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Immunomodulation Potential of *Andrographis Paniculata* and *Tinospora Cordifolia* Methanolic Extracts in Combination Forms

Yadav R[a*], Yadav N[a] and Kharya M D[b]

[a*]*Department of Pharmacy, SRMS, College of Engg. and Tech., Bareilly, U.P.*
[b]*Department of Pharmaceutical Sciences, Dr. H. S. Gour Central University, Sagar, M.P.*

Corresponding author*

Rajesh Yadav
Department of Pharmacy, SRMS, College of Engg.and Tech., Bareilly, U.P.
E-mail: raj_ishu78@rediffmail.com

Abstract

Objective: This work mainly focuses on the immunomodulatory effect of different drug combinations (DC-I to DC-V) of methanolic extracts of aerial parts of *Andrographis paniculata* (AP) and mature stems of *Tinospora cordifolia* (TC).
Materials and Methods: The coarse powder (40-mesh) of shade dried aerial parts of AP and mature stems of TC (500g each) was subjected separately to successive extraction with 1000ml each of petroleum ether (60-80^{o}C) followed by methanol. The doses of DC-I to DC-V were selected and were administered orally at doses of 200 mg/kg body weight to Albino Wistar rats and compared with the control group and standard drug i.e. cyclophosphamide (100mg/kg) respectively.
Results: Results suggest that although all the five tested combinations (DC-I to DC-V) prepared using different proportion of methanol extracts of *A. paniculata* and *T. cordifolia* (100:Zero, 75:25, 50:50, 25:75 and Zero:100) showed a significant increase in macrophase count, neutrophil adhesion(NA), haemagglutinating antibody (HA) titre and delayed type hypersensitivity (DTH) response. In rats immunized with sheep RBC, DC-I to DC-V enhanced the humoral antibody response to the antigen and significantly potentiated the cellular immunity by facilitating the footpad thickness response to sheep RBC in sensitized rats on chosen experimental models.
Conclusion: The values of macrophase, NA, HA, DTH and cellular responses of combination DC-II at a 200 mg/kg body weight were statistically significant as compared to other combinations. Combination DC-II exhibited best immunostimulant potential, indicating that it has promising immunomodulatory activity, as proposed from commercial point of view.
Keywords: *Andrographis paniculata*, Humoral and cellular responses, Immunostimulant, Methanol extract, Sheep RBC, *Tinospora cordifolia*.

1. Introduction

Indian traditional systems of medicines like Ayurveda and Siddha have the potential to the body's natural resistance to disease [1]. Recent studies with plants have revealed many compounds with potent antioxidant, antineoplastic, antiulcer, anti-inflammatory and immunostimulating potential [2]. It is believed that the immunomodulatory drugs promote positive health and maintain organic resistance against infections by establishing body's equilibrium and conditioning the body tissues [3]. The restorative and rejuvenating power of herbal remedies might be due to their action on the immune system and thereby responsible for the protection of the organism from extraneous substances and maintaining the homeostasis. Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal products to supplement the diet, mainly with the intention of improving the quality of life and preventing the diseases of elderly people [1]. India has been identified as a major resourceful area in the traditional and alternative medicines globally. Several plant products are known to exhibit immense medicinal value against human diseases. The plants use as an immunomodulator namely *Andrographis paniculata* and *Tinospora cordifolia*. They have good impact in the treatment and management of HIV-AIDS because these plant not only treats disease but also enhance the body vitality and immunity. The humoral and cell-mediated immune response was observed through delayed type hypersensitivity (DTH) model.

Andrographis paniculata, is an herbaceous plant of family Acanthaceae, also called King of Natural Bitters, is a traditional India, Southeast Asian and Chinese herb, used for centuries in Ayurvedic medicine. Since ancient times, *A. paniculata* is being used in Ayurvedic and traditional Siddha systems as well as in tribal medicine in India and some other countries for multiple clinical applications. It has three major spheres of influence, first by offering extensive immune system support, second to protect the liver and third to strengthen the cardiovascular system [4]. The herb has been revered for treating infectious diseases and is highly regarded for preventating many diseases, due to its powerful immune strengthening benefits. The global flu epidemic of 1918 was one of the most devastating infectious outbreaks in World

History. No country escaped its on-slaught except India where *Andrographis paniculata*, an amazing herb was credited with stopping the spread of the deadly virus. Its most active and major constituent-Andrographolide is a Lactone ring in basic diterpene glycoside molecules [5]. *Andrographis paniculata* is also reported to possess anti-inflammatory [6], Anti-Oxidation [7], Anti-hepatotoxicity [8], Anti-hyperglycemic effect [9], Anti-infection [10], Anti-cancer [11], Anti-atherosclerosis activity [12].

Tinospora cordifolia is a large extensively spreading, perennial climber belonging to the family Menispermaceae. It is widely distributed throughout tropical and subtropical India. In Hindi, the plant is commonly known as Giloya, Giloe or Amrita. Giloya is a Hindu mythological term that refers to the heavenly elixir which has saved celestial beings from old age and kept them eternally young. Guduchi, the Sanskrit name means one which protects the entire body [13]. The active adaptogenic constituents are diterpene compounds including tinosporone, tinosporic acid, cordifolisides A to E, syringen, the yellow alkaloid, berberine, giloin, crude giloininand, a glucosidal bitter principle as well as polysaccharides, including arabinogalactan polysaccharide [7-8]. It shows significant bactericidal activities. It improves bacterial clearance as well as improves phagocytic and intracellular bactericidal capacities of neutrophils. It also stimulates macrophage action. As a result it stimulates immune system of body [15]. In Ayurveda also called as Amrita, it is used as "*rasayana*" which has powerful immunostimulant activity [16]. Charaka described rasayana as antiaging, which increased the life span, promoted intelligence, improved memory and ensured freedom from diseases, indicating immunostimulant effect [17]. *T. cordifolia* is used to strengthen the immune system of the body by keeping the function of its various organs in harmony. It has great potency to alleviate impurity of body organs. *T. cordifolia* in Vedic age was considered as one of the most rejuvenating herbs working well on the entire seven Dhatus (the constituent elements of the body) keeping the bodies free from all types of illness [18]. Scientists realize that the effective life span of any antibiotic or synthetic molecule is limited so new sources especially plant sources need to be investigated. Therefore, in the today's world of modern medicine *T. cordifolia* is rightly called as "The Magical Rejuvenating Herb". Categorized as "rasayana" in traditional Indian System of Medicine it is used as general tonic because of its anti-inflammatory, anti-arthritic, anti-allergic, anti-malarial and immunomodulatory properties [19-21]. Its general adaptogenic and immunomodulatory activity was implicated in fighting infections.

Chemotherapeutic agents of today have mainly immunosuppressive activity and most of them are cytotoxic and exerts a variety of side effects. Then metabolism and clinical safety has not been clearly established. This has given rise to stimulation in the research for locating natural resources showing immunomodulatory activity. Presently due to variety of reasons, cases of immunity impairment and its associated problems are increasing. Unfortunately existing therapy does not provide any cure to handle such situation.

However *Andrographis paniculata and Tinospora cordifolia* have been described as over all rejuvenator in Ayurveda with its modifying effects on immunity, may provide a radical cure in such complications. It was therefore decided to investigate these plants the renowned drugs of Ayurveda for developing an immunomodulatory potential.

Although the literature revealed that *A. paniculata* and *T. cordifolia* possess good level of immunostimulant property. And based on the studies, these drugs have also been suggested for their clinical use **[**8-10], however as no study is available on immunomodulation where both of these drugs i.e. *A. paniculata* and *T. cordifolia* have been used together as combination.

The studies also revealed that although the drugs possess immunomodulatory activities, their mode of action can be different. Further, there are growing evidences that drugs capable of modulating single pathway or target are of limited value as immune related therapy and therefore system biology aiming at multi-valuable approaches are now gaining more interest/important. Keeping these facts in mind, it was thought to evaluate the immunomodulation potential of these two highly valued drugs in their combined form. And hence the study was planned accordingly.

The extracts from *A. paniculata* and *T. cordifolia* were subjected to evaluate the bio-actives following to preliminary phytochemical screening of extracts. After performing different chemical tests in each extracts of *A. paniculata* and *T. cordifolia*, it was found that the bio-actives were present in methanolic extracts of *A. paniculata* and *T. cordifolia*. Therefore, the petroleum ether extracts was not used and only the methanolic extracts were subjected to further studies. The five Drug combinations (DC-I to DC-V) prepared from methanol extracts of *A. paniculata* (MEAP) and *T. cordifolia* (METC) and evaluated for their immunomodulatory activity studies by using different animal models like Modulation of Macrophage Function, Humoral response in normal and cyclophosphamide induced immunodeficient rats, Neutrophil Adhesion (NA) Test, Haemagglutinating antibody (HA) Titre, Delayed type hypersensitivity (DTH) response in rats and compared with the control group and standard drug i.e. cyclophosphamide (100mg/kg) respectively. Therefore, present investigation is aimed at studying the immunomodulatory potential of DC-I to DC-V in order to justify the claims that the andrographolide, berberine present in the extracts of AP and TC may be responsible for its immunomodulatory potential.

2. Materials and Methods

2.1 Plant material:

The Aerial parts of *Andrographis paniculata* and selected mature stems of the *Tinospora cordifolia* were collected from medicinal plant garden of Department of Pharmaceutical Sciences, University Campus, Dr. H.S. Gour Vishwavidyalaya, Sagar (M.P.).The plant material was identified and authenticated taxonomically at the Botany Department of Dr. H.S Gour University, Sagar M.P. (Ref no-1417 and 1418, respectively, dated- 28.01.2011). A voucher specimen of the collected sample was deposited in the Departmental herbarium for future reference.

2.2Preparation of extracts:

The collected plant materials were washed with water to make them free from any dust or foreign matter. *A. paniculata* (aerial parts) was dried as such in open shade whereas for convenience of drying *T. cordifolia* (mature stems) were cut into small pieces, crushed and dried in open shade. After air drying the plant materials were powdered (40 mesh size). 500g of each powder was separately extracted by soxhlet successively with 1000ml each of petroleum ether (60-80^{o}C) followed by methanol. The appearance of colorless solvent in the siphon tube was taken as the end point of extraction. The successive extracts were separately filtered and concentrated at reduced temperature on a rotary evaporator and dried over a desiccator at room temperature to obtain total extracts i.e. petroleum ether and methanol extracts of *A. paniculata* and *Tinospora cordifolia*. After completion of extraction, extracts were weighted (w/w); percentage yield was calculated and abbreviated separately. The biologically potent methanol extract was prepared for herbal tablet formulation.

2.3 Preparation of Drug combinations from methanol extracts:

The studies also revealed that although the drugs possess immunomodulatory activities, their mode of action can be different. Further, there are growing evidences that drugs capable of modulating single pathway or target are of limited value as immune related therapy and therefore system biology aiming at multi-valuable approaches are now gaining more interest/important. Keeping these facts in mind, it was thought to evaluate the immunomodulation potential of these two highly valued drugs in their combined form.

2.4Animal selection:

Swiss Albino mice of either sex (20-25gms.) and Wistar albino rats of either sex (200-250gms.) were selected for carrying out Pharmacological activity. Animals were housed at room temperature (23±2^{0}C) with 12h light and 12h dark cycle and relative humidity (55±10%) and were given water (*ad libitum*) and were fed with rat pellet feed. Experiments on animals were conducted after getting approval from Institutional Animal Ethics Committee Dr. H.S. Gour Central University, Sagar, M.P. (Registration No. 379/01/ab/CPCSEA) which is registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.5Acute toxicity studies:

The acute oral toxicity studies and selection of dose was done as per guidelines of Organization for Economic Co-operation and Development (OECD), draft guidelines 423 received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Healthy albino mice of either sex were used for acute toxicity study to determine LD_{50} of investigating five different Drug combinations (DC-I to DC-V) of *A. paniculata* and *T. cordifolia*

The animals were randomly selected, marked to permit individual identification and kept in the cages for 7 days prior to dosing to allow for acclimatization to the laboratory conditions.

The test substances, Drug Combinations (DC-I to DC-V) of AP and TC were administered in a single dose by gavage. Three mice (n=3) were used in each category and starting dose lied in the range of 5-5000mg/kg body weight (OECD guideline 423). Dose ranging between 5, 300, 2000 and 5000mg/kg of body wt. of extract combinations were administered stepwise to the mice according to their weights. After the treatment, mice were observed individually once during the first 30 minutes, and then periodically during the first 24 hrs. There was no mortality till the dose of 2000mg/kg body weight in extract combinations. Considering this dose of 2000mg/kg body weight, 1/10th of this dose i.e. 200mg/kg body weight was taken as effective dose for five extract drug combinations (DC-I to DC-V) from methanol extracts of *A. paniculata* (MEAP) and *T. cordifolia* (METC) for carrying out their immunomodulatory screening.

2.6 Models for Immunomodulatory Screening of different Extract Combinations of AP and TC

2.6.1 Dose selection and Preparation for study:

After carrying out acute toxicity studies, it was observed that lethal dose for DC-I to DC-V combination was 2000mg/kg body weight and hence 1/10th of the dose i.e. 200mg/kg body weight was taken as effective dose for different Drug combinations (DC-I to DC-V) of extracts for performing the immunomodulatory screening.

2.6.2 Standard Drugs, Chemicals and reagents

The drugs, chemicals, and reagents procured from S.D. Fine Chemicals, (Mumbai, India) were of analytical

grade. **Cyclophosphamide** (German Remedies Limited, Kundaim Industrial Estate, Ponda, Goa) was used to produce immunosuppression in rats (Dose- 100mg/kg body weight). **Sheep Red Blood Cells (SRBCs)** at dose of 0.5ml were injected intraperitoneally for immunization and challenge to the rats. **Alsever's solution-** for collection of blood from sheep and **Phosphate Buffer saline (PBS -7.2pH)-** for collection of peritoneal fluid form mice were used.

2.6.3Sheep Red Blood Cells (SRBCs)

Blood from healthy Sheep was collected from local butcher house in **Alsever's solution**. It was washed three times with pyrogen free 0.9% normal saline and centrifuged at 3000 rpm for 5min. Supernatant was discarded. The settled SRBC was then suspended in normal saline and total SRBC counted using Neubauer chamber and RBC of this suspension was adjusted to a concentration of 5×10^9 SRBC (0.5ml) and injected intraperitoneally for immunization and challenge [26].

2.6.4Preparation of Alsever's solution

Formula:

Citric acid	0.055gm
Sodium citrate	0.8gm
Glucose	2.05gm
Sodium chloride	0.42gm
Distilled water to make volume up to	100 ml

All the above ingredients were weighed and dissolved in distilled water and the volume was made up to 100ml. The solution was stored in refrigerator **[**27].

2.6.5Preparation of Phosphate Buffer saline, (PBS) (7.2 pH)

Take 50ml of 0.2M of Pot. di hydrogen phosphate (KH_2PO_4), [27.218gms. KH_2PO_4 in 1000ml. water] was taken in 200ml. volumetric flask and 34.7ml. of 0.2M Sod. hydroxide was added and volume was made up to 200ml [28].

2.6.6Statistical Analysis of Experimental Data

All the experimental data for statistical analysis were presented as mean± SEM (n=4 in each group). Results obtained were statistically analyzed by using One-way ANOVA followed by Dunnett's comparision test. Statistical significance on comparison with control group was indicated by *mark, where *P<0.05 was considered as significant value.

2.6.7 Modulation of Macrophage Function

Albino male mice (20-25g) of either sex were housed under standard laboratory conditions prior to experimentation. They were fasted for a period of 24hrs. Allowing free access to drinking water, prior to p.o. drug administration.

Group-1: (control, n=4) received only clean tap water.

Group-2 to 6: (received Drug combination samples DC-I to DC-V, n=4) @ 200mg/kg body weight.

Peritoneal macrophage was isolated from the treated mice (n=4) on day 5^{th}, 10^{th} and 15^{th} consecutively and also from control mice. Peritoneal fluid was collected in Phosphate buffer saline (PBS, ph 7.2) and the macrophage count was done [29].

2.6.8 Humoral response in normal and cyclophosphamide induced immunodeficient rats

The depression in immune system associated with cancer, surgery, infection and certain drugs is characterized by the reduction in the number and function of neutrophils and macrophages as well as in intracellular bactericidal capacity of these cells. Cyclophosphamide is converted in the organism from a non-reactive to a highly reactive form. This alkylating agent, which is inactive *in vitro*, is activated *in vivo* by enzymatic cleavage of the phosphamide group, which releases the active portion of the compound once the molecule is split. The alkylating agents are thought to act by combining directly with certain intracellular molecules, particularly nucleic acid and proteins. It blocks the conversion of a precursor population (possibly small lymphocytes) to lyphoblasts and the thymus dependent lymphocytes are preferentially affected by cyclophosphamide.

Treatment schedule:

In this procedure male albino rats (200-250g) were divided into three groups.

Group- A: (–ve control, n=4) was administered tap water from day (-9) to day (+5) and on day (+2) 100mg/kg body weight of cyclophosphamide was administered orally in addition to water.

Group-B: (+ve control, n=4) was administered tap water from day (-9) to day (+5).

Group-C1 to C5: (Drug combination samples DC-I to DC-V, n=4) was administered 200mg/kg body weight of DC-I to DC-V once a day orally from day (-9) to day (+5) and on day (+2) 100mg/kg body weight of cyclophosphamide was administered orally in addition to DC-I to DC-V treatment.

- On day 0, rats in all groups were immunized (ip) with 0.5ml/100g body weight with SRBCs.
- On day (+6), blood was collected from each rat and serum separated.
- The value of highest serum dilution carrying visible hemagglutination was taken as the antibody titre[30].

Anti-SRBC-hemagglutination antibody titre in all drug combinations was found better (7.34±3.22 to 6.19±2.03) than positive control (4.32±2.64) but with the Drug combination (DC-II) treated cyclophosphamide induced rats it was found to be maximum (9.49±3.74) as compared to cyclophosphamide induced control rats (1.96±0.18).

2.6.9 Immunostimulant Activity of Drug Combination (DC-I TO DC-V) was performed on the following:

Immunostimulants, also known as immunostimulators, are substances (plants and nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components. Immunostimulant therapy may be beneficial for patients under a variety of settings that include prevention and treatment of various infectious diseases. It is important to know the appropriate use of such treatments so that the ideal immunostimulant preparation is selected for each individual patient. An ideal situation when a host is exposed to pathogen challenge (e.g. bacteria or virus) is to have optimal immunity that protects the host from disease. In many cases specific therapy in the form of antibacterial, antiprotozoal, antiparasitic or antifungal therapy will work in combination with the immune system to aid with pathogen clearance. In some instances, the addition of an immunostimulant will aid in "boosting" the immune response [31].

(a) Neutrophil Adhesion (NA) Test

(b) Hemagglutinating Antibody (HA) Titre

(c) Delayed Type Hypersensitivity (DTH) response in rats

Treatment: Albino Wistar male rats (200-250g) were used for the study. Animals were housed properly under standard conditions of temperature (23±2°C), 12:12h light/dark cycles and fed with standard pellet diet and water ad libitum. Fresh SRBC in Alsever's solution were obtained from authentic source. The animals were divided into six groups consisting of four animals per group. A group of four untreated rats was taken as control (Group 1). The Drug combinations (DC-I to DC-V) were fed orally for 14 days at a dose of 200mg/kg body weight (Group 2-6) for assessment of immunomodulatory effect. On 14th day, all groups of rats were challenged with SRBCs (0.5ml/100g body weight I.P.).

(a) Neutrophil Adhesion (NA) test

On 14th day of Drug combinations treatment, blood samples were collected (before challenge) by puncturing the tail-vein into heparanized vials and analyzed for total leukocyte counts (TLC) and differential leukocyte counts (DLC). After initial counts, blood samples were incubated with 80mg/ml of nylon fibers for 15min. at 37^{0}C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and percentage neutrophil gives Neutrophil Index (NI) of the treated and untreated blood samples and the difference was taken as index of neutrophil adhesion (NA)[32]. Percent neutrophil adhesion was calculated as below:-

$$\textbf{Neutrophil adhesion (NA) (\%)} = \frac{\textbf{NIu} - \textbf{NIt}}{\textbf{NIu}} \textbf{ X 100.}$$

Where NIu = Neutrophil index of untreated blood sample.

NIt = Neutrophil index of treated blood sample.

(b) Hemagglutinating Antibody (HA) titre

Rats of groups DC-I to DC-V were pretreated with Drug combinations for 14 days and each rat was immunized with SRBC (0.5ml/100g body weight I.P.), including control rats. The animals were treated with drug combinations for 14 more days and blood samples were collected from each rat on day 15 for HA titre. The titre was determined by titrating serum dilutions with SRBCs. The micro titre plates were incubated at room temperature for 2 hours and examined visually for agglutination. The highest number of dilution of serum showing hemagglutination was expressed as HA titre[33].

(c) Delayed Type Hypersensitivity (DTH) response

DTH response was determined by the significant decrease or increase in paw volume. All the six groups of SRBCs immunized rats were challenged by subcutaneous administration of SRBCs 0.5ml in right hind foot pad on 28th day and 0.2ml of 0.9% normal saline was similarly injected into left hind foot pad as control. The cell mediated immune response was measured at 24h after SRBCs challenged on 28th day in terms of increase in paw volume (plethysmometrically). The DTH response was expressed as the mean percent increase in paw volume between the right foot pad injected with SRBCs and left foot pad injected with normal saline[34].

3. Results

Immunomodulators are biologic response modifying compounds that affect the immune response in either a positive or negative fashion, where it results in an enhancement of immune reaction; it is named as immunostimulation which implies stimulation of non-specific system i.e. stimulation of the function and efficiency of granulocytes, macrophages and certain T-lymphocytes [37]. Immunosupression implies mainly to reduce resistance against infections, stress and may be because of environmental or chemotherapeutic factors. Immunostimulation and immunosuppression

both need to be tackled in order to regulate the normal immunological functioning. Hence, immunostimulating agents and immunosuppressing agents have their own standing. A number of disorders can be treated by biologic response modifiers; these include immunodeficiency diseases and autoimmune disorders. These drugs may work on cellular or humoral immune systems or both [38-39].

There are several herbal preparations used in the indigenous system of medicinewhich can enhance the body's im mune status. The drugs namely *A. paniculata* and *T. cordifolia* were collected and authenticated. The drugs were cut into pieces and powdered to a coarse consistency, which were subjected to extraction (soxhlet) by using petroleum ether (60-80) and methanol in succession as solvents. The extracts obtained were concentrated and dried in desiccators (Table 1 & 2).

Table 1: Extract detail of *A. paniculata* and *T. cordifolia*

Drug	Extract
A. paniculata **(Aerial parts)**	Petroleum ether(60-80ºC) extract
	Methanol extract
T. cordifolia **(Stems)**	Petroleum ether (60-80ºC) extract
	Methanol extract

Table 2: Yield of extraction derived extracts of *A. paniculata* and *T. cordifolia*

Plant material	*Extracted material*			
	Extract	Nature	Color	%Yield (w/w)
A. paniculata **(Aerial parts)**	Petroleum ether (60-80°C) Extract	Solid	Light -brown	1.06
	Methanol Extract	Solid	Dark brown	12.36
T. cordifolia **(stems)**	Petroleum ether (60-80°C) Extract	Solid	Light brown	2.31
	Methanol Extract	Semi solid	Light brown	11.58

All the individual dried extracts were checked for their active ingredients by proximate chemical analysis. For the design and preparation of different Drug Combinations (DC-I to DC-V), the individual dried extracts of *Andrographis paniculata* and *Tinospora cordifolia* were mixed in a requisite amount and 2% Tween 80 suspension was added to the each dried Drug combination in distilled water (100ml) as a suspending agent **(Table 3)**.

Table 3: Drug combinations of methanol extracts of *A. paniculata* and *T. cordifolia*

S.No.	Drug Combination	Quantity of (MEAP)	Quantity of (METC)
1	DC-I	100%	Zero
2	DC-II	75%	25%
3	DC-III	50%	50%
4	DC-IV	25%	75%
5	DC-V	Zero	100%

Note: The quantity of each Extract Combination is 5g.

The prepared Drug Combinations (DC-I to DC-V) were subjected to toxicity studies followed by dose selection was carried out as per guidelines of Organization for Economic Co-operation and Development (OECD), Draft guidelines 423 received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. During acute oral toxicity study, animals were observed for their Behavioural, Neurological and Autonomic profile. For acute oral toxicity studies of Five Drug Combinations (DC-I to DC-V) of methanol extracts of *A. paniculata* and *T. cordifolia* were administered in a single dose by gavage. Three animals were used in each category and starting dose lied in the range of 5-5000 mg /kg body weight (OECD guideline 423). $1/10^{th}$of the lethal dose was used as effective dose for immunomodulatory activity screening. It was observed that administration of single dose of different Drug combinations of methanol extract of *A. paniculata* herb and methanol extract of stem of *T. cordifolia* at dose of 200mg/kg, orally did not have any deleterious effects **(Table 4)**.

Table 4: Acute toxicity study for LD_{50} determination of Drug Combinations (DC-I to DC- V) of methanol extracts from *A. paniculata* and *T. cordifolia*

S.No.	Drug Combination	LD_{50} Cut-off	Vehicle
1	DC-I (100%)	2000 mg/kg	Tween-80
2	DC-II (75-25%)	2000 mg/kg	Tween-80
3	DC-III (50-50%)	2000 mg/kg	Tween-80
4	DC-IV (25-75%)	2000 mg/kg	Tween-80
5	DC-V (100%)	2000 mg/kg	Tween-80

Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating primarily the non-specific immune system i.e. stimulation of the function and efficiency of the macrophages and other complements. However, these drugs need to be subjected to systematic scientific studies to substantiate the therapeutic claims made with regard to their utility. The time dependent effect of different Drug Combinations (DC-I to DC-V) on morphometric functional changes in mice (peritoneal macrophages) were evaluated where Drug combination (DC-II) treated (200mg/kg b. wt. p.o.) animals showed a very significant increase in the macrophage count and the maximum number of macrophage cells (8,233±176.38, 9,243±208.17 and 9,891±218.58) were found to be on the 5th, 10th and 15th days respectively of extract administration as compared to control (3,233±497.77, 3,600±461.88 and 3,233±523.87).

The effects of Drug combination (DC-II) at dose 200mg/kg b.wt. p.o. of AP and TC treatment on morphometric and functional changes of macrophages in mice showed very significant increase ($p<0.01$) in the number of macrophage cells on the 5th, 10th and 15th days of drug administration. Thus it significantly activated macrophages and enhanced their function as compared to control, suggesting that (DC-II) combination possess potential immunostimulant effect (**Table 5, Figure 1)**.

Table 5: Effect of Drug Combinations (DC-I to DC-V) on morphometric and functional changes of macrophage in male albino mice

Macrophage count(Cell/mm3)	Days after Drug Combinations (DC-I to DC-V) (200mg/kg. P.O.)		
	Day 5th	Day 10th	Day 15th
Control Group	3,233±497.77	3,600±461.88	3,233±523.87
DC-I Group	6,033±185.59*	6,766±352.77*	7,600±305.5*
DC-II Group	8,233±176.38**	9,243±208.17**	9,891±218.58**
DC-III Group	7,366±233.33*	8,000±57.73*	8,533±233.33*
DC-IV Group	5,266±371.18	5,733±348.01	6,433±284.80*
DC-V Group	5,966±120.19	6,500±360.56*	7,333±240.37*

Values are expressed as mean±SEM, (n = 4), Comparison of Group I (Control) was made with all groups. **p<0.01 Very Significant compared to control group (ANOVA followed by Dunnett's test). *p<0.05 Significant compared to control group (ANOVA followed by Dunnett's test).

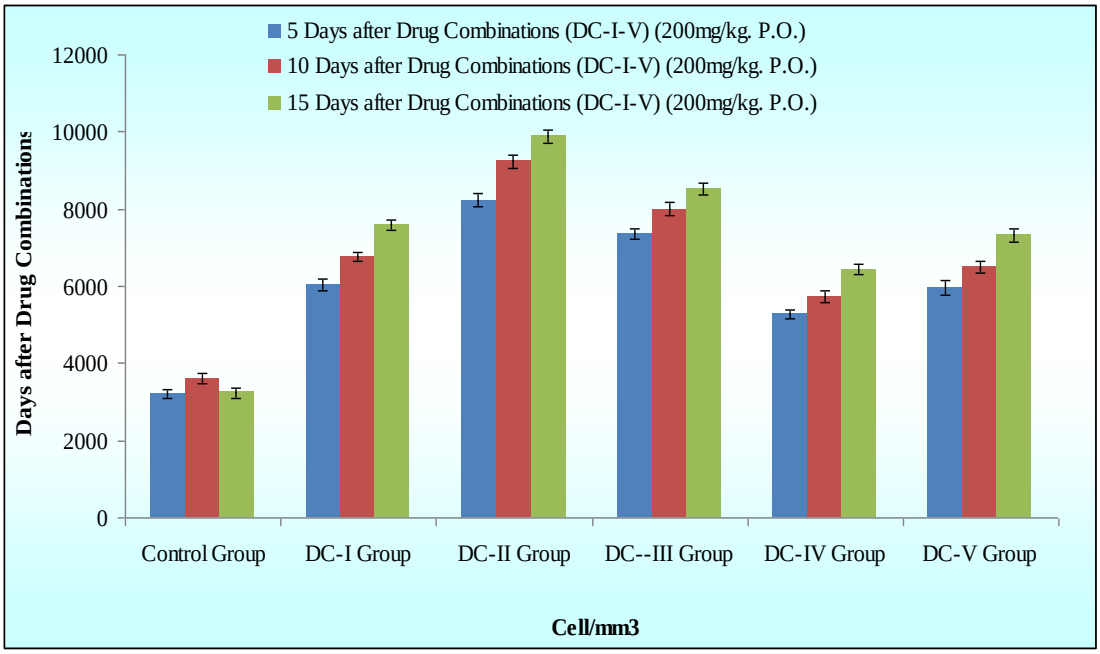

Figure 1: Effect of Drug Combinations (DC-I to DC-V) on morphometric and functional changes of macrophage in male albino mice.

Cyclophosphamide is modified nitrogen mustard which is converted in the organism from a non-reactive to a highly reacting form. Alkylation agents are thought to act by combining directly with certain intracellular molecules, particularly nucleic acid and proteins. Thus such molecules are denatured by formation of intracellular cells. It blocks the conversion of a precursor population (possibly small lymphocytes) to lyphoblasts. The thymus dependent lymphocytes are preferentially affected by cyclophosphamide.

The findings of present studies on cyclophosphamide induced immunosuppression model reveals that methanol extracts of both the chosen drugs i.e. *A. paniculata* and *T. cordifolia* on Anti-SRBC-hemagglutination antibody titre in the Drug combination-DC-II treated cyclophosphamide induced rats was found to be (9.49±3.74) as compared to cyclophosphamide induced control rats (1.96±0.18). The Anti-SRBC-hemagglutination antibody titre in the control rats was (4.32±2.64). The suppressive effect of cyclophosphamide was protected by animals treated with Drug combination-DC-I to DC-V and result revealed that administration of Drug combinations of AP and TC could stimulate the haemopoetic system.

It shows that the Drug combination-DC-II very significantly (P<0.01) protected cyclophosphamide induced humoral immunosuppression in rats as compared to control groups. This suggested a significant potentiating action of Drug combination (DC-II) on humoral immunity, as plant extracts significantly protected cyclophosphamide induced humoral immunosuppression in rats (**Table 6, Figure 2**).

Table 6: Effect of Drug combinations (DC-I to DC-V) on hemagglutination Antibody titre in Normal and Cyclophosphamide (CYMP, 100mg/kg) induced immunodeficient rats

Group	Treatment	Value
A	–ve Control (SRBC + CYMP)	1.96±0.18
B	+ve Control (SRBC)	4.32±2.64
C1	Drug combination (DC-I+SRBC+CYMP)	7.34±3.22*
C2	Drug combination (DC-II+SRBC+CYMP)	9.49±3.74**
C3	Drug combination (DC-III+SRBC+CYMP)	7.32±2.89*
C4	Drug combination (DC-IV+SRBC+CYMP)	6.80±2.21*
C5	Drug combination (DC-V+SRBC+CYMP)	6.19±2.03*

Values are expressed as mean±SEM, (n = 4), Comparison of Group I (Control) was made with all groups. **p<0.01Very Significant compared to control group (ANOVA followed by Dunnett's test). *p<0.05 Significant compared to control group (ANOVA followed by Dunnett's test).

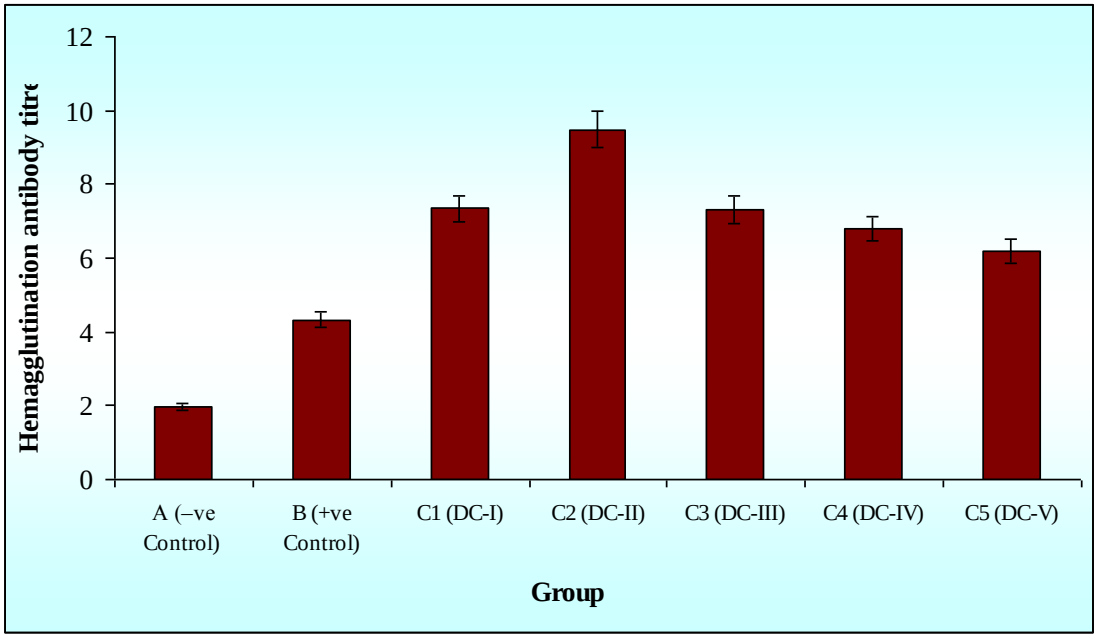

Figure 2: Effect of Drug combinations (DC-I to DC-V) on hemagglutination Antibody titre in Normal and Cyclophosphamide (CYMP, 100mg/kg) induced immunodeficient rats.

Neutrophil Adhesion Test is an indicative of the marginalization of phagocytic cells in the blood vessels, i.e. an indication of immunostimulation. As per the present findings on neutrophil adhesion, the % neutrophil adhesion in control group animals was 32.12, whereas in Drug combinations (DC-I,III,IV,V) treated group animals, at dose of 200mg/kg body weight, it was 39.84, 38.30, 37.50 and 35.91 respectively, while for Drug combination (DC-II) treated group animals at dose of 200mg/kg body weight, it was 54.76. The treatments showed very significant (P<0.01) increase at a dose of 200mg/kg body weight in Drug combination-DC-II treated group animals as compared to control, proving the immunostimulant action of the Drug combination (DC-II) of methanol extracts of AP and TC (**Table 7, Figure 3**).

Table 7: Effect of Drug combinations (DC-I to DC-V) on Neutrophil Adhesion in rats

S. No.	Group	TLC(10^3/mm^3) (A)		Neutrophil % (B)		Neutrophil Index (A X B) = (C)		Neutrophil Adhesion (%)
		UB	FTB	UB	FTB	UB	FTB	
1	Control	3.7±0.05	2.7±0.05	38.3±0.66	35.6±0.66	141.7±2.42	96.2±0.87	32.12
2	DC-I	3.7±0.02	2.7±0.04	38.3±0.33	31.6±0.66	141.8±2.51	85.3±0.36	39.84*
3	DC-II	4.6±0.06	3.1±0.05	48.6±0.33	32.6±0.57	223.5±2.81	101.1±1.52	54.76**
4	DC-III	5.7±0.05	4.8±0.33	51.3±0.57	37.6±0.57	292.4±1.87	180.4±2.21	38.30*
5	DC-IV	3.9±0.05	2.9±0.04	39.6±0.33	33.3±0.57	154.4±1.07	96.5±0.66	37.50*
6	DC-V	5.1±0.05	4.2±0.05	52.3±0.33	40.6±0.33	268.7±1.32	172.2±2.31	35.91*

Values are expressed as mean±SEM, (n = 4), Comparison of Group I (Control) was made with all groups. **p<0.01Very Significant compared to control group (ANOVA followed by Dunnett's test). *p<0.05 Significant compared to control group (ANOVA followed by Dunnett's test).

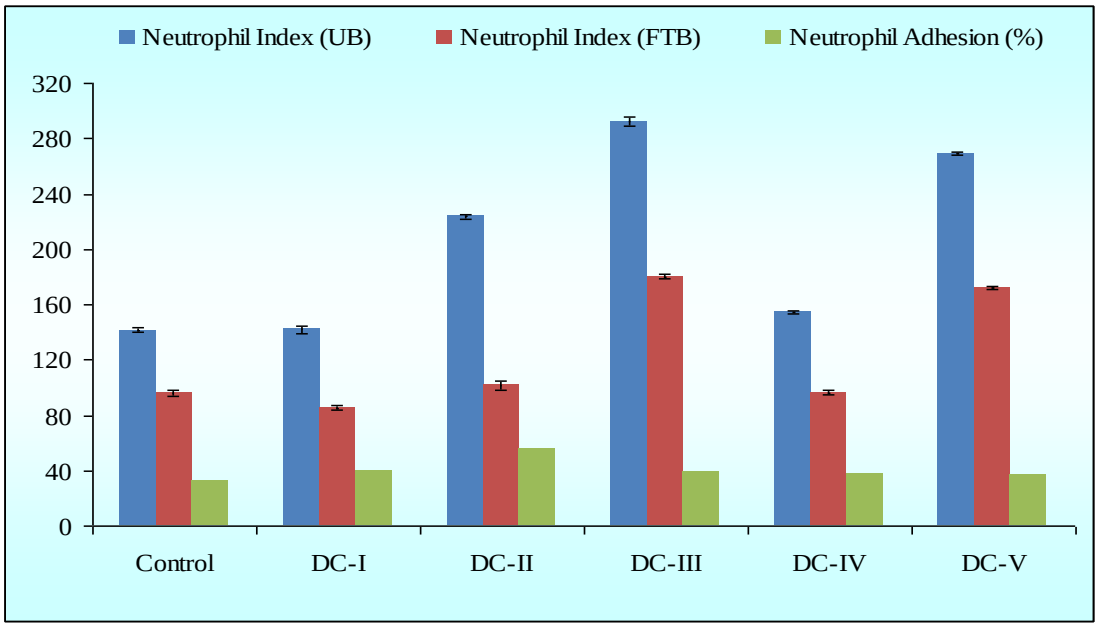

Figure 3: Effect of Drug combinations (DC-I to DC-V) on Neutrophil Adhesion in rats

Hemagglutination reaction: The antigen antibody reaction results in agglutination. The relative strength of an antibody titer is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The augmentation of humoral response by DC-I to DC-V, as evidenced by an enhancement of antibody responsiveness to SRBC in rats, indicated the enhanced responsiveness of macrophages and B lymphocytes subsets involved in antibody synthesis. Agglutination tests can be used to measure the level of antibodies to particulate antigens. In this test, serum containing antibodies was collected from animals of each group and serial dilutions were done in microtiter plate. For present study on Hemagglutinating Antibody (HA) titer, the animlas were treated with different Drug combinations (DC-I to DC-V) for 14 more days and blood samples were analyzed from each rat on day 15 for HA titre where values were very significantly (P<0.01) increased at the dose of 200mg/kg body weight for Drug combination (DC-II) (8.59±0.221) as compared to control (3.58±0.077), suggesting that 14 days pretreatment of drug combination-II of AP and TC was capable to enhance responsiveness of macrophages and lymphocytes inbolved in antibody synthesis and showed possible immunostimulant action of the Drug combination-DC-II of methanol extracts of AP and TC (**Table 8, Figure 4**).

Table 8: Effect of Drug combinations (DC-I to DC-V) on HA Titre to antigenic challenge by Sheep RBCs in rats

Group	H A Titre
Control	3.58±0.077
DC-I	6.90±0.233*
DC-II	8.59±0.221**
DC-III	6.42±0.231*
DC-IV	5.90±0.211*
DC-V	5.20±0.201*

Values are expressed as mean± SEM, (n = 4), Comparison of Group I (Control) was made with all groups. **p<0.01Very Significant compared to control group (ANOVA followed by Dunnett's test). *p<0.05 Significant compared to control group (ANOVA followed by Dunnett's test).

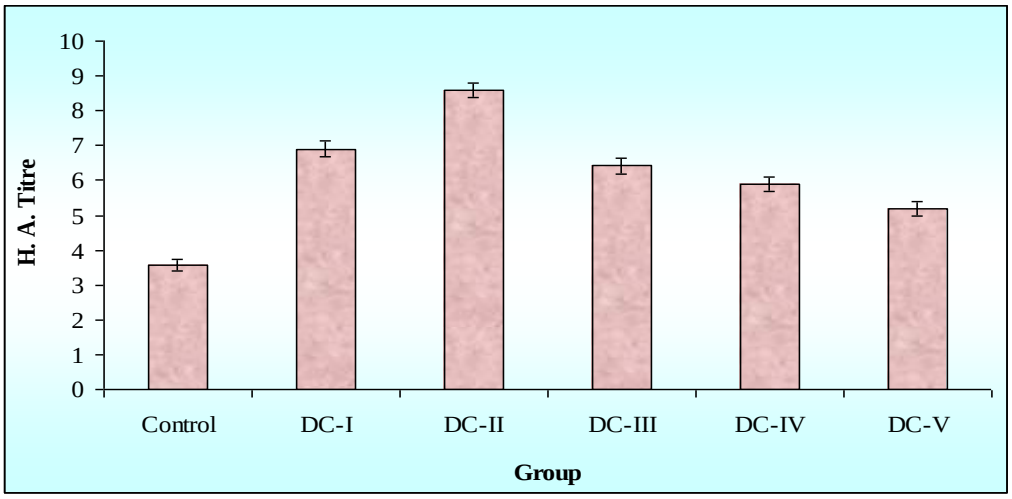

Figure 4: Effect of Drug combinations (DC-I to DC-V) on HA Titre to antigenic challenge by Sheep RBCs in rats

In the present investigation, DTH reaction was used to study the effect of Drug combinations (DC-I to DC-V) on cell mediated immunity, using SRBC as an antigen. On 28th day after 24h of challenge in the control group animals, paw edema was 2.03±0.141while in Drug combinations (DC-I to DC-V) treated group animals at dose of 200mg/kg body weight, it was 5.32±0.124, 8.82±0.228, 6.83±0.233, 6.12±0.201and 4.76±0.217respectively . Therefore, the peak edema after 24h of challenge was the evaluating parameter. The results with Drug combination (DC-II) of AP and TC (200mg/kg body weight) were statistically very significant (P<0.01) with regard to increase in paw volume compared to control treatment. Thus, the results obtained with Drug combination (DC-II) treatment concluded that the DC-II is able to stimulate the macrophages function to stimulate T cells for the hypersensitivity reaction in the immunized rats **(Table 9, Figure 5)**.

Table 9: Effect of Drug combinations (DC-I to DC-V) on DTH response to antigenic challenge by Sheep RBCs in rats

Group	DTH response (%increase in paw volume)
Control	2.03±0.141
DC-I	5.32±0.124*
DC-II	8.82±0.228**
DC-III	6.83±0.233*
DC-IV	6.12±0.201*
DC-V	4.76±0.217*

Values are expressed as mean±SEM, (n = 4), Comparison of Group I (Control) was made with all groups. **p<0.01Very Significant compared to control group (ANOVA followed by Dunnett's test). *p<0.05 Significant compared to control group (ANOVA followed by Dunnett's test).

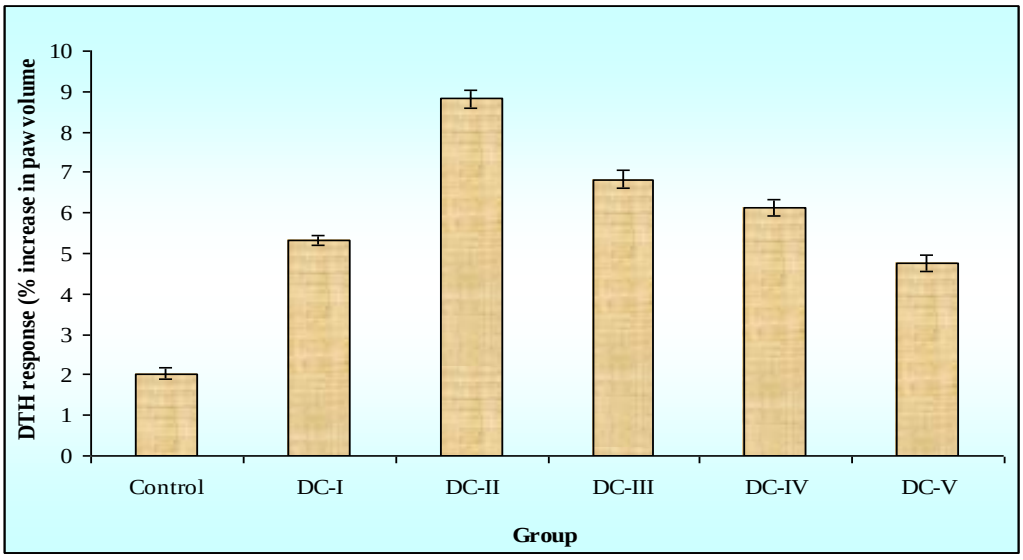

Figure 5: Effect of Drug combinations (DC-I to DC-V) on DTH response to antigenic challenge by Sheep RBCs in rats

Increase in DTH response indicated that DC-II of AP and TC has a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction i.e. cell mediated immunity [40].

4. Discussion

The literature revealed that although when used individually the *Andrographis paniculata* and *Tinospora cordifolia* exhibited immunomodulatory activities, however their immunomodulation potential, if used in combination, was yet to be evaluated.

A good number of herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity. Many plants used in traditional medicine also have immunomodulating activities. Some of these stimulate both humoral and cell-mediated immunity, while others activate only the cellular components of the immune system. Some of these plants also suppress both humoral and cell-mediated immunity [35]. The plants, *Andrographis paniculata* (Kalmegh) and *Tinospora cordifolia* (Giloya) are becoming increasingly popular for a variety of diseases and infective conditions, primarily influencing the host defence mechanism.
Immunity, both cell (Cellular) and antibody mediated (Humoral) are triggered by antigens. In Cellular responses, CD8+ T cells proliferate into "killer" T cells and directly attack the invading antigen while in humoral responses, B cells transform into plasma cells which synthesize and secrete specific proteins called antibodies or immunoglobulins. Antibodies bind to and inactivate a particular antigen. A cell-mediated immune response begins with activation of a small number of T cells (Lymphocytes) by a particular antigen. Once a T cell is activated, it can undergo proliferation and differentiation into a clone of cells, a population of identical cells that can recognize the same antigen and carry out some aspect of the immune attack. The body contains not only millions of different T cells but also millions of different B cells, each capable of responding to a specific antigen. Whereas cytotoxic T cells leave lymphatic tissue to seek out and destroy a foreign antigen. In the presence of a foreign antigen, specific B cells in lymph nodes, the spleen or lymphatic tissue in the gastrointestinal tract become activated. They differentiate into plasma cells that secrete specific antibodies, which then circulate in the lymph and blood to reach the site of invasion [36].

5. Conclusion

For catering the need of the hour for management of immune responses, the present study was designed to evaluate the combined immunomodulation potential of both these highly valued drugs. The present study was focused on their methanol extracts as they were found to possess important bio-actives with proven immunomodulatory activities. Subsequent in-depth screening, revealed better degree of immunomodulatory activity when methanol extracts of both of these drugs were mixed/combined together. Although all the five tested combinations (DC-I to DC-V) prepared using different proportion of methanol extracts of *A. paniculata* and *T. cordifolia* (100:Zero, 75:25, 50:50, 25:75 and Zero:100) revealed to modulate good level of activity on chosen experimental models, the combination DC-II showed best immunomodulatory activity. After arriving to a conclusion that combination DC-II exhibited best immunostimulant potential, as proposed, from commercial point of view so-that the formulation can be explored and exploited for the benefit of the people suffering from immunity related disorders.

The overall present studies revealed that methanol extracts of both the chosen drugs i.e. *A. paniculata* and *T. cordifolia* reported for possessing immunomodulatory activities, when used in combinations showed higher level of activity with broader level of spectrum profile.

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