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Research Article

Cardioprotective Effect of *Hibiscus syriacus* Extract on Isoproterenol Induced Myocardial Infarction in Rats

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ABSTRACT

The present research was designed to evaluate the cardio-protective role of chronic oral administration of *Hibiscus syriacus* flower extract against Isoproterenol-induced myocardial injury in rats and to compare it with α-tocopherol, a known cardioprotective antioxidant. Male Wistar albino rats were randomly divided into five groups (n = 6) and treated as per treatment protocol with two different doses of *H. syriacus* Methanolic extract (250 and 500 mg/kg body weight) 6 days per week for 4 weeks. At the end of the treatment, all the rats (except control rats) were administered with Isoproterenol (85 mg/kg) for two consecutive days and subjected to biochemical and histopathological estimation. Isoproterenol (group II) induced oxidative myocardial damage via alteration in the endogenous antioxidant enzymes and myocardial marker enzymes. *Hibiscus syriacus* extract in all two doses (group III, and IV) shows a protective mechanism via decreasing thiobarbituric acid reactive substance (TBARS) and enhancing the endogenous antioxidant enzymes (reduced glutathione (GSH), superoxide dismutase (SOD), and catalase). In *H. syriacus* treated groups significant increase ($p < 0.001$) of LDH in heart homogenate and a decrease of SGOT and LDH in serum were observed. Microscopic studies in ISO-treated animals revealed mitochondrial swelling, leukocyte infiltration, lipid inclusions, and myofibrillar loss, whereas the pre-treatment with *H. syriacus* led to a lesser degree of ISO-induced histological alterations. The extract effect was compared with the reference standard α-tocopherol which also offered similar protection in biochemical and histopathological changes. Thus, the study shows that *H. syriacus* Methanolic extract exhibits significant antioxidant activity and protects the heart from free radical-mediated toxicity of isoproterenol.

Introduction

Myocardial infarction is the acute condition of necrosis of the myocardium that occurs as a result of an imbalance between coronary blood supply and myocardial demand.[1] Oxidative stress resulting from increased production of free radicals is associated with decreased levels of antioxidants in the myocardium and plays a major role in cardiovascular diseases.[2] Damage to the myocardial cells arises due to the generation of toxic reactive oxygen species such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Isoproterenol (ISO) is an adrenergic agonist and acute administration of ISO in experimental animals causes necrosis of heart muscle.[3] ISO damages the myocardial via calcium accumulation in the cytosolic membrane, generation of reactive oxygen species, and procoagulant activity. ISO causes the patchy pathological changes in the myocardial tissue, which is almost clinically relevant to myocardial infarction of ischemic heart disease.[4]

Phytopharmaceuticals are gaining importance in allopathic as well as traditional medicine owing to their non-addictive and less toxic nature. Drugs to enhance the endogenous antioxidant enzymes to protect the heart from stress have been paid more attention. Natural antioxidants play a major role to reduce oxidative stress by scavenging the excess free radicals.[5] Administration of antioxidants during ischemic reperfusion injury ameliorates the

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severity of myocardial injury through augmentation of endogenous antioxidants, which might be a promising loom to treat heart disease.[6]

Several natural products have been reported to have protective roles against ISO-induced Myocardial infarction in rats. *H. syriacus* L. (Family-*Malvaceae*) is widely distributed all around the world as ornamental and green plants. Furthermore, it is also a medicinal plant traditionally it is used as an antipyretic, antihelminthic, diuretic, hypertension, asthma, hair problem, and antifungal agent in the orient. The flowers of *H. syriacus* were found to contain flavonoids such as apigenidine, pelargonidin, cyanidin, quercetin, crisantemin, and anthocyanine. The Juice of the flower contains glycosides, triterpenoids, lipids, terpenes, and beta-sitosterol.[7] *H. syriacus* has been reported to have antibacterial, antifungal, antimicrobial, hepatoprotective, anti-proliferative activity, anti-inflammatory, cytotoxic, and antidiabetic properties.[8-13] *H. syriacus* is also known for its antioxidant activity and effective scavenger of oxidative radicals.[14] The antioxidant activity of the plant can be attributed to the presence of phenolic compounds like delphinidin, petunidin, malvidin, and quercetin, which have already been shown to have antioxidant properties. The free radical scavenging property may be one of the mechanisms by which this drug is effective in traditional medicine.[15-18] *H. syriacus* is popular for its nutritional and medicinal values but no studies conducted in direction of the protective role of *H. syriacus* extract against ISO-induced cardiotoxicity may be via the antioxidant system. Hence, the present study was undertaken to find out the cardio-protective potency of methanolic extract of *H.syriacus* flowers (MEHS).

MATERIALS AND METHODS

Drugs and Chemicals

Flowers of *Hibiscus syriacus* were collected from Medicinal Garden of GRY Institute of Pharmacy, Borawan, Madhya Pradesh, India, and were authenticated by Dr. S. K. Mahajan M Sc, Ph.D., Department of Botany, Govt. P. G. College, Khargone, M.P., India. All chemicals were of analytical grade purchased from sigma chemicals, USA.

Extract preparation

Dried flowers of *H. syriacus* were coarsely powdered and 1kg of this powdered plant material was extracted with the help of the Soxhlet apparatus using different solvents. The solvent from the different extracts was removed under vacuum distillation; dried material was kept in a desiccator. A suspension of the flowers in 5% Tween 80 (Vehicle) was made daily.

Physicochemical Analysis

For physicochemical analysis, fresh plant material was collected and shade dried. Physical constants were determined following Indian Pharmacopoeia. It includes ash value, extractive value, and moisture content.

Preliminary Phytochemical analysis

H. syriacus extracts were analyzed for the various classes of phytoconstituents such as flavonoids, phenolic acids, anthocyanins, quinones, alkaloids, tannins, and saponins using standard phytochemical methods. Phytochemical tests were carried out following Shah and Quadry and Kokate.[19]

Experimental animals

Male wistar albino rats of body weight 150-200 g were obtained from the Institute Animal House. The rats were acclimatized in the Department of Animal House at an ambient temperature of 25°C, under a 12 hour dark -12 hour light cycle, for the whole study period. The animals were fed with a standard pellet diet and water *ad libitum*. The experiment was carried out according to the guidelines of the committee for the purpose of control and supervision of experimental on animals, New Delhi, India, and the research protocol was approved by the Institute animal ethical committee (1151/PO/Re/S/08/CPCSEA).

Experimental Protocol

The experimental rats were divided into five groups of 6 animals each. Group I, normal control rats treated with 2% Tween 80 in water (10 mL/kg). Group II, rats treated with Isoproterenol with 2% Tween 80 (10 mL/kg) Group III and IV, rats pretreated with *H. syriacus* flowers extract of two doses (250 mg/kg and 500 mg/kg body weight) and Group V rats treated with standard drug, α-tocopherol (60 mg/kg body weight) with the vehicle by oral gavages once a day for 4 weeks (6 days/week). At the end of the treatment period rats from all groups except the control group were administered Isoproterenol (ISO) 85 mg/kg i.p., for two consecutive days to induce myocardial injury. After 48 hours of the first dose of ISO the rats were sacrificed, hearts and blood samples were collected and immediately frozen in liquid nitrogen for biochemical estimation and in 10% buffered formalin for histological studies.[20]

Treatment protocol

The groups studied were:

Group I: Control, Vehicle + saline-injected rats

Group II: Vehicle + ISO treated rats (85 mg/kg)

Group III: 250 mg/kg of MEHS + ISO treated rats (85 mg/kg)

Group IV: 500 mg/kg of MEHS+ ISO treated rats (85 mg/kg)

Group V: α-tocopherol (60 mg/kg body weight, orally)

Estimation of biochemical parameters

The following biochemical parameters were studied in the heart homogenate.

Myocardial thiobarbituric acid reactive substances (TBARS)

TBARS levels in the myocardium were determined by the method described by Ohkawa *et al* (1979).[21] Hearts were homogenized with 10 mL of Trichloroacetic acid (TCA). 0.2 mL of the whole homogenate was taken to which 0.2 mL of 8.1% Sodium lauryl sulfate, 1.5 mL of 20% acetic acid, and 1.5 mL of 0.8% TBA were added. Volume was made up to 4 mL with double distilled water. It was heated at 950°C for 60 min. After cooling, 1 mL of double-distilled water and 5 mL of butanol: pyridine mixture were added and centrifuged at 4000 rpm for 10 min in a cold centrifuge. The organic layer was separated and absorbance was observed at 532 nm in a spectrophotometer.

Myocardial reduced glutathione

Myocardial GSH was estimated by the method of Ellman *et al*, (1959).[22] The reaction mixture contained 0.1 mL of supernatant, 2.0mL of 0.3M phosphate buffer (pH- 8.4), 0.4 mL of double-distilled water, and 0.5mL of 5, 5 dithiobis 2-nitrobenzoic acid. The reaction mixture was incubated for 10 min and the absorbance was measured at 412 nm. Data are expressed as mole per gram wet weight.

Superoxide dismutase

SOD levels in the hearts were determined by McCord and Firdovich method (1969) and modified by Kakkar *et al*, (1984).[23] A sample (100µl) was added to sodium pyrophosphate buffer (pH-8.3), followed by the addition of 0.1mL of 186 M phenazine methosulfate, 0.3mL of 300mM nitroblue tetrazolium, and 0.2mL of 780M NADH. The reaction mixture was incubated for 90 seconds at 300°C and the reaction was stopped by adding 1.0mL of acetic acid, 4.0mL of n-butanol was then added and centrifuged at 3000 g for 10min. The absorbance of the organic layer was measured at 560 nm. Data are expressed as units per mg protein.

Estimation of Catalase

Catalase level was estimated by the method described by Aebi *et al*.[24] Sample (50 µL) was added to a 3.0 mL cuvette that contained 1.95mL of 50mM phosphate buffer (pH 7.0). Then 1.0mL of 30mM hydrogen peroxide was added and changes in absorbance were followed for 30 seconds at 240 nm at an interval of 15 seconds. Catalase levels are expressed as units per mg of protein.

Cardiac biomarkers

Lactate dehydrogenase (LDH) and serum glutamic oxaloacetic transaminase (SGOT) activities in heart homogenate and serum were assayed by using Star 21 plus Biochemistry Auto Analyser (Cuesta Care Inc., Atascadero, USA).

Histological examination

The hearts of three animals from each group were removed, washed immediately with saline, and then fixed in 10% buffered formalin. The hearts were embedded in a paraffin section cut and stained with hematoxylin and eosin. These sections were then examined under the light microscope for histological changes.

Statistical analysis

All values are expressed as mean ± SD for 6 animals in each group. Data for various biochemical parameters were analyzed using a one-way analysis of variance followed by Tukey's multiple comparison tests (graph Pad Version 3.06, La Jolla, CA, USA). Significance is set at $p < 0.05$.

RESULTS AND DISCUSSION

Physicochemical parameters

After estimation of physical constants, the results obtained were total ash 17.9% w/w, acid Insoluble ash 7.12% w/w, water-soluble ash 10.33% w/w, water-soluble extractive value 40% w/w, alcohol soluble extractive 72% w/w and moisture content 4.8% w/w.

Phytochemical Investigation

Successful evaluation of botanical phytocompounds from plant material is largely dependent on The type of solvent used in the extraction procedure. Hence, our choice is methanol. The result of the phytochemical screen showed the presence of carbohydrates, glycosides, protein and amino acids, flavonoids, tannins, and steroids in flowers of *Hibiscus syriacus*. (Table. 1)

Pharmacological estimation

The results obtained in the different groups subjected to in-vivo myocardial injury are presented below.

Myocardial TBARS

A significant elevation of tissue TBARS level was seen in the isoproterenol-treated group ($p < 0.001$) as compared to normal animals. Administration of *Hibiscus syriacus* flower extracts (250 mg/kg and 500 mg/kg body weight) and α-tocopherol for 24 days resulted in a significant reduction in TBARS ($p < 0.001$) and the levels were almost similar to normal control rats. It is well known that Isoproterenol produces free radicals and these free radicals are involved in membrane damage, leading to elevated levels of TBARS.[25] Treatment with *Hibiscus syriacus* and α-tocopherol in isoproterenol-treated rats decreased the levels of TBARS and this action may be probably due to suppression of membrane damage and reduction in membrane fluidity (Table 2).

Myocardial GSH

Myocardial GSH levels were significantly reduced($p < 0.001$) in ISO-treated animals as compared to untreated animals. Pre-treatment with MEHS (500 mg/kg) and α-tocopherol showed a significant increase ($p < 0.001$) in GSH levels as compared to ISO treated group. Treatment of animals with a dose of 250 mg/kg of *Hibiscus syriacus* led

to an insignificant alteration in the levels of GSH (Table 2). Reduced glutathione is one of the most abundant non-enzymatic antioxidant bio-molecules present in the body.[26] Together with GSH-Px, glutathione reductase (GR), and CAT–SOD couple; it efficiently scavenges free radical species such as H_2O_2, superoxide anions, and alkoxy radicals.

Glutathione levels depleted by ISO-induced damage were significantly ($p < 0.001$) elevated by MEHS (250 and 500 mg/kg) and α- tocopherol pre-treatment. It may be understood that increased levels of GSH could be because of its enhanced synthesis or due to improved glutathione reductase activity in presence of MEHS.

Myocardial SOD

Myocardial SOD activity was significantly lower in the GII group ($p < 0.001$ than that in the control group. Pre-treatment with *Hibiscus syriacus* (250 mg/kg and 500 mg kg) significantly increased the SOD activity ($p < 0.001$) as compared to ISO-treated animals.

Myocardial catalase

ISO-induced myocardial necrosis produced a significant depletion in activities of antioxidant enzymes such as CAT ($p < 0.001$) and GSH-Px ($p < 0.001$) compared to normal animals. There was a slight increase in myocardial catalase levels in the GIII group (250 mg/kg) MEHS (500 mg/kg) and α- tocopherol pre-treatment to myocardial necrotic rats significantly restored the activities of CAT ($p < 0.001$) and GSH-Px ($p < 0.001$).

Cardiac biomarkers

ISO showed a significant ($p < 0.001$) decrease in the level of cardiac marker enzymes (SGOT and LDH) in heart homogenate with a corresponding increase in their levels in the serum when compared with normal control. An increase in the activity of these enzymes in serum could be due to leakage of these enzymes from the heart as a result of free radicals-induced necrosis.[27] In α- tocopherol and MEHS (250 and 500 mg/kg) treated groups significant ($p < 0.001$) increase of LDH in heart homogenate and a decrease of SGOT and LDH in serum were observed.

Histopathological finding

For histopathological studies, three heart samples from each group were extracted and examined under a light microscope. The histopathological finding showed the effect of plant *Hibiscus syriacus* on myocardial tissues of the ISO-induced rats. The histopathological finding of the ISO-induced myocardium showed infracted zone with edema and inflammatory cells and separation of cardiac muscle fibers (Fig. 1A-1E). Oral pre-treatment with *Hibiscus syriacus* 250 mg/kg showed myocardium with moderate edema and inflammatory cells with a decreased area of coagulative necrosis of myocardial fibers (Figure. 1D). Finally, treatment with *H. syriacus* 500 mg/kg showed mild myocardium with mild edema but no infarction and inflammatory cells and the cardiac fibers were within the normal limits (Fig. 1E). For all the parameters, oral pre-treatment of *H. syriacus* (250 mg/kg) to ISO-induced rats showed a significant improvement in the myocardial infarction and indicates the prophylactic cardioprotective effect of *Hibiscus syriacus*. Group IV-V (500 mg/kg and α-tocopherol) showed normal architecture of the heart tissue n=6; † $p < 0.001$ versus GI; a $p < 0.05$, bp < 0.01, cp < 0.001 versus GII; Values are obtained by one-way ANOVA followed by Bonferroni tests; GI: Normal saline (10 mL/kg), orally six days/week for four weeks; GII: Saline + ISO (10 mg/kg), i.p. injection after four weeks; GIII: MEHS (250 mg/kg), orally for four weeks + ISO i.p. injection after four weeks; GIV: MEHS (500 mg/kg), orally for four weeks + ISO i.p. injection after four weeks; GV: α- tocopherol (60 mg/kg), orally for four weeks + ISO i.p. injection after four weeks.

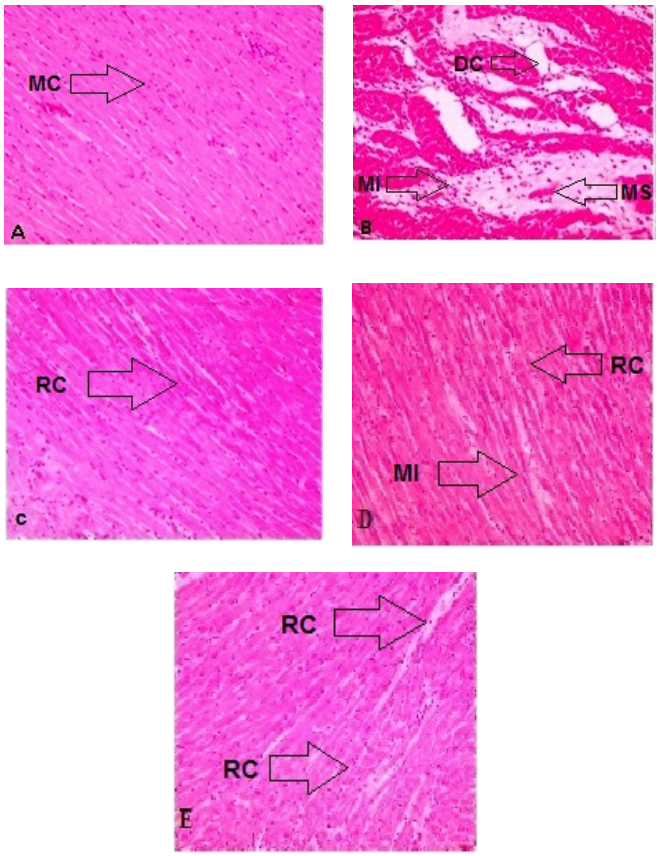

Fig. 1: Effect of methanolic extract of *H. syriacus*, flowers on myocardial morphology: (A) Control rat heart showed normal structure. (B) Rat treated with ISO showed Degenerative changes, mitochondrial swelling, and myocardial infraction. (C) α-tocopherol 60 mg/kg + ISO showed near-normal histological characteristics. (D) *H. syriacus* 250 mg/kg + ISO showed moderate regenerative changes in myocardial cells with a reduced myocardial infraction. (E) *H. syriacus* 500 mg/

Table 1: Qualitative Chemical Examination of Various Extracts (Obtained by Successive Solvent Extraction of *Hibiscus syriacus*)

Extract	*Alkaloid*	*Carbohydrate*	*Glycoside*	*Saponins*	*Proteins and Amino acids*	*Phyto---sterols*	*Fixed oils and Fats*	*Phenolic compounds and flavonoids*	*Tannins*
n-Hexane	-	+	-	-	-	+	+	-	-
Benzene	-	-	+	-	-	+	-	-	-
Chloroform	-	+	-	-	+	-	-	+	-
Acetone	-	-	+	-	-	+	+	+	+
Methanol	-	+	+	-	+	-	-	+	+
Chloroform water	-	+	+	-	+	+	-	+	+

Table 2: Biochemical parameters in different experimental groups

	Groups/unit	*GI*	*GII*	*GIII*	*GIV*	*GVI*
Biochemical	TBARS (µM/g tissue)	570.3 ± 5.57	1243.2 ± 4.91†	858.6 ± 6.9c	755.8 ± 8.0c	654.3 ± 12.2c
Parameters in heart	SOD (U/mg-protein)	1.76 ± 0.03	0.4 ± 0.03†	0.91 ± 0.01c	1.48 ± 0.04c	1.55 ± 0.04c
	GSH (µg/g tissue)	275.5 ± 1.60	203.0 ± 2.85†	238.1 ± 1.19c	253.5 ± 1.23c	255.3 ± 1.40c
	CAT (U/mg-protein)	55.15 ± 0.99	34.75 ± 0.92†	42.2 ± 1.52c	48.78 ± 1.22c	51.56 ± 0.87c
	GSH-PX (µg/g tissue)	273.5 ± 1.03	206.89 ± 1.06†	249.1 ± 1.7c	259.5 ± 2.51c	266.6 ± 1.70c
	SGOT (U/I)	205.0 ± 1.2	41.56 ± 1.18†	129.4 ± 1.06c	196.6 ± 1.8c	197.6 ± 1.84c
	LDH (U/I)	203.4 ± 1.59	74.21 ± 1.55†	149.1 ± 1.64c	181.1 ± 2.33c	198.7 ± 1.65c
Biochemical	TBARS (µM/g tissue)	234.1 ± 2.22	432.2 ± 3.13†	383.6 ± 2.87c	332.4 ± 2.71c	285.6 ± 4.58c
Parameters in serum	SGOT (U/I)	91.32 ± 1.33	213.32 ± 4.89†	166.8 ± 0.17c	117.1 ± 0.12c	102.76 ± 2.53c
	LDH (U/I)	75.12 ± 1.34	187.85 ± 1.05†	107.3 ± 1.55c	91.77 ± 1.34c	85.2 ± 1.99c

kg+ ISO showed significant regenerative changes with a significant reduction in myocardial infarction. (MC: Myocardial Cells, DC: Degenerative change, MI: Myocardial Infarction, MS: mitochondrial swelling, RC: Regenerative Changes)

DISCUSSION

The effect of ISO on the heart is mediated through beta receptors. Both adrenoceptors mediate the positive inotropic and chronotropic effects of beta-adrenoceptor agonists. It has been reported to cause severe stress in the myocardium resulting in infarct-like necrosis of the heart muscle. ISO-induced myocardial infarction serves as a well-standardized model to study the beneficial effects of many drugs and cardiac function. It is also well known to generate free radicals and stimulate lipid peroxidation, which may be a causative factor for irreversible damage to the myocardial membrane.[28] Extract from the flowers of *H. syriacus* extract, MEHS, contains flavonoids, protein, amino acid, glycoside, steroids, carbohydrates, and tannins, which are the most important active substances in the extract (Table.1). The most important flavonoids are glycosides of kaempferol, quercetin, and isorhamnetin with glucose or rhamnose. *Hibiscus syriacus* extract is well known for its antioxidant property, which may result from its ability to scavenge free radicals and neutralize ferryl ion-induced peroxidation. Several studies have reported that the antioxidant activity of plant extract could be helpful in the prevention and therapy of diseases and degenerative processes associated with oxidative stress.[29-30] However, there have been very limited studies on the cardio-protective activity of *Hibiscus syriacus* extract. It is reasonably hypothesized that *Hibiscus syriacus* extract may be helpful for the therapy of heart failure. In our laboratory, we observed the preventive effect of MEHS on cardiac marker enzymes, TBARS, GSH, SOD, and CAT in ISO-induced myocardial infarction in rats. In the present study administration of *H. syriacus* flowers extract caused a significant rise in myocardial endogenous antioxidants (SOD, GSH, and Catalase) and cardiac marker enzymes in heart homogenate (SGOT and LDH) (Table. 2) at the dose of 250 and 500 mg/kg. The increase in TBARS is indicative of enhanced oxidative stress, which in the absence of any evidence of cellular injury (as evidenced by histological studies), may be considered non-lethal. It is, therefore, possible that the increase in oxidative stress was nonlethal and might be responsible for cellular adaptive mechanisms. The principal finding of the present study is that cardiotoxicity was associated with oxidative stress, as evidenced by the increase in myocardial TBARS, depletion of myocardial endogenous antioxidant status (SOD, GSH, and Catalase), and cardiac marker enzymes (SGOT and LDH). Similar observations were made earlier by other studies.[31-34] chronic oral administration of *H. syriacus* flowers extract prevents oxidative stress and the structural changes associated with oxidative

stress. The mechanism of such protection of chronic oral administration of *H. syriacus* flowers extract may be due to myocardial adaptation, oxidative stress is mediated through augmentation of cellular antioxidants such as GSH, SOD, and CAT. Protection against oxidative stress through this mechanism may be one of the effective therapeutic approaches.

Histological examination of heart tissue of group 2 rats showed myocardial necrosis and separation of myocardial fibers with inflammatory mononuclear infiltrate whereas the examination of heart tissue of *H. syriacus* pretreated group (500 mg/kg) showed maximum protective effect by reduced histological changes as compared to ISO myocardial infracted rats. The protection might have been mediated through an extract of *H. syriacus*-induced increase in basal myocardium antioxidant enzyme activities. The cardioprotective effects of MEHS were compared with α-tocopherol as the standard natural antioxidant and also offered significant protection against ISO-induced depletion of marker enzymes and oxidative stress. This action may be probably due to the suppression of membrane damage and reduction in membrane fluidity. Histopathological examination of rat heart section treated with MEHS and α-tocopherol restored the myocardial damage with no evidence of focal damage produced by isoproterenol, which showed the cytoprotective action of MEHS.

The administration of antioxidant-rich natural drugs decreases mortality from cardiovascular diseases and also promises a therapeutic approach to combat oxidative stress associated with cardiac diseases. As per the phytochemical investigation, the MEHS contains flavonoids and phenolic compounds in high concentrations, which might be a responsible active principle for the cardioprotective action.

CONCLUSION

In conclusion, the present study suggests that the flowers of *H. syriacus* are particularly useful agents, as they could enhance myocardial and blood endogenous antioxidant levels without producing any cytotoxic effects. Therefore, the protection against myocardial injury in the treated rats is attributed to enhanced endogenous antioxidant activity. Further research to purify and identify the bioactive compounds responsible for the cardioprotection and antioxidative actions is in progress.

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ABBREVIATION USED:

ISO: Isoproterenol; MEHS: methanolic extract of *H. syriacus*; CPCSEA: Committee for the Purpose of Control and Supervision of Experimental on Animals; TBARS: thiobarbituric acid reactive substances; TCA: Trichloroacetic acid; GSH: reduced glutathione; SOD: Superoxide dismutase; CAT: catalase; NADH: nicotinamide adenine dinucleotide; SGOT: Serum glutamic oxaloacetic transaminase; LDH: Lactate dehydrogenase, GR: glutathione reductase.

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