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# Immuno-modulatory activity of *Triguna Makaradhwaja* — An Ayurvedic compound formulation

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The present study was carried out to evaluate immuno-modulatory activity of *Triguna Makaradhwaja* for humoral antibody formation and cell mediated immunity in established experimental models. Study was carried out in Wistar strain albino rats of either sex and test drug was administered orally at a dose of 3.15 mg/kg along with *Guduchi Ghana* and honey as adjuvant. Effect of test formulation on anti-body formation against sheep red blood cells was assessed for humoral antibody formation. The test drug and vehicles were administered for 10 consecutive days. On third day, 20% SRBC (Sheep Red Blood Cells) as sensitizing agent was injected subcutaneously to the rats of second, third and fourth groups in the dose of 0.5ml/100 g of body weight. Animals were sacrificed on 11$^{th}$ day. Parameters like haemagglutination titre; haematological, serum biochemical and histology of spleen, thymus and lymph nodes were studied to assess the effect on for humoral immunity. Immunological paw oedema was assessed for cell mediated immunity. Animals were sensitized with triple antigen subcutaneously on first day and the drug administration was continued for seven days. On 7$^{th}$ day animals were challenged with the same antigen by injecting to left hind paw and volume of immunological oedema thus produced was measured by volume displacement method after 24 and 48 hours. *Triguna Makaradhwaja* apparently enhanced antibody formation and cellularity of immunological organs, while it failed to show any significant impact on immunologically induced paw oedema. This study shows that *Triguna Makaradhwaja* is having marked immunostimulant effect and weak effect on cell mediated immunity (CMI). The adjuvant *Guduchi Ghana* and honey *per se* has immune-potentiating activity and it seems to be added to the therapeutic activity of the main drug.

**Keywords:** Cell mediated immunity, *Guduchi Ghana*, Haemagglutination, Honey, *Triguna Makaradhwaja*.

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## Introduction

Immunity is a biological term that describes a state of having sufficient biological defenses to avoid infection, disease, or other unwanted biological invasion. Immunity involves both specific and non-specific components. The non-specific components act either as barriers or as eliminators of wide range of pathogens irrespective of antigenic specificity. The main mechanism is to enhance different body defense mechanisms in a non-specific manner to endow it with better capacity to get adapted to different kinds of adverse conditions[1]. The term 'immunomodulation'[2] is used for describing, the effect of various chemical mediators, hormones and drugs on the immune system. Immunomodulation is a therapeutic approach in which intervene in auto regulating processes of the defense system is tried. Immunomodulation is very important in a homotoxicological treatment protocol, especially dealing with a chronic disease. To activate or regulate immune reactions, it not only put the defense system on the right purposeful level of action, but also keeps the clinical symptoms of inflammation within for the patient acceptable levels, or stimulates a non-reactive immune system[3].

Use of herbs or herbo-mineral formulations for improving the overall resistance of body against common infections and pathogens has been a guiding principle of Ayurveda[4] and for this there is a separate class of immunomodulatory drugs known as *Rasayana*. They are supposed to have the ability of

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protecting the body against external factors that induce disease. This implied resistance against disease may represent the modern concept of immunity[5]. *Makaradhwaja* is one such popular metalo-mineral formulation predominantly in manufacturing of which herb are also as inevitable and vital part, and is most popular and effective in *Kupipakwa* preparation (a specific type of pharmaceutical practice in which medicines are prepared by gradually increasing heating patterns using a vertical electric muffle furnace) of Ayurvedic medicine. The main components being *Swarna* (Gold), *Parada* (Mercury) *Gandhaka* (Sulfur) in the ratio of 1:8:16 generally[7]. As per classical literature, this drug is mainly used for *Rasayana* (Rejuvenator) purposes[8]. This drug is also well-known for its propensity to modulate immune system.

*Triguna Makaradhwaja*[9] (TM) is a herbo-minaral compound, levigated by two herbal drugs viz. *Gossypium arboreum* Linn. and *Aloe barbadensis* Mill. As the quantity of sulphur varies in the manufacturing of *Makaradhwaja* so the properties enhances, thus excess *Gandhaka* is assimilated/ digested in *Parada* (Mercury) to form a therapeutically extremely effective compound, a polysulfide form probably is termed as *Gandhaka Jarana*, which plays an important role in the detoxification of mercury. As the quantity of sulphur varies in the manufacturing of *Makaradhwaja* so the properties enhances and name of product also differs a bit. As in context to present research work *Gandhaka* was digested thrice the *Parada*. *Gandhaka Jarana* (The process of digestion of excess of sulphur in mercury) plays an important role in the detoxification of mercury. It has been claimed that *Parada* without the process of *Gandhaka Jarana*, cannot be able to cure the disease[10], so it must be treated with *Gandhaka* to make it highly effective i.e. to acquire many pharmacological and therapeutic properties. Here, *Makaradhwaja* was prepared using three times of *Gandhaka* than that of *Parada* i.e. *Triguna Makaradhwaja* and evaluated for immuno-modulatory activity.

## Materials and Methods

All the raw drugs used in the formulation were procured and authenticated by pharmacy Gujarat Ayurved University, Jamnagar. *Triguna Makaradhwaja* was prepared using *Shuddha Swarna* (processed gold), *Parada* and *Gandhaka* in the proportion of 1:8:16 using levigating medias as *Rakta Karpasa Pushpa* (flowers of *Gossypium arboreum*) and *Kumari Swarasa* (expressed juice of *Aloe barbadensis* Mill.) by following the classical guidelines[11] in Electric muffle furnace with the help of gradual heating pattern in eighteen hours[11], in the department of Rasashastra and Bhaishajya Kalpana of IPGT & RA. *Guduchi Ghana*[12] (GG – water extract of *Tinospora cordifolia*), which is used as adjuvant as per classical guidelines was also prepared by crushing fresh stem pieces of *Guduchi* [*Tinospora cardifolia* (Willd.) Miers ex Hook.f.] and kept for overnight by soaking in water followed by preparation of *Kwatha* (decoction) using total eight times of water. The *Kwatha* is subjected to gradual heat to prepare *Guduchi Ghana*[12] as per classical procedure. Honey which was used as vehicle as advocated in classical texts was procured from local market.

### Animals

Wistar strain albino rats (*Rattus norvegicus*) of either sex were obtained from animal house attached to Pharmacology laboratory of our institute. Six animals were housed in each cage made up of poly-propylene with stainless steel top grill. The dry wheat (post hulled) waste was used as bedding material and was changed every morning. The animals were exposed to 12 h light and 12 h dark cycle with the relative humidity of 50 to 70% and the ambient temperature during the period of experimentation was 22 ± 03ºC. Animals were fed with Amrut brand rat pellet feed supplied by Pranav Agro Mills Pvt. Limited. For their drinking purpose tap water *ad libitum* was used. The experiments were carried out in conformity with the Insttitutional animals Ethics Committee (IAEC) after obtaining its permission (Approval number: IAEC/07/2010/01/MD).

### Dose fixation

The general clinical dose of *Makaradhwaja* is 35mg[13] along with *Guduchi Ghana* (463 mg) as adjuvant and honey as *anupana* (Vehicle). The animal dose was calculated by extrapolating the human dose to animal dose based on the body surface area ratio by referring to the table of Paget and Barnes (1964)[14]. Thus the dose of *Triguna Makaradhwaja* was 3.15 mg/kg rat and the dose of *Guduchi Ghana* (GG) was 45 mg/kg. The test drug was weighed carefully in an electronic digital balance and stock solution was prepared in honey at suitable concentration to enable administration of 0.1 ml for 100 g rat and administered to animals orally based on the body weight. The amount of vehicle honey was decided on

the basis of observation of clinical study where the required quantity of it is to be added with the drug and given for licking. The previous research works have shown that honey as *Anupana* has considerable effect in reducing toxicity of mineral drugs[15].

**Chemicals**

All the chemicals and reagents used in the experimental study were procured from standard and reputed firms and are of analytical grade (EXLR), regularly used in the laboratory. Triple antigen was procured from Serum Institute of India, Pune.

**Effect on humoral anti-body formation**

The effect of test drugs on anti-body formation against sheep red blood cells (SRBC) was studied as described by Puri *et al*, (1994)[16]. Wister strain albino rats of either sex weighing between 200 ± 30 g were selected and divided into four groups. First group received tap water and served as the normal control to which SRBC was not injected. Second group received tap water and served as SRBC control to which SRBC was injected. Third group received GG with honey and served as vehicle control group. Calculated dose of TM was administered to fourth group. The test drug and vehicles were administered for 10 consecutive days. On third day, Sheep blood was collected from the city slaughter house in a sterilized bottle containing Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride) aseptically so that agglutination of blood does not take place. The collected sheep blood was thoroughly washed with sterile normal saline through repeated centrifugation until the supernatant fluid became colorless and made to 20% SRBC solution. This sensitizing agent was injected subcutaneously to the rats of second, third and fourth groups in the dose of 0.5 ml/100 g of body weight.

On the 11$^{th}$ day blood was collected by puncturing supra-orbital plexus by capillary tubes under ether anaesthesia for estimation of haematological and biochemical parameters. Blood (0.08 ml) was mixed with 0.02 ml of EDTA (33.33 mg/ml) and fed to the auto analyzer (ERBA CHEM-5, Trans Asia) which was automatically drawn in to the instrument for estimating different haematological parameters. Then the animals were sacrificed by ether over dose and the blood was collected in sterile test tubes. Serum was separated from it and complement in it was inactivated by incubating it for 30 minutes at 56°C in a serological water bath. Serum total protein (Biurate method)[17] and serum albumin (BCG Dye method)[18] were also estimated. Serum globulin was calculated from serum protein and serum albumin values, A/G ratio was calculated from the above values. Serum IgG was estimated by using ELISA method.

**Estimation of antibody titre**

The micro-titer plate was filled with 0.1 ml sterile normal saline and serial two fold dilutions of 0.1 ml of the serum in sterile saline solution were made in the micro-titer plate up to 16 times. 0.1 ml of thrice saline washed 3% SRBC was added to each well of the tray. Blood from the same animal (Sheep) was used for both sensitization and to determine antibody titer. The trays were covered and placed in refrigerator overnight. Antibody titer (heamagglutination titer)[19] was noted on the next day. The titer was converted to $\log_2$ values for easy comparison.

Spleen, lymph node and thymus were dissected out from the sacrificed animals and transferred to 10% formaldehyde solution for fixation and later on processed for histological studies. The histopathological slides of different organs were prepared by referring standard procedure[20].

**Effect on cell mediated immunity**

Effect on cell mediated immunity was evaluated by following the procedure of Bhattacharya (1993)[21]. Wistar strain albino rats of body weight ranging from 180 ± 30 g were used as experimental animals. The selected animals were divided into three groups of six animals in each group. First group received tap water and served as the normal control. Second group received GG with honey and served as vehicle control group. Calculated dose of TM was administered to third group. All the animals were sensitized subcutaneously (0.5 ml/100g body weight) on first day of drug administration by following solution; Triple antigen (DPT)-1 ml, Normal saline (0.9%) -4 ml and potash alum (10%)-1 ml. The *p*H of this solution was maintained between 5.6 - 6.8 using 10% sodium carbonate. The drug administration was continued for seven consecutive days. On 7$^{th}$ day one hour after drug administration the initial paw volume of left hind paw was noted and 0.1 ml of above solution was injected in to plantar aponeurosis of same paw. Volume of immunological oedema thus produced was measured by volume displacement method (Bhatt *et al*, 1977)[22] after 24 and 48 h of injection using Plethysmometer (Electronic-IITC). Percentage increase in paw volume, which is the index of oedema formation over initial value, was calculated.

**Statistical analysis**

Results were presented as Mean ± SEM, difference between the groups was statistically determined by unpaired Student's 't' test[23]. $P<0.05$ was considered as statistically significant.

## Results

Administration of GG and TM apparently increased the anti-body titre value in comparison to SRBC control rats, however only the observed increase of antibody titre of TM treated group is found to be statistically significant. Further when the value from TM treated group was compared with values of vehicle control (GG) group, test drug produced statistically non-significant increase in anti-body titre (Table 1).

SRBC sensitization significantly increased serum IgG, total protein and albumin levels. Treatment with test drugs did not affect the total protein level to significant extent in comparison to SRBC control group. TM treated group significantly decreased serum albumin level, while GG and SM did not affect it to significant extent. Treatment with GG and TM decreased serum IgG level, however the decrease observed in GG group is found to statistically significant (Table 1).

SRBC sensitization significantly decreased eosinophil and monocyte count to significant extent in comparison to normal control group. Administration of vehicle and test drug non-significantly attenuated eosinophil and monocyte counts. Other haematological factors are non-significantly affected by SRBC sensitization (Table 2).

Administration of GG and TM failed to suppress immunological paw oedema in 24 h, however, they suppressed paw oedema non-significantly at 48 h after triple antigen injection (Table 3).

Table 1—1: Effect on haemogglutination titre and serum biochemical parameters

| Groups | Antibody titer ($Log_2$ values) | Total protein (g/dl) | Albumin (g/dl) | Globulin (g/dl) | Serum IgG |
|---|---|---|---|---|---|
| Control | --- | 6.35 ± 0.13 | 3.17 ± 0.062 | 3.18 ± 0.18 | 381.0 ± 5.26 |
| SRBC | 4.505 ± 0.52 | 7.08 ± 0.06$^{\#}$ | 3.67 ± 0.033$^{\#\#\#}$ | 3.41 ± 0.05 | 423.67 ± 10.55$^{\#}$ |
| GG | 6.007 ± 0.42$^{¥}$ | 6.85± 0.22 | 3.43± 0.17 | 3.41± 0.17 | 382.66± 12.36* |
| TM | 6.354 ± 0.27$^{¥¥}$ | 6.80± 0.56 | 3.25± 0.17* | 3.550± 0.41 | 381.50± 21.66 |

$^{\#}$ P<0.05, $^{\#\#}$P<0.001 (Compared with normal control), *P<0.05 (Unpaired t test Vs SRBC control)

$^{¥}$P<0.05, $^{¥¥}$P<0.01 (One Way ANOVA, P=5.37 Vs SRBC control)

Table—2: Effect on hematological parameters

| Parameters | Control | SRBC | GG | TM |
|---|---|---|---|---|
| Hemoglobin ( g/dl) | 15.12 ± 0.22 | 15.28 ± 0.32 | 15.517±0.389 | 16.183 ± 0.37 |
| WBC ($10^3$ L) | 8250.0 ± 734.3 | 7766.6 ± 249.8 | 6016.67± 446.78* | 7350.00± 684.96 |
| Neutrophils ($10^3$ L) | 22.17 ± 5.06 | 19.00 ± 1.82 | 22.33 ± 1.94 | 18.16 ± 1.86 |
| Lymphocyte ($10^3$ L) | 72.00 ± 5.03 | 78.00 ± 1.69 | 74.167± 2.22 | 79.33± 2.09 |
| Eosinophil ($10^3$ L) | 3.17 ± 0.17 | 1.83 ± 0.31$^{\#}$ | 2.000± 0.25 | 1.33± 0.21 |
| Monocyte ($10^3$ L) | 2.67 ± 0.21 | 1.17 ± 0.17$^{\#\#}$ | 1.50± 0.22 | 1.16± 0.16 |
| RBC ($10^6$/ L) | 8.45 ± 0.09 | 8.59 ± 0.31 | 8.82± 0.16 | 9.08± 0.17 |
| MCV (C -M) | 57.96 ± 0.38 | 58.05 ± 0.63 | 56.61± 0.66 | 57.55± 0.61 |
| MCH (pg / l) | 17.88 ± 0.19 | 17.85 ± 0.40 | 17.58± 0.16 | 17.81± 0.25 |
| MCHC (g/dl) | 30.85 ± 0.19 | 30.71± 0.37 | 31.06± 0.18 | 30.95± 0.23 |

$^{\#}$ P<0.05, $^{\#\#}$P<0.01 (comparison to normal control) *P<0.05 (comparison to SRBC control)

Examination of sections of thymus from normal control rats exhibited normal cytoarchitecture (Plate 1A). In sections from SRBC control group increase in cellularity was observed in comparison to normal control group sections (Plate 1B). In sections from GG and TM treated groups also mild increased cellularity was observed (Plate 1C & 1D).

Sections of spleen from normal control rats exhibited normal cytoarchitecture (Plate 2A). The cytoarchitecture of SRBC control group was found to be similar to normal control group sections (Plate 2B). In sections from GG and treated groups shows increase white pulp proportion in comparison to SRBC control group (Plate 2C & 2D).

Sections of lymph node from normal control rats exhibited normal cytoarchitecture (Plate 3A). The cytoarchitecture of SRBC control group was found to be similar to normal control group sections (Plate 3B). In sections from GG and TM treated groups moderate increased cellularity was observed (Plate 3C & 3D).

## Discussion

*Makaradhwaja* is among the most popular and effective *Kupipakwa* preparation of Ayurvedic medicine. It is believed that after *Jarana* of three times of *Gandhaka* in it, effectiveness further potentiates and the formulation is termed as *Triguna Makaradhwaja*. As per classical literature, this drug is mainly used for *Rasayana* purposes. *Rasayana* drugs are well-known for their propensity to modulate immune system. The safety profile of this formulation was reported by Patgiri *et al*[24] on acute and chronic administration to the rats by adopting battery of parameters like ponderal changes, behavioural profile, hematological, biochemical and histopathological parameters and found to be relatively safe.

Table—3: Effect on immunological paw edema

| Groups | 24 h | % change | 48 h | % change |
|---|---|---|---|---|
| Control | 24.82 ± 4.74 | -- | 15.99 ± 4.50 | -- |
| GG | 33.95 ± 5.15 | 36.85↑ | 11.99 ± 1.46 | 25.04↓ |
| TM | 25.90 ± 4.30 | 04.40↑ | 14.28 ± 7.59 | 10.67↓ |

Data: Mean ± SEM ↑- Increase ↓- Decrease

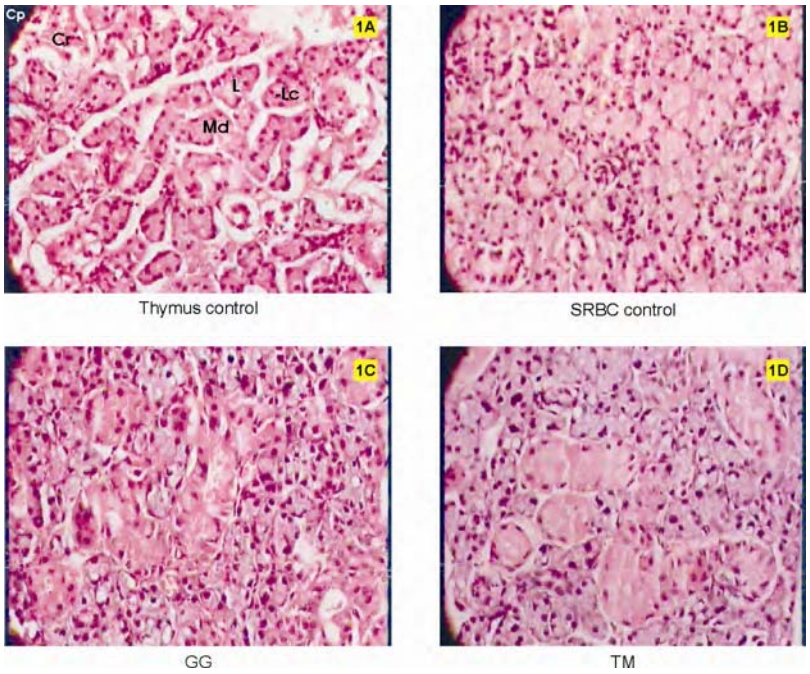


Plate 1 (A-D)— A. Photomicrographs of thymus from control group (1×400 magnification); **Cp**- Capsule. **Cr**- Cortex. **Md**-Medulla. **L**-Lobule. **Lc**-Lymphocytes; **Note:** Normal cytoarchitecture;
B. Photomicrographs of thymus from SRBC control group (1×400 magnification); **Note:** Slightly increased cellullarity;
C. Photomicrographs of thymus from GG treated group (1×400 magnification); **Note:** Increased cellullarity;
D. Photomicrographs of thymus from TM treated group (1×400 magnification); **Note:** Marked increase in cellullarity

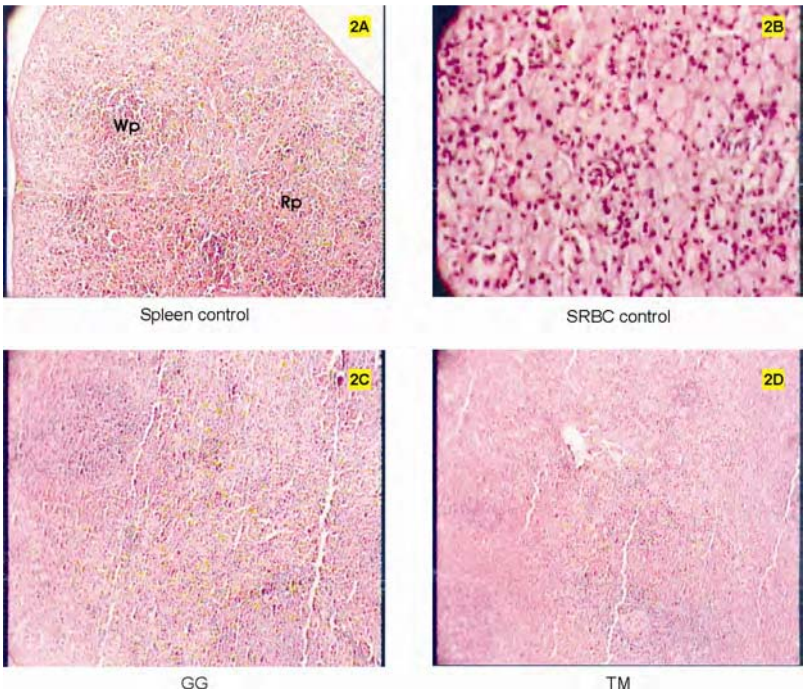


Plate 2 — A. Photomicrographs of spleen from control group (1×100 magnification); **Rp** -Red pulp, **Wp**-White pulp; **Note**: Normal cytoarchitecture;
B. Photomicrographs of spleen from SRBC control group (1×100 magnification); **Note:** Normal cytoarchitecture;
C. Photomicrographs of spleen from GG treated group (1×100 magnification); **Note:** Increased white pulp proportion;
D. Photomicrographs of spleen from TM treated group (1×100 magnification); **Note:** Increased white pulp proportion

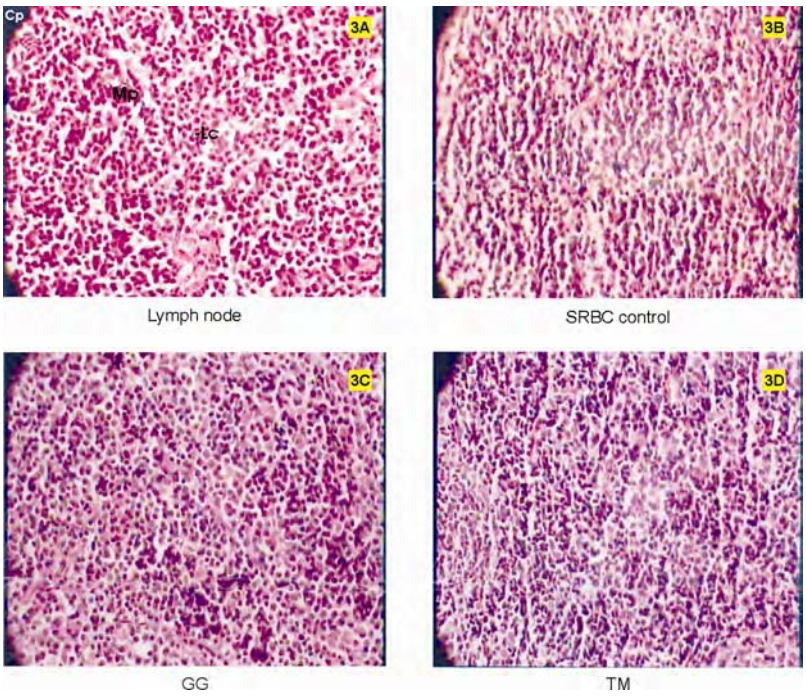


Plate 3 — **A.** Photomicrographs of lymph node from control group (1×400 magnification); **Cp**- Capsule, **Cr**- Cortex, **Md**-Medulla, **Lc**-Lymphocytes; **Note:** Normal cytoarchitecture;
B. Photomicrographs of lymph node from SRBC control group (1×400 magnification); **Note:** Slightly increased cellullarity;
**C.** Photomicrographs of lymph node from GG treated group (1×400 magnification); **Note:** Increased cellullarity;
**D.** Photomicrographs of lymph node from TM treated group (1×400 magnification); **Note:** Marked increase in cellullarity

Haemagglutination antibody titre is a primary parameter for studying the humoral response. Antibody molecules which are secreted by plasma cells mediate the humoral immune response. Treatment with TM significantly increased antibody titer, whereas in GG treated group only a non-significant increase was observed. It is to be noted that because of this non-significant increase the antibody elevation observed with test formulation was found to be statistically non-significant in comparison to GG group. This indicates that adjuvant does contribute, although moderately, to the antibody increasing effect. It may have contributed to the elevation observed in TM group.

Among the 10 hematological parameters studied, antigen injection leads to significant changes only in two parameters. They were significant decrease in eosinophil and monocyte counts in SRBC control group. Treatment with test drugs did not produce any significant impact on these two parameters.

Among the biochemical parameters, serum total protein and albumin levels are significantly increased in SRBC control group. Vehicle administration did not affect them to significant extent, while TM significantly reversed the antigen induced elevation in serum albumin level. Further serum IgG was found to be significantly elevated by sensitization of SRBC. However, this does not correlate well with anti-body titre changes since in GG group in which moderate elevation in antibody titre was observed a decrease in serum IgG level was observed. This may be reflective of effect on other types of antibodies also. As thymus and spleen were lymphoid organs and play a vital role in immune responses, administration of antigen may bring about some changes in their anatomical and physiological features. Administration of TM significantly enhanced cellularity of all the three organs studied as revealed by histological examination, this further indicates the immune stimulant property of the test drug.

Cell mediated immunity do not involve increased formation of antibodies. But through direct effect this reaction neutralizes intracellular pathogens (such as viruses), fungi, malignant cells and grafts of foreign tissue. The test drug did not affect immunological oedema which is used as representative test for cell mediated immunity. Normally those drugs which enhance anti-body formation depress cell mediates immunity, such an effect was not observed. This indicates complex nature of the immunomodulation observed with the test formulation.

The first step in the immune reaction is the recognition of the foreign antigen by macrophages and helper T cells, which become activated and are specific. These activated T cells, which are antigen specific, divide many times to form memory T cells and cytotoxic (killer) T cells (also called CD8 T cells). The memory T cells will remember the specific foreign antigen and become active if it enters the body again. Two pathways mediate immune response depending up on the type of T helper cells involvement. The two are Th1 helper and Th2 helper cell mediated pathways. Th1 pathway is involved in the elaboration of cell mediated immunity through secretion specific cytokines[25] where as Th2 pathway is involved in the enhancement of anti-body formation through plasma cell activation. In the present study no significant effect was observed on CMI while elevation in antibody titre and IgG secretion was observed. This indicates that the test formulation has got specific stimulatory effect on the Th2 pathway of immune reaction. Further studies would be required to elucidate the exact mechanism of this effect. However, it can be suggested that this stimulation may involve enhanced antigen processing by the antigen presenting cell and or through increased formation of cytokines like IL-$_4$ and tissue growth factor-b (TGB-b) both of which stimulate B-lymphocytes to proliferate[32]. Further, it is well established that *Guduchi*[26-28] and honey[29-31] are reputed for their immune-potentiating activity. Combining adjuvant with primary drug leads to further moderate increase in anti-body formation. The combination of these adjuvants with TM might have lead to further increase in observed immune stimulation activity. However, further studies in other models of immune reaction and evaluation in immune-suppressed state would throw more light on the probable mechanism involved. It would be useful to also study the effect on formation of different cytokines and modulation of factors involved in their expression.

## Conclusion

*Triguna Makaradhwaja* was evaluated in two models of immune reactions. The data obtained indicate significant anti-body formation stimulation with the test formulation. This inference is supported by the elevation observed in serum IgG level. The immunological paw odema which was employed as representative of CMI was not affected to significant extent. Weak stimulation observed with the adjuvants indicates their role at least partially in the anti-body formation observed with the test formulation. It is

suggested that this increase in anti-body might be due to increased formation of cytokines mediating Th2 pathway of immune reaction.

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