

## IN VIVO EVALUATION OF ANTIOXIDANT ACTIVITY OF ALCOHOLIC EXTRACT OF *RUBIA CORDIFOLIA* LINN. AND ITS INFLUENCE ON ETHANOL-INDUCED IMMUNOSUPPRESSION

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### ABSTRACT

**Objective:** To evaluate the *in vivo* antioxidant activity of alcoholic extract of the roots of *Rubia cordifolia* Linn. (RC) and to study its influence on ethanol-induced impairment of immune responses.

**Methods:** The ethanol-treated (2 g/kg, 20% w/v, *p.o.*, daily for four weeks) rats concurrently received either RC or a combination of vitamin E and C (each 100 mg/kg, *p.o.*) daily for the same period. The 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.

**Results:** Chronic administration of ethanol decreased the humoral and cell-mediated immune response, phagocytosis, phagocytosis index, TLC, GSH, CAT and SOD activities and increased the LPO. These influences of ethanol were prevented by concurrent daily administration of RC and the effect was comparable with that of the combination of vitamin E and C.

**Conclusion:** The ethanol-induced immunosuppression is due to oxidative stress and *Rubia cordifolia* can prevent the same by virtue of its *in vivo* antioxidant property.

**KEY WORDS** Ethyl alcohol      impaired immunity      oxidative stress

### INTRODUCTION

The immunocompromized condition of chronic alcoholics has been clinically evident since long time<sup>1,2</sup>. The impairment in the humoral and cell-mediated immune responses have been experimentally demonstrated in ethanol-administered rats<sup>3</sup>. Most of the adverse effects of ethanol are attributed to its ability to generate the cytotoxic free radicals as the same can be prevented by antioxidant therapy<sup>4,5</sup>.

*Rubia cordifolia* Linn. (RC) has been reported to possess a significant antioxidant activity in *in vitro* studies<sup>6</sup>. However, it is not known whether it is equally effective *in vivo*. Moreover, one of the earlier studies has shown that RC can also prevent the polluted air-induced immunosuppression<sup>7</sup>. Whether it can

attenuate the ethanol-induced immunosuppression has not yet been evaluated. Hence, it was proposed to evaluate the efficacy of RC to prevent the ethanol-induced immunosuppression and to find out whether it is related to its antioxidant activity *in vivo*.

### MATERIALS AND METHODS

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 activities of superoxide dismutase (SOD) and catalase (CAT). The results were compared with that of vitamin E and C, the known natural antioxidants.

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**Alcoholic extract of roots of *Rubia cordifolia***

**Linn.:** The roots of RC were collected from the forest of Dandeli (Karnataka) and authenticated by the of Department of Botany, Government Institute of Science, Nagpur. The powder of air-dried roots was extracted with ethanol by cold maceration. The suspension of solvent dried extract (11% w/w yield) in gum acacia was administered orally for four weeks at a dose of 100 mg/kg/day. This dose is a minimum and nontoxic (LD<sub>50</sub>>2 g/kg) dose at which RC has been shown to prevent the immunosuppression induced by polluted air<sup>7</sup>.

**Chemicals:** Pyrogallol and hydrogen peroxide were obtained from S.D. Fine Chemicals Ltd., India. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), Hank's balanced salt solution (HBSS), phosphate buffer and Tris buffer were obtained from Sigma, USA. All other reagents used were of analytical grade.

**Animals:** The Institutional Animal Ethics Committee approved the animal studies. The Sprague-Dawley rats (National Institute of Nutrition, Hyderabad) of both sex and average weight (150-200 g) were housed at controlled temperature (25±2°C) with food and water *ad libitum*.

The experimental group was subdivided in such a manner that all subgroups concurrently received ethanol (20% w/v, 2 g/kg)<sup>8</sup> and either the alcoholic extract of RC (100 mg/kg each, orally) or the vitamin C (100 mg/kg, orally) and E (100 mg/kg, orally)<sup>9</sup> or vehicle. The above treatments were given daily for four weeks. The control groups received vehicle (distilled water) instead of ethanol.

**Immunological studies<sup>7</sup>:** Blood was withdrawn from the jugular vein of a sheep (Government Veterinary College, Nagpur) 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 (0.5x 10<sup>9</sup> cells/ 100 g, *i.p.*), with first challenge on day 14 and second on 20th day of the experiment.

**Humoral immune response:** On day 20 and 27, the blood was withdrawn from retro-orbital plexus of all antigenically challenged rats. 25 µl of serum was serially diluted with 25 µl of phosphate-buffered saline. Sheep RBC (0.025 x 10<sup>9</sup> cells) were added to each of

these dilutions and incubated at 37°C for one hour. The rank of minimum dilution that exhibited hemagglutination was considered as the antibody titer. The level of antibody titer on day 20 of the experiment was considered as the primary humoral immune response whereas the one estimated on day 27 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 x 10<sup>9</sup> cells), in the subplantar region of right hind paw on day 27, was assessed after 48 h *i.e.* on day 29. 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.

**Phagocytosis<sup>10</sup>:** On day 28, 3 ml of HBSS containing 10% bovine serum albumin (BSA), was injected into the peritoneal cavity of the animal and the same was recovered by gentle aspiration. The macrophages (3 x 10<sup>5</sup> / 600 µl), present in the aliquots, were incubated on glass plates at 37°C for 30 min in a humidified chamber. The cells, adhered to the glass, were incubated with live cells of *Candida albicans*, previously opsonized in autologous plasma (2x10<sup>6</sup> / 250 µl), at 37°C for 30 min, then washed with HBSS, again incubated for 30 min with autologous plasma and finally stained with Wright's dye. A total of 300 cells were counted under microscope and results are reported as: a) Phagocytosis percent, b) Phagocytosis index, and c) Digestion index.

**Blood parameters:** Following parameters were assessed in the blood, withdrawn from the retro-orbital plexus, at the end of 4th week *i.e.* on 29th day.

**Total leukocyte count (TLC):** TLC was assessed by routine hematological method.

**Lipid peroxidation (LPO)<sup>11</sup>:** 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. One ml of 1% thiobarbituric acid was added to 4 ml of supernatant, heated in boiling water for 60 min and cooled immediately. The absorbance was measured spectrophotometrically at 532 nm. The lipid peroxidation was calculated on the basis of the

**Table 1.** Effect of ethanolic extract of *Rubia cordifolia* Linn. (RC) on immune response in rats (mean±SEM, n=6).

Groups	Treatments	Humoral immune response (Mean antibody titer levels)		Cellular immune response (% increase in paw volume)	Phagocytosis percentage	Digestion index	Total index	Leukocyte count (cells/mm <sup>3</sup> )
		Primary	Secondary					
Control	Vehicle	8.33±0.47	11.27±0.25	34.37±2.65	89.75±4.98	2.90±0.33	0.93±0.02	6714.33±53.77
	RC	7.66±0.51	11.90±0.9	35.27±2.7	87.50±1.64	2.89±0.37	0.99±0.06	6887.67±37.15
	Vitamin C and E	7.85±1.06	11.50±0.67	36.2±3.2	79.67±2.68	2.75±0.52	0.90±0.05	6695.21±23.56
Ethanol-treated	Vehicle	4.33±0.47*	8.10±0.3*	12.14±1.28*	57.60±4.7*	0.9±0.1*	0.21±0.01*	4572.17±19.38*
	RC	7.33±0.37#	10.80±0.5#	30.50±1.1#	80.89±2.82#	2.79±0.56#	0.87±0.02#	6644.17±74.88#
	Vitamin C and E	7.31±0.51	10.30±0.4	28.85±2.33	75.64±6.82	2.48±0.69	0.79±0.09	6564.67±46.82
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
	P	0.0011	0.0005	<0.0001	0.0002	0.039	<0.0001	<0.0001

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

molar extinction coefficient of malondialdehyde (MDA) ( $1.56 \times 10^5$ ), and expressed in terms of nanomoles of MDA/g Hb.

**Superoxide dismutase (SOD)**<sup>12</sup>: It was estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 µl of the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min by spectrophotometer (Schimadzu 1601, Japan). One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD is expressed as units/mg protein.

**Catalase (CAT)**<sup>13</sup>: Catalase activity was determined in erythrocyte lysate using Aebi's method with some modifications. 50 µl of the lysate was added to a cuvette containing 2 ml of phosphate buffer (pH 7.0) and 1ml of 30 mM H<sub>2</sub>O<sub>2</sub>. Catalase activity was measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H<sub>2</sub>O<sub>2</sub>, 43.6 M cm<sup>-1</sup> was used to determine the catalase activity. One unit of activity is equal to one millimoles of H<sub>2</sub>O<sub>2</sub> degraded per minute and is expressed as units per milligram of protein.

**Reduced glutathione (GSH)**<sup>14</sup>: 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 X g for 5 min and 1 ml of the filtrate was added to 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.

**Statistical analysis:** All data were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. 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 (2 g/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

**Table 2.** Effect of ethanolic extract of *Rubia cordifolia* Linn. (RC) on oxidative stress in rats (mean±SEM, n=6).

Group	Treatment	Lipid peroxidation nmMDA/g Hb	Glutathione µmDTNB conjugated/g Hb	Superoxide dismutase units/mg protein	Catalase units /mg protein	
Control	Vehicle	70.79±1.64	4.69±0.25	29.37±0.62	283.44±6.32	
	RC	65.65±2.45	5.02±0.45	29.24±0.35	278±5.65	
	Vitamin C and E	69.36±3.89	4.98±0.98	29.32±0.65	291±10.52	
Ethanol-treated	Vehicle	170.38±1.89*	2.59±0.16*	16.12±0.83*	192.87±4.25*	
	RC	72.65±1.68 <sup>#</sup>	4.71±0.24 <sup>#</sup>	19.89±0.65 <sup>#</sup>	232.56±8.67 <sup>#</sup>	
	Vitamin C and E	99.30±2.82 <sup>@</sup>	4.18±0.17	20.12±0.32	252.64±9.32	
One-way ANOVA		F	258.61	3.78	100.02	23.17
		df	5, 30	5, 30	5, 30	5, 30
		P	<0.0001	0.0090	<0.0001	<0.0001

\*P< 0.05 when compared with respective control group. <sup>#</sup>P< 0.05 when compared with vehicle treated experimental group. <sup>@</sup>P<0.05 when compared with RC treated experimental group.

further observed that the daily treatment with alcoholic extract of RC (100 mg/kg) significantly ( $p<0.05$ ) prevented these effects of ethanol.

The oxidative stress marker studies (Table 2) revealed that the chronic administration of ethanol increased the levels of LPO, decreased the activities of SOD and CAT and reduced the content of GSH. Pearson correlation analysis revealed that the immunotoxic and oxidative stress-generating effects of ethanol possess a 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 RC prevented the above ethanol-induced changes in the markers of oxidative stress.

Influence of RC on ethanol-induced changes were more or less similar and comparable with the vitamin E and C treatment.

## 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 E and C significantly attenuated these ethanol-induced

changes, it appears that the immunotoxic effects of ethanol may be due to oxidative stress. The literature has documented free radical generation during the metabolism of ethanol<sup>4</sup>. The level of the markers of oxidative stress, observed in ethanol-treated rats, substantiate the possibility of extensive generation of free radicals. It is further observed that administration of alcoholic extract of RC, 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 RC may be subsequent to the antioxidant activity, which it possesses. The observed *in vivo* antioxidant activity of RC is in substantiation of its earlier reported activity in *in vitro* studies<sup>6</sup>.

The mechanism of free radical-induced impairment of immune system is not yet properly delineated. Reduced glutathione, a free radical scavenger, plays a key role in the activation of T cells and macrophages<sup>15</sup>. The present investigations have revealed that chronic treatment with ethanol depleted the glutathione, reduced the digestion index and impaired phagocytosis. It is further observed that alcoholic extract of RC or the mixture of vitamin E and C prevented the above influences of ethanol on glutathione levels, digestion index and phagocytosis. Hence, the observed impairment of phagocytosis by ethanol may be subsequent to the depletion of

glutathione. Since the antioxidant therapies could reverse these influences of ethanol, the free radicals appear to be involved in this effect of ethanol.

In fact, free radicals are the signaling entities in T cell activation. However, continued generation of free radicals, over a long period was shown to downregulate the activation of T cells and NF-kappa B<sup>6</sup>. Similar downregulation of T cell activation has been observed when nitric oxide, a free radical generator, is constantly generated by macrophages<sup>17</sup>. It has also been shown that NO synthase inhibitor attenuates this downregulation<sup>17</sup>. Ethanol is also known to generate nitric oxide<sup>4</sup>. Hence, the reduction in oxidative stress, by the antioxidant effect of ethanolic extract of RC or the mixture of vitamin E and C, appear to be the underlying mechanism for the observed immunomodulation.

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