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Effect of Gossypin on Isoproterenol – Induced Myocardial Infarction in Albino Rats

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ABSTRACT

The present study was aimed at investigating the cardioprotactive activities of the Gossypin was isolated from *Hibiscus vitifolius by* methanolic extract. The cardioprotactive activities of extract have been evaluated by using A portion of heart tissue was preserved in 10% formalin (pH 7.2) and subjected to histopathological studies .The heart sections were made 2mm thick and infarction area was evaluated by 2,3,4-Triphenyltetrazolium chloride(TTC) staining and biochemical analysis such as lactate dehydrogenase (LDH), creatine kinase (CK-MB), thiobarbituric acid reactive substances (TBARS), (GSH),(SOD),CAT were estimated in serum and heart of experimental animals using standard biochemical kits. Normal group showed normal cardiac fibre arrangements without necrotic fibres. ISO group showed intense necrosis, damage and irregular arrangements and morphological disturbances of myofibrils with increased interfibre distance. While standard (Losartan20mg/kg) and treated groups showed significant reduction in the damage produce by ISO group. The heart homogenate from the control group showed significant reduced activities of, SOD *and* increase activity of LPO compared to normal group. The Gossypin treated 5, 10, 20mg/kg groups showed significant protection by reducing elevated levels of LPO and increase level of antioxidant enzymes SOD , the non-enzymatic antioxidant GSH significantly as compared to control group. The blood serum from control group showed significant increase in enzyme marker LDH level as compared to normal groups. In contrast Gossypin (5, 10,20mg/kg) treated groups and standard group animals showed significant reduction in serum LDH level as compared to control. The blood serum from control group showed significant increase in enzyme marker LDH level as compared to normal groups. In contrast Gossypin (5, 10,20mg/kg) treated groups and standard group animals showed significant reduction in serum LDH level as compared to control. The results show the Cardioprotactive activity of Gossypin.

Keywords: Cardioprotactive activity, Gossypin, *Hibiscus vitifolius*

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INTRODUCTION

The ability of catecholamines, when administered in supraphysiologic dosages, to induce morphological alterations of the heart resembling severe myocardi subcutaneous injections of the synthetic beta-adrenoceptor agonist isoproterenol produced infarct-like lesions of the myocardium in the rat. The biochemical and histologic changes occurring after administration of this agent in rats have been well documented. Myocarditis or left ventricular (LV) hypertrophy was already noted early in this century. In 1959, Rona and Chappel showed that subcutaneous injections of the synthetic beta-adrenoceptor agonist isoproterenol produced infarct-like lesions of the myocardium in the rat. The biochemical and histologic changes occurring after administration of this agent in rats have been well documented. The exact mechanism of isoproterenol-induced myocardial damage has not been clarified, but a mismatch of oxygen supply versus demand following coronary hypotension and myocardial hyperactivity may offer the best explanation for the complex morphological alterations observed in the presence of a patent coronary vasculature. The pharmacologic effect of isoproterenol is believed to be associated with its 3-adrenergic effect, which increases heart rate, decreases blood pressure, and diminishes the oxygen supply to the myocardium. As early as 6 min after intraperitoneal isoproterenol injection, histologic changes occur, including myofilament fragmentation, contraction-band formation, and hyalinization associated with dilation of the sarcoplasmic reticulum. Focal necrosis, extensive inflammation, and infiltration by polymorphonuclear leucocytes were found in the damaged heart at 24 hr after the injection. These changes resemble those observed in human myocardial infarction. Comparable biochemical alterations such as shifting electrolytes, serum enzyme level increased etc., have also been reported. All of these studies suggest that this experimental lesion is comparable to the infarct found in humans.

Gossypin was isolated from *Hibiscus vitifolius.* The yellow part of the petals of *Hibiscus vitifolius (*Malvaceae*)* (1.5g) extracted with methanol for 3 to 4 hrs concentrated to small volume under vacuum and was kept in refrigerator for 24 hrs. A large amount of yellow solid separated, which was filtered and washed with methanol. The yellow solid has a melting point 228-230^{O}C. Based on the spectral data (UV, IR, NMR and Mass) the compound was identified as gossypin. The flavonoid gossypin has been reported to exhibit anti-inflammatory action through inhibition of arachidonic acid metabolism. Gossypin significantly reduces the effect of paw edema and increased vascular permeability induced by histamine, 5-HT, bradykinin and Prostaglandin-E. Gossypin has also been shown to possess anti-hyaluronidase activity Antinoceptive activity of gossypin was also reported.

MATERIALS AND METHOD

Chemicals

Isoproterenol hydrochloride was purchased from Sigma chemical company. Diagnostic kits used for the estimation of marker enzymes CK-MB, LDH and for plasma profile reagents were Procured from ERBA Manheim manufactured by transasia biomedicals LTD, Baddi, Dist. Solan (HP), India. All other biochemical reagents and chemicals were of Analytical grade.

Animals

Healthy Wistar albino rats of either sex weighing between 180-220 g were taken for the study. All the animals were procured from the Animal House of the H S K. College of Pharmacy and research centre, Bagalkot, Karnataka. The animals were acclimatized by keeping them in the animal house facility of H S K College of Pharmacy, Bagalkot for a week. They were housed in polypropylene (32 x 24x16 cm) cages containing husk as bedding material and maintained under controlled conditions of temperature (25±2 c), humidity (55±5%) and 12h light and 12h dark cycles. The animals were fed with standard pellet diet and water *ad libitum*. Approval of the Institutional Animals Ethics Committee (IAEC) of H.S.K College of Pharmacy, Bagalkot was taken for conducting cardioprotective activity (Ref.No. IAEC HSKCP/IAEC, Clear / 2013-14 / 1-12**)**

Natural product

Gossypin was obtained as pure drug from Sigma chemical Co. USA

Procedure

Albino Wistar rats either sex were divided into six groups (n = 6). Gossypin and Standard drug were treated for 12 days. At the end of the treatment period, groups II, III, IV, V and VI were administered with isoproterenol at a dose of 85 mg/kg body wt.SC interval of 24.

Groups	Treatment
Group I	Normal (normal saline)
Group II (85 mg/kg,SC)	Isoproterenol
Group III	Losartan +ISO
Group IV (5 mg/kg)	Gossypin +ISO
Group V (10mg/kg)	Gossypin+ ISO
Group VI (20mg/kg)	Gossypin +ISO

Post treatment investigations

Twenty four hours after the last injection of ISO, the animals were euthanized under light ether anesthesia. Blood was collected and serum was separated by centrifugation. Hearts were dissected out and immediately washed in ice-cold saline and a homogenate was prepared in phosphate buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used

for biochemical estimations. A portion of heart tissue was preserved in 10% formalin (pH 7.2) and subjected to histopathological studies .The heart sections were made 2mm thick and infarction area was evaluated by 2,3,4-Triphenyltetrazolium chloride(TTC) staining.

Biochemical Analysis

Marker enzymes, such as lactate dehydrogenase (LDH) and creatine kinase (CK-MB**)** were estimated in serum and heart of experimental animals using standard biochemical kits. Myocardial thiobarbituric acid reactive substances (TBARS) were estimated as a marker of lipid peroxidation. Myocardial reduced glutathione (GSH) was estimated by the method of Ellman et al. Superoxide dismutase (SOD) levels in heart were estimated by the method of Kakkar et al and catalase **(**CAT) by the method of Chance & Maehly. Other myocardial endogenous antioxidant enzymes.

Lipid peroxidation (LPO)

Thiobarbituric acid reactive substances (TBARS) in the homogenate were estimated by using standard protocol. Briefly, the 0.5 ml of 10% homogenate was incubated with 15% TCA, 0.375% TBA and 5N HCl at 95 ºC for 15 min, the mixture was cooled, centrifuged and absorbance of the supernatant measured at 532 nm against appropriate blank. The amount of lipid peroxidation was determined by using $\varepsilon = 1.56 \times 10^{5\ -1}cm^{-1}$ and expressed as TBARS nmoles /mg of protein.

Superoxide dismutase (SOD)

Superoxide dismutase activity was determined based on the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH. Briefly, 25 µl of the supernatant obtained from the centrifuged heart homogenate was added to a mixture of 0.1 mM epinephrine in carbonate buffer (10.2) in a total volume of 1ml and the formation of adrenochrome was measured at 295 nm. The SOD activity (U/mg of protein) was calculated by using the standard plot.

Catalase (CAT)

Catalase activity was assayed by the method of Claiborne[23] (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M), and 0.05 ml homogenate (10%, w/v) in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nM H_2O_2 consumed/ min /mg protein.

Total thiols

This assay is based on the principle of formation of relatively stable yellow colour by sulfhydryl groups with DTNB[24] Briefly, 0.2 ml of heart homogenate was mixed with phosphate buffer (pH 8), 40 µl of 10 mM DTNB and 3.16 ml of methanol. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blanks. The Total thiol content was calculated by using $\varepsilon = 13.6 \times 10^{31}\ cm^{-1}\ M^{-1}$ [9].

Glutathione (GSH)

GSH was estimated in various tissues by the method of Sedlak and Lindsay, 1968. Briefly, 5% tissue homogenate were prepared in 20 mM EDTA, pH 4.7, and 100 µl of the homogenate or pure GSH was added to 0.2 M Tris-EDTA buffer (1.0 ml, pH 8.2) and 20 mM EDTA, pH 4.7 (0.9 ml) followed by 20 µl of Ellman's reagent (10 mmol/l DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. Samples were centrifuged before the absorbance of the supernatants was measured.

Total protein

The total protein contents of 10% heart homogenates were determined by using the modified Lowry's method. Briefly, 9 ml of chilled ethanol were added to 1 ml of heart homogenate and centrifuged. The precipitates were dissolved in 1 ml of 0.1N NaOH. The dark blue colour produced by addition of alkaline mixture and phenol-folin reagent, was measured at 610 nm by using spectrophotometer.

Lactate dehydrogenase (LDH)

The enzyme lactate dehydrogenase (LDH-L) is distributed in tissues particularly heart, liver muscle, and kidney. LDH –L catalyzes the oxidation of lactate to pyruvate in the presence of NAD which is subsequently reduced to NADH. The rate of NADH formation measured at 340 nm is directly proportional to serum LDH-L activity.

Reagent composition

L-Lactate 75 mM; NAD 5.5 mM; Buffer 80mM; pH 9.0±0.1 (30˚ C); Non-reactive stabilizers and filter.

Assay parameters

Method	Kinetic
Wavelength	340
Temperature	37˚ C
Sample Volume	25µl
Reagent Volume	1000 µl
Factor	6592

Unit IU/L

Procedure

Lactate dehydrogenase was estimated in serum by using standard enzyme kit supplied by Aspen Laboratories. Add the sample 25 µl in tube marked containing Regent Composition of L-Lactate 75 mM; NAD 5.5 mM; Buffer 80 mM. Mix and incubate at 37˚C for 60 Sec and take absorbance at 340 nm

RESULTS AND DISCUSSION

Effect of Gossypin on biochemical estimation

The heart homogenate from the control group showed significant reduced activities of, SOD (P<0.05) and increase activity of LPO (p<0.001) compared to normal group. The Gossypin treated 5, 10, 20mg/kg groups showed significant protection by reducing elevated levels of LPO (p<0.001) and increase level of antioxidant enzymes SOD (P<0.05), the non-enzymatic antioxidant GSH (p<0.05) significantly as compared to control group.

Effect of Gossypin on enzyme markers

The blood serum from control group showed significant increase in enzyme marker LDH (p<0.01) level as compared to normal groups. In contrast Gossypin (5, 10,20mg/kg) treated groups and standard group animals showed significant reduction (p<0.01) in serum LDH level as compared to control.

Table 1: Effect of Gossypin on enzyme markers

Treatment Groups	Lipid peroxidation (nm/mg of protein)	Catalase (U/mg of protein)	Total Thiols (µm/mg of protein)	SOD (U/mg of protein)
Normal	23.63 ± 9.054	0.518± 0.115	13.82±1.574	86.73±1.008
Control	83.53 ± 14.82^{a}	0.117 ±0.030^{c}	1.625±0.545^{a}	47.16±13.19^{c}
Losartan(20 mg/kg)	31.92 ± 5.63***	0.583 ± 0.056**	11.88±1.217**	87.21± 6.055*
Gossypin(5 mg/kg)	27.28 ± 6.42***	0.224± 0.094^{ns}	2.738±1.270^{ns}	46.55± 10.66^{ns}
Gossypin(10 mg/kg)	22.11 ± 0.90 ***	0.127 ± 0.021^{ns}	7.248±2.815^{ns}	29.39 ± 0.98^{ns}
Gossypin(20mg/kg)	20.46 ± 5.00 ***	0.470± 0.133*	8.515±2.434*	37.96 ± 4.222^{ns}

All values are expressed as a Mean ± SEM, n=6, One Way Analysis of Variance (ANOVA), followed by multiple comparison Dunnet's test. The minimum value of ^{a}p<0.05, ^{b}p<0.01 as compared to normal group, *p<0.05, **p<0.01, ***p<0.001 as compared to control group.

Effect of gossypin on myocardial infarction

Normal group showed visible myocardial tissue which was stained brick red due to formation of red formazan with LDH of myocardial tissue. In ISO treated group scattered patches of necrotic tissue were clearly visible as unstained infarcted region. Treated groups and standard (Losartan20mg/kg) group shown markedly reduced infarction in myocardial tissue of heart.

Figure 2: Effect of gossypin on myocardial infarction

Treatment Groups	LDH (IU/L)	CK-MB(IU/L)
Normal	136.4 ± 14.03	228.5±44.14
Control	587.0 ± 42.30^{b}	288.2 ± 21.68^{ns}
Losartan(20mg/kg)	386.0 ± 67.09**	59.67 ± 17.55**
Gosypin(5 mg/kg)	526.6 ± 85.96^{ns}	173.8 ± 35.44^{ns}
Gossypin(10 mg/kg)	397.6 ± 66.78**	247.3 ± 52.57^{ns}
Gossypin(20mg/kg)	171.6 ± 149.1**	263.2 ± 49.84^{ns}

All values are expressed as a Mean ± SEM ,n=6, One Way Analysis of Variance (ANOVA), followed by multiple comparison Dunnet's test The minimum value of $^{b}p<0.01$, as compared to normal group, $^{**}p<0.01$, as compared to control

Effect of the Gossypin on histopathological studies

Normal group showed normal cardiac fibre arrangements without necrotic fibres. ISO group showed intense necrosis, damage and irregular arrangements and morphological disturbances of myofibrils with increased interfibre distance. While standard (Losartan20mg/kg) and treated groups showed significant reduction in the damage produce by ISO group.

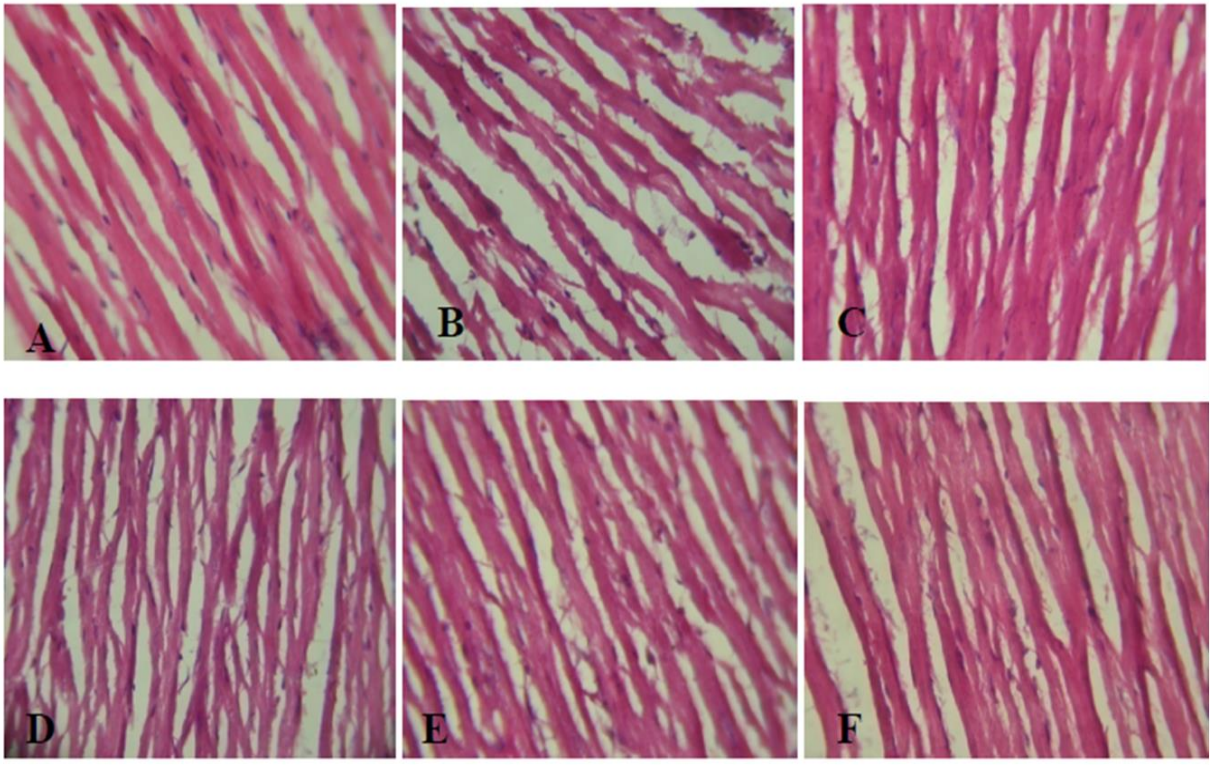

Figure 1: The effect of Gossypin against ISO induced damage in rats

The effect of Gossypin against ISO induced damage in rats Photographs of heart sections were prepared (6 µm thickness) from different treatment groups stained with Haemotoxylin and Eosin, 40x. Plates; **A**: Normal group showed normal cardiac artitecture and arrangement of myofibril, absence of interfibrillar necrosis, regular and normal multinuclear myofibrils arrangement and

vacuolization, macrovesicular fatty changes. **B**: ISO induced group animals exhibited intense interfibrillar necrosis, vacuolization, macroveisicular fatty changes and damage and irregular arrangement and morphological change of myofibrils associated with increased interfibrillar distance. **C**, **D**, **E, and F**: Losartan 20 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg of Gossypin exhibited significant cardiac remodeling activity against ISO induced injury in isolated rat heart preparation by normal cardiac architecture, arrangement of myofibrils and absence of interfibrillar necrosis.

Statistical analysis

All values were expressed as mean ± SEM. The results were statistically evaluated using One-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests using Graph Pad software .Significance for difference between groups was evaluated for student's *t*-test to come to final conclusion.

CONCLUSION

Myocardial Infarction (MI) or damage is a major public health concern and common presentation of ischemic heart diseases. It is a clinical syndrome arising from sudden and persistent curtailment of myocardial blood supply resulting in necrosis of the myocardium. Isoproterenol-induced myocardial infarction is a standardized model to study the beneficial effects of many drugs and antioxidants. ISO produced reactive oxygen species (ROS) *via* its auto-oxidation and subsequently produced oxidative stress. Higher level of catecholamines depletes the energy reserve of cardiac muscle cells, leading to complex biochemical and structural changes that cause irreversible cellular damage and ultimately necrosis. Several mechanisms of isoproterenol induced myocardial infarction have been reported. Isoproterenol acts both on β1 and β2 adrenoceptors, activation of which leads to positive inotropic and chronotropic effects. Thus, isoproterenol produces relative ischemia due to myocardial hyperactivity and coronary hypotension. Other probable mechanisms include increased cyclic adenosine monophosphate, increased intracellular Ca^{++} overload, depletion of high energy phosphate stores and oxidative stress[12].

Increased generation of cytotoxic free radicals, due to the auto-oxidation metabolic products of isoproterenol, is one of the well recognized mechanisms of isoproterenol induced myocardial necrosis. Isoproterenol, upon auto-oxidation produces quinones which react with oxygen to produce superoxide anion (O_2 –) and H_2O_2.

The production of superoxide radical results in secondary formation of H_2O_2 and Hydroxy radicals In the present study, with the focus on the protective effects of Gossypin pre-

treatment improve against isoproterenol induced myocardial infarction in rats. It showed that pre-treatment with of these indigenous plants could prevent myocardial infarction induced by ISO in the rats .Free radical scavenging enzymes such as SOD, catalase and are the first line of cellular defense system against oxidative stress, eliminating reactive oxygen radicals such as superoxide and hydrogen peroxide and preventing the formation of more deteriorating hydroxyl radicals. The equilibrium between antioxidants and free radicals is an important process for the effective removal of oxidative stress in intracellular organelles. However, in pathological conditions like myocardial infarction, the generation of reactive oxygen species can dramatically disturb this balance with an increased demand of the antioxidant defense system. As discussed earlier, isoproterenol auto-oxidation leads to generation of enormous amounts of reactive oxygen species. These reactive oxygen species may attack any type of molecules, but their main target appears to be polyunsaturated fatty acids (PUFAs) within membranes forming peroxyl radicals. These radicals then attack adjacent fatty acids within membranes causing a chain reaction of lipid peroxidation.

In this present study isoproterenol administration resulted in marked elevation in SOD, and GSH. Activities of antiperoxidative enzymes (SOD) was decreased significantly in the heart tissue of isoproterenol injected animals.

SOD is a class of enzymes, which catalyses the dismutation of two superoxide radicals to form hydrogen peroxide and molecular oxygen. It was observed a decreased concentration of GSH in the heart and decreased activities of glutathione dependent enzymes such as GST in the heart of isoproterenol injected rats. GSH, a tripeptide, is one of the most abundant non-enzymatic antioxidant bio molecules present in the body Cytosolic enzymes CK-MB, LDH, which serve as the diagnostic markers leak out from the damages tissue to blood stream when cell membrane becomes permeable or rupture. The amount of these cellular enzymes in serum reflects the alterations in plasma membrane integrity and or permeability. Further, histopathological findings confirmed the induction of myocardial infarction by isoproterenol and the protection rendered by extracts treatment to the cardiac muscle.

Histopathological examination of myocardial tissue in normal illustrated clear integrity of the myocardial cell membrane and no inflammatory cell infiltration was observed. Isoproterenol injected rats showed coagulative necrosis, separation of cardiac muscle fibers and infiltration of inflammatory cells. The reduced inflammatory cell infiltration and normal cardiac muscle fiber architecture further confirmed the cardioprotective effect of gossypin.

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