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Protective effect of *Ginkgo biloba* on ethanol - induced immunosupression in rats

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ABSTRACT

The present investigation deals with the study of protective effect of Ginkgo biloba (GB) aqueous dried leaf extract on ethanol-induced impairment of immune responses. The ethanol treated (2 g/kg, 20% w/v p.o.), rats concurrently received either the dried leaf extracts of Ginkgo biloba (300 mg/kg each, orally) or a combination of vitamin C and E (100 mg/kg each, orally) or vehicle for the same period. The various parameters like phagocytosis, total leukocyte count (TLC), humoral and cell-mediated immune responses, lipid peroxidation (LPO), reduced glutathione (GSH) content, superoxide dismutase (SOD) and catalase (CAT) activities were assessed. Chronic administration of ethanol decreased the humoral and cell-mediated immune response, phagocytosis, phagocytosis index, TLC, GSH, CAT and SOD activities and increases the LPO. These influences of ethanol were prevented by concurrent daily administration of extract and the effect was comparable with that of the combination of vitamin C and E. The ethanol-induced immunosupression is due to oxidative stress and Ginkgo biloba can prevent the same by virtue of its antioxidant property.

Key words: Immunosupression, Ethanol, Ginkgo biloba, Oxidative stress.

INTRODUCTION

The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of many diseases. Modulation of immune response to alleviate the disease has been interest for many years [1]. The function and efficacy of immune system may be influenced by many exogenous factors like food and pharmaceuticals, physical and psychological stress and

hormones etc. resulting in either immune stimulation or immunosupression. Indian medicinal plants are rich source of substances, which are claimed to induce para immunity, the nonspecific immunomodulation. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of impaired immune responsiveness. Plant drugs which have been used in traditional medicine for their antiviral, antioxidant or antitumor activities are good candidates for screening immunostimulant potency. Also there are large numbers of compound like alkaloids, terpenoids, saponins, simple phenolic compounds, essential fatty acids, vitamins, polysaccharides having potential immunostimulating potency [2].

Ginkgo biloba L. (GB) (Ginkgoaceae) is an important herb of the Chinese traditional system of medicine [3]. It is known to be endowed with a variety of biological and pharmacological properties due to the high content of secondary metabolites. The GB acts as a scavenger of reactive oxygen species (ROS) [4], peroxyl radical [5] and nitric oxide [6] due to its high phenolic content. Previously, cardioprotective, antiasthmatic, antidiabetic and potent central nervous system activities have been reported [7, 8, 9]. Furthermore, the extract and its ingredients exhibit an antagonistic effect on platelet-activating factor [10] and the inhibitory effects on expression of inducible nitric oxide synthase as well as nitric oxide production [11].

Since long time there are clinical evidences for immune compromised condition of chronic alcoholics [12, 13]. The impairment in the humoral and cell mediated immune responses have been experimentally demonstrated in ethanol administered rats [14]; it may be attributed to generation of ROS. Present literature revealed the scope of GB as an immunomodulator based on this; an attempt has been made to evaluate the immunomodulatory potency of GB in ethanol-induced immunosupression to evaluate the pharmacological basis for their activity.

MATERIALS AND METHODS

Chemicals - Pyrogallol and hydrogen peroxide are obtained from S.D. Fine Chemicals Ltd. (India). Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), 5, 5'- dithiobis (2-nitrobenzoic acid) (DTNB), Hank's balanced solution (HBSS), phosphate buffer and Tris buffer were obtained from Rajesh Chemicals (Mumbai, India). All other chemicals and regents were of analytical grade.

Plant material – The aqueous extract of GB leaf was gifted by Ranbaxy Labs., Delhi (India).

Animals - Sprague-Dawley rats of either sex weighing 120-150g were housed in a standard environmental condition fed on standard diet, water ad libtum at $24 \pm 2^{\circ}$ C and day-night cycle 06:00 h to 18:00 h. All the animal experimentations were out carried after prior permission from the Institutional ethical committee of the Govt. College of Pharmacy, Amravati (MS) India.

Immunological studies

The animals were majorly divided into two groups i.e. normal control and ethanol treated (20% w/v, 2g/kg) [15]. Further each group was subdivided into vehicle, extract (GB, 300 mg/kg p.o.) and drug treated (Vit. C and E, 100 mg/kg, p.o.). The above treatments were given daily for four weeks. The control groups received vehicle (distilled water) instead of ethanol. The immune

function was assessed using humoral and cell mediated immune responses, phagocytosis and total leukocyte count (TLC) in rats. Whereas the oxidative stress was assessed by estimating lipid peroxidation (LPO), reduced glutathione content (GSH) and the activity of superoxide dismutase (SOD) and catalase (CAT). The results were compared with that of vitamin C and E, the known natural antioxidants.

The sheep blood was withdrawn from the juglar vein (Slaughter house) and RBCs were preserved in Alsever solution. It was then suspended in phosphate buffered saline for further use. All rats were antigenically challenged twice with sheep RBC (0.5×10^9 cells/100 g, i.p.), with first challenge on 14th day and second on 20th day of the experiment [16, 17].

Humoral immune response: On day 20th and 27th, the blood was withdrawn from retro-orbital plexus of all antigenically challenged rats. 25 μ l of serum was serially diluted with 25 μ l of phosphate-buffered saline. Sheep RBC (0.025 × 10⁹ cells) were added to each of these dilutions and incubated at 37 °C for 1 hr. The rank of minimum dilution that exhibited hemagglutination was considered as the antibody titer. The level of the antibody titer on 20th day of the experiment was considered as the primary humoral immune response, where as the one estimated on day 27th of the experiment was the secondary humoral immune response.

Cellular immune response: This was assayed by footpad reaction method in rats. The increase in the paw volume induced by an injection of sheep RBC (0.025×10^9 cells), in the sub planter region of right hind paw on day 27, was assessed after 48 h. The mean percent increase in paw volume was considered as delayed type of hypersensitivity reaction and considered as an index of cell-mediated immunity. The volume of left hind paw, injected similarly with phosphate buffered saline, served as control. The percentage reduction in paw volume was taken as a measure of cellular immune response.

Phagocytosis: On day 28, 3 ml of HBBS containing 10% bovine serum albumin (BSA), was injected into the peritoneal cavity of the animal and the same was recovered by aspiration. The macrophages $(3 \times 10^5 / 600 \,\mu)$, present in the aliquots, were incubated on glass plates at 37°C for 30 min in humidified chamber. The cells adhered to the glass, were incubated with live cells of *Candida albicans*, previously opsonized autologous plasma ($2 \times 10^6 / 250 \,\mu$), at 37°C for 30 min, then washed with HBSS, again incubated for 30 min with autologous plasma and finally stained with Wrights dye. A total of 300 cells were counted under microscope and results are reported as Phagocytosis percent, Phagocytosis index and Digestion index [18]. The blood, withdrawn from the retro orbital plexus, at the end of 4th week and TLC was assessed by routine hematological method.

Antioxidant activity

The blood withdrawn from the retro orbital plexus, at the end of 4th week were studied for oxidative stress, was assessed by estimating LPO, SOD, CAT and GSH.

Lipid peroxidation (LPO)

To 2 ml, 5% suspension of separated RBC in 0.1 M phosphate buffered saline, 2 ml of 28% trichloroacetic acid was added and centrifuged. 1 ml of 1% thiobarbituric acid was added to 4 ml of supernatant, heated in boiling water for about 60 min and cooled immediately. The absorbance

Shirish P. Jain et al

was measured spectrophotometrically at 532 nm. The lipid peroxidation was calculated on the basis of the molar extinction coefficient of malondialdehyde (MDA) (1.56×10^5) , and expressed in terms of nanomoles of MDA/g Hb [19].

Superoxide dismutase (SOD)

It was estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 μ l of the lysate, 75mM of Tris-HCl buffer (pH 8.2), 30mM EDTA and 2mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm. One unit of enzyme activity is 50% inhibition of the rate of the autooxidation of pyrogallol as determined by change in absorbance / min at 420 nm. The activity of SOD is expressed as units/mg protein [20].

Catalase (CAT)

Catalase activity was determined in erythrocyte lysate using Aebi's method with some modifications. 50 μ l of the lysate was added to the cuvette containing 2 ml of phosphate buffer (pH 7.0), 1 ml of 30mM H₂O₂ and was measured at 240 nm. Molar extinction coefficient of H₂O₂, 43.6 M cm⁻¹ was used to determine the catalase activity. One unit of activity is equal to one mM of H₂O₂ degraded per minute and is expressed as units per mg of protein [21].

Reduced glutathione (GSH)

Blood glutathione was measured by addition of 0.2 ml of whole blood to 1.8 ml distilled water followed by 3.0 ml of precipitating mixture (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl to make 100 ml of solution). It was centrifuged at 5000 RPM for 5 min and to 1 ml of the filtrate was added 1.5 ml of the phosphate solution, followed by the addition of 0.5 ml of DTNB reagent. The optical density was measured at 412 nm using spectrophotometer [22].

Statistical analysis

All data were analyzed with one-way ANOVA followed by Dennett's multiple comparison tests. The intergroup difference was considered significant when p<0.05. The correlation between oxidative stress and immunological parameters was checked by Pearson correlation analysis.

RESULTS

Table 1 exhibits that prolonged oral treatment of rats with ethanol (2g/kg, 20% w/v) significantly (P<0.05) decreased both, the antibody titer and the increase in paw volume. Similarly, there appears to be a parallel decrease in the percentage and index of phagocytosis, digestion index and total leukocyte count. It was further observed that the daily treatment with dried aqueous leaf extract of GB (300 mg/kg) significantly (P<0.05) prevented effects of ethanol.

The oxidative stress marker (Table 2) studies revealed that the chronic administration of ethanol increase the level of LPO, decrease the activity of SOD and CAT and reduced the content of GSH. Pearson correlation analysis revealed that the immunotoxic and oxidative stress-generating effects of ethanol posses significant correlation with each other (Humoral immunity *vs* Lipid peroxidation, r = -0.961; Cellular immunity *vs* Lipid peroxidation, r = -0.982; and Phagocytosis percentage *vs* Lipid peroxidation, r = -0.948, all at p< 0.05). The concurrent treatment of ethanol-administered rats with GB prevented the above ethanol-induced changes in

Shirish P. Jain et al

the marker of oxidative stress. Effects of GB on ethanol-induced changes were more or less similar and comparable with the vitamin E and C treatment.

Groups	Treatments	Humoral immune response (Mean antibody titer levels)		Cellular immune response (%increase in paw	Phagocytosis percentage	Digestion Index	Total index	Leukocy te count (cells/ mm ³)
		1 milar y	secondary	volume)				11111 <i>)</i>
Control	Vehicle	8.30 ± 0.74	10.96 ± 0.23	33.90 ± 2.65	90.10 ± 4.98	3.10 ± 0.33	0.96 ± 0.02	6817.33 ± 53.77
	GB	7.69 ± 0.51	12.05 ± 0.9	35.90 ± 2.7	84.50 ± 1.64	2.92 ± 0.37	1.10 ± 0.06	6790.67 ± 37.15
	Vitamin C and E	7.88 ± 1.06	11.47 ± 0.67	36.23 ± 3.2	79.70 ± 2.68	2.78 ± 0.52	0.87 ± 0.05	6715.18 ± 23.56
Ethanol treated	Vehicle	4.36 ± 0.47*	8.13±0.3*	12.11 ± 1.28*	57.57 ± 4.7*	$0.9\pm0.1*$	$0.23 \pm 0.01*$	4472.21 ± 19.38*
	GB	7.30 ± 0.37 [#]	$10.83 \pm 0.5^{\#}$	$30.47 \pm 1.1^{\#}$	$80.86 \pm 2.82^{\#}$	$2.79\pm0.56^{\#}$	$0.87 \pm 0.02^{\#}$	$6544.20 \pm 74.88^{\#}$
	Vitamin C and E	7.22 ± 0.51	10.45 ± 0.4	29.10 ± 2.46	76.10 ± 6.82	2.52 ± 0.69	0.85 ± 0.09	64590.7 ± 44.75
One-way ANOVA	F	5.48	6.13	14.79	7.11	2.74	32.17	353.89
	df	5,30	5,30	5,30	5,30	5,30	5,30	5,30
	Р	0.0011	0.0005	< 0.0001	0.0002	0.039	< 0.0001	< 0.0001

 Table 1. Effect of aqueous dry leaf extract of Ginkgo biloba (GB) on immune response in rats

 (mean SEM, n=6)

 ${}^{*}P < 0.05$ when compared with respective control group. ${}^{\#}P < 0.05$ when compared with vehicle treated experimental group.

Table 2. Effect of aqueous dry leaf extract of *Ginkgo biloba* (GB) on oxidative stress in rats (mean SEM, n=6).

Group	Treatments	Lipid peroxidation (NmMDA/g Hb)	Glutathione µ mDTNB (conjugated /g Hb)	Superoxide dismutase (units/mg protein)	Catalase (units/mg protein)
Control	Vehicle	72.96 ± 1.98	5.37 ± 0.70	30.37 ± 0.87	285.23 ± 7.47
	GB	64.50 ± 2.32	6.22 ± 0.37	28.10 ± 0.53	277.97 ± 6.15
	Vitamin C and E	70.68 ± 2.88	5.08 ± 0.82	28.87 ± 0.45	290.18 ± 12.56
Ethanol treated	Vehicle	$174.57 \pm 2.07*$	$3.09 \pm 0.22*$	$17.23 \pm 0.98*$	$193.21 \pm 5.38*$
	GB	$71.86 \pm 1.89^{\#}$	$5.79 \pm 0.56^{\#}$	$20.07 \pm 0.02^{\#}$	$234.20 \pm 8.88^{\#}$
	Vitamin C and E	$98.10 \pm 3.72^{@}$	5.52 ± 1.06	21.13 ± 0.99	148.67 ± 10.55
One wer	F	259.72	4.03	101.12	24.06
ANOVA	df	5,30	5,30	5,30	5,30
	Р	< 0.0001	0.0090	< 0.0001	< 0.0001

^{*}P < 0.05 when compared with respective control group. [#]P < 0.05 when compared with vehicle treated experimental group. [@]P < 0.05 when compared with GB treated experimental group.

DISCUSSION

The present study has shown that the administration of ethanol, over a period of four weeks, not only impaired the immune responses but also produced oxidative stress in rats. Since, the mixture of vitamin C and E significantly attenuated these ethanol-induced changes; it appears that the immunotoxic effects of ethanol may be due to oxidative stress. The literature has

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documented free radical generation during the metabolism of ethanol. The level of the marker of the oxidative stress, observed in ethanol treated rats, substantiate the possibility of extensive generation of the free radicals. It is further observed that administration of GB extract, prevented the ethanol-induced changes of immunological and oxidative stress parameters, and the effect was comparable to that of vitamin E and C. Hence, the immunomodulatory effect of GB may be subsequent to the antioxidant activity, which it possesses.

The mechanism of free radical-induced impairment of immune system is not yet properly understood. Reduced glutathione, a free radical scavenger, plays a key role in the activation of T cells and macrophages [23]. The present investigations have revealed that chronic treatment with ethanol depleted the glutathione, reduced the digestion index and impaired the phagocytosis. It is further observed that aqueous dry leaf extract of GB or the mixture of vitamin C and E prevented the above influences of ethanol may be subsequent to the depletion of glutathione. Since the antioxidant therapies could reverse these influences, the free radicals may involve in this effect of ethanol.

In fact, free radicals are the signaling in T cell activation. However, continued generation of free radicals, over a long period was shown to down regulate the activation of T cell and NF kappa-B [24]. Similar down regulation of T cell activation has been observed when nitric oxide, a free radical generator, is constantly generated by macrophages. It has also been shown that NO synthase inhibitor attenuates this down regulation [25]. Previously it was reported, that GB extract and its ingredient show inhibitory effect on the expression of inducible nitric oxide. Recent reports showed that GB attenuates oxidative stress in macrophages and endothelial cell [26]. Hence the reduction in oxidative stress, by the antioxidant effect of aqueous dry leaf extract of GB or the mixture of vitamin C and E, appear to be the underlying mechanism for the observed immunomodulation.

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