Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

Available online at www.ijpsdronline.com

Research Article

Evaluation of Immunomodulatory Effect of T-Ayu-Hm Premium in Experimental Animal Models

Atul M. Desai[1*], Hemshree A. Desai[1], Rutvij A. Desai[2], Ankit K. Merai[3], Chirag Desai[3], Maryam Khan[3], Arindam Paul[3]

[1]*Dhanvantari Clinic, Ayurveda Health Care and Research Centre, Vyara, Gujarat, India*
[2]*Master of Science; Manila Central University, Philippines*
[3]*Department of Pharmacology: ROFEL Shri G.M. Bilakhia College of Pharmacy, Vapi, Gujarat, India*

ARTICLE INFO

Article history:
Received: 22 June, 2021
Revised: 14 July, 2021
Accepted: 18 July, 2021
Published: 30 July, 2021

Keywords:
Immunomodulatory study,
Levamisole,
Neutrophil adhesion,
T-AYU-HM Premium.

DOI:
10.25004/IJPSDR.2021.130409

ABSTRACT

To evaluate the immunomodulatory effect of T-AYU-HM Premium in experimental animal models. Female Wistar rats were divided into four groups in each model and acclimatized under standard conditions. The control group received vehicle (0.5% CMC), the standard group received Levamisole (50 mg/kg), Test-1 group received T-AYU-HM Premium (600 mg/kg), and Test-2 group received T-AYU-HM Premium (1200 mg/kg). The effect of T-AYU-HM Premium as an immunomodulator was studied by evaluating the neutrophil adhesion model, carbon clearance model, cyclophosphamide-induced neutropenia model, and haemagglutinating antibody titer model. The neutrophil adhesion in standard, Test-1, and Test-2 are 55.39%, 23.32%, and 38.06%, respectively. The %reduction of TLC and DLC in standard and both test groups were significant compared to the control group after nylon fiber treatment. The phagocytic index of standard, test-1 and test-2 was found to be 0.01735, 0.01416, and 0.00923, respectively, this indicates that the test drug affects the reticuloendothelial system. A significant decrease in cyclophosphamide-induced neutropenia implies that the cyclophosphamides' influence on the hemopoietic system is lessened. The percentage reduction in TLC of standard and both test drug groups are 11.12%, 20.66%, and 13.43%, respectively. The %reduction in DLC of standard and both test drug groups were significant compared to a control group. Haemagglutinating antibody (HA) titer test determines the effect of the drug on humoral immunity. Primary HA titer values of standard, test-1 and test-2 were 5.33, 2.66, and 4.50, respectively. Secondary HA titer values of standard, test-1, and test-2 were 13, 7.16, and 11.16, respectively. HA titer value significantly increases in standard and test groups compared to control in both primary and secondary evaluation. In the current study, results show that the T-AYU-HM Premium tablets exhibit Immunomodulatory activity.

Introduction

In the Covid-19 pandemic, boosting the immune system becomes most vital for the population to fight against infection. Before the vaccine arrived in 2021, many herbal formulations were studied and evaluated for effectiveness against COVID-19 and immunomodulatory ability. Considering the advantage of being safe and long-term use, many herbal formulations are under study for immunomodulation activity.[1]

Red Blood Cells (RBCs) are prominent modulators of the innate immune system and inflammation. Mammalian erythrocytes retain the ability to interact with endogenous and exogenous inflammatory molecules in the blood. Chemokines, Nucleic Acids, and Pathogens in blood circulation are bind and scavenged by erythrocytes. Internal components of erythrocytes such as hemoglobin and heme are also formidable facets of innate immunity, capable of generating antimicrobial reactive oxygen species (ROS) to defend against invading hemolytic microbes and

[*]**Corresponding Author:** Atul M. Desai

Address: Dhanvantari Clinic, Ayurveda Health Care and Research Centre, Vyara, Gujarat, India

Email ✉**:** dratuldesai@gmail.com

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

promoting pathologic inflammatory and autoimmune responses.[2] The components of RBC like DARC (Duffy antigen/chemokine receptor), heme, GYPA (glycophorin A), and TLR-9 (toll-like receptor-9) act as an immunomodulator by assisting or ceasing the immune system.[2]

T-AYU-HM Premium is a novel herbo-mineral formulation that possesses anti- sickling activity.[3] The preclinical studies have already been established for this formulation.[4] Each T-AYU-HM Premium tablet (300 mg) contains: *Terminalia chebula* (25 mg), *Zingiber officinale* (25 mg), *Asparagus racemosus* (25 mg), *Punica granatum* (12.5 mg), *Myristica fragrans* (25 mg), *Piper longum* (37.5 mg), *Tinospora cordifolia* (37.5 mg), *Leptadenia reticulata* (37.5 mg). Calyx of Mica (25 mg), Calyx of iron (12.5 mg).[3] The clinical trial was also performed on sickle cell anemia. The effect of T-AYU-HM Premium in novel coronavirus was reported in case studies, and safety and efficacy were evaluated through CTRI registered clinical trial.[3,5] The ability of the formulation to sustain the cellular integrity and prevent lysis of red blood corpuscle cells might help the body sustain oxygen saturation and avoid complications related to it. The ingredients present in the formulation have established already reported immunomodulatory activity like *leptadeniareticulate* (100, 200 mg/kg, oral), *Terminalia chebula* (100–500 mg/kg, oral), *Punica grantum* (100 mg/kg, oral), *piper longum* extract of piperine (10 mg/dose/animal).[6-10] Therefore, in the present study, the immunomodulatory effect of the T-AYU-HM Premium tablet in experimental animal models was evaluated to come up with the preclinical scientific data to assist the clinical use of formulation.

Methodology[11-13]

Experiments were conducted on healthy female Wistar rats aged 8–12 weeks. Animals were housed in the animal house of ROFEL Shri G.M. Bilakhia College of Pharmacy, Vapi under standard conditions as per CPCSEA guidelines – temperature 22 ± 3°C, relative humidity 30–70%, 12 hours light-dark cycle and free to access standard feed and purified R.O. water ad libitium. All experiment procedures for evaluating the immunomodulatory effect of T-AYU-HM Premium in experimental animal models were carried out after obtaining approval by the Institutional Animal Ethics Committee of ROFEL Shri G.M. Bilakhia College of Pharmacy-Vapi- Protocol No. ROFEL/IAEC/2021/0002.

Neutrophil Adhesion Test

In this model, drug treatment (test drug, standard, or vehicle) was given for 14 days orally to Albino Wistar Rats mentioned in Table 1. On the 14th day, blood samples were collected from the retro-orbital plexus into heparinized vials. After initial counts, blood samples were incubated with 80 mg/mL nylon fibers for 10 minutes at 37°C to determine total leukocyte count (TLC) and differential leukocyte count (DLC). The total leukocyte count and differential leukocyte count of the incubated samples were then determined. The following formula was used to compute the percentage of neutrophil adhesion:

Percentage (%) neutrophil adhesion = Niu – Nit x 100/Niu.

Where; Nit = Neutrophil index of treated blood samples.

Niu = Neutrophil index of untreated blood samples.

Macrophage Phagocytosis by Carbon Clearance Method

A carbon clearance test was performed to evaluate the effect of the T-AYU-HM Premium tablet on the reticulo-endothelial system (RES) mentioned in Table 2. RES is a diffuse system consists of phagocytic cells. An increase in the phagocytic index indicates more phagocytosis of colloidal carbon particles in systemic circulation by macrophage. Phagocytosis has the primary role of amputating foreign bodies and micro-organisms also dead cells and injured cells. The carbon clearance method is necessary to evaluate the immunomodulatory effects of drugs. The control group received the vehicle, while other groups received test compounds. The rate of carbon clearance is calculated by phagocytic index (K) by the following equation, K = (lnOD1-lnOD2)/t2 - t1. OD1 and OD2 are the optical density at times 0 and 15 minutes after blood collection from the tail vein, respectively.

Table 1: Groups of animals for neutrophil adhesion test

Groups	*Name*	*No. of Rats*	*Treatment (Dose mg/kg)*
I	Control	6	Vehicle (0.5 % CMC) 1 mL/kg for 14 days orally
II	Standard	6	Levamisole 50 mg/kg for 14 days orally
III	Test 1	6	T-AYU-HM Premium 600 mg/kg for 14 days orally
IV	Test 2	6	T-AYU-HM Premium 1200 mg/kg, for 14 days orally

Table 2: Groups of animals for carbon clearance test

Groups	*Name*	*No. of Rats*	*Treatment (Dose)*
I	Control	6	Vehicle (0.5 % CMC) 1 mL/kg for 5 days orally
II	Standard	6	Levamisole 50 mg/kg for 5 days orally
III	Test 1	6	T-AYU-HM Premium 600 mg/kg for 5 days orally
IV	Test 2	6	T-AYU-HM Premium 1200 mg/kg for 5 days orally

Cyclophosphamide-induced Neutropenia Test

In this model mentioned in Table 3, test drugs, standards, or vehicles were given orally for 10 days to Albino Wistar Rats. A neutropenic dose of cyclophosphamide (200mg/kg, subcutaneous) was administered on the 10th day, and this day was designated as zero-day. Then blood samples were collected via retro-orbital plexus of each animal and total leukocyte count, differential leukocyte count, and % neutrophil was performed before and on the 3rd day after cyclophosphamide injection. Values of total leukocyte counts and differential leukocyte count of treated groups were compared with control groups.

Haemagglutinating Antibody (HA) Titer Test

In this model mentioned in Table 4 test drugs, standards, or vehicles were given orally for 14 days to Albino Wistar Rats before immunization, and then each rat was immunized with 0.5 x 10^9 Sheep Red Blood Cells (SRBCs) via intraperitoneal route including the control group. Day on immunization was labeled as day zero. Then again, drug treatment was continuing another 14 days. Blood samples were collected on the immunization day and the last dose of the treatment for performing haemagglutination titer. The titer was analyzed by titrating serum dilutions with SRBC (0.025 x 10^9 cells) in microtiter plates. The microtiter plates were incubated for 2 hours at room temperature before being visually checked for agglutination. The minimum volume of serum showed haemagglutination was expressed as haemagglutination titer.

Result and Discussion

Neutrophil Adhesion Test

Neutrophils are frontal cells in the immune system as they can recognize, phagocytose, and destroy foreign agents. The ability of neutrophils to adhere to endothelial surfaces and migrates to an inflammatory site is important for preventing bacterial infection was estimated by percentage neutrophil adhesion to nylon fibers. An increase in percentage neutrophil adhesion is an indication of the stimulation of immune response towards antigens. [11,13]

%Eosinophil, %Monocytes, and %Basophils was found to be 2%, 1%, and 0% before treatment of nylon fiber and 1%, 0%, and 0% after nylon fiber treatment respectively. There was a significant increase in percentage neutrophil adhesion in standard, test-1, test-2 when compared to the control group. Also, there was a significant percentage reduction in total leukocyte counts and differential leucocyte count after nylon fiber treatment in standard and both doses of T-AYU-HM Premium tablet groups shown in Tables 5 and 6. This indicates stimulation of the immune system towards antigens.

Table 3: Groups of animals for cyclophosphamide-induced neutropenia test.

Groups	*Name*	*No. of Rats*	*Treatment (Dose)*
I	Control	6	Vehicle (0.5 % CMC) 1 mL/kg for 10 days orally
II	Standard	6	Levamisole 50 mg/kg for 10 days orally
III	Test 1	6	T-AYU-HM Premium 600 mg/kg for 10 days orally
IV	Test 2	6	T-AYU-HM Premium 1200 mg/kg for 10 days orally

Table 4: Groups of animals for haemagglutinating antibody titer test.

Groups	*Name*	*No. of Rats*	*Treatment (Dose for 28 days)*
I	Control	6	Vehicle (0.5 % CMC) 1 mL/kg orally
II	Standard	6	Levamisole 50 mg/kg orally
III	Test 1	6	T-AYU-HM 600 mg/kg orally
IV	Test 2	6	T-AYU-HM 1200 mg/kg orally

Table 5: Effect of T-AYU-HM tablets on TLC and DLC.

		Total Leucocytes Count (WBC/mm^3)		*% Neutrophils*		*% Lymphocytes*	
Groups	*Treatment*	*Before*	*After*	*Before*	*After*	*Before*	*After*
Control	0.5% CMC	7483.33± 533.80	7150 ± 501.83	49.5 ± 0.56	47 ± 0.68	34.66 ± 1.40	32.66 ± 0.98
Standard	Levamisole	13550 ± 71.87$^{\#**}$	11533.33 ± 197.76$^{\#**}$	67.33 ± 0.84$^{\#**}$	35.33 ± 1.02$^{\#**}$	66.33 ± 0.71$^{\#**}$	47.83± 0.98$^{\#**}$
Test-1	T-AYU-HM (600mg/kg)	11066.67 ± 262.88$^{*\#**}$	10283.33 ± 235.82$^{*\#**}$	53.16 ± 1.01$^{*\#}$	43.66 ± 1.05$^{*\#}$	40.66 ± 0.42$^{*\#**}$	36.5± 0.76$^{*\#}$
Test-2	T-AYU-HM (1200mg/kg)	12433.33 ± 125.60$^{*\#\$**}$	11150 ± 92.19$^{*\#**}$	60.16 ± 2.02$^{*\#\$**}$	41.33 ± 0.49$^{*\#**}$	53.83 ± 0.98$^{*\#\$**}$	43± 1.84$^{*\#\$**}$

All values are expressed as Mean ± SEM, where n=6 animal in each group
Data were analyzed by one-way ANOVA followed by a t-test.
$^{\#}$P <0.05 and **P <0.01 when compared with control
*P <0.05 when compared with standard and $^{\$}$P <0.05 when compared with test-1

Carbon Clearance Test

Administration of standard drug and both doses of test drug (600 and 1200 mg/kg) significantly increase phagocytic index compared to control, shown in Table 7. Significant increases in the phagocytic index indicate an increase in clearance of colloidal carbon particles from circulation by phagocytic cells.[11,13]

Cyclophosphamide-induced Neutropenia Test

Cyclophosphamide is an anticancer drug with side effects such as hepatotoxicity, immune toxicity, hepatotoxicity, and mutagenicity. It causes an inability to form new blood cells that leads to leucopenia and thrombocytopenia. As a result, a decrease in cyclophosphamide-induced neutropenia implies that the cyclophosphamides' influence on the hemopoietic system is lessened.[11,13] The effect of the T-AYU-HM tablets on the hemopoietic system was assessed using the cyclophosphamide-induced neutropenia test.

%Eosinophil, %Monocytes and %Basophils was found to be 2%, 1%, and 0% before treatment of cyclophosphamide and 1%, 0%, and 0% cyclophosphamide treatment respectively. There was a significant percentage reduction of differential leucocyte counts in standard and test groups compared to control, as shown in Table 8 and 9. This indicates T-AYU-HM Premium tablets might affect the hemopoietic system.

Haemagglutinating Antibody (HA) Titer Test

A haemagglutinating antibody (HA) titer test was performed to evaluate the effect of the T-AYU-HM Premium tablet on the humoral immune system. The haemagglutination antibody is a reaction between antigen and antibody molecules that give a product of B cells and

Table 6: Effect of T-AYU-HM tablets on neutrophil adhesion and TLC and DLC

Groups	*Treatment*	*%Reduction in Total Leucocytes Count (WBC/mm^3)*	*%Reduction in Differential Leucocytes Count (%)*		*%Neutrophil Adhesion*
			%Neutrophils	*%Lymphocytes*	
Control	0.5% CMC	4.41 ± 0.54	7.72 ± 1.81	5.55 ± 1.23	11.83 ± 1.37
Standard	Levamisole	14.88 ± 1.41$^{\#**}$	47.53 ± 1.31$^{\#**}$	27.88 ± 1.35$^{\#**}$	55.39 ± 0.92$^{\#**}$
Test-1	T-AYU-HM (600mg/kg)	7.04 ± 1.04$^{*\#}$	17.62 ± 3.12$^{*\#}$	10.27 ± 1.19$^{*\#}$	23.32 ± 3.59$^{*\#}$
Test-2	T-AYU-HM (1200mg/kg)	10.28 ± 0.99$^{*\#\$**}$	30.86 ± 2.70$^{*\#\$**}$	20.09 ± 3.13$^{*\#\$**}$	38.06 ± 2.09$^{*\#\$**}$

All values are expressed as Mean ± SEM, where n=6 animal in each group
Data were analysed by one way ANOVA followed by a t-test.
$^{\#}$P <0.05 and **P <0.01 when compared with control
*P <0.05 when compared with standard and $^{\$}$P <0.05 when compared with test-1

Table 7: Effect of T-AYU-HM tablets on phagocytic index by carbon clearance test

Groups	*Treatment*	*Phagocytic Index*
Control	0.5% CMC	0.00647 ± 0.00082
Standard	Levamisole	0.01735 ± 0.0011$^{\#**}$
Test-1	T-AYU-HM (600mg/kg)	0.01416 ± 0.00045$^{*\#**}$
Test-2	T-AYU-HM (1200mg/kg)	0.00923 ± 0.00012$^{*\#\$**}$

All values are expressed as Mean ± SEM, where n=6 animal in each group
Data were analyzed by one way ANOVA followed by a t-test.
$^{\#}$P <0.05 and **P <0.01 when compared with control
*P <0.05 when compared with standard and $^{\$}$P <0.05 when compared with test-1

Table 8: Effect of T-AYU-HM tablets on TLC and DLC

		Total Leucocytes Count (WBC/mm^3)		*% Neutrophils*		*% Lymphocytes*	
Groups	*Treatment*	*Before*	*After*	*Before*	*After*	*Before*	*After*
Control	0.5% CMC	7483.33 ± 533.80	3666.66 ± 421.63	45.33 ± 1.52	24.16 ± 1.01	34.66 ± 1.40	21.5 ± 0.67
Standard	Levamisole	12816.66 ± 177.79$^{\#**}$	11400 ± 305.50$^{\#**}$	65.33 ± 1.28$^{\#**}$	54 ± 0.63$^{\#**}$	63.33 ± 1.08$^{\#**}$	60.16 ± 1.10$^{\#**}$
Test-1	T-AYU-HM (600mg/kg)	11016.67 ± 291.45$^{*\#**}$	8750± 359.39$^{*\#**}$	51.5 ± 1.08$^{*\#**}$	32.33 ± 1.42$^{*\#**}$	46.66 ± 1.17$^{*\#**}$	36.83 ± 0.79$^{*\#**}$
Test-2	T-AYU-HM (1200mg/kg)	12233.33 ± 158.46$^{*\#\$**}$	10583.33 ± 200.69$^{*\#\$**}$	58.33 ± 2.62$^{*\#\$**}$	41.83 ± 0.54$^{*\#\$**}$	57 ± 1.26$^{*\#\$**}$	51.5 ± 1.38$^{*\#\$**}$

All values are expressed as Mean ± SEM, where n=6 animal in each group
Data were analysed by one way ANOVA followed by a t-test.
$^{\#}$P <0.05 and **P <0.01 when compared with control
*P <0.05 when compared with standard and $^{\$}$P <0.05 when compared with test-1

Table 9: Effect of T-AYU-HM Premium on TLC and DLC by CYC-induced neutropenia

Groups	*Treatment*	*% Reduction in Total Leucocytes Count (WBC/mm^3)*	*% Reduction in Differential Leucocytes Count (%)*	
			% Neutrophils	*% Lymphocytes*
Control	0.5% CMC	51.27 ± 4.26	46.47 ± 2.53	37.67 ± 2.17
Standard	Levamisole	11.12 ± 1.29$^{\#**}$	17.14 ± 2.20$^{\#**}$	4.99 ± 0.74$^{\#**}$
Test-1	T-AYU-HM (600mg/kg)	20.66 ± 1.75$^{*\#**}$	37.30 ± 1.84$^{*\#}$	20.81 ± 2.62$^{*\#**}$
Test-2	T-AYU-HM (1200mg/kg)	13.43 ± 1.84$^{*\#\$**}$	27.44 ± 3.79$^{*\#\$**}$	9.62 ± 1.71$^{*\#\$**}$

All values are expressed as Mean ± SEM, where n=6 animal in each group
Data were analyzed by one way ANOVA followed by a t-test.
$^{\#}$P <0.05 and **P <0.01 when compared with control
*P <0.05 when compared with standard and $^{\$}$P <0.05 when compared with test-1

Table 10: Effect of T-AYU-HM Premium tablet on humoral immunity by HA titer test

Groups	*Treatment*	*Haemagglutination titer (HA)*	
		Primary	*Secondary*
Control	0.5% CMC	1.66 ± 0.21	2.33±0.33
Standard	Levamisole	5.33 ± 0.21$^{\#**}$	13 ±0.36$^{\#**}$
Test-1	T-AYU-HM Premium (600mg/kg)	2.66 ± 0.21$^{*\#**}$	7.16 ±0.74$^{*\#**}$
Test-2	T-AYU-HM Premium (1200mg/kg)	4.50 ± 0.22$^{*\#\$**}$	11.16±0.30$^{*\#\$**}$

All values are expressed as Mean ± SEM, where n=6 animal in each group
Data were analysed by one way ANOVA followed by a t-test.
$^{\#}$P <0.05 and **P <0.01 when compared with control
*P <0.05 when compared with standard and $^{\$}$P <0.05 when compared with test-1

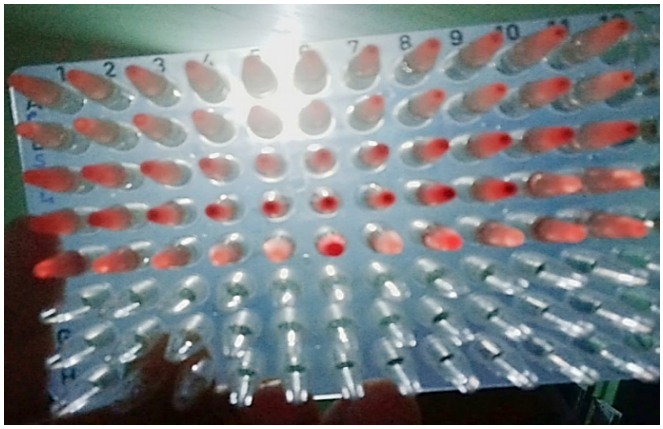

Fig. 1: Haemagglutinating antibody (HA) titer test.

plasma cells and give agglutinin. This is a central role in the humoral immune system. Thus, forming agglutinin titer indicates stimulation of the humoral immune system. Result of HA titre shown in Fig. 1.[11, 13]

Results in Table 10 indicate that HA titer value significantly increases in standard and test groups when compared to control in both primary and secondary evaluation. This indicates that the T-AYU-HM Premium tablet might affect stimulating the humoral immune system.

Conclusion

In the current study, results show that the T-AYU-HM Premium tablets exert Immunomodulator activity as an Immunostimulant effect in all four experimental animal models of study. Observing the results of all four experimental models concludes that T-AYU-HM Premium might affect cellular and humoral immunity. Hence T-AYU-HM Premium tablets might have a potential therapeutic value in vital clinical conditions to alleviate many diseases by acting as an immunomodulator drug. This immunomodulation activity of T-AYU-HM Premium might become useful in fighting against various viral infections too.

Acknowledgment

Authors are expressing their sincere thanks to ATBU Harita Pharmaceuticals, Vyara-Gujarat, India, for providing a sample for the study.

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HOW TO CITE THIS ARTICLE: Desai AM, Desai HA, Desai RA, Merai AK, Desai C, Khan M, Paul A. Evaluation of Immunomodulatory Effect of T-Ayu-Hm Premium in Experimental Animal Models. Int. J. Pharm. Sci. Drug Res. 2021;13(4):432-437. **DOI:** 10.25004/IJPSDR.2021.130409