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Chronic oral administration of *Ocimum sanctum* Linn. augments cardiac endogenous antioxidants and prevents isoproterenol-induced myocardial necrosis in rats

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Abstract

Wistar rats (200–250 g) of either sex were fed with fresh leaf homogenate of *Ocimum sanctum* by oral gavage in two different doses, 50 mg kg^{-1} (Os 50) and 100 mg kg^{-1} (Os 100), daily for 30 days. This was followed by isoproterenol administration (85 mg kg^{-1} s.c. two doses at 24 h intervals) in both control and *O. sanctum*-fed rats to induce myocardial necrosis. Hearts were isolated for estimation of endogenous myocardial antioxidants (superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and glutathione peroxidase (GPx) and myocardial lipid peroxidation) and light microscopic study. Increased basal myocardial antioxidant SOD (9.3 ± 1.2 vs 3.7 ± 0.7 units mg^{-1} protein; $P < 0.05$) and catalase activities (34.3 ± 5.4 vs 17.9 ± 5.1 units mg^{-1} protein; $P < 0.05$) were observed in the Os 50 group only without any evidence of cellular injury in both the groups. In control rats, isoproterenol administration caused significant depletion of myocardial SOD (1.7 ± 0.2 units mg^{-1} protein) and GPx (104 ± 2 mU mg^{-1} protein) activities and increase in GSH (551.7 ± 30.9 μg g^{-1} wet weight of tissue) level, with evidence of myocardial necrosis. Isoproterenol-induced changes in myocardial SOD, GPx and GSH were prevented by both the doses of *O. sanctum*, however cellular injury was minimal only with 50 mg kg^{-1}. The results indicate that long-term feeding of *O. sanctum* offered significant protection against isoproterenol-induced myocardial necrosis through a unique property of enhancement of endogenous antioxidants.

Introduction

Ocimum sanctum Linn. (Tulsi) is a well-known plant that is grown all over India. Several medicinal properties have been attributed to the plant in Indian traditional medicine, in which it is mostly used as an aqueous extract of leaves (Godhwani et al 1987, 1988). The leaf juice has been used for chronic fever, hemorrhage, dysentery, dyspepsia and skin diseases (Agrawal et al 1996; Maity et al 2000). Recently, some studies have also demonstrated that *O. sanctum* possesses good antioxidant activity and this has been mainly attributed to the presence of compounds such as flavonoids, tannins, ascorbic acid and carotenoids (Ganasoundari et al 1997).

Catecholamines play an important role in normal cardiac function. Nevertheless, excess release of catecholamines is responsible for the development of various cardiac dysfunctions, e.g. in cardiac remodelling following acute myocardial infarction (Piano & Prasun 2003), myocyte cell death in heart failure (Carelock & Clark 2001; Goldspink et al 2003) and emotional stress (Klein 2001; Ueyama et al 2003). Higher levels of catecholmines deplete the energy reserve of cardiac muscle cells, leading to complex biochemical and structural changes that cause irreversible cellular damage and ultimately necrosis (Rona 1985). Catecholamines are also known to cause increased reactive oxygen species generation via oxidation and subsequent oxidative stress (Yates & Dhalla 1975), which leads to myocardial necrosis (Bors et al 1978; Dhalla et al 1978). Isoproterenol, a selective beta-adrenergic agonist, is a widely used model to produce infarct-like lesions of the myocardium in rat (Rona et al 1959).

Augmentation of key endogenous antioxidants (superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and glutathione peroxidase (GPx)) has been identified as a unique mode of protection against oxidative stress (Engelman et al 1995). This property has been reported to be present in some compounds, such as

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monophosphoryl A (Lawson et al 1993) and probucol (Siveski-Iliskovic et al 1995), and has generated scientific interest. Some plant products have also been demonstrated to cause augmentation of myocardial antioxidants following chronic administration (Maslova et al 1993; Gauthaman et al 2000; Banerjee et al 2003; Rajak et al 2004).

The present study was designed to investigate whether or not chronic oral administration of fresh leaves of *O. sanctum* has any effect on the basal level of some key myocardial antioxidants and subsequent isoproterenol-induced myocardial necrosis.

Materials and Methods

Plants

Fresh *O. sanctum* (Tulsi) leaves were purchased from a local market and botanical authentication was carried out by the Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India (voucher No. 45). The leaves were ground in double-distilled water to a final concentration of 25 mg mL^{-1}. The freshly prepared aqueous homogenate was fed daily in two different doses of 50 and 100 mg kg^{-1} by gavage within 30 min of its preparation.

The leaves of *O. sanctum* are rich in various bioactive compounds, such as 70% eugenol, an essential oil, and flavonoids, such as apigenin and luteolin (Wagner et al 1994). Qualitative estimations of the compounds were carried out and the leaf homogenate was found to be positive for flavonoids, alkaloids, terpenoid and essential oil. Keeping this in mind along with the fact that in India *O. sanctum* leaves in fresh form have traditionally been prescribed for prophylaxis against various diseases, the present study was designed to evaluate the effect of a fresh leaf homogenate of *O. sanctum* on isoproterenol-induced myocardial necrosis.

Animals

The study was approved by the institute animal care ethics committee (Ethical clearance reference number 137/IAEC/01), which conforms to USA NIH guidelines for animal research (NIH Publication 85–23, 1985). Age-matched laboratory-bred Wistar rats of both sexes weighing between 150 and 200 g, maintained under standard laboratory conditions at 25 ± 2°C, relative humidity 50 ± 15% and normal photo period (12 h dark/12 h light), were used for the experiment. Commercial pellet diet and water were provided ad libitum. Commercial pellet diet (Ashirwad, India) contained 24% protein, 5% fat, 4% fibre, 55% carbohydrate, 0.6% calcium, 0.3% phosphorous, 10% moisture and 9% ash w/w.

Chemicals

All chemicals were of analytical grade and were obtained from Sigma Chemicals (St Louis, MO). Double-distilled water was used for all biochemical assays.

Experimental protocol

Aqueous *O. sanctum* leaf homogenate was fed by oral gavage every day at a fixed time (10.00 h) for 30 days in two different doses, 50 (Os 50; n = 12) and 100 mg kg^{-1} (Os 100; n = 12). Control rats (C; n = 12) were fed by oral gavage normal saline daily for 30 days. At the end of 30 days, six rats each from group (C, Os 50 and Os 100) were administered isoproterenol 85 mg kg^{-1} s.c. (dissolved in double-distilled water) twice, at 24-h intervals (ISO; Os 50 + ISO; Os 100 + ISO; Rona 1985). Twenty-four hours after the last injection the animals were anesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and euthanized. The body weight of the rats in all the groups was measured throughout the experimental period at regular intervals. Food and water intake were also monitored. Hearts were removed and stored in liquid nitrogen for estimation of biochemical parameters and in 10% buffered formalin (pH 7.2) for light microscopic studies.

Biochemical parameters

Myocardial thiobarbituric acid reactive substances (TBARS) were estimated as a marker of lipid peroxidation and myocardial endogenous antioxidants, e.g. GSH, SOD, catalase and GPx were estimated. A decrease in all or some, with or without a rise in TBARS, was considered as oxidative stress. The TBARS assay is used to evaluate lipid peroxidation under conditions of oxidative stress. This is widely used for ex-vivo and in-vitro measurements (Pettenuzzo et al 2003; Sahin & Gumuslu 2004).

Tissue preparation (Bruce & Baudry 1995)

Heart was minced and homogenized in 0.05 M ice-cold phosphate buffer (pH 7.4; 1:10 w/v) by using a Teflon homogenizer. A 0.2 mL portion of the homogenate was used for estimation of TBARS. The remaining part of the homogenate was divided into two parts. One part was mixed with 10% trichloroacetic acid (1:1), centrifuged at 5000 *g* (4°C, for 10 min) and the supernatant was used for GSH estimation. The remaining part of the homogenate was centrifuged at 15 000 *g* at 4°C for 60 min, and the supernatant was used for SOD, catalase, GPx and protein estimation.

Enzyme assays

Myocardial catalase

Catalase activity was estimated by the method described by Aebi (1974). Supernatant (50 μL) was added to a 3.0 mL cuvette that contained 1.95 mL of 50 mM phosphate buffer (pH 7.0). Then 1.0 mL of 30 mM hydrogen peroxide was added and changes in absorbance were followed for 30 s at 240 nm at intervals of 15 s. Catalase activity was expressed as units per milligram protein as compared to the standard. One unit decomposes 1.0 μmol of H_2O_2 to O_2 and H_2O per minute at pH 7.0 at 25°C at a substrate concentration of 50 mM H_2O_2.

Myocardial SOD
SOD activity in the hearts was determined by the modified method of Kakkar et al (1984). Aliquots of the supernatant (100 μL) were added to sodium pyrophosphate buffer (pH 8.3) followed by addition of 0.1 mL of 186 μM phenazine methosulphate, 0.3 mL of 300 mM nitroblue tetrazolium and 0.2 mL of 780 μM NADH. The reaction mixture was incubated for 90 s at 30°C and the reaction stopped by adding 1.0 mL of acetic acid. Four millilitres of n-butanol was then added and centrifuged at 3000 *g* for 10 min. The absorbance of the organic layer was measured at 560 nm. One unit is defined as the enzyme concentration required to inhibit the optical density at 560 nm of chromogen production by 50% in 1 min under the assay conditions, and expressed as specific activity in milliunits mg^{-1} protein. Because the assay was carried out for 90 s, the factor 2/3 was applied for calculating units.

Myocardial GPx
GPx activity was determined by the method described by Wendel (1981). Supernatants were assayed for GPx activity. GPx activity was assayed in a 1.0 mL cuvette containing 400 μL of 0.25 M potassium phosphate buffer (pH 7.0), 200 μL of sample, 100 μL of 10 mM GSH, 100 μL 2.5 mM NADPH and 100 μL of glutathione reductase (6 U mL^{-1}). The reaction was started by adding 100 μL of 12 mM hydrogen peroxide and changes in absorbance at 366 nm at 30-s intervals were measured for 60 s. GPx activity was expressed as units mg^{-1} protein as compared to the standard. One unit catalysed the oxidation by H_2O_2 of 1.0 μmol of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25°C.

Myocardial TBARS
TBARS in tissue was measured as described by Okhawa et al (1979). To 0.2 mL of the homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid and 0.6 mL of distilled water were added and the mixture was incubated at 95°C in a water bath for 60 min then TBARS was extracted with a mixture of butanol:pyridine (15:1 v/v) and the absorbance read at 532 nm.

Myocardial GSH content
GSH content in the tissue was measured according Ellman (1959). The homogenate was mixed with 10% trichloroacetic acid in a 1:1 ratio and then centrifuged for 10 min at 5000 rpm. The supernatant was mixed with 2 mL of 0.3 M phosphate buffer (pH 8.4), 0.4 mL of double-distilled water and 0.5 mL of DTNB (5,5-dithiobis-(2-nitrobenzoic acid)). The reaction mixture was incubated for 10 min and the absorbance was measured at 412 nm. The level of GSH was determined from the standard curve with commercial available GSH (Sigma Chemicals). Data were expressed as μmol per gram wet weight.

Histopathological study

Myocardial tissue was fixed in 10% buffered formalin (pH 7.2), routinely processed and embedded in paraffin. Paraffin sections (7 μm) were cut on glass slides and stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) reagent and examined under a light microscope by a pathologist blinded to the groups studied.

Statistical analysis

All values were expressed as mean ± s.e.m. Data were analysed using one-way ANOVA followed by the post-hoc Bonferroni multiple range test for analysis of biochemical data using Spss (10.0) statistical software. Values were considered statistically significant when $P < 0.05$.

Results

There was no mortality and no changes in body weight or food and water intake patterns of rats in any group.

Biochemical parameters (Figure 1)

Baseline studies (groups Os 50 and Os 100)
Myocardial TBARS. There was no significant change in myocardial TBARS in groups Os 50 (100.9 ± 7.5 nmol g^{-1} wet wt tissue) and Os 100 (118.7 ± 7.4 nmol g^{-1} wet wt tissue) as compared to group C (97.5 ± 10.2 nmol g^{-1} wet wt tissue).

Myocardial GSH. There was no significant change in myocardial GSH levels in groups Os 50 (381.2 ± 34.4 μg g^{-1} wet wt tissue) and Os 100 (355 ± 37.8 μg g^{-1} wet wt tissue) as compared to group C (415.5 ± 35.7 μg g^{-1} wet wt tissue).

Myocardial catalase activity. A significant ($P < 0.05$) increase in myocardial catalase activity was observed in

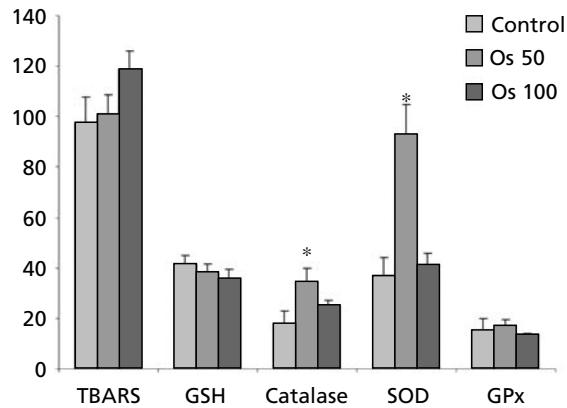

Figure 1 Effect of chronic intake of *O. sanctum* on TBARS (nmol g^{-1} wet wt tissue), GSH ($\times 10^{-1}$ μg g^{-1} wet wt tissue), catalase (units mg^{-1} protein), SOD ($\times 10$ units mg^{-1} protein) and GPx ($\times 10^{-1}$ munits mg^{-1} protein) in rat heart. All values are expressed as mean ± s.e.m. (n = 6). *$P < 0.05$ vs C (one-way ANOVA followed by Bonferroni post-hoc test).

group Os 50 (34.3 ± 5.4 units mg^{-1} protein) in comparison to group C (17.9 ± 5.1 units mg^{-1} protein), whereas no significant change was observed in myocardial catalase activity in group Os 100 (24.9 ± 2.1 units mg^{-1} protein).

Myocardial SOD activity. A significant ($P < 0.05$) rise in myocardial SOD activity was observed in Os 50 group (9.3 ± 1.2 units mg^{-1} protein) in comparison to group C (3.7 ± 0.7 units mg^{-1} protein) whereas no significant change was observed in myocardial SOD activity in group Os 100 (4.1 ± 0.7 units mg^{-1} protein).

Myocardial GPx activity. There was no significant change in myocardial GPx activity in group Os 50 (168 ± 28 mU mg^{-1} protein) and group Os 100 (137 ± 1 mU mg^{-1} protein) as compared to group C (153 ± 45 mU mg^{-1} protein).

Isoproterenol administration in O. sanctum pretreated rats (Os 50 + ISO and Os 100 + ISO) (Figure 2)

Myocardial TBARS. There was no significant change in myocardial TBARS in group ISO (119.9 ± 18.7 nmol g^{-1} wet wt tissue) vs group C (97.5 ± 10.2 nmol g^{-1} wet wt tissue). Myocardial TBARS in group Os 50 + ISO (101.8 ± 5.4 nmol g^{-1} wet wt tissue) and Os 100 + ISO (112.6 ± 32.2 nmol g^{-1} wet wt tissue) were not significantly changed.

Myocardial GSH. There was a significant ($P < 0.05$) increase in myocardial GSH in group ISO in comparison to group C (551.7 ± 30.9 vs 415.5 ± 35.7 $\mu g\, g^{-1}$ wet wt tissue). This rise was absent in group Os 50 + ISO (396.3 ± 20.3 $\mu g\, g^{-1}$ wet wt tissue; $P < 0.01$) and group Os 100 + ISO (346 ± 24.1 $\mu g\, g^{-1}$ wet wt tissue; $P < 0.001$).

Myocardial catalase activity. No significant change in myocardial catalase activity was observed in the ISO group. However, myocardial catalase activity was significantly ($P < 0.05$) high in the Os 50 + ISO group (29.8 ± 3.8 units mg^{-1} protein vs group ISO 14.5 ± 1.5 units mg^{-1} protein). No significant change in myocardial catalase activity was observed in group Os 100 + ISO (17.9 ± 1 units mg^{-1} protein) in comparison to group ISO.

Myocardial SOD activity. A significant reduction ($P < 0.05$) of myocardial SOD activity was observed in group ISO (1.7 ± 0.02 units mg^{-1} protein) when compared to group C (3.7 ± 0.7 units mg^{-1} protein). Myocardial SOD activity was significantly preserved in Os 50 + ISO (3.3 ± 0.6 units mg^{-1} protein) with no change in group Os 100 + ISO (3.1 ± 0.7 units mg^{-1} protein) in comparison to group ISO.

Myocardial GPx activity. A significant reduction ($P < 0.01$) in myocardial GPx activity was observed in group ISO (104 ± 2 mU mg^{-1} protein) when compared to group C (153 ± 45 mU mg^{-1} protein). There was a significant preservation of GPx activity in group Os 50 + ISO (135 ± 38 mU mg^{-1} protein; $P < 0.01$) and in group Os 100 + ISO (139 ± 2 mU mg^{-1} protein; $P < 0.001$) as compared to group ISO.

Histopathology

Figure 3 shows the light micrograph of control heart showing normal architecture. No significant change in the light micrograph was observed in the Os 50 and Os 100 groups. There was confluent necrosis of cardiac muscle fibres with infiltration of acute and chronic inflammatory cells along with extravasation of red blood cells in the ISO group (Figure 4). There was evidence of mild inflammation with occasional foci of lymphocytes in group Os 50 + ISO with no sign of muscle necrosis (Figure 5). In the Os 100 + ISO group there was focal necrosis of muscle fibre with evidence of moderate inflammation as compared to the Os 50 + ISO group (Figure 6).

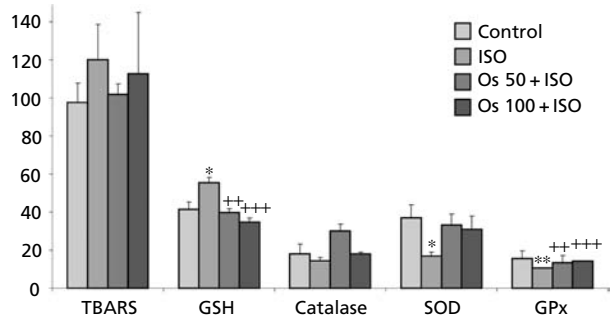

Figure 2 Effect of chronic intake of *O. sanctum* on TBARS (nmol g^{-1} wet wt tissue), GSH ($\times 10^{-1}$ $\mu g\, g^{-1}$ wet wt tissue), catalase (units mg^{-1} protein), SOD ($\times 10$ units mg^{-1} protein) and GPx ($\times 10^{-1}$ munits mg^{-1} protein) in rat heart after isoproterenol-induced injury. All values are expressed as mean ± s.e.m. (n = 6). $^{*}P < 0.05$ vs C, $^{+}P < 0.05$ vs ISO, $^{++}P < 0.01$ vs ISO, $^{+++}P < 0.001$ vs ISO (one-way ANOVA followed by Bonferroni post-hoc test).

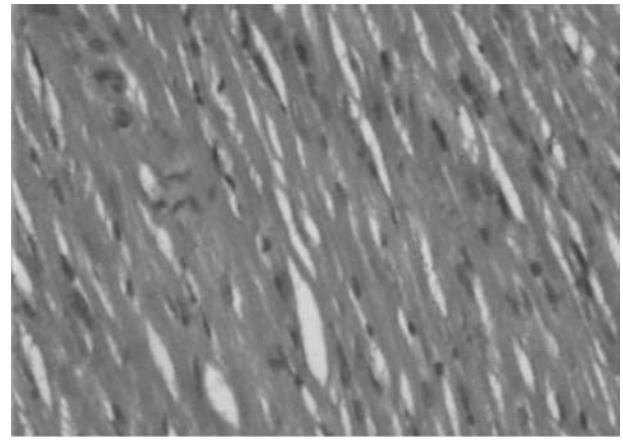

Figure 3 Light micrograph of control rat heart (C) showing normal architecture of myocyte (×10, H&E).

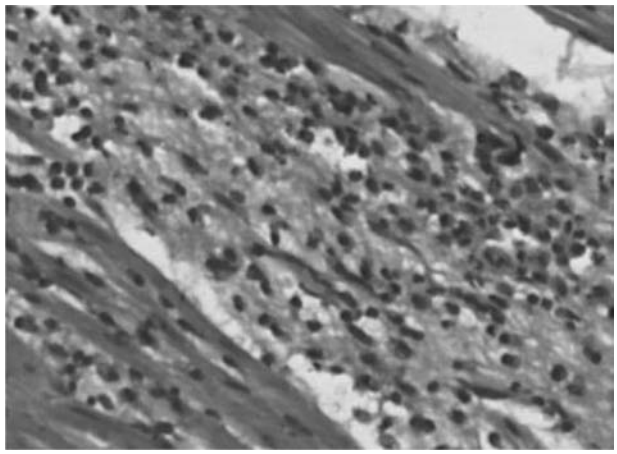

Figure 4 Light micrograph of rat heart treated with isoproterenol (ISO; 85 mg kg^{-1}, s.c.) showing focal confluent necrosis of muscle fibre with acute and chronic inflammation and myophagocytosis. Extravasation of red blood cells is also present (×10, H&E).

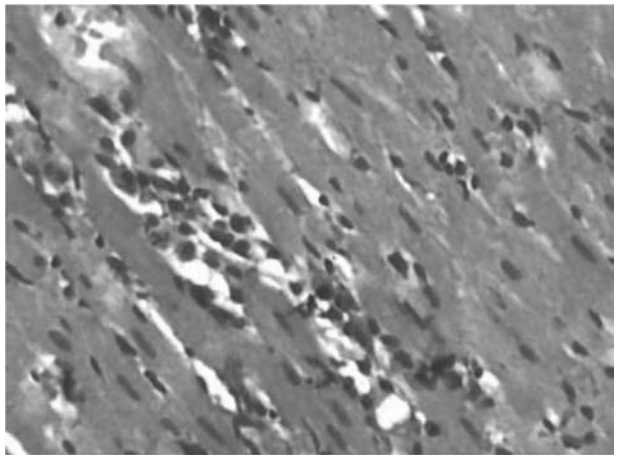

Figure 6 Light micrograph of rat heart pretreated with *O. sanctum* 100 mg kg^{-1} and administered isoproterenol (OS 100 + ISO) showing evidence of moderate inflammation with focal necrosis of muscle fibres with neutrophil infiltration (×10, H&E).

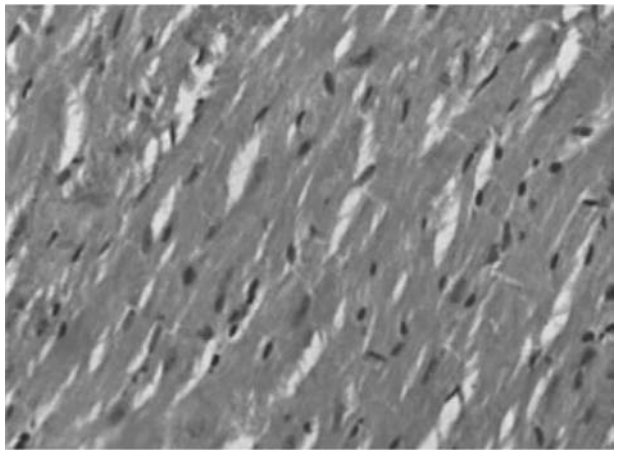

Figure 5 Light micrograph of rat heart pretreated with *O. sanctum* 50 mg kg^{-1} and administered isoproterenol (Os 50 + ISO) showing evidence of mild inflammation with occasional foci of lymphocytes (×10, H&E).

Discussion

In the present study chronic feeding fresh leaf homogenate of *O. sanctum* in a 50 mg kg^{-1} dose caused augmentation of endogenous myocardial antioxidants (150% SOD and 90% catalase activities). Although the antioxidant activity of *O. sanctum* has been reported in previous studies (Banerjee et al 1996; Maulik et al 1997; Devi & Ganasoundari 1999), augmentation of endogenous antioxidants by *O. sanctum* is being reported here for the first time. However, this property was not observed in the higher dose.

Pharmacological augmentation of endogenous myocardial antioxidants has been identified as a promising therapeutic approach in diseases associated with increased oxidative stress (Lawson et al 1993; Siveski-Iliskovic et al 1995). An increase in SOD activity has been reported to be beneficial in the event of increased free radical generation (Yen et al 1996). However, a simultaneous increase in catalase and/or GPx activity is essential for an overall beneficial effect of an increase in SOD activity (Harman 1991; Engelman et al 1995; Das & Maulik 1995; Schaefer et al 1998). An increase in SOD activity alone, without a concomitant rise in the activity of catalase and/or GPx, might be detrimental since SOD generates hydrogen peroxide as a metabolite, which is more cytotoxic than oxygen radicals and must be scavenged by catalase and/or GPx. In this context, augmentation of both SOD and catalase activities appears to be of more biological significance.

In the present study, isoproterenol (a catecholamine) caused oxidative stress in rat heart as evidenced by the reduction in myocardial SOD and GPx activities along with widespread myocyte injury and neutrophil infiltration, which is consistent with similar findings in earlier studies (Kumari & Menon 1987; Kaul & Kapoor 1989; Ithayarasi & Devi 1997; Sharma et al 2001; Banerjee et al 2003). The metabolism of isoproterenol produces quinones, which react with oxygen to produce superoxide anions and hydrogen peroxide, leading to oxidative stress and depletion of the endogenous antioxidant system (Bors et al 1978; Dhalla et al 1978; Rathore et al 2000). However, a rise in myocardial GSH level without any change in the myocardial TBARS level and catalase activity appears to be paradoxical. Similar effects of isoproterenol have been reported in previous studies (Rathore et al 1998; Banerjee et al 2003). The mechanism of such an effect appears to be compensatory.

In the *O. sanctum* 50 mg kg^{-1} dose, significant protection against isoproterenol-induced oxidative stress was observed in terms of preservation of endogenous antioxidants. The degree of myocardial necrosis and loss of muscle fibre was also significantly less in this group. The protection might

have been mediated through an *O. sanctum*-induced increase in basal myocardial SOD and catalase activities. In the *O. sanctum* 100 mg kg^{-1} dose preservation of antioxidants and prevention of isoproterenol-induced myocyte injury was less significant. This could possibly be due to less than optimal augmentation of endogenous antioxidants with *O. sanctum* in the 100 mg kg^{-1} dose. A previous study (Sharma et al 2001) has also reported a protective effect of *O. sanctum* in the 50 mg kg^{-1} dose against isoproterenol-induced myocardial injury. However, the study differed in respect to the formulation of *O. sanctum* used (hydroalcoholic extract vs fresh leaf homogenate in our study) and the duration of administration (12 days vs 30 days in our study).

The choice of formulation used in our study appears to be more rational as it is in this form that *O. sanctum* has been used in the Indian system of medicine for ages for its beneficial effects.

Conclusion

It can be concluded that chronic oral administration of fresh leaf homogenate of *O. sanctum* augments endogenous myocardial antioxidants and protects rat heart against isoproterenol-induced myocardial necrosis and associated oxidative stress.

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