tissue from the group II showed focal myocyte degeneration with dense infiltration of

Table 3—Mean wt. (g) of animals observed during the study

Days	Normal	Group I	Group II	Group III
Day 1	122.2±1.2	121.5±1.1	123.2±0.8	121.8±1.3
Day 5	123.7±0.9	122.9±1.1	124.6±1.2	122.9±1.1
Day 10	125.6±1.1	124.2±1.5	125.5±1.2	123.6±0.9
Day 14	127.2±0.8	125.3±1.2	127.1±1.0	125.2±1.1

[Values are expressed as Mean ± SD for three animals. Groups: I, Control; II-IV, Treated with 100, 250 and 500 mg/kg of body wt., respectively]

Table 4—Biochemical analysis for acute toxicity studies

Biochemical parameters	Groups			
	I	II	III	IV
AST (U/L)	5.2 ±0.1	5.4 ±0.2*[a]	5.7±0.1*[b]	5.7±0.2*[b]
ALT (U/L)	10.7 ±0.3	11.5±0.4*[a]	11.7±0.5*[b]	11.8±0.3*[b]
Cholesterol (mg/dL)	157±2.6	158±4.4*[a]	171±8.5*[b]	172±6.7*[b]
RBC ($\times10^6$/mm^3)	6.8±0.2	6.5±0.2*[a]	6.2±0.3*[b]	6.1±0.3*[b]
WBC ($\times10^3$/mm^3)	3.8±0.3	3.9±0.2*[a]	3.9±0.1*[b]	3.8±0.2*[b]
Hemoglobin (g/dL)	14.1±0.3	13.8±0.6*[a]	13.8±0.5*[b]	13.6±0.6*[b]
Creatinine (mg/dL)	0.62±0.01	0.66±0.01*[a]	0.72±0.02*[b]	0.74±0.02*[b]

[Groups: I, Control; II-IV, Treated with 100, 250 and 500 mg/kg of body wt., respectively. Values are given as mean Mean± SD for groups of three animals each and statistically significant at P <0.05. [a]Comparison made with Group I and Group II, [b]Group III and Group IV compare with Group II; *Values are from this data obtained it was found that the cardio tonic did not show any toxic effects in the animal of all groups. In order to carry out the cardiac protective studies 250 mg/kg of cardiotonic was administered to rats for further studies]

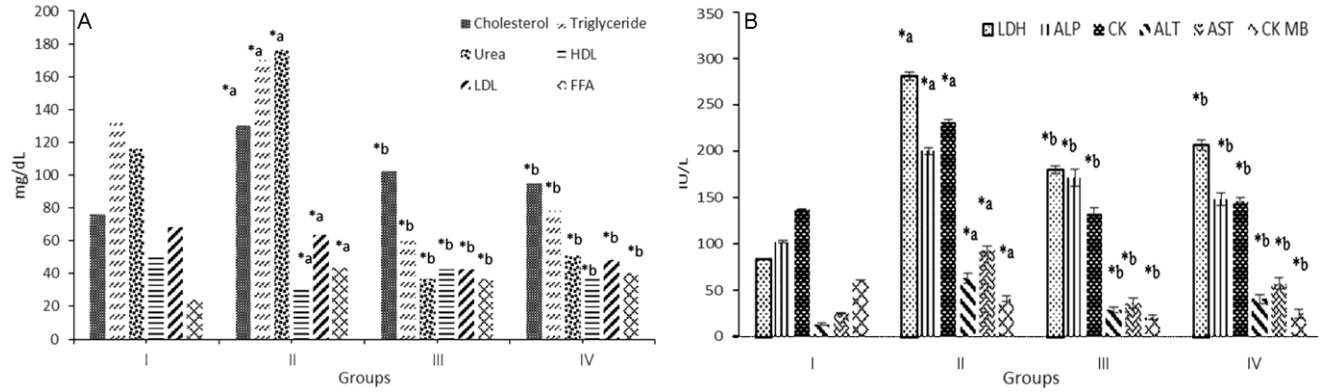

Fig. 2—Effect of cardiotonic on (A) various biochemical parameters [total cholesterol (TC), triglyceride (TG), urea, HDL, LDL and free fatty acids levels]; and (B) cardiac marker enzymes (LDH, ALP, CK, ALT, AST, and CK-MB) in serum of experimental rats. [Values are the mean ± SD for six animals in each group. Values are statistically significant at *P <0.05; statistical significance was compared within the groups as follows. a, Myocardial Infarction rats were compared with normal rats; and b, Zocor and cardiotonic treated rats were compared with Myocardial Infarcted rats.

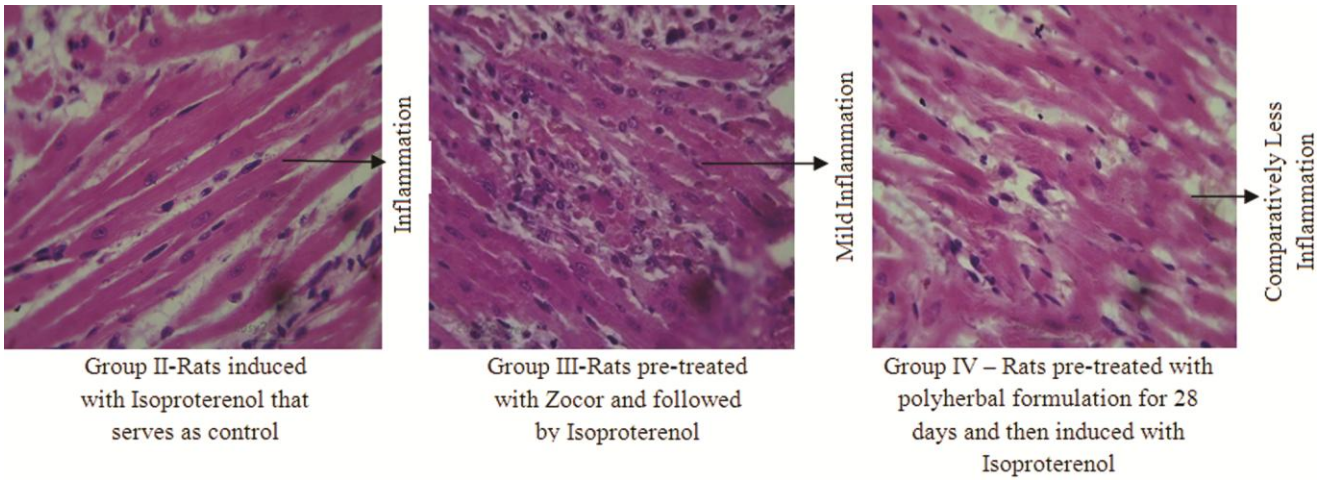

Fig. 3—Histological images of hematoxyline & eosin stained paraffin sections of experimental rat hearts, Magnification 40 X

lymphocytes and macrophages. The blood vessels showed congestion and mild edema. Group III animals also showed mild edema with few scattered lymphocytes, however, there was no myocyte degeneration observed in them. The tissues from group IV showed no pathology in the myocytes. There was no focal accumulation and the blood vessels showed congestion. The depletion of myocardiocytes and inflammation in group IV were less as compared with the group II and III animals.

Discussion

Myocardial cell death due to prolonged ischemia leads to myocardial infarction. It takes several hours before myocardial necrosis can be identified by macroscopic or microscopic examination. The entire process leading to a healed infarction usually takes at least 5-6 weeks[3]. Isoproterenol causes sub-endocardial myocardial ischemia, hypoxia, finally necrosis that result in cardiac dysfunction due to modify physiological balance between production of free radicals and anti-oxidative defense systems[39]. Isoproterenol administration also reduces blood pressure, which triggers reflex tachycardia, thereby increasing myocardial oxygen demand. Moreover, its positive inotropic and chronotropic actions further increase the myocardial oxygen demand, which leads to ischemic necrosis of myocardium in rats similar to human myocardial infarction[40,41]. It also causes irretrievable damage to myocardium by elevation of lipid peroxidation and decreases the antioxidant mechanism[6,30].

The diagnostic marker enzymes of myocardial infarction are creatine kinase, lactate dehydrogenase, alanine transaminase and aspartate transaminase[10,42,43]. It has been reported that myocardial necrosis show membrane permeability alterations which bring about the loss of function and integrity of myocardial membranes. The macromolecules that leak from the injured tissues, enzymes, because of their tissue specificity and catalytic activity, are the excellent markers of tissue damage. Improved activities of these marker enzymes like AST, ALT, ALP, CK, CK-MB in the serum are indicative of cellular damage, severity of necrotic damage and loss of functional integrity of cell membrane[42,44]. Increase in serum level of AST, ALT, ALP as observed in this study may reflect the damage of cells.

The levels of these enzymes are reported to be directly proportional to the number of necrotic cells present in the cardiac tissue[45]. In this context, it has been reported that CK-MB is present in higher proportion and concentration in the myocardium and also is referenced as early marker of MI[46]. Increased levels of ALT, AST, LDH and CK in serum 'the diagnostic markers', may be due to the outflow of these enzymes as a result of necrosis induced by isoproterenol in rats[47]. Treatment with CPHF (250 mg/kg) in isoproterenol induced rats reduced the activity of marker enzymes which clearly indicates the cardioprotective effect of the polyherbal formulation against MI.

In the isoproterenol group, the significant increase in CK-MB indicated myocyte injury. A minor increase in CK-MB after isoproterenol administration in the CPHF-treated group indicates cardioprotective activity of polyherbal formulation. Our experimental data also showed lowered activities of these enzymes

in polyherbal treated rats compared to the isoproterenol treated rats, indicating protection against necrosis of the myocardial membrane.

The lower LDH level in CPHF treated group, although is not significant, following isoproterenol injection, further supports the protective effect. Rise in the serum level of AST in the isoproterenol alone group compared to the control group indicates myocardial necrosis. Polyherbal treated rats had AST in lower levels, indicating cardio protection. The action of polyherbal formulation also seems to preserve the structural and functional integrity of the myocardial membrane, as apparent from the reduction in the levels of these serum marker enzymes in rats pretreated with CPHF when compared to the isoproterenol alone treated group (Fig. 2B), thus establishing the cardio protective effect of CPHF.

Moreover, LDH levels elevate up on tissue inflammation and necrosis. Numerous researchers have also reported elevated levels of CK-MB and LDH in MI induced by isoproterenol[42]. Findings of the present study are in accordance with the above experimental evidences whereby treatment with the cardio tonic significantly decreased the levels of CK-MB and LDH as compared to the isoproterenol control animals, signifying the improvement in necrotic injury induced by isoproterenol. Rats pre-treated with polyherbal formulation showed a significant rise in myocardial CK-MB, and LDH and thereby demonstrated that polyherbal formulation rendered the myocytes less leaky by preventing myocardial membrane disruption and disorganization subsequent to reduced lipid peroxidation[48].

Isoproterenol treated rats showed broad necrosis, due to lipid peroxidation the leakage of enzymes from the heart. Reduced necrotic damages in CPHF treated animals may be the reason for the decreased activities of the marker enzymes in treated groups. Large number of studies has demonstrated that free radicals initiate lipid peroxidation resulting in alteration of membrane integrity, fluidity and permeability[49].

The polyherbal formulation has been mentioned in Ayurvedic text as a rasayana; agents that promote longevity and prevent diseases by providing strength and immunity. This is similar to the modern classification of adaptogens, as stress plays an important role in aetiopathogenesis of heart diseases; many rasayanas were reported to exhibit cardioprotective activity[50]. Thus, it is possible that the multitude of cardio-friendly activities in the polyherbal formulation may render resistance as well as impart strength and stabilization to the myocardium against necrotic damage[48]. The active alkaloids present in the CPHF contributes as the antioxidant potential and these in turn arrest the reactive oxygen species and prevents the myocardial damage[51,52].

In our study, isoproterenol administration results in adversely depleted myocardial CK-MB isoenzyme and LDH activities, which clearly supports the occurrence of isoproterenol-induced myocardial injury. Furthermore, on histoarchitectural examination of myocardial tissue, the presence of focal loss of myofibers, myonecrosis, marked edema, and infiltration of chronic inflammatory cells were observed. Thus, the biochemical and histopathological findings of the present study confirm that isoproterenol produces cardio toxic effects, and the suitability of this model has been established for studying the cardioprotective effect of CPHF.

The cardio tonic used in the present investigation was in crude form and likely to contain any alkaloid. The alkaloids presence was confirmed by HPTLC analysis. The study needs further investigation in order to know the exact mechanism behind the cardioprotective effect of cardiotonic.

In conclusion, this study has further confirmed the cardioprotective nature of the polyherbal formulation (CPHF) consisting of *Allium sativum* (pulp), *Andrographis paniculata* (leaves), *Boerhavia diffusa* (leaves), *Moringa oleifera* (bark), *Piper betle* (leaves) and *Piper longum* (seeds). The oral administration of CPHF to the experimental animals showed decreased biochemical parameters (low density lipoprotein, cholesterol, urea, free fatty acids) and activity of cardiac enzymes (ALT, ALP, AST, CK-MB) as compared with the control group. The increase in high density lipoprotein in the treated group may help in preventing deposition of cholesterol in cardiac tissues and the damage that may occur in cardiomyocytes. This protective effect could be due to the preventive actions of phytochemicals such as alkaloids in CPHF.

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