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Further investigations on the antioxidant activity of *Ocimum sanctum* using different paradigms of oxidative stress in rats

S.K. Bhattacharya[1]*, A. Bhattacharya[2], K. Das[3], A.V. Muruganandam[1], K. Sairam[1]

1. Department of Pharmacology, Institute of Medical Scienes, Banaras Hindu University, Varanasi 221005
2. Drug Research & Development Centre, 1078 Jessore Road, Calcutta 700028
3. Indian Institute of Chemical Biology, Calcutta 700032

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Abstract

Objective : To extend the investigations on the reported antioxidant activity of *Ocimum sanctum*. Materials and methods : The oxidative stress parameters used in rats were the isolated heart ischaemia-reperfusion injury, chronic (28 days) exposure to cigarette smoke, chronic footshock stress (21 days) and iron-overload hepatotoxicity. The parameters of oxidative stress included the estimation of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), and lipid peroxidation (LPO). A standardized extract of *O. Sanctum* (OS) and vitamin E (VE), a standard antioxidant agent, were used as the test drugs. Results : OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.) administered for 5 days prior to induction of ischaemia-reperfusion reversed the depletion of SOD, CAT and GPX, and the increase in LPO induced in heart by this model of oxidative stress. Likewise, both OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.), administered for 28 days, concomitant with exposure to cigarette smoke (CS) reversed the changes induced by CS in rat heart and lung SOD, CAT, GPX and LPO. A similar effect was noted when these doses of OS and VE were administered for 21 days along with exposure to chronic stress induced oxidative stress in rat brain frontal cortex and striatum. Iron overload induced increase in hepatic lipid peroxidation was attenuated by these doses of OS and VE, administered for 5 days prior to iron overload. Conclusion : OS exhibited significant antioxidant activity against several paradigms of oxidative stress induced by a variety of techniques in different rat tissues, which was comparable to that induced by VE. The results confirm the antioxidant activity of OS and indicate that, like VE, it is not tissue specific. It also provides a basis for the clinical use of OS in several clinical conditions involving oxidative stress.

Key words:- *Ocimum sanctum*, vitamin E, oxidative stress, antioxidant action

1. Introduction

Ocimum sanctum Linn (Tulsi), known as holy Basil in the Western world, is a widely grown plant in India and is considered sacred by Hindus. Medicinal properties have been attributed to the plant in several ancient systems of medicine, including Ayurveda, Siddha, Unani, Greek, Egyptian

* Corresponding Author
E-mail: salil@banaras.ernet.in

and Roman (1). The leaves of the plant (OS) have been used as expectorant, diaphoretic, antiemetic, anti-infective, analgesic and for their Rasayana properties (2).

Rasayanas are a group of plant derived drugs which form an important constituent of the Ayurvedic concept of preventive medical care, aimed at improving the quality of life, while promoting longevity. Thus, they are claimed to improve physical and mental health, increase the resistance of the body to infection and other external factors which tend to perturb the homeostasis of the human physiological system, promote revival of physiological functions after debilitating diseases and to augment intellect and other cognitive function (3).

There is, thus, a remarkable similarity between the Ayurvedic concept of rasayanas and the modern concept of adaptogens. The latter are a group of plant-derived drugs which appear to induce a state of non-specific resistance of the body to diverse aversive assaults which threaten internal homeostasis.

Pharmacological investigations on OS indicate that the plant has significant anti-stress (4), immunostimulant (5), anabolic (6), cognition-facilitating (7), anti-inflammatory (8), radioprotective (9), anti-gastric ulcerogenic (10), antidiabetic (11), anti-hyperlipidaemic (12), antifertlity (13) and anti-carcinogenic (14) activities.

In view of the wide range of pharmacological actions exhibited by OS, search for a likely common modality led to the investigation of its anti-oxidant action, since several of these pharmacological actions and their clinical connotations are known to involve oxidative stress (15) induced by excessive accumulation of toxic oxidative and non-oxidative free radicals and/or by deficient antioxidant defence (15).

A recent investigation (16) indicated that an OS leaf extract, similar to the one used in this study, exhibited significant antioxidant activity in rat brain frontal cortex and striatum after oral administration for 14 and 21 days. Earlier, *in vitro* studies had demonstrated significant antioxidant effect of OS (17, 18).

The present investigation was conducted to investigate the *in vivo* effectiveness of OS as an antioxidant against a variety of rat models of oxidative stress, including ischaemia-reperfusion cardiac injury, chronic cigarette smoke induced pulmonary and cardiac injury, chronic stress induced changes in frontal cortex and striatum, and iron overload induced hepatic injury. In the first three paradigms, the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were assessed, whereas lipid peroxidation (LPO), as the final determinant of oxidative stress, was assessed in all the investigative models used.

2. Materials and methods

The investigations were conducted on CF strain albino rats (120-160g), of either sex. The animals were housed in colony cages (4-5 rats/cage) at an ambient temperature of 25 ± 20°C and 45-55% relative humidity, with a 12 h light/12 h dark cycle. The rats had free access to standard pellet chow and drinking water. Experiments were conducted between 9 h and 14 h. 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985) guidelines were followed.

Fresh leaves of OS were collected from the Ayurvedic Garden of this Institute, and authenticated by the Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University. The leaves were shade dried and a 50% methanol extract (16) was used. The extract which was 28.4% (w/w) in terms of the dried leaves, was standardized on the basis of its eugenol content (15-18%) (16). Aqueous suspension of the extract with 0.3% carboxymethylcellulose as the suspending agent, was used.

Control animals received the vehicle, 0.3% carboxymethylcellulose in distilled water (2.5 ml/kg, p.o.). OS was administered in the doses of 50 and 100 mg/kg, p.o. Vitamin E (VE) (200 mg/kg, p.o.) (Torrent Laboratories, India) was used as the standard antioxidant for comparison. The duration of treatment varied in the different paradigms used and has been given under the specific methods.

The following experimental paradigms of oxidative stress were used;

2.1 Ischaemia-reperfusion myocardial injury (IRMI)

The isolated rat heart perfusion technique (19) was used. Rats were anaesthetized with pentobarbitone sodium (40 mg/kg, i.p.) and the hearts were dissected out. The aorta was cannulated and perfused with Kreb-Heinslet solution at 37°C on a Langendorf's apparatus. The perfusion cycle used to induce IRMI was a slight modification of the method described earlier (19). The 30 min perfusion cycle consisted of normal perfusion for 5 min, followed by stoppage of the perfusion (ischaemia) for 10 min, and finally restoration of perfusion (reperfusion) for 15 min. Control rat hearts were perfused for 30 min continuosly. At the end of each cycle, the heart was removed, weighed and processed for the biochemical estimations.

OS (50 and 100 mg/kg, p.o.), VE (200 mg/kg, p.o.) and the vehicle were administered for 5 days, the last administration being one hour prior to the perfusion experiments.

2.2 Chronic exposure to cigarette smoke (CS) (20)

Rats were exposed to CS for 2 h each day, including Sundays, for 28 days. The apparatus was a 2 litre glass cylinder (diameter 7.5 cm) with an inlet at the bottom and outlet at the top. The top of the cylinder, below the outlet, was packed lightly with cotton wool to provide oxygenation from the atmosphere.

A volunteer was made to smoke cigarettes (Charminar brand, nicotine content 2.5 mg/cigarette) with a high tar content. 8-10 cigarettes were smoked and the smoke was exhaled in to a glass container connected to the inlet of the cylinder. The outlet was connected to a slow suction pump. On day 28, 1 h after the last 2 h exposure to CS, the rat was sacrificed by spinal dislocation, the heart and lungs were dissected out, weighed and processed for the biochemical estimations.

OS (50 and 100 mg/kg, p.o.), VE (200 mg/kg, p.o.) and the vehicle were administered for all the 28 days, 1 h prior to exposure to CS.

2.3 Chronic stress

The rats were subjected to chronic footshock-induced stress with an added element of unpredictability (21). The animals were administered 1 h footshock daily for 21 days through a grid floor in a perspex box with no route for escape. The duration of each shock (2 mA) and the intervals between the shocks was randomly programmed between 3-5 sec and 10-110 sec, respectively. The rats were sacrificed on day 21, 1 h after the last exposure to stress. The brain was dissected out and the striatum and frontal cortex were removed, weighed and processed for the biochemical estimations.

OS (50 and 100 mg/kg, p.o.), VE (200 mg/kg, p.o.) and the vehicle were administered for 21 days, 1 h prior to exposure to stress.

2.4 Iron overload induced hepatotoxicity

Iron overload was induced by the administration of ferrous sulphate (30 mg/kg, i.p.) (22). The rats were sacrificed 1 h later, the liver was removed, washed with saline perfusate, weighed and processed for estimation of LPO.

2.5 Biochemical estimations

The tissues obtained from the different experimental groups were homogenized in 2 ml of ice cold triple distilled water and sonicated for 16 sec. Thereafter, the homogenates were centrifuged (10,000 x g for 2 min) and the supernatants were used for the estimation of SOD, CAT and GPX. For estimation of LPO activity, the tissues were homogenized in cold potassium chloride (0.5 M). The following biochemical estimations were done:

2.5.1 SOD activity (23)

The assay was based on the ability of SOD to inhibit the spontaneous oxidation of adrenaline to adenochrome. Results are expressed as units (U) of SOD activity/mg protein. One unit of SOD induced approximately 50% inhibition of auto-oxidation of adrenaline.

2.5.2 CAT activity (24)

The assay was based on the ability of CAT to induce the disappearance of hydrogen peroxide (H_2O_2),

which was followed spectrophotometrically. One unit (U) of CAT was defined as the amount of the enzyme required to decompose 1 μM of H_2O_2 per min, at 25°C and pH 7.0. Results are expressed as units (U) of CAT activity/mg protein.

2.5.3 GPX activity (25)

H_2O_2 was used as the substrate. Sodium azide (1 mM) was added to the reaction mixture to inhibit remnant CAT activity. One unit of GPX was defined as the amount of the enzyme decomposing 1 mol H_2O_2 per min, at 25°C and pH 7.0. Results are expressed at units (U) of GPX activity/mg protein.

2.5.4 LPO activity (26)

LPO was determined by estimating the accumulation of the peroxidative product, thiobarbituric acid-reactive substances (TBARS), using a standard curve of 1,1,3,3-tetramethoxypropane, and was expressed as nmol TBARS/g tissue.

2.5.5 Protein estimation

Protein was estimated by the method of Lowry *et al.* (27)

2.6 Statistical analysis

It was done first by one-way analysis of variance, followed by post-hoc use of unpaired Student's *t*-test. A probability value of <0.05 was accepted as being statistically significant.

3. Results

3.1 Ischaemia-reperfusion myocardial injury (IRMI)

The results are summarized in Table 1. OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.), administered once daily for 5 days, tended to increase cardiac SOD, CAT and GPX concentrations, with concomitant decrease in LPO.

However, the results remained statistically insignificant in normally (30 min) perfused hearts. IRMI produced marked decrease in SOD (47.5%), CAT (58.9%) and GPX (39.3%) concentrations, which was accompanied by an increase in LPO (67.8%). 5 day pretreatment with OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.) significantly reversed the IRMI induced decreases in SOD activity by 28.2%, 56.4% and 36.3%, respectively, the decrease in CAT activity by 39.5%, 75.3% and

Table 1.
Effects of *Ocimum sanctum* (OS) and vitamin E (VE) on cardiac superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and lipid peroxidation (LPO) in isolated rat heart model of ischaemia-reperfusion injury (IRI). (Data represent mean ± SEM)

Groups* (mg/kg, p.o.)	**N**	**SOD (U/mg protein)**	**CAT (U/mg protein)**	**GPX (U/mg protein)**	**LPO (n mol TBARS/g)**
Normal perfusion (30 min, NP)	8	23.6 ± 3.2	39.4 ± 3.9	0.84 ± 0.09	49.4 ± 3.9
OS (50) + NP	6	25.6 ± 2.6	42.7 ± 2.2	0.91 ± 0.16	43.2 ± 2.9
OS (100) + NP	6	29.2 ± 3.0	46.9 ± 3.8	0.99 ± 0.18	40.2 ± 3.6
VE (200) + NP	6	26.4 ± 2.2	44.5 ± 2.6	0.96 ± 0.14	44.2 ± 2.0
Ischaemia-reperfusion (IRI)	12	12.4 ± 1.6[a]	16.2 ± 1.2[a]	0.51 ± 0.04[a]	82.9 ± 4.2[a]
OS (50) +IRI	8	15.9 ± 1.1[b]	22.6 ± 0.9[b]	0.66 ± 0.06[b]	60.2 ± 2.7[b]
OS (100) +IRI	8	19.4 ± 0.8[b]	28.4 ± 1.4[b]	0.79 ± 0.09[b]	44.8 ± 3.2[b]
VE (200) +IRI	8	16.9 ± 0.9[b]	26.2 ± 1.6[b]	0.69 ± 0.08[b]	66.6 ± 2.0[b]

*The vehicle and the test drugs were administered once daily for 5 days prior to the perfustion experiments.
[a]$p < 0.05$ different from normal perfusion (NP) group; [b]$p<0.5$ different from ischaemia-reperfusion (IRI) group.

Table 2.
Effects of *Ocimum sanctum* (OS) and vitamin E (VE) on chronic (28-days) cigarette smoke (CS) exposure induced changes in rat heart and lung superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and lipid peroxidation (LPO) (data represent mean ± SEM)

Treatments* (mg/kg, p.o.)	N	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	LPO (n mol TBARS/g)
HEART					
Vehicle (V)	8	3.9 ± 0.4	14.4 ± 0.9	0.08 ± 0.006	39.4 ± 1.9
OS (50) + V	6	4.2 ± 0.9	15.0 ± 1.2	0.09 ± 0.008	36.4 ± 1.4
OS (100) + V	6	5.4 ± 0.6[a]	18.4 ± 0.9[a]	0.16 ± 0.006[a]	30.2 ± 1.4[a]
VE (200) + V	6	5.0 ± 0.3[a]	16.9 ± 0.6[a]	0.12 ± 0.005[a]	33.9 ± 0.9[a]
V + CS	10	5.9 ± 0.4[a]	10.4 ± 0.6[a]	0.04 ± 0.004[a]	69.4 ± 1.6[a]
OS (50) + CS	8	4.2 ± 0.3[b]	14.9 ± 0.8[b]	0.06 ± 0.003[b]	42.9 ± 1.4[b]
OS (100) + CS	8	5.9 ± 0.6[b]	16.0 ± 0.9[b]	0.08 ± 0.005[b]	62.4 ± 2.2[b]
VE (200) + CS	8	4.8 ± 0.7[b]	15.2 ± 0.8[b]	0.06 ± 0.004[b]	52.9 ± 1.6[b]
LUNGS					
Vehicle (V)	8	10.9 ± 0.9	26.7 ± 1.8	0.16 ± 0.008	50.4 ± 2.9
OS (50) + V	6	12.4 ± 0.8	28.4 ± 1.4	0.19 ± 0.009	46.4 ± 1.8
OS (100) + V	6	14.2 ± 0.6[a]	32.4 ± 1.1[a]	0.22 ± 0.008[a]	42.4 ± 1.6[a]
VE (200) + V	6	12.8 ± 0.9[a]	30.1 ± 1.4[a]	0.2 ± 0.004[a]	44.8 ± 0.9[a]
V + CS	10	16.4 ± 1.1[a]	14.9 ± 0.9[a]	0.08 ± 0.003[a]	98.4 ± 5.2[a]
OS (50) + CS	8	12.9 ± 0.9[b]	18.4 ± 0.8[b]	0.12 ± 0.006[b]	72.4 ± 3.2[b]
OS (100) + CS	8	12.0 ± 0.8[b]	23.9 ± 1.2[b]	0.16 ± 0.008[b]	60.4 ± 2.6[b]
VE (200) + CS	8	13.4 ± 0.9[b]	21.4 ± 0.9[b]	0.14 ± 0..006[b]	66.9 ± 3.4[b]

*The vehicle and test drugs were administered once daily for 28 days one hour prior to exposure to cigarette smoke
[a]$p < 0.05$ different from vehicle-treated group; [b]$p<0.5$ different from CS group.

61.7%, respectively, and the decrease in GPX activity by 29.4%, 54.9% and 35.3%, respectively. IRMI induced increase in LPO activity was attenuated by these doses of OS and VE by 27.4%, 46.0% and 19.7%, respectively.

3.2 Chronic exposure to cigarette smoke (CS)

The results are summarized in Table 2. OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.), administered once daily for 28 days tended to increase both cardiac and lung SOD, CAT and GPX levels, with concomitant decrease in LPO activity. The results induced by the higher dose of OS and that of VE were statistically significant. CS for 28 days increased heart and lung SOD activity by 51.3% and 50.4%, respectively. On the contrary, cardiac CAT activity decreased by 27.8% and that of lung CAT by 44.2%, and cardiac and lung GPX activities decreased by 50%.

The LPO activities in heart and lung were increased by CS to the extent of 76.1% and 95.2%, respectively. OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.), administered for 28 days along with exposure to CS, tended to reverse the CS effects in both heart and lung SOD, CAT, GPX and LPO activities, the effects being statistically significant.

3.3 Chronic footshock stress

The results are summarized in Table 3. Twenty one (21) day exposure to footshock stress induced significant increase in rat brain frontal cortex (79.3%) and striatal (53.9%) SOD activity. On the contrary there was significant decrease in frontal cortex CAT and GPX activities (47.2% and 25%, respectively), and that of striatal CAT and GPX activities (54.3% and 44.4%, respectively). Frontal cortex and striatal LPO activity was increased by 67.6% and 41.2%, respectively, following exposure

to stress. 21 day treatment with OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.), given prior to stress exposure, significantly reversed stress induced changes in SOD, CAT, GPX and LPO activities in both the rat brain regions investigated.

3.4 Iron overload induced hepatoxicity

The results are summarized in Table 4. OS (50 and 100 mg/kg, p.o.) and VE (200 mg/kg, p.o.) administered once daily for 5 days, tended to decrease hepatic LPO activity but the effects were statistically insignificant. Iron overload (ferrous sulphate, 30 mg/kg, i.p.) significantly increased (88.5%) hepatic LPO activity which was attenuated by both doses of OS (29.6% and 44.9%, respectively) and by VE (26.5%).

4. Discussion

Oxidative stress, implicated in the pathogenesis of a wide variety of clinical disorders, refers to the cytologic consequence of a mismatch between the production of free radicals and the ability of the cell to defend against them. Oxidative stress can thus occur when the generation of free radicals is augmented, scavenging of free radicals or repair of oxidatively modified macromolecules decreases, or both. This imbalance results in the build up of oxidatively modified molecules, predominantly superoxide (O_2^{-}.) and hydroxyl (OH^{-}.) species. Hydrogen peroxide (H_2O_2) and peroxynitrate ($ONOO^{-}$), although not themselves free radicals, contribute to the cellular redox state.

Together, these molecules are referred to as reactive oxygen species (ROS). ROS can produce functional alterations in lipids, proteins and DNA. Oxidative lipid damage, termed lipid peroxidation, produces a progressive loss of cell membrane fluidity, reduces membrane potential and increases permeability to ions like Ca^{2+}. In addition the toxic peroxidation metabolites can damage cell proteins and DNA (28). An array of cellular defence systems exist to counteract ROS.

These include enzymatic and non-enzymatic antioxidants that lower the steady concentrations of ROS and help repair oxidative cellular damage. SOD catalyzes dismutation of the superoxide radical, and is the only enzyme known to use a free radical as a substrate. ($2O_2^{-}$.+ $H_2O = H_2O_2 + O_2 + 2OH^{-}$.). Apart from the hydroxyl radical, H_2O_2, generated by the action of SOD, is highly toxic by itself and

Table 3.
Effects of *Ocimum sanctum* (OS) and vitamin E (VE) on chronic footshock stress (CS) induced changes in rat brain superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and lipid peroxidation (LPO) (data represent mean ± SEM)

Treatments* (mg/kg, p.o.)	N	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	LPO (n mol TBARS/g)
FRONTAL CORTEX					
Vehicle (V)	8	16.4 ± 1.3	19.3 ± 1.6	0.08 ± 0.004	3.09 ± 0.3
V + CS	8	29.4 ± 0.9[a]	10.2 ± 0.8[a]	0.02 ± 0.001[a]	5.18 ± 0.9[a]
OS (50) + CS	6	21.2 ± 0.8[b]	13.4 ± 0.6[b]	0.04 ± 0.002[b]	3.86 ± 0.6[b]
OS (100) + CS	6	18.2 ± 0.8[b]	17.4 ± 0.9[b]	0.06 ± 0.003[b]	4.82 ± 0.6[b]
VE (200) +CS	6	20.4 ± 0.7[b]	15.4 ± 0.8[b]	0.04 ± 0.004[b]	4.06 ± 0.9[b]
STRIATUM					
Vehicle (V)	8	20.4 ± 1.2	23.2 ± 1.6	0.09 ± 0.006	3.98 ± 0.6
V + CS	8	31.4 ± 0.9[a]	10.6 ± 0.8[a]	0.04 ± 0.003[a]	5.62 ± 0.6[a]
OS (50) + CS	6	24.9 ± 1.0[b]	14.8 ± 0.9[b]	0.06 ± 0.004[b]	4.39 ± 0.4[b]
OS (100) + CS	6	19.0 ± 0.8[b]	19.2 ± 0.8[b]	0.08 ± 0.006[b]	4.06 ± 0.6[b]
VE (200) + CS	6	20.4 ± 0.9[b]	17.2 ± 0.9[b]	0.06 ± 0.004[b]	4.28 ± 0.9[b]

*The vehicle and test drugs were administered once daily for 21 days one hour prior to footshock stress (CS);
[a] $p < 0.05$ different from vehicle-treated group (V); [b]$p<0.5$ different from chronic stress (CS) group.

can generate hydroxyl radicals by the so-called Fenton reaction, by reacting with ferrous ions. Hydroxyl radicals are highly toxic and induce lipid peroxidation of cell membranes. H_2O_2 is neutralized by the enzymes CAT and GPX.

Thus, the free radical scavenging activity of SOD is effective only when it is followed up by increased CAT and/or GPX activity (29). Potential antioxidant therapy should, therefore, include either natural ROS scavenging antioxidant enzymes or agents which are capable of augmenting the actions of these enzymes. Since it is virtually impossible to attenuate the generation of ROS, attention has been concentrated on increasing antioxidant defence in attempting to limit oxidative tissue damage and, thereby, prevent or ameliorate oxidative stress induced diseases and retard their progression (29).

Ayurvedic rasayanas have been extensively used to promote health and increase longevity by increasing defence of the body against disease and adverse environmental factors, by arresting the aging process and by revitalizing the body in debilitated conditions (30). At least part of the rasayana action may be due to an antioxidant activity. Several of these rasayanas, including *O.sanctum* (16), *Withania somnifera* (31,32), *Emblica officinalis* (33-35), *Bacopa monniera* (36) and *Shilajit* (37), have been reported to augment antioxidant defence in experimental situations.

In the present study, OS, which had earlier been reported to increase rat brain striatal and frontal cortex SOD, GPX and CAT after treatment for 14-21 days (16), was investigated for confirmatory antioxidant activity by using diverse models of oxidative stress in rats. Vitamin E (VE), a well accepted antioxidant agent, was used for comparison.

Myocardial infarction is a major cause of mortality caused by ischaemic/ hypoxic injury to cardiac muscles. It has now been conclusively demonstrated that reoxygenation of the myocardium induced by reperfusion, following a brief period of ischaemia, aggravates the ischaemic insult and may even result in cardiac arrhythmias. The cause of ischaemia-reperfusion myocardial injury (IRMI) is postulated to be due to the increased availability of oxygen in the ischaemic-hypoxic area, resulting in excessive accumulation of ROS, particularly O_2^- and H_2O_2, brought about by xanthine oxidase and catalytic transition metals. Depletion of ATP in hypoxic tissue leads to accumulation of hypoxanthine, which can be oxidized by xanthine oxidase, following reperfusion-reoxygenation of the tissue, resulting in rapid and marked generation of ROS.

Furthermore, the released transition metals, following anoxia, can promote the formation of hydroxyl radicals from H_2O_2. This results in augmented lipid peroxidation and extensive cellular damage (37). In the present study, IRMI resulted in significant depletion of all the antioxidant enzymes, SOD, CAT and GPX, resulting in marked increase in lipid peroxidation. These IRMI induced effects were reversed significantly by OS, and by VE, supporting earlier evidence that antioxidants can reduce cardiac toxicity following ischaemia-reperfusion (37).

Cigarette smoke is a highly complex mixture of toxic agents, some of which are either free radicals themselves or free radical precursors. Semiquinones present in cigarette smoke can generate O_2^- from O_2 and the transition metal ions present in the smoke and in tobacco can promote the generation of hydroxyl ions from H_2O_2. It is estimated that 1 μg of iron is inhaled per pack of ten cigarettes. Lung macrophages and respiratory tract secretions in smokers have elevated iron levels. This iron overload can induce increased generation of hydroxyl ions from H_2O_2, resulting in augmented lipid peroxidation and tissue injury.

Cigarette smoke can also release iron from ferritin in the lungs (38). Antioxidants like VE and vitamin-C afford protection against cigarette smoke oxidative stress induced dyslipidaemia in rats as evidence by attenuated lipid peroxidation in serum and tissues (38). Erythrocytes of smokers were found to have increased peroxidase activity which could be reversed by VE (39).

In the present study, chronic exposure to cigarette smoke (CS) over a 4-week period, induced significant increase in heart and lung SOD, with

Table 4.
Effects of *Ocimum sanctum* (OS) and vitamin E (VE) on iron overload-induced hepatic lipid peroxidation in rats ± data represent mean ± SEM)

Treatments* (mg/kg, p.o)	n	Lipid peroxidation (nmol TBARS/g tissue)
Vehicle (V)	12	0.52 ± 0.09
OS (50) + V	6	0.46 ± 0.08
VE (100) + V	6	0.32 ± 0.09
VE (200) + V	6	0.41 ± 0.06
Iron overload (IO)	8	0.98 ± 0.12[a]
OS (50) +IO	8	0.69 ± 0.12[b]
VE (100) + IO	8	0.54 ± 0.09[b]
VE (200) + IO	8	0.72 ± 0.14[b]

*Vehicle, OS and VE were administered for 5 days, one daily, prior to iron overload
[a]$p < 0.05$ different from vehicle (V) treated group; [b]$p<0.05$ different from iron overload (IO) group.

depletion of CAT and GPX, together with increased lipid peroxidation, in these tissues. These CS induced changes were reversed following concomitant treatment with OS and VE. This appears to be the first investigation on the effect of cigarette smoke on tissue antioxidant enzymes.

An earlier study from this laboratory indicated that chronic footshock stress for 21 days induced significant increases in striatal and frontal cortex SOD levels, with concomitant reduction in CAT and GPX activity, and marked increase in lipid peroxidation, in rats. These brain areas were selected in view of their vulnerability to oxidative stress induced neurodegeneration (32).The present investigation confirms these observations.

It has been postulated that several of the stress related diseases may result from induced oxidative stress (40). Cold-restraint gastric ulcers in rats have been correlated with increased gastric mucosal SOD activity and augmented lipid peroxidation (41).

The model of chronic stress used in this study has earlier been shown to induce a number of biochemical, behavioural and physiological perturbations in rats (21), some of which have been linked to oxidative stress, and include immunosupression, cognitive deficits, male sexual dysfunction, glucose intolerance and peptic ulceration. These stress-induced effects were counteracted by a rasayana formulation consisting of ingredients known to exert significant antioxidant effects, like OS, *W. somnifera*, *E. officinalis* and *shilajit* (21). In the present study OS and VE reversed the changes induced by chronic stress in rat brain, confirming the earlier study conducted with *E. officinalis* in rats (32).

Iron overload is associated with lipid peroxidation induced liver damage, characterized by massive iron deposition in hepatic parenchymal cells, leading to fibrosis and eventually to hepatic cirrhosis (22). Iron overload, induced by i.p. administration of ferrous salts, induce peroxidation by reacting with H_2O_2, derived from the action of SOD, to form the highly and toxic hydroxyl radical by the Fenton's reaction (41). These hydroxyl radicals can attack all biological molecules, including lipid membranes to initiate lipid peroxidation. The toxic peroxidative products cause widespread cellular injury.

In a recent study (34), iron overload was shown to induce marked hepatic lipid peroxidation which was accompanied by significant increase in serum concentrations of alanine amino-transferase, aspartate aminotransferase and lactate dehydrogenase, indicating iron overload induced hepatic damage.

The present study confirms iron overload induced augmented hepatic lipid peroxidation (34, 22).

Pretreatment with OS and VE significantly attenuated iron overload induced hepatic lipid peroxidation, confirming the antioxidant activity of these agents.

The data emanating from the present investigation indicates that both OS and VE had marginal *per se* effects on the antioxidant enzymes, SOD, CAT and GPX, and on LPO, when the treatment was restricted to 5 days.

However, when these agents were administered for 21 or 28 days, they produced discernible increase in the levels of the antioxidant enzymes and reduction in LPO. This observation is in keeping with an earlier report, wherein OS was shown to increase levels of SOD, CAT and GPX in rat brain areas only after treatment for 2 weeks and beyond (16).

Another interesting observation was the increase in SOD activity induced by CS and chronic stress paradigms, in contrast to reduction noted in the IRMI group. It appears that when the oxidative stress is of a short duration, there is a depletion of SOD, whereas in oxidative stress of a chronic nature, there is increase in activity of the enzyme, possibly by enzyme induction.

Increased SOD activity, concomitant with augmented LPO activity has been noted in cold-restraint gastric ulcers (41), in Down's syndrome in human brain (42) and in some brain areas of aged rats, consonant with the ROS hypothesis of aging (43). Chronic footshock induced stress in rats was found to be associated with increased SOD activity and increased LPO activity in rat brain areas (32).

Administration of antioxidants caused significant reduction of gastric ulcerations concomitant with reduced LPO activity (41). Likewise, the antioxidant tannoid principles of *E. officinalis* reversed chronic stress effects on rat frontal cortex and striatal SOD and LPO (32).

Similarly, long term administration of a biocatalyser, known to have an antiaging effect, reduced brain LPO activity and decreased the augmented SOD activity in aged rat brain (43). Transgenic mice over-expressing the SOD gene suffer from massive oxidative stress manifested as augmented lipid peroxidation in brain and skeletal muscles (44).

This apparent paradox where increased levels of a major antioxidant enzyme, SOD, results in increased oxidative stress induced lipid peroxidation, has been elucidated. Increased levels of SOD would lead to increased dismutation of the superoxide (O_2-.) radical leading to excessive production of H_2O_2 and the highly toxic hydroxyl (OH.) radical. H_2O_2 can also generate hydroxyl radicals by reacting with transition metals.

The oxidative enzymes required to scavenge H_2O_2 and hydroxyl radicals, namely CAT and GPX, being depleted in all the paradigms of oxidative stress investigated, would result in the accumulation of hydroxyl radicals leading to increased lipid peroxidation and resultant cellular injury. It has been postulated that the ratio between SOD on one hand and CAT and GPX on the other, is critical in determining oxidative stress (42).

It has been further shown that excess SOD can catalyze surrogate reactions, which include a peroxidase function, capable of generating hydroxyl radicals from H_2O_2 (42). It has been noted that Ayurvedic rasayanas have marked similarity with the modern concept of adaptogens and that, a major part of their pharmacological actions and clinical uses can be explained on the basis of their antioxidant activity (40).

Thus, several of the reported pharmacological actions of OS, including its antistress (4), immunostimulant (5), cognition-facilitating (7), antiulcerogenic (10), anti-inflammatory (8), antidiabetic (11), antihyper-lipidaemic (12), radioprotective (9) and anticarcinogenic (14) actions can be rationalized, at least in part, by its antioxidant activity.

The present investigations, using a variety of experimental models of oxidative stress in several tissues, indicates that OS, like vitamin E, has potent and non-tissue specific antioxidant action. It is, therefore, likely that OS may prove to be beneficial in clinical disorders induced by oxidative stress where the clinical presentation is not confined to a single physiological system. OS has been subjected to extensive chemical investigations. Eugenol, the

major chemical entity identified in OS leaf extracts and seed oil, has been found to exert significant antioxidant activity (45). However, the OS flavonoids, including orientin and vicenin, isolated from OS leaves, exhibit significant ROS scavenging activity and marked radioprotective effects (46).

The present investigation provides conclusive *in vivo* evidence for the antioxidant action of OS against several models of oxidative stress with diverse etiologies. However, clinical investigations are required to confirm these observations. Such investigations will help in rationalizing the Ayurvedic uses of the plant, particularly in conditions where oxidative stress is likely to be involved as part of the disease etiopathogenesis.

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