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Immunomodulatory effect of alcoholic extract of *Terminalia chebula* ripe fruits

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Abstract:
Most of the synthetic chemotherapeutic agents are immunosuppressants and exerts variety of side effects. The herbal based immunomodulators are employed as supportive or adjuvant therapy to overcome the undesired effects of chemotherapeutics agents. Herbal drugs will be significantly more effective and highly efficacious supplement to use as a general adaptogen and immune strengthener in healthy people and as a very useful, safe and effective nutrient support tonic for those challenged by disease. The present work described that *Terminalia chebula* alcoholic extract shows immunomodulator activity. The various parameters determined were differential leukocyte count (DLC), phagocytic activity and zinc sulphate turbidity test. Oral administration of *T. chebula* alcoholic extract (100 mg/kg, p.o.) was found to increase the neutrophils and lymphocytes as compared to vehicle and cyclophosphamide treated groups. *T. chebula* alcoholic extract showed linear time dependent significant phagocytic activity as compared to SRBC sensitized and cyclophosphamide treated group. In zinc sulphate turbidity test *T. chebula* treated rats serum showed more turbidity (cloudy) which indicate the increase in the immunoglobulin level as compared to vehicle, SRBC sensitized and cyclophosphamide treated group. Finally it can be concluded that *Terminalia chebula* ripe fruits show potent immunomodulatory action.

Key words: *DLC, Immunomodulator, Phagocytic activity, Terminalia chebula, Zinc Sulphate Turbidity Test.*

Introduction:

In recent years, there has been growing interest in the field of herbal medicines research and search for promising male wister rats remains a potential area of investigation of immunomodulatory agents from natural products [1, 2]. *Terminalia chebula* Retzius (*T. chebula)* belonging to Combretaceae family, is the native plant of India and South East Asia. The dried ripe fruit of *T. chebula*, commonly known as black myrobalan in English and *Harad* in Hindi, is being used either alone or as an adjuvant to other medicines like Triphala and Menosan, and is a popular folk medicine in India and Asia. *T. chebula* has been reported to exhibit a variety of biological activities including antiviral [3], antibacterial [4], antidiabetic, renoprotective [5], radioprotective [6], anticancer [7], antioxidant and adaptogenic activity [8, 9]. Chemical constituents of *Terminalia* species have been identified as tannins, flavonoids, sterols, amino acids, fructose, resin and fixed oils. In varying compositions, compounds like anthraquinones, gallic acid, chebulinic and chebulagic acid, ellagic and ethaedioic acid, 4, 2, 4 chebulyl- β-D-glucopyranose, terpinenes and terpineols have been described for this species [4, 7, 10]. It can be concluded that *T. chebula* posse's significant immunomodulatory activity.

Plant Material and Preparation of Extract:

The fresh ripe fruits of *T. chebula* were shade dried and the coarse powders (335g) were extracted in soxhlet apparatus using alcohol for 32 hrs. The extract was then concentrated to dryness under reduced pressure by using rotary evaporator at 42-45°C, yielded 16g of dry extract and preserved in a dessicator for further use.

Animals:

Male Wister rats weighing 150-180 g were procured from Laboratory Animals Resources, Division of Animal Genetics, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly Reg No. CPC-196 and acclimatized to laboratory condition at Animal House, IFTM, Moradabad at room temperature 23±5^{0}C with a 12/12h/light/dark cycle and relative humidity (55±10%). The Institutional Animal Ethical Committee reviewed the animal protocol prior to the experiment. All rats were treated in accordance with the guideline for the care and use of laboratory animals (NIH Publication N0.86-23, revised 1985) with

the permission of Institute Animal Ethical Committee (Proposal No.11 2010).

Animal Grouping:

For experimental procedure, Male Wister rats were divided in the following four groups containing six rats in each group.

Group I (n=6): Negative control: Rats treated with 2 ml of 1% gum acacia solution in distilled water.

Group II (n=6): Positive control: Sensitized rats (by administrating $1x10^8$ SRBCs, i.p.) treated with 1% gum acacia solution orally.

Group III (n=6): Rats treated with cyclophosphamide 100 mg/kg/p. o.

Group IV (n=6): Sensitized rats treated with *Terminalia chebula* alcoholic extracted 100 mg/kg/p. o. in the following regimens.

a) 4 days prior to sensitization (days -3, -2, -1, 0).

b) 7 days after sensitization (days +1, +2, +3, +4, +5, +6, +7).

Preparation of Sheep Red Blood Cells (SRBC):

From healthy sheep blood was collected from local butcher house and mixed with sterile Alsever's solution (1:1). It was thoroughly mixed and centrifuged at 3000 rpm for 5 min. Supernatant was discarded, SRBC pellets were washed with sterilized phosphate buffer saline (pH 7.2) 2-3 times. Then the SRBC pellets were prepared in phosphate buffer saline (pH 7.2) and total SRBC was counted using Neubauer chamber, finally $1x10^8$ SRBCs (0.5ml) were injected intraperitoneally for sensitization and challenging the rats [11].

Blood Profile for Study of Immunomodulatory Activity:

Rats were divided into four groups as described earlier. After 7 days treatment, blood was collected from rats by retro-orbital plexus for study of different parameters.

Determination of Differential Leukocyte Count (DLC):

A drop of blood drop was added on the centre line of the glass slide about 1 cm from one end and blood smear was prepared. Then smear was stained with diluted Leishman's stain for 30 min and washed with distilled water and dried at room temperature. For counting of DLC the slide was examined under microscope at 100x using Cedar wood oil. Finally total number of Neutrophils, Lymphocytes and Monocytes in the 100 cells were counted and results were expressed in percentage [12].

In vitro Phagocytic Activity:

Preparation of Blood PMN cells

Separation of blood PMN cells was done as per the method described by (Daley *et al* 1991). Blood sample (1ml) was collected by retro orbital plexus in heparinised sterile tubes (20 IU heparin / ml of blood). One part of blood was diluted with two parts of sterile Tris- ammonium chloride buffer (pH 7.3) and thoroughly mixed for 1-2 minutes and was kept for 20 minutes at room temperature. Blood samples were centrifuged at 3000 rpm for 20 minutes. The supernantant was discarded and the cell pellets were removed with 5 ml sterile chilled phosphate buffer solution (PBS) (pH 7.4). Then the solution was further centrifuged for 10 minutes in the same manner twice to get PMN cell pellet. The pellet obtained was resuspended in 1 ml of sterile cold PBS [13].

Preparation of Microorganism

Escherichia coli (NCIM 2391) was grown and kept on a slant agar media. Before use, the microorganism was inoculated in 100 ml of 2.5% nutrient broth media for 18hrs at 37±2°C. The culture was then washed twice with sterile PBS (pH 7.2) and re-suspended in 1 ml gelatin HBSS (Hank's Buffered Salt Solution) to get a concentration of $1x10^7$ cells/ml. During each experiment, the numbers of viable microorganisms were

determined by counting colony forming units (CFU), using nutrient agar plates [14].

Viable PMN cell count

The viable cell counts were determined by Trypan blue exclusion techniques. 20 µl each of cell suspension and 0.1 % trypan blue were mixed and kept for 2 minutes at room temperature. A drop of mixture was loaded on haemocytometer, the viable (unstained) and dead (stained) cells were counted in WBC counting chamber. The viable cell count was expressed as per the method reported earlier [15].

Microbiological Assay for the Phagocytosis Activity

To assess phagocytosis and *T. chebula* alcoholic extract (100µg/ml) in the final volume of 0.1ml were incubated with 2 ml of PMNCs suspension ($1x10^7$ cells/ml) and 2 ml of microorganism ($1x10^7$ cells/ml) at 37 ± 2^0C for 1hr in 5% CO_2 atmosphere in a slanting position. 1ml of the standard drug, cyclophosphamide (100 mg/ml) was incubated with fetal calf serum in the same conditions. At 30 min intervals upto 120 min, 0.5 ml aliquot of the suspension was removed and added to 1.5 ml of the ice-cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 100g for 4 min. Under this condition, the non-ingested microorganisms remained in the supernatant fluid. The viable count of the microorganisms was done using the colony counter. Phagocytosis was expressed as the percentage decrease in the initial number of viable extracellular bacteria [16].

Determination of Humoral Immunity by Zinc Sulphate Turbidity Test (ZSTT):

The rats were divided in four groups as described, six hours after the last dose blood was collected and the serum was used for estimation of immunoglobulin levels using method devised by (Mullen *et al.*, 1975).

Zinc Sulphate Solution Preparation

The triple distilled water was boiled for 15 min. to remove dissolved CO_2 and was used to prepare zinc sulphate solution (208 mg/liter). The $ZnSO_4$ solution was kept in an aspirate bottle to protect uptake of carbon dioxide. This was achieved by insertion of soda lime tube into the stopper. A tubing to deliver 6 ml per vial was connected to the aspirator bottle.

Test procedure

A control vial containing 6 ml distilled water and test vial containing 6 ml zinc sulphate solution were taken and added to 0.1 ml serum sample. The solutions were gently shaken to ensure complete mixing and reading was taken spectrophotometrically at 580nm.

Statistical analysis:

The results were expressed as mean ± S.D. and statistical evaluation of the data was done using students t-test and $P<0.05$ was considered as significant.

Results:

Determination of Differential Leukocyte Counts:

Table 1 showed that on the zero day the neutrophil were 62.32, 63.21, 67.04 and 63.87% in the vehicle treated, SRBC sensitized, cyclophosphamide treated and *T. chebula* alcoholic extract treated group respectively. On 14^{th} day percentage of neutrophil in the *T. chebula* alcoholic extract group (63.13%) were almost equal to vehicle treated group (62.52%), but the percentage of neutrophil in the positive control group was 61.62% (SRBC sensitized) i.e. lower as compared to vehicle treated group (62.32%) at 7^{th} day treatment. In case of *T. chebula* alcoholic extract treatment percentage of lymphocytes on the zero day, 7^{th} and 14^{th} day were 30.22, 29.94 and 29.22 respectively. On the 14^{th} day the lymphocyte percentage declined more rapidly in SRBC treated group as compare to *T. chebula* alcoholic extract treated group.

Table 1: Immunomodulatory effect of *T. chebula* alcoholic extract on Differential Leukocyte Count (DLC)

0 day			
Groups	Neutrophils (%)	Lymphocyte (%)	Monocyte (%)
Vehicle	62.32±0.62	28.03±0.31	3.82±0.02
SRBC sensitized	63.21±0.68	25.02±0.19	2.58±0.03
Cyclophosphamide	62.01±0.23	27.12±0.11	3.03±0.06
T. chebula	63.87±0.11*	30.22±0.13*	4.01±0.02*
7 day			
Vehicle	62.32±0.72	28.76±0.23	3.48±0.06
SRBC sensitized	61.62±0.92	24.27±0.18	2.21±0.1
Cyclophosphamide	56.32±0.53	21.41±0.31	2.92±0.04
T. chebula	65.41±0.43*	29.94±0.22*	3.01±0.01*
14 day			
Vehicle	62.52±0.20	28.15±0.83	3.05±0.04
SRBC sensitized	64.68±0.68	23.85±0.23	2.01±0.01
Cyclophosphamide	52.13±0.52	17.87±0.73	1.43±0.08
T. chebula	63.13±0.28*	29.22±0.32*	3.08±0.02*

*P< 0.05 when compared with SRBC sensitized and cyclophosphamide treated groups.

Similarly, monocyte percent in *T. chebula* alcoholic extract treated group increased (4.01%) as compare to SRBC treated group (Table 1). However response in case of *T. chebula* treated group was better than SRBC sensitized, cyclophosphamide treated group. Further, the cyclophosphamide treated group showed rapid decrease in the neutrophil, lymphocytes and monocytes as compared to other groups due to its immunosuppressive action.

Phagocytic Activity Determined by Blood Polymorphonuclear Cells:

Table 2 showed that the phagocytic index significantly (P<0.05) increased after 30, 60, 90 and 120 min intervals. Maximum phagocytic index was observed in *T. chebula* alcoholic extract group after 120 min incubation as compared to SRBC sensitizes rats (89.53%) and cyclophosphamide treated animals (75.32 %) incubation.

Zinc Sulphate Turbidity Test (ZSTT) for Determination of Humoral Immunity:

Table 3 showed that significant increase in the serum immunoglobulin levels in *T. chebula* alcoholic extract treated group (26.118±0.2132) whereas SRBC sensitized rats did not showed any significant increase in the serum immunoglobulin levels (18.215±0.4852) as compared to vehicle (21.398±0.8543) and cyclophosphamide treated (20.191±0.1184) rats respectively.

Discussion:

Previous studies on cyclophosphamide showed that in rats lymphocytes decrease due to immunotoxic effect as well as decreases in the activity of lymphoid cells especially the $CD4^+$ lymphocytes [18]. Whereas the present study shows that *T. chebula* alcoholic extract (Table 1) increases the neutrophils and lymphocytes as compared to vehicle and cyclophosphamide

Table 2: Phagocytic Activity Determinations

Treatment group	Dose	Phagocytosis index (%)			
		30 min	60min	90min	120min
Vehicle	2 ml of 1% gum acacia solution	60.12±1.21	71.14±1.34	82.16±1.69	91.37±1.83
SRBC sensitized	0.2ml/animal, i p.+2 ml of gum acacia solution orally	60.98±1.58	70.13±1.12	82.45±1.17	90.18±1.67
Cyclophos-phamide	100 mg/kg. orally	57.15±1.34	60.12±1.24	68.25±1.22	75.32±1.34
T. chebula	100mg/kg p.o.	64.45±1.56*	72.33±1.43*	80.65±1.32*	89.53±1.33*

*P<0.05 when compared with control, SRBC sensitized and cyclophosphamide treated groups.

Table 3: Zinc Sulphate Turbidity Test

Treatment group	Dose	Serum immunoglobulin level (ZST units)
Vehicle	2 ml of 1% gum acacia solution	21.398±0.8543
SRBC sensitize	0.2ml/animal, i p.+2 ml of gum acacia solution orally	18.215±0.4852
Cyclophosphamide	100 mg/kg. orally	20.191±0.1184
T. chebula	100 mg/kg p .o.	26.118±0.2132*

*P<0.05 when compared with control, SRBC sensitized and cyclophosphamide treated groups.

treated groups, which indicated immunostimulatory acivity of *T. chebula.*

The phagocytosis and intracellular killing of microorganisms by polymorphonuclear phagocytes was determined by the direct measurement of the microbicidal activity [19]. Phagocytosis was expressed as the phagocytic index in which the percent decrease in the initial number of viable extracellular was determined microbiologically after incubation with polymorphnuclear leukocytes. Polymorphonuclear cells engulf and destroy the foreign substances with their intracellular killing mechanism. When the innate immune response fails the next level of defense is provided by B cells (antibody mediated immune response) and T cells (cell mediated immune response). Any means by which these defense systems can be catalysed/enhanced will prove to boost the overall immune response and well being of all hosts [20].

Present study showed that the *T. chebula* alcoholic extract has showed the significant phagocytic activity increase in time dependent manner as compared to control, SRBC sensitized and cyclophosphamide treated group. However *T. chebula* showed the more phagocytic index as compared to control, SRBC sensitized and cyclophosphamide treated group. From the obtained data, it can be concluded that *T. chebula* alcoholic extract comprises of more immunostimulant activity than control and cyclophosphamide treated group.

The estimation of serum immunoglobulin levels was used to evaluate the increase in serum immunoglobulin concentration after administration of drug. Immunoglobulins are antibodies that react specifically with SRBC antigen and formation of cloudy serum is concentratic immunomodulatory property [21]. In the present study (Table 3) the *T. chebula* treated rats serum showed the more turbidity (cloudy) which indicate the increase in the immunoglobulin level after *T. chebula* alcoholic extract treatment as compared to vehicle, SRBC sensitized and cyclophosphamide treated group. The turbidity was expressed as ZST units which in terms indicate the amount of immunoglobulins present in sample. This indicates the immunomodulatry property of *T. chebula.*

Conclusion:

In conclusion, it is revealed that the alcoholic extracts of *T. chebula* obtained from the dried ripe fruits possess good immunomodulatory activity. Although the ongoing research work is still under progress in order to explore the cellular changes and other pharmacological and biotechnological investigations in male wister rat.

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