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Evaluation of immunomodulatory potential of *Ocimum sanctum* seed oil and its possible mechanism of action

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Abstract

The present study investigates the effect of *Ocimum sanctum* seed oil (OSSO) on some immunological parameters in both non-stressed and stressed animals. An attempt has also been made to explore the possible mechanism of immunomodulatory activity. OSSO (3 ml/kg, ip) produced a significant increase in anti-sheep red blood cells (SRBC) antibody titre and a decrease in percentage histamine release from peritoneal mast cells of sensitized rats (humoral immune responses), and decrease in footpad thickness and percentage leucocyte migration inhibition (LMI) (cell-mediated immune responses). Restraint stress (RS) produced a significant reduction in the anti-SRBC antibody titre, foot pad thickness and percentage LMI (% LMI). The effects of RS on humoral as well as cell-mediated immune responses were effectively attenuated by pretreating the animals with OSSO. Co-administration of diazepam (1 mg/kg, sc), a benzodiazepine (BZD), with OSSO (1 ml/kg, ip) enhanced the effect of OSSO on RS-induced changes in both humoral and cell-mediated immune responses. Further, flumazenil (5 mg/kg, ip), a central BZD receptor antagonist inhibited the immunomodulatory action of OSSO on RS-induced immune responsiveness. Thus, OSSO appears to modulate both humoral and cell-mediated immune responsiveness and these immunomodulatory effects may be mediated by GABAergic pathways. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Ocimum sanctum* seed oil; Humoral immune response; Cell-mediated immune response; Restraint stress

1. Introduction

Stress, an integral part of a human life has been reported to produce several disease states (Wolff et al., 1950; Solomon and Amkraut, 1981) Alleviation of such stressful situations by psychotherapy or drugs is the key to the treatment of conditions whose etiological basis stems from stress. Physiological stress is known to bring about a wide range of biochemical and behavioral changes in the organism, which is now better known as Selye's Stress Adaptation Syndrome (Kulkarni, 1989). During recent years much attention has been focused on the immunological changes occurring during stress and various studies have reported that stressful situations in fact alter humoral, as well as cell-mediated immune responses (Dantzer and Kelley, 1989; Mediratta, 1994; Mediratta and Sharma, 1997).

Millions of people around the world use traditional systems of medicine for developing immunity, resis-

tance against infections/diseases, to prevent or alleviate the symptoms of the disease or cure it. The main factors that make natural products attractive candidates for human use include their ease of availability, cost effectiveness and presumed safety. Efforts are now being made to unravel the mechanism of action of these natural products.

Ocimum sanctum Linn (OS) commonly known as 'Tulsi' has been extensively used in Ayurvedic system of medicine for various ailments and has been shown to possess significant adaptogenic/anti-stress properties (Bhargava and Singh, 1981). Different parts of the plant are claimed to be effective in a number of diseases (Satyavati, 1987). A steam distilled extract of OS leaves has been shown to enhance anti-sheep red blood cells (SRBC) and IgE antibody titre and to reduce antigen-induced histamine release from peritoneal mast cells (Mediratta et al., 1988). The fixed oil obtained from OS seeds is reported to possess significant anti-inflammatory, antipyretic, analgesic and antiarthritic activities (Singh and Majumdar 1995a,b, 1997; Singh et al.,

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1996). However, the effect of OS seed oil (OSSO) on stress-induced immune responsiveness has not been studied. The present study was, therefore, undertaken to investigate the effect of OSSO on some humoral and cell-mediated immunological parameters in stressed animals. Further, the effect of OSSO has also been studied on these immunological parameters in non-stressed animals. Also, attempts have been made to explore the possible mechanism of immunomodulatory activity of OSSO.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–220 g), male Swiss albino mice (25–28 g) and guinea pigs (700–1000 g) were used in the study. They were kept in 12-h light:12-h dark cycles (07:00–19:00 h) and temperature controlled (22 ± 2 °C) condition. Food and water were available ad libitum unless otherwise indicated. The care of the animals was as per the 'Guidelines for the Care and Use of Animals in Scientific Research' prepared by Indian National Science Academy, New Delhi (Anonymous, 2000).

2.2. Plant material

The dried OS seeds were collected from Maidan Garhi, New Delhi, India and authenticated by a resident botanist, Department of Genetics, Indian Council of Agricultural Research. The voucher specimen has been deposited in the Indian Agricultural Research Institute (IARI), Pusa, New Delhi, India. The seeds were crushed and cold macerated in petroleum ether (40–60 °C) for 3 days. The extract was taken out and petroleum ether evaporated. The oil thus obtained was filtered and then stored at room temperature in light-protected bottles for experimental use.

2.3. Experiments in non-stressed animals

2.3.1. Humoral immune responses

1. Haemagglutination titre to sheep red blood cells. The rats were immunized with SRBC (0.5×10^9 cells per ml per 100 g, ip) on day 0. The animals were then divided in two groups, each group comprising of eight rats. Animals in one group were injected with normal saline while the second group received OSSO in a dose of 3 ml/kg per day, ip from day 1 to 6. On day 7 the animals were lightly anaesthetized with ether and blood was collected from retroorbital plexus. The serum was separated and the haemagglutination titre was estimated using

microtitre plates. Two-fold dilutions (0.025 ml) of sera were made in the microtitre plates with saline. To each well 0.025 ml of 1% (V/V) SRBC was added. The plates were incubated for 1 h at 37 °C and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre, which was expressed in a graded manner, the minimum dilution (1/2) being ranked as 1. The mean ranks of different groups were compared for statistical analysis.

2. Histamine release from peritoneal mast cells of sensitized rats. The animals were sensitized by subcutaneously injecting 0.5 ml egg albumin (25 mg/ml) along with 0.5 ml Freund's complete adjuvant and triple antigen (0.5 ml containing $20\,000 \times 10^6$ B pertussis organisms) on day 0. The rats were divided into two groups of eight animals each. Group I was treated with normal saline and group II with OSSO 3 ml/kg/day, ip from day 1 to 13. On the 14th day animals were sacrificed by exsanguination and peritoneal mast cells were extracted in heparinized Krebs's Ringer solution and finally suspended in the non-heparinized Krebs's Ringer solution containing, in addition, 1 mg/ml of human serum albumin. Cells were usually pooled from two sensitized rats and the cell suspension was divided into 8–10 samples. The samples were incubated at 37 °C for 10 min under gentle shaking followed by addition of antigen, i.e. egg albumin (0.5 mg/ml) and incubated for another 10 min. The reaction was terminated by placing the sample in ice-cold water. The released histamine was bioassayed on atropinized guinea pig ileum and the spontaneous release values were deducted from the results.

2.3.2. Cell-mediated immune responses

1. Leucocyte migration inhibition (LMI) test in rats. The animals were sensitized by subcutaneous injection of 0.5 ml egg albumin (25 mg/ml) along with 0.5 ml of Freund's complete adjuvant on day 0. The control group was administered normal saline while the test group was injected with OSSO 3 ml/kg per day, ip from day 1 to 13. On day 14 all animals were lightly anaesthetized with ether and the chest was opened. About 5–6 ml of blood was collected in a syringe containing 250 U of heparin, by direct cardiac puncture and LMI test was performed (Mediratta and Sharma, 1997).
2. Footpad thickness test in mice. The animals were immunized with SRBC (1×10^8 cells, sc) on day 0. The mice were then divided into two groups, each comprising of 10 animals. Group I received normal saline and acted as control. Animals in group II were injected OSSO in a dose of 3 ml/kg per day, ip from day 1 to 5. On day 5 all animals received

1×10^8 SRBC in the right hind paw and normal saline in the left hind paw. The difference in the footpad thickness of the two paws was measured 24-h later by fluid displacement method.

2.4. Experiments in stressed animals

2.4.1. Humoral immune response

The rats were sensitized by injecting SRBC (0.5×10^9 cells per ml per 100 g, ip) on day 0. On day 7 they received the same dose of antigen, i.e. booster dose and the animals were then divided into different groups of eight rats each. On day 8 one group was administered vehicle and kept in home cages; they were not subjected to RS and acted as 'no stress' (NS) control, however, like stressed animals they were also deprived of food and water. The 2nd, 3rd, 4th, 5th, and 6th groups were injected with vehicle, OSSO (3 ml/kg), diazepam (5 mg/kg), diazepam (1 mg/kg) + OSSO (1 ml/kg) and flumazenil (5 mg/kg) + OSSO (3 ml/kg), respectively, and then subjected to RS in Plexiglas restrainers (Inco, Ambala) at room temperature (22 ± 2 °C). During the period of stress animals were deprived of food and water. Then, 24 h later all the animals were lightly anaesthetized with ether, and blood was collected from retroorbital plexus. Serum was separated and haemagglutination titre was estimated as above.

2.4.2. Cell-mediated immune responses

1. LMI test. The rats were sensitized with 0.5 ml egg albumin (25 mg/ml) and 0.5 ml Freund's complete adjuvant given sc on day 0. The animals were then divided into two main groups. The rats in one group were subjected to RS for 24 h at room temperature (22 ± 2 °C) on day +1 and +13 (1st and 13th day after the day of sensitization, i.e. day 0). Vehicle, OSSO (3 ml/kg), diazepam (5 mg/kg), diazepam (1 mg/kg) + OSSO (1 ml/kg) or flumazenil (5 mg/kg) + OSSO (3 ml/kg) was administered just prior to subjecting the animals to RS on day +1 and +13. During the period of stress, the rats were deprived of food and water. Animals in the other group were not subjected to RS and acted as a 'no stress' (NS) control. However, like stressed animals they were also deprived of food and water. On the 14th day all the animals were anaesthetized with ether and the chest was opened. About 5–6 ml of blood was withdrawn in heparinized syringe by cardiac puncture and LMI test was performed (Mediratta and Sharma, 1997).
2. Footpad thickness test; mice were immunized by injecting 1×10^8 SRBC sc in the back and then divided into different groups of 10 animals each. The non-stressed animals were injected vehicle from day 1 to 5 and kept in home cages. The animals of

the stressed group were treated with vehicle, OSSO (3 ml/kg) diazepam (5 mg/kg, diazepam (1 mg/kg) + OSSO (1 ml/kg) or flumazenil (5 mg/kg) + OSSO (3 ml/kg) and then subjected individually to RS for 1 h every day in round pipe mouse restrainers from day 1 to 5 at room temperature (22 ± 2 °C). On the 5th day, animals in all the groups were challenged with SRBC 1×10^8 SRBC sc in the right hind foot pad, whereas normal saline was injected in the left hind paw. Increase in foot pad thickness was measured 24 h after the challenge by fluid displacement method.

2.5. Statistical analysis

The data were analyzed using Mann–Whitney 'U' test, Student's *t*-test and χ^2 -square test whenever appropriate. A 'P' value of < 0.05 was used as the level of significance in all the statistical tests.

3. Results

3.1. Effects in non-stressed animals

3.1.1. Humoral immune responses

In rats sensitized with SRBC on day 0, administration of OSSO (3 ml/kg per day, ip) from day 1 to 5 produced a significant ($P < 0.05$) increase in anti-SRBC antibody titre (Fig. 1). On the other hand OSSO (3 ml/kg per day, ip) injection from day 1 to 13 significantly ($P < 0.01$) inhibited antigen induced histamine release from the peritoneal mast cells of rats sensitized with egg albumin along with Freund's complete adjuvant and triple antigen (Fig. 1).

3.1.2. Cell-mediated immune responses

OSSO (3 ml/kg per day, ip) produced a significant reduction of the increase in paw volume in mice ($P < 0.05$) and % LMI in rats ($P < 0.01$) as compared with the control normal saline treated group (Table 1).

3.2. Effects in stressed animals

3.2.1. Humoral immune response

RS produced a significant ($P < 0.05$) reduction in the anti-SRBC antibody titre. The antibody titre decreased from 7.8 ± 0.4 (mean \pm S.E.) in the NS group to 5.6 ± 0.3 in the RS group. Pretreating the animals with OSSO (3 ml/kg) or diazepam (5 mg/kg) immediately prior to subjecting them to stress effectively blocked the effect of RS on the anti-SRBC antibody titre. Coadministration of low doses of diazepam (1 mg/kg) with OSSO (1 ml/kg) potentiated the immunomodulatory action of OSSO on RS-induced immunosuppression. Further, flumazenil, a specific central benzodiazepine (BZD) re-

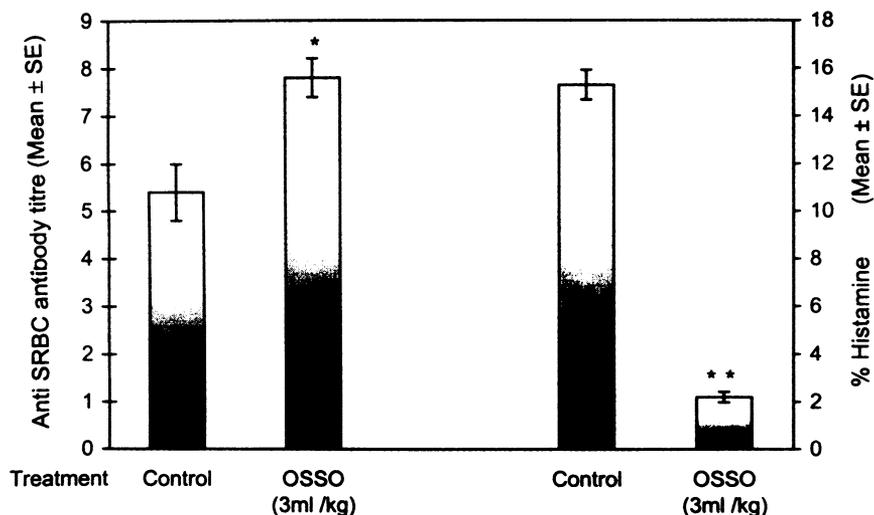


Fig. 1. Effect of *Ocimum sanctum* seed oil (OSSO) on anti-SRBC antibody titre and % histamine release in non-stressed rats; * $P < 0.05$; ** $P < 0.001$.

ceptor antagonist also attenuated the OSSO-induced antagonism of reduction in the anti-SRBC antibody titre produced by RS (Fig. 2, Table 2).

3.2.2. Cell-mediated immune responses

Similar to the effect on humoral immune response, RS caused a significant suppression of cell-mediated immunity, the % LMI and footpad thickness responses were significantly reduced as compared with the NS group. Pretreating the animals with OSSO (3 ml/kg) or diazepam (5 mg/kg) prior to stress procedure attenuated the effect of RS on both % LMI and foot pad thickness. Like humoral immune response, co-administration of diazepam (1 mg/kg) with OSSO (1 ml/kg) potentiated the effect of OSSO on both footpad thickness and % LMI. The immunomodulatory effect of OSSO on both parameters of cell-mediated immune responses was effectively blocked by pretreating the animals with flumazenil (5 mg/kg) (Table 3).

4. Discussion and conclusion

Stress is an important environmental factor, which can affect a number of body functions. It is known that stressful conditions that modify the susceptibility of an individual to a variety of illnesses also influence the immune processes (Solomon and Amkraut, 1981) and alleviation of such stressful situations by psychotherapy or drugs is the key to the treatment of conditions whose etiological basis stem from stress. Keeping this in view the present study was carried out to investigate the effect of OSSO on some immunological parameters in both non-stressed and stressed animals. Pretreating the rats with OSSO produced a significant increase in the anti-SRBC antibody titre and a decrease in the antigen-

induced histamine release from the peritoneal mast cells of the sensitized non-stressed animals. Thus, OSSO exhibited a positive/beneficial effect on humoral immunity in naive non-stressed animals. In the case of cellular immunity, both the studied parameters, i.e. foot pad thickness and % LMI were significantly reduced after OSSO treatment. During the cell-mediated immune response the sensitized T-lymphocytes, on being challenged with the antigen secrete a number of lymphokines including LMI factor (Mustaffa, 1992). These lymphokines attract scavenger cells to the site of reaction, which are then immobilized to promote defensive (inflammatory) reaction. The results of the present study indicate that there was an inhibition of release of lymphokines such as LMI factor on OSSO administration, allowing the inflammatory cells to move away from the site of reaction. Thus OSSO, besides having a direct anti-inflammatory effect as reported earlier (Singh and Majumdar, 1997), may reduce inflammation by inhibiting cell-mediated immune response.

Stress has been reported to produce suppression of both humoral and cell-mediated immune responses

Table 1
Effect of *Ocimum sanctum* seed oil (OSSO) on cell-mediated immune responses in non-stressed animals

| Treatment (ml/kg) | Footpad thickness test in mice (increase in paw volume in mm) (mean \pm S.E.) ^b | LMI test in rats % inhibition ^c |
|-------------------|--|--|
| Normal saline | 0.07 \pm 0.005 | 58 |
| OSSO(3) | 0.034 \pm 0.004 ^a | 30 ^a |

$n = 8$.

^a $P < 0.001$.

^b Student's t -test.

^c χ^2 -square test.

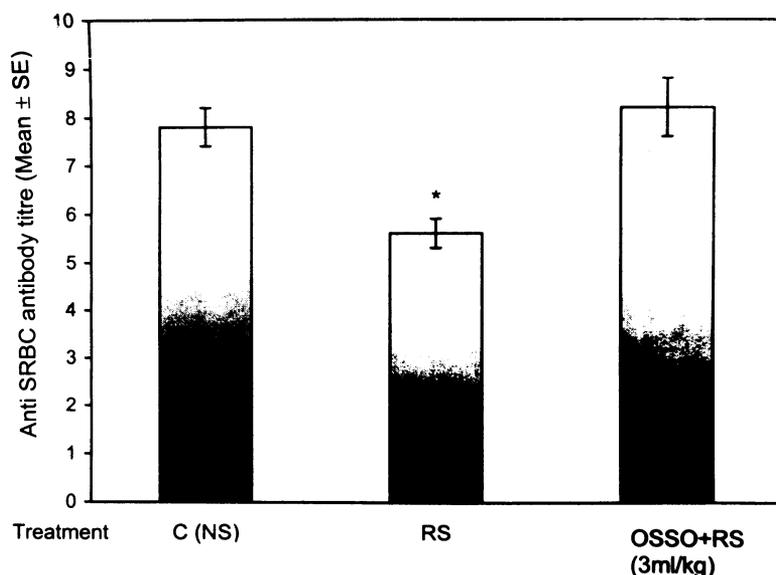


Fig. 2. Effect of restraint stress (RS) and its modulation by *Ocimum sanctum* seed oil (OSSO) on anti-SRBC antibody titre in rats; * $P < 0.05$; NS, no stress.

(Dantzer and Kelley, 1989; Mediratta, 1994; Mediratta and Sharma, 1997). In consonance, the results of the present study also show that RS produced a reduction of antibody titre as well as footpad thickness and % LMI. Pretreating the animals with OSSO effectively blocked the immunosuppressive effect of RS on both humoral and cell-mediated immune responses. For antagonism of suppression of humoral immunity produced by RS, its positive immunomodulatory activity as observed in non-stressed animals where OSSO administration produced an increase in anti-SRBC antibody titre, may be working in the same direction to oppose the stress-induced humoral immunosuppression. However, the same may not be true for the cell-mediated immune responses where both % LMI and footpad thickness appeared to decrease in non-stressed animals but seemed to be stimulated in stressed animals, i.e. inhibition of the RS-induced inhibition of % LMI and change in paw volume. This effect on cell-mediated immunity may be related to immunological allostasis which explains that an organism is capable of allostatic regulation of the immune responses to avoid immunological dissonance which can lead to death from disorders such as excessive inflammatory response syndrome, or conversely, the compensatory anti-inflammatory response syndrome (McEwen, 1988; Bone, 1996). In the context of the present study if inflammatory or anti-inflammatory connotations are substituted for 'stress' and 'anti-stress' conditions, the results on both humoral and cell-mediated immune response in stressed animals suggest that OSSO has acted to maintain the immunological allostasis.

To investigate the mechanism of immunomodulatory effect of OSSO, its interaction with diazepam, a BZD

which is known to exert anti-anxiety and anti-stress activity (Mediratta, 1994; Mediratta and Sharma, 1997; Tripathi, 1991) was studied. BZDs produce their effect by augmenting GABA receptor mediated chloride ion conductance (Havoundjian et al., 1987; Tripathi, 1991). In the present study, diazepam potentiated the immunomodulatory activity of OSSO on RS-induced immunosuppression. Further, the effect of OSSO on stress-induced immunological parameters was significantly antagonised by flumazenil, a central BZD receptor antagonist (Brodgen and Goa, 1991). These results indicate that OSSO may be exerting its immunomodulatory effect by modulating GABAergic activity. Thus, OSSO appears to influence both humoral and cell-mediated immunological parameters in naive non-stressed,

Table 2

Effect of restraint stress (RS) and its modulation by *Ocimum sanctum* seed oil (OSSO), diazepam and flumazenil on anti-SRBC antibody titre

| Treatment (mg/kg) | Anti-SRBC antibody titre (mean ± S.E.) |
|----------------------------------|--|
| Control (NS) | 7.8 ± 0.4 |
| RS | 5.6 ± 0.3 ^a |
| OSSO (3 ml)+RS | 8.2 ± 0.6 ^{**b} |
| Diazepam (5 mg)+RS | 8.4 ± 0.4 ^{**b} |
| Diazepam (1 mg)+OSSO (1 ml)+RS | 8.8 ± 0.6 ^{**b} |
| Flumazenil (5 mg)+OSSO (3 ml)+RS | 6.2 ± 0.4 ^c |

NS, No stress; $n = 8$; * $P < 0.05$; ** $P < 0.001$ (Mann-Whitney 'U' test).

^a Compared with control (NS) group.

^b Compared with RS group.

^c Compared with OSSO+RS group.

Table 3
Effect of restraint stress (RS) and its modulation by *Ocimum sanctum* seed oil (OSSO), diazepam and flumazenil on cell-mediated immune responses

| Treatment (ml–mg/kg) | Footpad thickness test in mice (increase in paw volume in mm) (mean \pm S.E.) ^a | LMI test in rats % inhibition ^b |
|----------------------------------|--|--|
| Control (NS) | 0.067 \pm 0.003 | 46.0 |
| RS | 0.042 \pm 0.004 ^{*c} | 20.0 ^{**c} |
| OSSO (3 ml)+RS | 0.062 \pm 0.003 ^{*d} | 39.0 ^{**d} |
| Diazepam (5 mg)+RS | 0.061 \pm 0.002 ^{*d} | 41.5 ^{**d} |
| Diazepam (1 mg)+OSSO (1 ml)+RS | 0.072 \pm 0.003 ^{**d} | 61.5 ^{**d} |
| Flumazenil (5 mg)+OSSO (3 ml)+RS | 0.048 \pm 0.003 ^{*c} | 28.2 ^{**c} |

$n = 8-10$; $*P < 0.05$; $**P < 0.001$.

^a Student's *t*-test.

^b χ^2 -test.

^c Compared with NS group.

^d Compared with RS group.

^e Compared with OSSO+RS group.

as well as stressed animals and these immunomodulatory properties could be mediated via GABAergic pathways.

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