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Immunomodulatory activity of ethanolic leaf extract of *Terminalia chebula*

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ABSTRACT

Terminalia chebula, a plant rich with phytochemicals, was selected in the present investigation for evaluating its immunomodulatory activities. Extract of the leaves were prepared in various solvents. Ethanolic extract was found to contain significant amount of phytochemicals. Hence, it was selected to study immunomodualtory activity. Silica gel column chromatography of ethanolic extract was performed. The fractions were further subjected to TLC and most active fractions were administrated to balb/C mice for evaluating immunomodulatory studies. Out of the five fraction (S1 – S5), S3 was found to possess immunostimulant activity. Provision of Ethanol Extracts of *Terminalia chebula* on balb/C mice can increase the antibody titers IgM and IgG.

1. INTRODUCTION

Rising interest in medicinal herbs has increased scientific analysis of their therapeutic potentials and safety. Some of the medicinal plants are believed to enhance the natural resistance of the body to infections [1]. Immunostimulation and immunosuppression both need to be tackled in order to regulate normal immunological functioning. Therefore, stimulatory or suppressive agents which possess activity to normalize or modulate pathophysiological processes are called immunomodulatory agents. There is a rise in the usage of herbal plants to treat diseases of the immune system over the last century. Besides, compared to synthetic drugs, herbal drugs are frequently considered to be less toxic and with fewer side effects. Therefore, the search for more effective and safer agents that can possess immunomodulatory activity has intensified across the world [2]. *Terminalia chebula* has immunomodulatory (3), antifungal and anti bacterial (4), antioxidant (5), anti-viral (6) anti-inflammatory activities (7) and anti-diabetic and anti-proliferative (8) cardioprotective (9) and radioprotective activities [10].

The purification and identification of the compounds responsible for the biological activity detected in the extract is a crucial step. Structural complexity in natural products is diverse, going from the very simple to the highly complex in developing natural products as useful drugs.

A number of studies are undertaken to separate immunomodulatory compounds by thin layer chromatography (TLC) and column chromatography (CC). The stationary phase most commonly used is silica gel with different solvent systems including benzene, toluene, diethyl ether, butanol, ethyl acetate, acetone, ethanol, chloroform, acetic acid, hexane and water for chromatographic separations [11, 12].

The aim of the present study was screening of solvent system for extraction of immunomodulatory compounds from leaf of *Terminalia chebula* using non-polar to polar solvent for complete extraction, isolation and purification of immunomodulatory compounds by column chromatography.

2. MATERIALS AND METHODS

Animals:
Female Balb/c mice 6- 8 weeks old were obtained from National Centre for laboratory Animal sciences (NCLAS), NIN, Hyderabad, Telangana, India. The animals are fed with food and water *ad libitium*. 12 hours of light and dark conditions were maintained. All the animal experiments were carried according to CPCSEA rules (GU/GIS/IAEC/2013/Protocol No.10/2013).

Chemicals:
Ovalbumin (OVA), O-phenylene diamine (OPD), goat anti-mouse IgG and IgM were procured from sigma aldrich, Mumbai, 96 well microtiter flat bottom Enzyme-Linked Immunosorbent Assay (ELISA) plates (Nunc, Denmark) for determining the humoral mediated immune responses. All other chemicals were procured from local vendors and they are of analytical grade.

Collection and Preparation of plant material:
Fresh leaves of *Terminalia chebula* were collected in sterile bags and carried to the laboratory. This plant was abundantly found in Kakinada (Latitude-16^{o}93'N, Longitude-82^{o}33'E), East Godavari district, North Coastal region of Andhra Pradesh and authenticated by Dr. S. B Padal, Department of Botany, Andhra University, Visakhapatnam. Specimen of the same was deposited in Botany Department Herbarium and voucher number was BDH-22201.
The fresh leaves were washed with tap water and then thoroughly cleaned with distilled water and shade dried for a week. Then the dried leaves were grinded to a fine powder by using mortar and pestle. Shade dried and finely powdered leaves of *T.chebula* (200 g) were extracted with ethanol for 48 hours nearer to the solvent's boiling point with Soxhlet extractor yielding 35.6g of crude extract. The crude ethanolic extract was then concentrated to dryness using rotary evaporator (Superfit PBV-6) that resulted in 24.5 g dried extract. (13)

Purification:
Purification of immunomodulatory compounds using Silica Gel column:
The crude ethanolic leaf extract of *T.chebula* (5 g) was subjected to further purification by loading onto silica gel column to separate the extract into different component fractions. Silica gel was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase (14). The glass column (60cm in length and 7cm in diameter) was used for purification of immunomodulatory compounds. A glass wool was placed at the bottom of the column and it was fixed to stand vertically using clamps. Then 1/3 rd of the column was filled with n- hexane. Meanwhile, silica gel with mesh size 60-120 was activated by placing over night in hot air oven at 110°C and suspended in n-hexane to form slurry. The column was carefully packed with silica gel slurry with constant tapping. A 5 g of ethanolic extract was triturated with silica gel (1:2 w/w) and loaded onto the column. The active fractions were eluted at a rate of 5mL/min. The collected fractions were read through UV spectrophotometer 200-700nm. Alike fractions were pooled and further TLC has been carried out for the purified fractions (15).

Thin Layer Chromatography:
The homogeneity of the fractions was examined using thin layer chromatography. Aliquots of fractions were spotted on base line of precoated silica gel TLC sheets and allowed to dry for few minutes. Then the TLC plates were placed into TLC chamber saturated with solvent system (16). Ethyl acetate and benzene were used as solvent systems in different ratios. The developed

chromatogram was subjected to Iodine vapours for the detection of spot. Retention factor (R_f) can be calculated:

$$R_f = \frac{\text{Distance travelled by sample fraction}}{\text{Distance travelled by solvent front}}$$

The purified fractions eluted from silica gel column which shown single spots in TLC were further subjected to immunomodulatory studies.

Immunization:
Ethanolic fractions of TC obtained from silica gel column chromatography were studied by a 49 day protocol as shown in figure.1 was carried out (17). A 28 female Balb/c mice were divided into seven groups (4 mice per group).
Group I: Negative control contains Saline
Group II: Positive control contains Ovalbumin
Group III – VII: Fractions eluted from silica gel column.
Sample was injected to mice by intra peritoneal method (10µg in 0.5ml for each mice). Immunization was done on 0^{th} day, 21^{st} and 42^{th} days.

Collection of serum:
At the appropriate time intervals i.e., 7^{th}, 28^{th}, 49^{th} days after the immunization, the mice were exposed to infra red light and bled from the tail vein into a glass centrifuge tube and the blood was allowed to clot. The clotted blood was rimmed and the serum was separated after centrifugation at 8000 rpm (REMI R-8C) and stored at -20°C for further use.

Effect of purified fractions on anti-ova IgG and anti-ova IgM antibody levels by ELISA:
A 100µl of antigen ovalbumin (200ng) solution is coated to 96 well micro titre plate and incubated over night at 4°c. Plate was washed thrice with wash buffer (PBS-T) and wells were blocked with blocking agent (2% skimmed milk powder) and incubated for 2 hours at 37°C. After washing, plates were incubated with 100 µl of primary antibody (200µg) incubated for 1 hr at 37°C. Again washed thrice, then the plates were incubated with 100µl secondary conjugated antibody (10 µg) for 1 hour at 37°C. After washing, plates were incubated with 100 µl substrate solution (14mg OPD+H_2O_2) and then the reaction was stopped after 3 minutes by 50 µl of stop solution (8N H_2SO_4). Optical density was measured at 492nm using ELISA reader (Thermo scientific Multi scan FC). The data expressed was the mean of Optical Density (OD) of the triplicates (17).

3. RESULTS AND DISCUSSION

In the present study, ethanolic extract of *T.chebula* leaves was subjected to TLC and silica gel column chromatography for further purification of immunomodualtory compounds. A total of 25 different solvent systems have been used for TLC with different ratios. Out of which, 9 solvent system showed better separation of compounds (**Table 1**). Among 9 solvent system, one i.e., ethyl acetate and benzene (2:8) shown good retardation (**Figure 2**). Total 226 fractions were collected by gradient elution of silica gel column containing 5g of ethanolic extract using 4 different ratios of benzene: ethyl acetate (10:0, 9:1, 8:2, 7:3) and represented in (**Table 2)**. The fractions from 87-94 were eluted with benzene (R_f value 0.67), 34-40 fractions eluted from benzene: ethyl acetate (9:1; R_f value 0.75), 24-26 and 44-48 fractions eluted from benzene: ethyl acetate (8:2; R_f value 0.79 and 0.64 respectively), 1-27 fractions eluted from benzene: ethyl acetate (7:3; R_f value 0.72) shown single spots and were pooled respectively. These fractions were named as S1 to S5 respectively.
The five purified fraction shown single spot (**Figure 3**) were intraperitoneal administrated was found to be enhanced the induction of OVA-specific primary IgM and secondary IgG antibody responses in Balb/c mice as determined by ELISA (**Figure 4 & 5**). The OVA-specific IgG and IgM antibody responses of sample-3 (S3) were found to increase with 10 mg dose of extract and

confirming the stimulatory activity of the *T.chebula.* Due to high stimulatory activity of pooled fractions of S3, further analysis was carried out to this S3 fraction.

Table 1: Separation of immunomodulatory compounds by TLC using different solvent system

S.No	Solvents	Ratio	No. of spots	R_f value
1	Ethyl acetate + benzene + chloroform	4:3:3	3	0.5, 0.52,0.78
2	Ethyl acetate + toluene	2:8	1	0.47
3	Ethyl acetate	--	1	0.42
4	Ethyl acetate + chloroform + ethanol	4:4:2	2	0.76, 0.7
5	Ethyl acetate + chloroform + water	4:4:2	2	0.69, 0.77
6	Ethyl acetate + water	5:5	1	0.51
7	**Ethyl acetate + benzene**	**2:8**	**4**	**0.77, 0.59, 0.31, 0.32**
8	Ethyl acetate + benzene + acetic acid	4:3:3	No spots	---
9	Ethyl acetate + water + diethyl ether	4:3:3	No spots	---

Table 2: Silica gel column chromatography elution profile.

Fraction No.		Total Volume collected (mL)	TLC spots and R_f value
Benzene	1-20	100	
	21-60	400	
	61-65	25	
	66-86	100	
	87-94	35	Single spot Rf value 0.67
Benzene + ethyl acetate (8:2)	1-23	115	
	24-26	15	Single spot Rf value 0.79
	27-43	80	
	44-48	25	Single spot Rf value 0.64
Benzene + ethyl acetate (7:3)	1-27	135	
	28-40	60	
	41-44	20	Single spot Rf value 0.72
Benzene + ethyl acetate (9:1)	1-6	30	
	7-23	80	
	24-33	40	
	34-40	35	Single spot Rf value 0.75

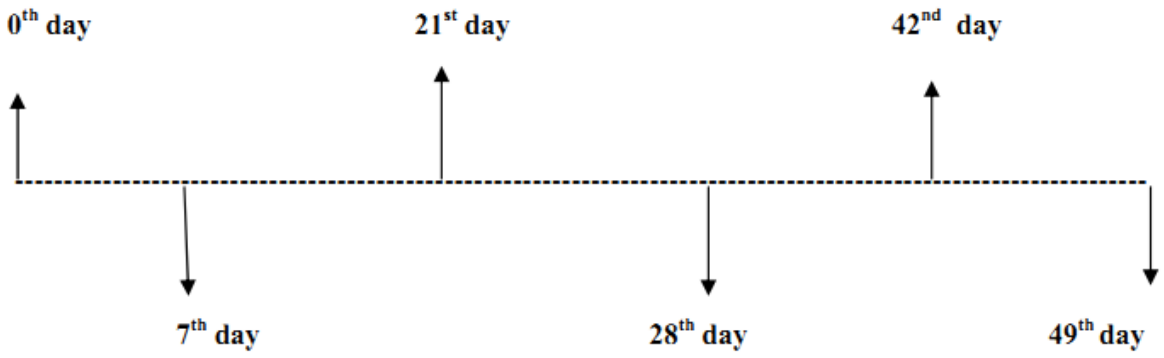

Figure 1: Immunization protocol to determine the levels of anti-ova IgG and IgM in Balb/c mice

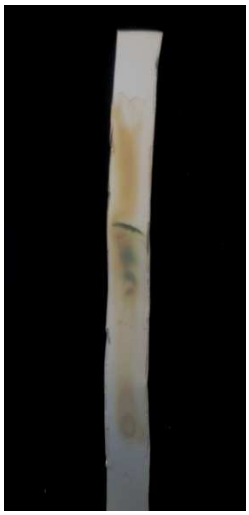

Figure 2: TLC sheet showing good retardation of crude sample with ethyl acetate and benzene (2:8).

Figure 3: TLC plate showing single spots of purified samples (S1-S5)

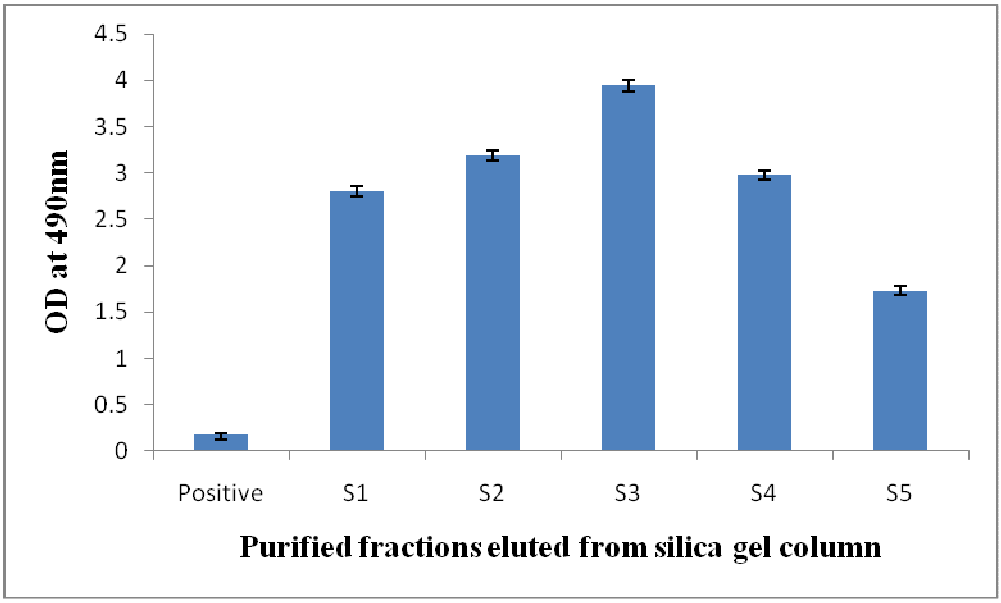

Figure 4: Effect of purified ethanolic fraction of *T.chebula* on anti-ova IgG response in Balb/c (2^o anti serum)

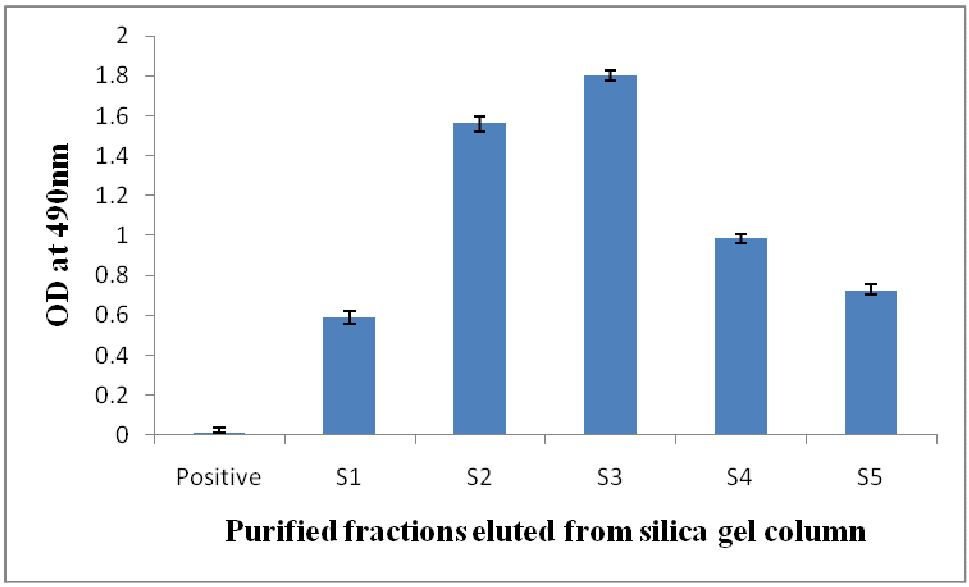

Figure 5: Effect of purified ethanolic fraction of *T.chebula* on anti-ova IgM response in Balb/c (1^o anti serum)

4. CONCLUSION

In the present study, the immunomodulatory activity was identified in the ethanolic leaf extract of *T.chebula* plant. The fraction was eluted on the silica gel column for further purification. The purified fraction obtained from silica gel column exhibited immunostimulant activity. It was found that *T. chebula* is enhancing antigen induced response in Balb/c mice so this can pave way for designing new adjuvant molecules. Studies are on for further purification and structural elucidation.

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