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The ameliorative effects of *Averroha carambola* on humoral response to sheep erythrocytes in non-treated and cyclophosphamide-immunocompromised mice

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ABSTRACT

Objective: To evaluated immunomodulatory and antioxidant activity of the methanol extract of *Averrhoa carambola* (*A. carambola*) leaves in mice. **Methods:** The assessment of immunomodulatory activity on specific and non-specific immunity was studied by administration of test extract by oral feeding canula to the test groups. Hemagglutinating antibody (HA) titer, delayed type of hypersensitivity (DTH) response, hematological profile (Hb, WBC, RBC), lipid per oxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) were determined by in vivo experiments. **Results:** The evaluation of immunomodulatory potential of *A. carambola* (800 and 1 200 mg/kg, p.o.) evoked a dose-dependent increase in antibody titer values and DTH reaction induced by SRBC was also found significant ($P<0.001$). Also it caused increase in hematological profile, GSH, SOD, CAT activity and significantly decreased LPO levels in cyclophosphamide-induced immunosuppressed mice. Result shows that the extract treated animals showed up regulation of (IL-6 and TNF- α) cytokines. **Conclusions:** Immunomodulators are being used as an adjuvant in conditions of immunodeficiency in cancer and to a limited extent in acquired immunodeficiency syndrome. The results obtained in this study indicate that *A. carambola* possesses potential immunomodulatory and antioxidant activity.

1. Introduction

In today's fast food life, our immune system is under constant attack at all times from countless antigens, whether it is from the food we eat, the air we breathe, the water we drink or nutritional imbalance in the diet. Daily rhythms can have a profound effect on immune response through hormonal modifications. Immunological diseases are growing at epidemic proportions that require aggressive and innovative approaches to the development of new treatments. Some of the factors leading to a weakened immune system include stress, exposure to environmental toxins, sedentary lifestyle, inadequate sleep, alcohol and tobacco abuse, malabsorption, antibiotics, chemotherapy, birth control pills, cortisone, and other drug therapies. Plants are invaluable sources of new drugs. There is an ever-growing interest for investing different species of plants to identify their potential therapeutic applications. Scientific studies on plants used in ethno-medicine have led to the discovery of many valuable drugs. Many more plants are still to be explored and offer scope for further investigation[1].

The mammalian immune system is composed of many cell types including B cells, T cells, NK cells, neutrophils, basophils, eosinophils, macrophages, mast cells, and mediators, such as cytokines, that interact with non-immune cell types and each other in complex and dynamic networks to ensure protection against foreign pathogens. When tissue homoeostasis is disturbed, macrophages and

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mast cells immediately release soluble mediators, such as proinflammatory cytokines like TNF, and interleukins[2].

Cytokines are elaborated by different types of cells, may act in autocrine, paracrine or endocrine manner, stimulate or regulate the growth and functions of nearby cells through specific receptors, and are highly potent as they are effective in picomolar quantities. They are truly language of communication between cells of the immune system. Immunomodulators can regulate the cytokine production such as tumor necrosis factor, interleukins and interferons and these cytokines may, inturn activate T-cells or NK cells[3]. Immunosuppression is a common consequence of long term cyclophosphamide (CP) therapy in cancer patient. Immunosuppressants have already been employed in transplantation surgery. Reduction of immunosuppressive effects may thus become beneficial to patients undergoing CP-chemotherapy[4]. In this study, we, therefore, assessed the protective effects of *Averrhoa carambola* (*A. carambola*) against cyclophosphamide-induced immunosuppression.

A. carambola L. (Oxalidaceae) is commonly known as 'Kamrakh', Carambola apple, or 'Star fruit' [5]. The plant is a small, handsome evergreen tree about 9.0 m in height with close dropping branches. *A. carambola* is traditionally found to be useful for many ailments. The leaves are antipruritic, antipyretic, anthelmintic and are also useful in scabies, intermittent fevers and elimination of intestinal worms. The genus Averrhoa contains two species, bilimbi (*Averrhoa bilimbi* L.) and carambola (*A. carambola* L.). *A. carambola* is considered the more important of two species[6-8]. Star fruit and has been used extensively for treatment of some diseases like inflammation[9], hypotensive[10], hepatotoxicity[11] and antioxidant[12]. It is an ingredient of various herbal formulations, which are used to support the immune system. Naturally occurring flavanoids, phenols, benzoquinones dihydrobesic alcohol, carotenoids and lutein are the main constituents of the plant *A. carambola*. It also contains two alkyl phenols, namely, 2, 5-dimethoxy-3-undecylphenol and 5-methoxy-3-undecylphenol, with two known benzoquinones, 5-O-methylembelin and 2-dehydroxy-5-O-methylembelin from the wood of *Averrhoa carambola*[13] and ζ-carotene, β-cryptoflavin ,mutatoxanthin, β-carotene, β-apo-8′-carotenal, cryptoxanthin, cryptochrome and lutein were present in fruit of carambola[14]. Even though this plant has gained scientific importance, however, there is no scientific report available in the literature against the effect of these phytoconstituents enriched extract in immune modulation. Therefore, the present study was undertaken to assess the *in vivo* antioxidant and immunomodulatory activity of *A. carambola* leaves extract against cyclophosphamide-induced immunosuppression.

2. Materials and methods

2.1. Plant material collection and authentication

The fresh leaves of *A. carambola* were collected from the field area of Bahraich, district U.P. India. For identification and taxonomic authentication, sample of plant material was given to National Botanical Research Institute (NBRI) Lucknow, India. The plant material was confirmed and authenticated by Dr. A. K. S. Rawat, Scientist and Head, Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute Lucknow, India with report no. CIF-RB-2-132.

2.2. Preparation of methanol extract of A. carambola (MEAc)

Shade-dried and coarsely powdered *A. carambola* leaves (100 g) were soaked in 500 mL of methanol [methanol/drug mass ratio 5:1] and kept at room temperature for 48 h with intermittent mixing. Methanol extract of plant obtained after 48 h of soaking was filtered using Whatman Qualitative Grade-1 filter paper and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried. It was stored at 4 ℃ for further use.

2.3. Quality control and chemo profiling of MEAc

Extract complied with WHO limits on safety and purity with respect to physico-chemical evaluations (loss on drying, ash values, extractive values) and preliminary phytochemical screening as per WHO recommended guidelines for standardizations[15]. The qualitative analysis of the MEAc was performed on HPTLC comprising of densitometer (CAMAG Model-3 TLC scanner) equipped with win CATS 4 software, semi automatic sampler (Linornat-5) and documentation was carried out by CAMAG PROSTER 3 at 366 nm (CAMAG, Berlin, Germany).

2.4. Drugs

Cyclophosphamide (Endoxan from Cadila Healthcare limited, India) 50 mg/kg b.w. was used. The doses used in the present study were selected based on previous studies[11-17]. All drugs were dissolved in pyrogen-free isotonic saline. Suspension of the MEAc was prepared in carboxy methyl cellulose (CMC, 0.3%) using pyrogen-free isotonic saline.

2.5. Test animals

Healthy Swiss albino mice (25-30 g) of either sex were selected for the study. The animals were fed on commercial diet (Hindustan lever pellets, Bangalore) and water ad libitum. Each animal was used once. The experiments were

performed between 10.00 and 16.00 h. The experimental protocol has been approved by the institutional ethical committee with approval no IU/Pharm/PhD./CPCSEA/12/06.

2.6. Antigenic materials

For the present study, the antigenic material used was sheep RBCs (SRBC). Fresh blood was collected from sheep sacrificed in the local slaughter house. It was mixed with Alsever's solution in 1:1 proportion and was stored at 4 ℃ in refrigerator.

Composition of Alsever's solution:

Contents	% W/ V
Glucose	2.05
Sodium chloride	0.42
Sodium citrate	0.80
Citric acid	0.55

During the experimentation, adequate amount of blood was taken from the above stock solution (*i.e.* SRBCs, stored in Alsever's solution) and was allowed to stand at room temperature. It was washed three times with pyrogen free normal saline (0.9% w/v NaCl). The RBC count of this suspension was determined by hemocytometer using Neubauer Chamber. The known amount of RBCs (0.5×10^9 cells/mL/100 g) was injected intraperitoneally to the mice as an antigenic challenge.

2.7. Immunosuppressant

In the present study cyclophosphamide (CP) 50 mg/kg ip was used as immunosuppressing agent.

2.8. Dosing schedule

Animals were divided into six groups (I-VI). Each group comprised of a minimum of six animals. Group I (control) received normal saline for 7 consecutive days, group II (CP) animals were injected with a single dose of CP on 6 day of initiation of experiment, group III (Plant extract AC1) animals and group IV (Plant extract AC2) animals were administered with plant extract for 7 consecutive days. Group V animals (Plant extract AC1+ CP) and group VI animals (Plant extract AC2+CP), were given plant extract treatment for 7 days with a single injection of CP ip on 6th day. For humoral response animals of all groups will be challenged with 0.2 mL of 10% SRBC ip on 5th day.

2.9. Immunization schedule

The mice belonging to the above groups were antigenically challenged with SRBC (0.5×10^9 cells/mL/100 g) on 5th day intraparitoneally.

2.10. Humoral immune response model

Measurement of antibody titer by hemagglutination reaction was performed by using method of Bin-Hafeez *et al* with some modification. The mice were lightly anesthetized with anesthetic ether. A fine capillary was gently inserted into the lower angle of eye at 45 ° and the blood was obtained from retro-orbital plexus. The blood was collected into vial and centrifuged for separating serum. The serum of mice was used for determination of hemagglutination titer. Micro titration plate having 96 cups was used for carrying out titration. Each cup was filled with (25 ± 1) of normal saline. (25 ± 1) of serum obtained from mice blood was added to 1stcup and was mixed with (25 ± 1) of normal saline present in microtitration plate. In this way serial two fold dilutions of serum were prepared. To each cup (25 ± 1) of 1% v/v SRBC was added. The plate was incubated at 37 ℃ for 1 h. and then was observed for agglutination. The antibody titer was expressed in terms of maximum dilution, which gave positive hemagglutination reaction[18,19].

2.11. Cellular immune response model

Cell mediated immune response was assessed by footpad reaction test. On 7th day, after measuring volume of footpad of both legs, SRBC (0.025×10^9 cells) were injected in right paw and 0.025 mL of saline was injected in left paw. On 8th day after 24 h, the paw volume was measured again to check the increase or decrease in volume. The increase in paw volume was considered as an index of cell mediated immunity[20,21].

2.12. Blood parameters

The blood withdrawn from the above antigenically challenged mice were used to estimate the hematological parameters like hemoglobin (Hb), RBC and WBC by usual standardized laboratory method[19].

2.13. Body weight and relative organ weight determination

Animals of all groups were sacrificed 24 h after the last dose. Body weight gain and relative organ weight (organ weight/100 g of body weight) of kidney, liver, and spleen were determined for each animal[18].

2.14. Assessment of antioxidant parameters

In all group animals, spleen were collected after the scarification and washed immediately with ice cold saline to remove blood. Spleen tissues of mice were homogenized (10%) in phosphate buffer (pH 7.4). The homogenate was centrifuged at 12 000 *g* for 20 min at 4 ℃ to obtain supernatant and it was used for the estimation of LPO and

reduced glutathione (GSH) [22,23]. The activity of catalase (CAT) and superoxide dismutase (SOD) was measured by the methods described by Aebi, Kakkar, and Rotruck[24-26].

2.14.1. Assay of TBARS

Lipid peroxidation is a free radical mediated event. The primary products of such damage are a complex mixture of peroxides which then breakdown to produce carbonyl compounds. The malondialdehyde (MDA) is one such carbonyl compound, which forms a characteristic chromogenic adduct with two molecules of TBA. The colourimetric reaction of TBA with MDA, a secondary product of lipid peroxidation, has been widely accepted for measuring lipid peroxidation. The total protein which was present in the homogenate was estimated by following the method which was described by Lowry et al[27] The units of the TBARS activity which were determined were expressed in terms of nmoles MDA/mg protein.

2.14.2. Assay of glutathione

This spectrophotometric procedure was based on the method of Ellman i.e. DTNB [5, 5′-dithiobis-(2-nitrobenzoic acid)] is reduced by -SH groups to form one mole of 2-nitro-5- mercaptobenzoic acid per mole of -SH, as described by Sedlak and Lindsay. The units of the GSH activity which were determined were expressed in terms of nmoles μg/mg protein[28].

2.14.3. Assay of SOD

The assay of SOD was carried out, based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol, as described by Marklund and Marklund. The total protein which was present in the homogenate was estimated by following the method which was described by Lowry *et al*[27]. The units of the SOD activity which were determined were expressed in terms of milligram of the total protein[29].

2.14.4. Assay of catalase

In the UV range, H_2O_2 shows a continuous increase in the absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in the absorbance at 240 nm. The difference in absorbance (ΔA) per unit time is a measure of the catalase activity. The units of the CAT activity which were determined were expressed in terms of nmol H_2O_2/mg protein[30].

2.15. Determination of TNF- α , and IL-6 level

The concentrations of TNF- α and IL-6 in the mice serum were determined specific quantitative sandwich ELISA kits according to the instruction of the manufacturer purchased from eBioscience and Cayman Chemical USA.

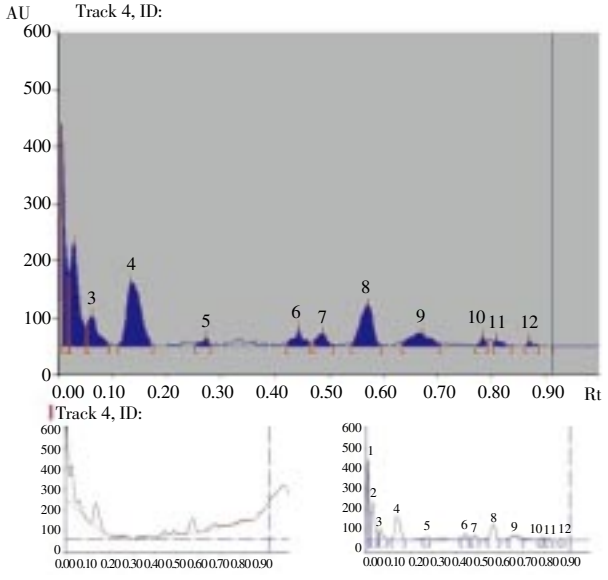
2.16. Statistical analysis

Data were statistically analyzed using student′s *t*-test to determine significant differences in data of various groups; *P* values less than 0.05 were considered significant. The values are expressed as means ± SE.

3. Results

3.1. Standardization of extract

The physico-chemical characters such as total alcohol soluble extractive, water soluble extractive, ash value, acid insoluble ash, water-soluble ash, loss on drying and foreign matter analysis was done and results are presented in Table 1. The preliminary phytochemical investigation of the methanol extract of *A. carambola* showed the presences of phytosterols, flavanoids, acidic compound, carbohydrates, glycosides and alkaloids, results are tabulated in Table 2. The extract was analyzed qualitatively by HPTLC. Finger printing studies on methanol extract showed presence of various phytoconstituents with different intensities, with their respective R_f values (Figure 1). Sample was applied on TLC plate (10 × 20 cm Silica gel $60F_{254}$, Merck) by semi automatic TLC sampler using 100 μL syringe. The plate was developed in pre-saturated horizontal chamber with solvent system comprising of *n*-butanol: Acetic acid: water (2:4:6). The plates were visualized under UV at 366 nm



Peak	Start Rt	Start Height	Max Rt	Max Height	Max %	End Rt	End Height	Area	Area %	Assignod sutostarce
1	0.01	376.5	0.01	376.5	40.47	0.02	122.5	2367.1	19.04	unknown*
2	0.02	124.1	0.03	182.2	19.59	0.05	34.5	2675.9	21.52	unknown*
3	0.05	35.2	0.06	54.1	5.81	0.09	3.2	912.9	7.34	unknown*
4	0.11	1.1	0.14	112.3	12.07	0.17	0.9	2666.3	21.44	unknown*
5	0.25	3.8	0.27	5.3	1.64	0.28	2.4	185.3	1.49	unknown*
6	0.42	4.0	0.44	31.7	3.41	0.46	6.6	478.0	3.84	unknown*
7	0.47	7.7	0.49	22.1	2.37	0.51	2.3	391.5	3.15	unknown*
8	0.54	3.2	0.57	73.0	7.85	0.60	0.0	1538.9	12.38	unknown*
9	0.63	6.1	0.67	23.6	2.53	0.71	4.6	825.1	6.64	unknown*
10	0.77	0.1	0.78	17.2	1.85	0.79	9.0	151.8	1.22	unknown*
11	0.80	5.8	0.81	11.7	1.26	0.84	0.3	156.7	1.26	unknown*
12	0.86	0.8	0.87	10.6	1.14	0.89	0.8	83.8	0.67	unknown*

Figure 1. HPTLC Finger printing of methanol extract of *A. carambola* leaves.

Table 1
Physicochemical parameters of *A. carambola* leaves.

Quantitative parameter	Values obtained (%) w/w
Alcohol soluble extractive	12.2
Water soluble extractive	16.6
Total ash	8.5
Acid insoluble ash	1.7
Water – soluble ash	4.0
Loss on drying	11.3
Swelling index	2.0
Foreign matter	1.2

Table 2
Preliminary phytochemical analysis of methanol extract of *A. carambola* leaves.

S.No.	Constituents	*A. carambola* leaves
1	Alkaloids	+
2	Carbohydrate	+
3	Glycoside	+
4	Phenolic compound and tannins	+
5	Flavonoides	+
6	Proteins and amino acids	–
7	Sterols	+
8	Acidic compound	+
9	Lipids/fats	–

3.2. Hemagglutinating antibody (HA) titer

The HA titer was used to assess humoral immune response. The CP treatment produced decrease in the HA titer value after 2 h incubation with SRBCs (Table 3). Administration of both the tested doses (800 and 1 200 mg/kg, respectively) produced a significant increase in HA titer as evident from hemagglutination after incubation of the serum with SRBCs. The increase was found highly significant. Thus, the plant extract showed protective effects on humoral immunity.

Table 3
Effect of *A. carambola* (AC) extract on humoral immune response.

Group	Treatment	Mean antibody titer a (in terms of rank of cups of titer plate)± S.E.
Group I	Control (Normal saline)	9.25±0.30
Group II	Normal saline + CP	3.17±0.16[b]
Group III	AC 800 mg/kg	10.50±0.22[c]
Group IV	AC 1 200 mg/kg	11.25±0.25[e]
Group V	AC 800 mg/kg+CP	5.30±0.55[d]
Group VI	AC 1 200 mg/kg+CP	7.60±0.20[d]

[a]Values are expressed as mean ± S.E. of 6 mice, [b]P<0.001 Statistical significance versus Group I, [c]P<0.01 Statistical significance versus Group I, [d]P<0.001 Statistical significance versus Group II, [e]P<0.05 Statistical significance versus Group I.

3.4. Delayed type hypersensitivity (DTH) reactions

The cell-mediated immune response of A.carambola leaves extract was assessed by DTH reaction, i.e. foot pad reaction, as shown in Figure 2, the test extract produced a significant, dose-related increase in DTH reactivity in mice. Increase in DTH reaction in mice in response to cell dependent antigen revealed the stimulatory effect of *A. carambola* extract on T cells. The change in percentage deduction in footpad volume was also found significant.

Table 4
Effect of *A. carambola* extract on relative organ weight.

Group	Treatment	Relative organ weighta (g) ± S.E.		
		Liver	Kidney	Spleen
Group I	Control (Normal saline)	5.14±0.01	1.367±0.45	0.622±0.05
Group II	Normal saline + CP	4.82±0.17[b]	1.115±0.26[c]	0.334±0.06[b]
Group III	AC 800 mg/kg	5.04±0.74[e]	1.325±0.02[e]	0.500±0.10[c]
Group IV	AC 1 200 mg/kg	5.12±0.05[e]	1.330±0.26[c]	0.611±0.08[b]
Group V	AC 800 mg/kg + CP	4.89±0.11[d]	1.352±0.25[f]	0.451±0.09[d]
Group VI	AC 1 200 mg/kg + CP	4.90±0.07[f]	1.340±0.53[d]	0.511±0.10[f]

[a]Values are expressed as mean ± S.E. of 6 mice, [b]P<0.001 Statistical significance versus Group I, cP<0.01 Statistical significance versus Group I, [d]P<0.001 Statistical significance versus Group II, [e]P<0.05 Statistical significance versus Group I, [f]P<0.01 Statistical significance versus Group II.

Table 5
Effect of *A. carambola* extract on hematological parametersa.

Group	Treatment	RBC ($\times 10^6$/mm^3)	WBC($\times 10^3$/mm^3)	Hb (g/dL)
I	Control: Normal saline	9.26±0.33	6.26±0.28	13.76±0.48
II	Normal saline + CP	9.22±0.21	1.52±0.17[c]	12.52±0.78[b]
III	AC 800 mg/kg	9.53±0.14[c]	7.65±0.85	13.36±0.92[c]
IV	AC 1 200 mg/kg	9.47±0.38	7.93±0.40[b]	13.65±0.51[c]
V	AC 800 mg/kg + CP	9.24±0.21	3.21±0.53	12.33±0.28[d]
VI	AC 1 200 mg/kg + CP	9.29±0.57	3.99±0.30[d]	12.39±0.31[d]

[a]Values are expressed as mean ± S.E for 6 mice, [b]P<0.001 Statistical significance versus Group I, [c]P<0.01 Statistical significance versus Group I, [d]P<0.001 Statistical significance versus Group II, RBC (million/mm^3), WBC (thousand/mm^3), Hemoglobin (g/dL)

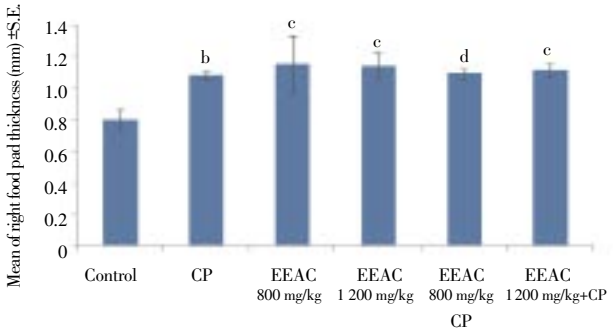


Figure 2. Effect of *A. carambola* extract on Delayed Type Hypersensitivity Response. Values are expressed as mean ± S.E. of 6 mice, [b]*P*<0.001 Statistical significance versus Group I, [c]*P*<0.01 Statistical significance versus Group I, [d]*P*<0.001 Statistical significance versus Group II, [e]*P*<0.05 Statistical significance versus Group II.

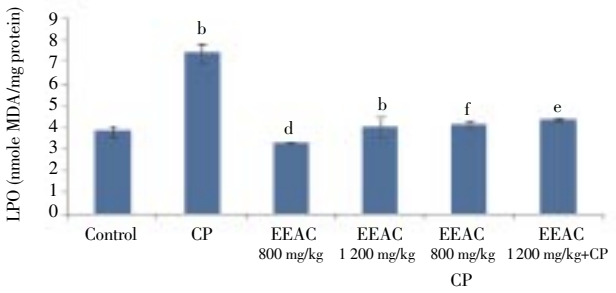


Figure 3. Effect of MEAc on oxidative stress parameter (LPO), MEAC= methanol extract of *A. carambola* CP=cyclophosphamide, LPO=lipid peroxidation, aValues are expressed as mean ± S.E. of 6 mice, [b]*P*<0.05 Statistical significance versus Group I, [c]*P*<0.01 Statistical significance versus Group I, [d]*P*<0.001 Statistical significance versus Group I, [e]*P*<0.01 Statistical significance versus Group II, [f]*P*<0.001 Statistical significance versus Group II.

3.5. Effect on relative organ weight

CP injection caused a prominent reduction in relative organ weight (kidney, liver, and spleen) of the mice (Table 4) recovery was also observed significant in each organ.

3.6. Effect on hematological parameters

Cyclophosphamide at the dose of 50 mg/kg, *i.p.* caused a significant reduction in the hemoglobin, RBCs and WBCs (Table 5). Combined treatment of cyclophosphamide and *A. carambola* extract, restoration of bone marrow activity as compared with cyclophosphamide alone treated mice was observed.

3.7. Effect of A. carambola leaves extract on LPO and levels of antioxidant enzymes

The oxidative stress marker studies revealed (Figure 3-6) that the administration of cyclophosphamide significantly increased the levels of LPO, decreased the activities of SOD, CAT and reduced the content of GSH as compared to the control group. The *A. carambola* leaves extract showed a moderate effect on LPO, SOD, CAT, and GSH. The cyclophosphamide treatment along with *A. carambola* leaves extract decreased TBA reactive products as compared to the cyclophosphamide-exposed group. Moreover, a significant elevation in the CAT and GSH content was also observed in comparison to cyclophosphamide-treated animals. SOD activity also increased when treated with *A. carambola* extract (MEAc) as compared to the cyclophosphamide control group.

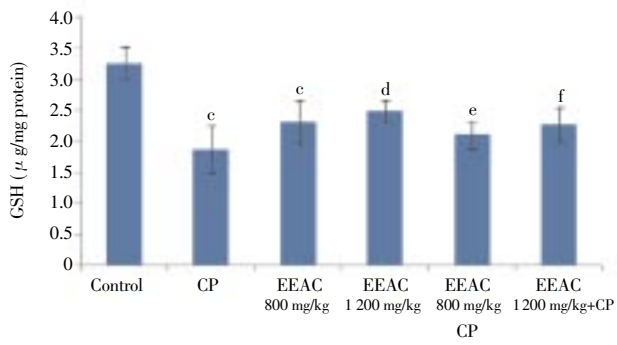


Figure 4. Effect of MEAc on oxidative stress parameter (GSH), MEAC = methanol extract of *A. carambola* CP=cyclophosphamide, GSH=reduced glutathione., [a]Values are expressed as mean ± S.E. of 6 mice, [b]*P*<0.05 Statistical significance versus Group I, [c]*P*<0.01 Statistical significance versus Group I, [d]*P*<0.001 Statistical significance versus Group I, [e]*P*<0.01 Statistical significance versus Group II, [f]*P*<0.001 Statistical significance versus Group II.

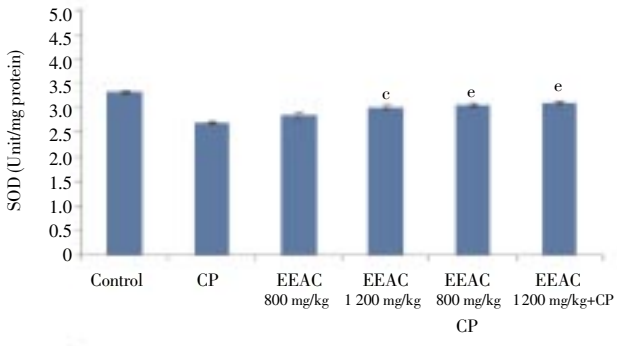


Figure 5. Effect of MEAc on oxidative stress parameter (SOD), MEAC = methanol extract of *A. carambola* CP=cyclophosphamide SOD=superoxide dismutase, [a]Values are expressed as mean ± S.E. of 6 mice, [b]*P*<0.05 Statistical significance versus Group I, [c]*P*<0.01 Statistical significance versus Group I, [d]*P*<0.001 Statistical significance versus Group I, [e]*P*<0.01 Statistical significance versus Group II, [f]*P*<0.001 Statistical significance versus Group II.

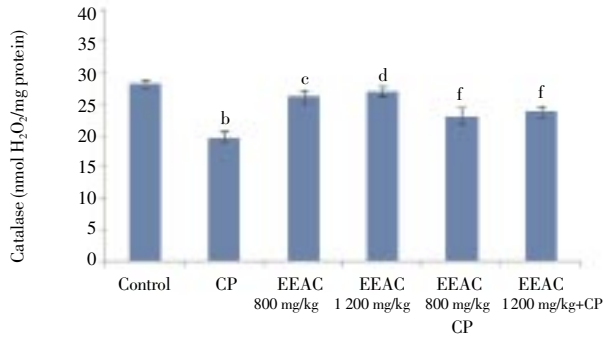


Figure 6. Effect of MEAc on oxidative stress parameter (CAT), Values are mean±S.E. of 6 mice. MEAC = methanol extract of *A. carambola* CP=cyclophosphamide CAT=catalase. [b]P<0.05 Statistical significance versus Group I, [c]P<0.01 Statistical significance versus Group I, [d]P<0.001 Statistical significance versus Group I, [e]P<0.01 Statistical significance versus Group II, [f]P<0.001 Statistical significance versus Group II.

3.8. Effect of MEAc on proinflammatory cytokine

Effect of test drugs on proinflammatory cytokine production showed that the extract treated animals showed significant up regulation of (IL-6 and TNF- α) cytokines in a dose-dependent manner in comparison to normal control animal. (Figure 7&8) While levels of TNF- α and IL-6 were decreased by the treatment with cyclophasphamide which was then significantly increased in comparison to model control animals, by the administration of *A. carambola* extract along with cyclophasphamide.

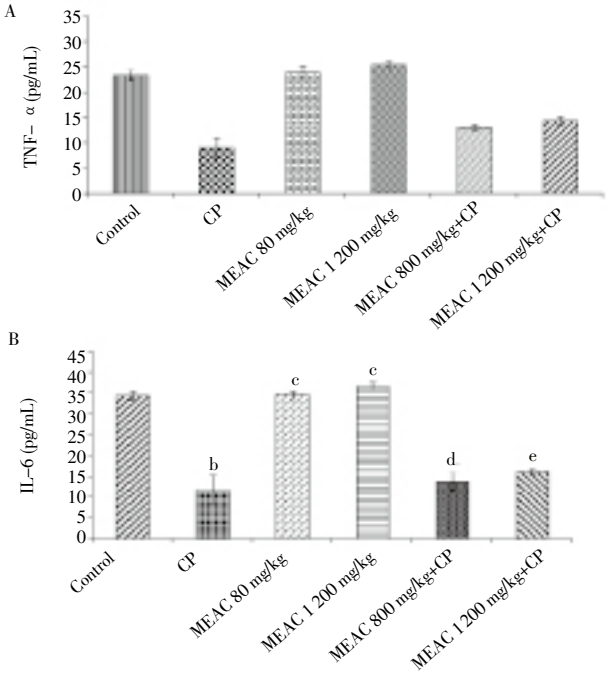


Figure 7. Effect of *A. carambola* methanol extract (MEAc) on proinflammatory cytokine level (A) Tumor necrosis factor- α (TNF-α); (B) Interleukin-6 (IL-6), Concentration was expressed in pg/mL. Data were expressed as the mean ± SEM of n = 6 animals per group. [b]P<0.05 Statistical significance versus Group I, [c]P<0.01 Statistical significance versus Group I, [d]P<0.01 Statistical significance versus Group II, [e]P<0.001 Statistical significance versus Group II.

4. Discussion

Considering the structural data, we could state that *A. carambola* posses the immunostimulatory principles. Immunomodulators are being used as an adjuvant in conditions of immunodeficiency in cancer and to a limited extent in acquired immunodeficiency syndrome. Many of the presently available immunomodulators such as levamisole, glucans, L-fucose as well as Corynebacterium parvum bacterium, are not free from side effects, which include fever, neutropenia, leucopenia and, at times, allergic reactions. Hence, screening for new immunomodulators is an urgent need. Immunomodulatory agents of plant origin enhance the immune responsiveness of the organism against a pathogen by activating the immune system. However, these agents should be subjected to systemic studies to substantiate the therapeutic claims made with regard to their clinical utility.

MEAc has ability to modulate humoral immune responses by acting at various levels in immune mechanism such as antibody production, release of mediators of hypersensitivity reactions, and tissue responses to these mediators in the target organs. In our study, foot volume was enhanced after *A. carambola* treatment, suggesting cell-mediated immune enhancement. The high values of hemagglutinating antibody titer obtained in the *A. carambola* leaves extract indicated that immunostimulation was achieved through humoral immunity. Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). The CMI responses are critical to defend against infectious organisms, infection of foreign grafts, tumor immunity and delayed type hypersensitivity reactions. The results obtained shows that *A. carambola* (MEAc) given to the mice increases the number of antibodies in their serum while produces an inhibitory effect on cell-mediated immune response. As the results the drug can be suggested for its therapeutic usefulness in various infections and inflammatory diseases. In case of chemotherapy of cancer patients, it can be a beneficial tool to reduce the humoral immunosuppressive effects. Humoral antibodies that are capable of killing free tumor cells in blood and in serosal cavities have been suggested to play a very important role in cancer. Cyclophosphamide (CP) is an alkylating agent commercially used in antineoplastic chemotherapy. Both experimental and clinical results have demonstrated an apparently paradoxical effect of CP on the tumor-host immune response. The better anti-tumor effect of CP depends on the larger dose of CP administered. However, along with a reduction of the tumor mass, large doses of CP usually bring an impairment of the host defense mechanisms, leading to immunosuppressive and cytotoxic effects[31-33]. To explore the possibility for adjuvant immunotherapy in severely immunosuppressed mice, the immunomodulatory activity of *A. carambola* (MEAc) was evaluated by the model of intensive chemotherapy with CP

in mice, and its effects on cellular and humoral immunity were investigated. In the present study, MEAc exhibited beneficial actions on the specific and nonspecific immunity of immunosuppressed mice at the optimal dose. The actions of CP are primarily directed toward the depletion of T/B lymphocytes and the deficiency of macrophages[34]. A significant increase in white blood cell count was observed in methanol extract- treated mice as compared with cyclophosphamide treatment alone. Extract significantly ameliorated the RBC count, hemoglobin and also restored the suppressive effects induced by cyclophosphamide.

Toxicities of cyclophosphamide include the suppression of white blood cells, RBC, Hb, nausea, vomiting, gonadal atrophy, liver, renal, and bladder injury. Several visual symptoms, such as lethargy, alopecia, unusual weakness and anorexia. During chemotherapy, cylophosphamide also causes hepatic damage as well as urotoxicity, which is a problem associated with internal organs due to its cytotoxic nature[35,36]. Significant improvements were found in relative organ weights of kidney, liver, and spleen; therefore, MEAc could be suggested for the drug-induced immunopathy in the organs.

While screening the immunomodulatory activity, most of the studies employ agents like cisplatin, cyclophosphamide or corticosteroids in order to induce the immunosuppression in the experimental animals[37]. These agents are known to generate free radicals in the biological system and thereby cause oxidative stress[38]. However, several workers have demonstrated that pro-oxidants suppress the immune responses in experimental animals[39]. In addition, it has been documented that the immunomodulators isolated from plant sources possess antioxidant activity[40]. The present study had shown that the administration of cyclophosphamide not only impair the immune responses but also produce oxidative stress in mice. In view of this, it appeared that cyclophosphamide which is a strong generator of superoxide radicals might impair the immune response through oxidative stress. It is further observed that administration of *A. carambola* leaves extract prevented the cyclophosphamide-induced changes of immunological and oxidative stress parameters. Hence, the immunomodulatory effect of *A. carambola* extract may be subsequent to the antioxidant activity which it possesses.

The inflammatory response in the body is mediated by the proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6). The level and persistence of TNF- α and IL-6 cytokines play an important role in determining the behavior of a given factor in immunomodulation. IL-6 plays a key role in host immune responses, such as acute protein synthesis, and the maintenance of homeostasis also acts as both a pro-inflammatory and anti-inflammatory cytokine[41]. Our result shows that test drugs induce cytokine production (including IL-6 and TNF- α) in a dose-dependent manner.

Investigations characterizing the secondary metabolites of leaves of *Averrhoa carambola* have identified C-glycoside flavones, such as apigenin-6-C- β -L-fucopyranoside and apigenin-6-C-(2″- O- α -L-rhamnopyranosyl)- β -L-fucopyranosid. It is also known as carambolaflavone[15]. It might be possible that the immunostimulatory activity of leaves of *Averrhoa carambola* was due to the presence of these phytoconstituents. Because in previous reports flavanoids and terpenes mediate production of inflammatory cytokine[42] and shows its protective effect of on ethanol - induced immunosupression in rats[43].

In conclusion this study shows that the methanol extract of leaves of *A. carambola* (MEAc) could possibly have both antioxidant and immunostimulatory capabilities. The reactive oxygen species and hyper immune activation are thought to be associated with the pathogenesis of chronic diseases such as inflammatory diseases and HIV/AIDS, and HIV infected individuals have impaired antioxidant defenses, the inhibitory effect of the *A. carambola* extract (MEAc) on free radicals and humoral immune response stimulation may partially explain why the *A. carambola* leaves extract is beneficial in ameliorating disease conditions. Protection of immune system by dietary antioxidants may play an important role in preserving the immune function and achieving healthy ageing. The accruing knowledge from this research is opening up new avenues for targeting cytokines and their receptors, or subunits shared by cytokines or their receptors, to treat undesirable inflammatory conditions. Further studies on the specific mechanism of action of *A. carambola* extract and its semisynthetic derivatives, in order to establish its therapeutic potential for the prevention of immune diseases are required.

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