

## Amelioration of Cyclophosphamide Induced Immunosuppression by the hydro-Alcoholic Extract of *Gymnema Sylvestre* Leaves in Albino Rats

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Cyclophosphamide (CP), the most commonly used anti-neoplastic agent causes immunosuppression and toxic effects on various organs that are the limiting factors of cancer treatment. It can be hypothesized that addition of new immunopotentiating agents with detoxification properties would have beneficial role in cancer therapy. Many researchers have proved that, if certain plant products are combined with cancer chemotherapeutic agents, reduce toxicities and improve tumour response. In Ayurveda, *Gymnema sylvestre* is commonly used for diabetes, obesity and asthma. Also it possesses anti-inflammatory, astringent and digestive properties. Reports on the immunostimulatory activity of *Gymnema sylvestre* leaves are available from some in vitro and in vivo experiments. With this background the present study was undertaken to evaluate the potential beneficial role of hydro-alcoholic extract of *Gymnema sylvestre* leaves (GSE) on cyclophosphamide induced immunosuppression in rats. In this experiment, five groups (n=6 in each) of wistar albino rats were randomly divided to receive drugs and vehicle orally for 21 days. Gr I and II received vehicle. Gr III, IV and V were administered with Levamisole 50 mg/kg, GSE 25mg/kg and GSE 50 mg/kg respectively. Except Gr I rats, all rats were injected intraperitoneally with Cyclophosphamide (100mg/kg) on day 9<sup>th</sup> and 16<sup>th</sup> of drug treatment. The effects on various organ weights, rise in Haemagglutination titre to Sheep RBC Antigen, delayed type of hypersensitivity (DTH) response to Sheep RBC, percentage of neutrophil adhesion to nylon fibre and phagocytic index from carbon clearance test were evaluated. Humoral and cellular immunity were measured from HA titre and DTH response respectively. It has been observed that, GSE 50 mg/kg significantly increased the antibody titre, percentage neutrophil adhesion and phagocytic index in CP induced immunosuppressed rats. It also restored the CP induced changes in organ weights and the DTH response at 24 and 48 hours of antigen challenge. But these effects were not comparable to that of Levamisole. Our study shows that *Gymnema sylvestre* reduced the CP induced immunotoxicities and therefore, it could be a safe supplement to cyclophosphamide chemotherapy.

**Keywords:** *Gymnema sylvestre*, Cyclophosphamide, Immunomodulation, Albino rats.

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Several cytotoxic drugs are being used as cancer chemotherapeutics as well as for long term immunosuppressive therapy in organ transplant subjects.<sup>1</sup> Cyclophosphamide is most commonly used chemotherapeutic drug against a variety of cancers and disorders like systemic

lupus erythematosus, rheumatoid arthritis and multiple sclerosis but has toxic effects on normal cells too.<sup>2-4</sup> Apart from its therapeutic efficacy, its immunosuppressive and cytotoxic effects bring an impairment of host defence mechanism leading to significant morbidity and mortality which is

a major limiting factor in cancer treatment.<sup>5</sup> The toxic effects of cyclophosphamide (CP) are mainly due to generation of its active metabolites namely phosphoramidate mustard and acrolein, the most toxic agent<sup>6</sup>. The molecular mechanism of CP inducing immunosuppression has been explained by Huang and Li in 2013<sup>7</sup> As IL-10 and IFN- $\gamma$  play an important role in cyclophosphamide induced immunosuppression, the model of CP induced immunosuppression is employed to evaluate the potential immunomodulatory effects of test substances.<sup>8-10</sup> Hence, discovery of new immunopotentiating agents with detoxification properties would have potential beneficial effects in cancer treatment.

A large number of plants such as *Tinospora cordifolia*, *Moringa indica*, *Ocimum sanctum*, *Azadirachta indica*, *Abutilum indicum*, *Curcuma longa*, *Moringa Olifera*, *Centilla asiatica* have been shown to possess a wide array of immunomodulatory effects in rat and mice.<sup>11</sup> Many studies have reported that combining certain plant products with chemotherapy may improve quality of life, tumour response and reduce toxicities of chemotherapy, eg *Ganoderma lucidum*,<sup>5</sup> *Kigelia africana*<sup>12</sup>, Onion lectin<sup>1</sup> hemicellulose of bamboo shavings,<sup>8</sup> *Decalepis hamiltonii*<sup>13,14</sup> *Roscorea procera* rhizomes<sup>15</sup> etc.

*Gymnema sylvestre* R Br. (Family Asclipeceae) leaves, commonly known as Gudmar has been widely used in Indian Ayurveda traditional medicine to treat diabetes, obesity and asthma.<sup>16</sup> The leaves of this plant has also been used as anti-inflammatory, astringent, anodyne, digestive and liver tonic.<sup>17</sup> The effect of *Gymnema* leaf extract on fat and glucose metabolism is also reported.<sup>18</sup> In some in - vitro experimental models the immunostimulatory effect of *Gymnema* has been observed<sup>11</sup> and hence this activity in in-vivo models needs to be explored. With this background, the present study was aimed to investigate the immunomodulatory activity of hydro-alcoholic extract of *Gymnema sylvestre* leaves using experimental models of cyclophosphamide-induced immunosuppression in Wistar albino rats.

## MATERIALS AND METHODS

### Plant Material

The hydroalcoholic extract of leaves of

plant *Gymnema sylvestre* were procured from Indian Herbs Saharanpur (UP)

### Animals

Thirty wistar albino rats of either sex of 2-3 months old (weighing between 120-150gms) were procured from a registered breeder. (no.526/02/bc/CPCSEA) The animals were housed in central animal house of MKCG Medical College Berhampur (Regd No. 472/CPCSEA/) and maintained at 22 $\pm$ 1 $^{\circ}$  C with 55 $\pm$ 10% relative humidity and 12/12 hour light/ dark cycle. All rats were fed with standard pellet diet and water *ad libitum*. The animals were allowed to acclimatize to laboratory conditions one week prior to the day of experimentation. Prior to the experimentation, the experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and care of animals was taken as per CPCSEA guidelines.

### Drugs and chemicals

All the drugs and chemicals were of analytical grade. Levamisole (Khandewal pharmaceutical Ltd. Mumbai), cyclophosphamide (Biochem Pharmaceutical, Mumbai), colloidal carbon (Indian Ink, camel India pvt. Limited), Alsevers solution and Phosphate buffer saline were procured from Hi-Media Laboratories Pvt. Limited.

### Test compound preparation

The dilution of hydro-alcoholic extract of *Gymnema sylvestre* leaves was prepared in 1% gum acacia prior to oral administration. Freshly prepared solution was used for experiment.

### Acute toxicity study

Acute toxicity study was done as per OECD guidelines 423 [limit test]<sup>19</sup>. The extract at doses 5, 50, 1000 and 2000 mg/kg were given orally to different group of overnight fasted rats. The animals had free access to water only. The animals were observed for 24 hours. There was no mortality or behavioural changes observed during the study period.

### Phytochemical screening of plant material

Preliminary phytochemical screening of hydro-alcoholic extract of this plant revealed presence of alkaloids, triterpenoids, flavonoids, steroids, tannins and phenolic compounds.

### Experimental Design

The rats were randomly divided into five different groups for each experimental model. All the drugs and vehicles were given

orally for 21 days. On 9th and 16th day of study, cyclophosphamide was injected intraperitoneally (i.p) at a dose of 100mg/kg to all the rats except that of Gr-I (control)<sup>20</sup>

Gr.I- (Control) Received vehicle, 1% gum acacia  
Gr.II- (Disease control) received Cyclophosphamide -100mg/kg ip and 1% gum acacia orally  
Gr.III- (standard) Cyclophosphamide + Levamisole- 50mg/kg

Gr.IV -Cyclophosphamide+Gymnema sylvestre -25mg/kg

Gr.V - Cyclophosphamide+Gymnema sylvestre -50mg/kg

### Experimental procedure

#### Preparation of sheep RBC antigen

For this purpose, 5 ml of sheep blood was collected in sterile Alsever's solution in 1:1 preparation and centrifuged at 2000rpm for 10 minutes. Then it was washed with buffer saline (PBS) for 4-5 times and kept in refrigerator for further use. On the day of experiment, the RBC suspension was adjusted to a concentration of  $0.5 \times 10^9$  cells after RBC count in Neubers chamber. This suspension was used for immunisation and antigen challenge.<sup>21, 22</sup>

### Body and Organ weight

The initial body weight of each animal was weighed before starting the experiment and weighed at weekly interval after administration of drug or vehicle. At the end of experiment, the animals were sacrificed. The liver, spleen and kidney were removed and weighed immediately.

### Haemagglutination Ab (HA) titre

On 14th day of drug treatment, rats of all the groups were immunized with  $0.5 \times 10^9$  sheep RBC intraperitoneally. The day of immunization was referred as day 0. The drug treatment was continued for another 7 more days. On 21st day, 1 hour after the last test dose, blood samples were collected from retro orbital plexus and serum was separated.

The Ab titre was determined by challenging 25  $\mu$ L of two fold diluted sera with  $0.025 \times 10^9$  Sheep RBC suspensions in 96 well microtitre plates. PBS was used as diluents in all samples. The plates were incubated at 37°C for 1hr and then observed for haemagglutination. The highest dilution showing haemagglutination was taken as Ab titre.<sup>23</sup>

**Table 1.** Effect of different drugs and vehicle on relative organ weights of rats

Group SEM	Drug and dose	Mean relative organ weight (mg/ 100gm B.W.) $\pm$ SEM		
		Spleen	Liver	Kidney
I (Control)	1% Gum Acacia- 0.5ml/ Rat orally	0.52 $\pm$ 0.029	4.77 $\pm$ 0.293	0.87 $\pm$ 0.027
II- CP control	100mg/kg (i.p) +GA 0.5 ml orally	0.21 $\pm$ 0.029 <sup>a</sup>	2.46 $\pm$ 0.233 <sup>a</sup>	0.56 $\pm$ 0.024 <sup>a</sup>
III	CP+LEV -50 mg/kg	0.41 $\pm$ 0.0149 <sup>c</sup>	4.16 $\pm$ 0.197 <sup>c</sup>	0.76 $\pm$ 0.202 <sup>c</sup>
IV	CP+GSE-25mg/kg	0.30 $\pm$ 0.009 <sup>b</sup>	3.40 $\pm$ 0.178 <sup>b</sup>	0.65 $\pm$ 0.021
V	CP+GSE-50mg/kg	0.39 $\pm$ 0.013 <sup>c</sup>	3.81 $\pm$ 0.152 <sup>b</sup>	0.72 $\pm$ 0.021 <sup>c</sup>

n=6 in each group. One way ANOVA test reveals - a: p<0.05 vs normal control, b- p<0.05, c- p<0.001 vs CP control group

**Table 2.** Effect of different drugs and vehicle on haemagglutination titer, percentage neutrophil adhesion and phagocytic index in rats

Group	HA titer	% NA	Phagocytic Index
I(control)	138.7 $\pm$ 25.69	28.47 $\pm$ 2.06	0.068 $\pm$ 0.0633
II(CP)	24.0 $\pm$ 3.58 <sup>a</sup>	13.25 $\pm$ 1.18 <sup>a</sup>	0.026 $\pm$ 0.0031 <sup>a</sup>
III(CP + LEV)	106.7 $\pm$ 13.4 <sup>c</sup>	24.52 $\pm$ 1.27 <sup>c</sup>	0.061 $\pm$ 0.0041 <sup>c</sup>
IV(CP+ GSE 25mg/kg)	53.33 $\pm$ 6.75	16.92 $\pm$ 1.45	0.037 $\pm$ 0.0026
V(CP+GSE-50 mg/kg)	85.33 $\pm$ 13.49 <sup>b</sup>	21.83 $\pm$ 1.34 <sup>b</sup>	0.047 $\pm$ 0.0035 <sup>b</sup>

n=6 in each group. One way ANOVA test reveals - a : p<0.05 vs normal control, b- p<0.05, c- p<0.001 vs CP control group.

**Delayed Type Hypersensitivity (DTH) response**

On 7th day of treatment the paw volume of right hind foot pad of all rats were measured using plethysmometer by mercury displacement method. The animals were then immunized by injecting 20 $\mu$ l of 0.5X10<sup>9</sup> SRBC's intra-peritoneally. On 21<sup>st</sup> day of drug treatment, all rats were challenge with 0.025X10<sup>9</sup> SRBC S.C into right hind paw. After 24 and 48 hr of challenge, the right hind paw volumes were measured again. The difference between pre and post challenge paw volumes was expressed as DTH reaction.<sup>23, 24</sup>

**Neutrophil adhesion test**

On day 14, from all rats the blood samples from retro orbital puncture were collected in EDTA containing vials and analyzed for TLC, Differential count(DLC). After initial count, blood samples were incubated with 80mg/ml of nylon fiber at 37°C for 15mins. The incubated blood samples were again analyzed for TLC and DLC

The product of TLC and % Neutrophil gives Neutrophil index of the sample.<sup>25</sup>

$$\text{Neutrophil adhesion \%} = \left[ \frac{\text{Neutrophil index of untreated blood sample} - \text{Neutrophil index of treated blood sample}}{\text{Neutrophil index of untreated blood sample}} \right] \times 100$$

**Carbon clearance test**

The animals were divided into five groups and drug or vehicle treatment was given for 21 days exactly as per the above experimental design. On 21<sup>st</sup> day, 3 hours after the last dose all the animals of each group were injected intravenously (tail vein) with carbon ink suspension (1:50 dilution of Indian ink, Camel) in a dose of 0.5 ml/ 100gm body weight. Blood was withdrawn from retro-orbital venous plexus (25 $\mu$ l) at 0 and 15 minutes after injection of colloidal carbon ink and was lysed with 0.1% of sodium carbonate solution (3ml). The optical density was measured spectrophotometrically at 650 nm.<sup>24, 26</sup> The Phagocytic index (K) was calculated using the formula:

$$K = (\ln OD_1 - \ln OD_2) / t_1 - t_2$$

Where OD<sub>1</sub> and OD<sub>2</sub> are the optical densities at time t<sub>1</sub> and t<sub>2</sub> respectively.

**Statistical analysis**

The data were analyzed by One-way ANOVA with post hoc Tukey's multiple comparison 't' test using Graph pad Prism version

7.0 Values of p < 0.05 were considered to be the minimum level of significance.

**RESULTS****Effect on lymphoid organ weight**

The relative weights of liver spleen and kidney with respect to their body weights in CP control rats were significantly reduced in comparison to normal control rats. (p<0.001) With treatment of levamisole, and GSE 25 as well as 50 mg/kg the relative organ weights were significantly increased in comparison to that of CP treated rats as shown in Table-1.

**Effect on Humoral immunity**

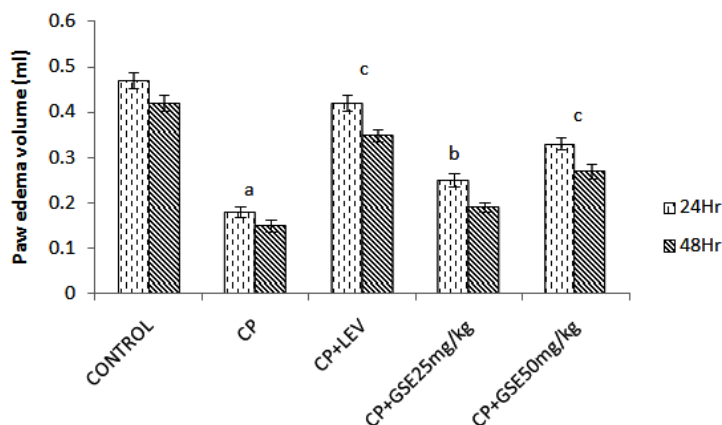
Administration of Cyclophosphamide decreased HA titre to a highly significant extent as compared to that of control rats. (p <0.001) The mean Ab titre of control rats was 138.7 $\pm$ 25.69 whereas the CP treated rats had 24.0 $\pm$ 3.58. Levamisole increased HA titre (106.7 $\pm$ 13.4) to a highly significant extent in CP induced immunosuppressed rat when compared to that of CP treated control rats. (p<0.001) But this effect is not comparable to that of normal rats. GSE with 50 mg/kg, significantly increased the HA titre to 85.33 $\pm$ 13.49 in comparison to CP control group of rats (p<0.01) revealing stimulation of humoral immune response to sheep RBC. (Table-2)

**Effect of *Gymnema sylvestre* on cell mediated immunity**

Administration of Cyclophosphamide (100mg/kg) i.p. produced a significant decrease in paw edema at 24 and 48 hours of sheep antigen challenge to a highly significant extent(p<0.01) in comparison to control animals. The mean paw edema volume of control rats at 24 and 48 hours were 0.47 $\pm$ 0.018ml and 0.42 $\pm$ 0.02 ml respectively. The Standard drug Levamisole with CP elicited a highly significant increase in paw edema volume to 0.41  $\pm$  0.022 ml and 0.35 $\pm$  0.013 ml at 24 and 48 hours respectively.(p<0.001) *Gymnema sylvestre* 25 and 50mg/kg also elicited a significant increase in Delayed hypersensitivity response in cyclophosphamide treated immunosuppressed rats suggesting activation of cellular immunity. (p<0.05). (Figure-1)

**Effect on %Neutrophil adhesion (NA)**

In this test, the percentage of neutrophil adhesion in normal control and CP control rats



n=6 in each group. One way ANOVA test reveals - a :  $p < 0.05$  vs normal control, b-  $p < 0.05$ , c-  $p < 0.001$  vs CP control group.

**Fig. 1.** Paw oedema of various treatment groups at different time intervals

were  $28.47 \pm 2.06$  and  $13.25 \pm 1.18$  respectively which revealed a highly significant decrease in neutrophil adhesion with administration of Cyclophosphamide. ( $p < 0.001$ ) Levamisole and *Gymnema sylvestris* (50mg/kg) increased % neutrophil adhesion to a significant extent in CP induced immunosuppressed rats when compared with that of CP control group but in comparison to Control rats, this effect was not significant. (Table-2) With Levamisole, GSE-25mg/kg and GSE-50 mg/kg, the percentage NA were  $24.52 \pm 1.27$ ,  $16.92 \pm 1.45$  and  $21.83 \pm 1.34$  respectively. Increase in % neutrophil adhesion shown by GSE correlates with margination of cells in blood vessels.

#### Effect on Phagocytic index

The phagocytic index with CP treatment was significantly reduced to  $0.026 \pm 0.0031$  in comparison to normal control group of rats ( $0.068 \pm 0.0633$ ). Levamisole treated rats had phagocytic index of  $0.061 \pm 0.0041$  which was a highly significant increase as compared to CP controlled rats. Administration of GSE 50 mg/kg b.w. significantly enhanced phagocytic activity showing phagocytic index of  $0.047 \pm 0.0035$  ( $p < 0.05$ ) The results therefore indicate an improvement in immunity by treatment with GSE (Table 2).

## DISCUSSION

Immune response is a complex system that involves a network of biochemical mechanisms.

Immunosuppression may occur due to diseases or certain chemotherapeutic substances which reduce resistance against infection and stress. Immunostimulation implies the stimulation of some non specific system i.e. Macrophage, complement, granulocytes and T-lymphocytes. In recent years, research in ethnopharmacology has focused on the role of various plant constituents in boosting immunological response.

Cyclophosphamide (CP) has been widely used in various cancer chemotherapeutic regimens and prevention of Graft rejection which also suppresses immunity. The immunosuppressive models have been used to evaluate the immunoregulatory properties of various drugs and medicinal plant products.<sup>7</sup> Hence in the present study, cyclophosphamide (CP) induced immunosuppression in wistar rats has been used as a suitable model for investigating the immunomodulatory potential of hydroalcoholic extract of *Gymnema sylvestris* leaves.

In the present study, Levamisole and GSE restored the changes in relative weights of lymphoid organs like spleen, liver, kidney in CP induced immunosuppressed rats. (Table 1)

In the present study Cyclophosphamide significantly suppressed the antibody titer in response to sheep RBC. Levamisole and *Gymnema sylvestris* leaf extract (GSE) at 50 mg/kg augmented the humoral immune response by increasing antibody titer in CP injected rats. (Table-2.) The

humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting cells. Thus rise in antibody titre to sheep RBC antigen shows the index of humoral immunity.<sup>27</sup> Our observations corroborate with that of P.K.M. Nagarathna *et al* (2014) who have reported the immunostimulant properties of *Kigelia africana*.<sup>12</sup>

DTH is a part of process of graft rejection, tumor immunity and mainly responsible for development of immunity to intracellular microorganisms. It is an immuno-inflammatory reaction in which TH1 and macrophages take an important role. DTH is mediated by a specific antigen which activate T-Lymphocytes and subsequently release cytokines. In the present study, Sheep RBC was used as an antigenic substance which elicits hypersensitivity reaction. In comparison to CP control group, rats treated with Levamisole, GSE (25 and 50 mg/kg) significantly enhanced DTH reaction as measured from increase in hind foot paw edema volume (Fig.1) suggesting infiltration of macrophages to inflammatory site. Macrophages are one of the important phagocytic cells and play a vital role in innate immunity response, also activate adaptive immune response against any foreign object invading host cells including tumor cells<sup>28</sup> The index of macrophage activation and pro-inflammatory response is reflected from rise in level of NO, TNF- $\alpha$  and COX-2. It has been shown in the study of V.P. Kumar *et al* that *Allium cepa* agglutinin increased the level of NO, TNF- $\alpha$  and COX-2 in CP treated immunosuppressed rats.<sup>1</sup> In many other studies also the effect of various plant extracts have been shown to increase DTH response reflecting their immunostimulant effect.<sup>29</sup>

In the neutrophil adhesion test, the adhesion of neutrophils to nylon fibres describes the margination of cells to blood vessels and that is mediated through the interaction of  $\beta_2$  integrins present on the surface of the neutrophils.<sup>30</sup> Neutrophils are also capable of wide range of responses in particular chemotaxis, phagocytosis, exocytosis also both intracellular and extracellular killing. In this test Levamisole and GSE (50mg/kg) significantly increased % of neutrophil adhesion to nylon fibers as compared to CP treated control group but this effect was not comparable to that of normal control rats. (Table-2) This effect of *Gymnema* might be due to the upregulation of  $\beta_2$

integrins through which neutrophils firmly adhere to nylon fibres. Thus it can be explained that GSE causes stimulates margination of neutrophils towards the site of inflammation. Such effect with plant extracts of *Trichopus zeylanicus* was also observed by R.S Bachhav.<sup>29</sup>

In Carbon clearance test, the phagocytic activity was enhanced by Levamisole and GSE 50mg/kg b.w. It indicates an improvement in non specific immunity involving R.E. system. Such a correlation was explained in the study of V. Sharma *et al* 2010 who have experimented with the plant *Anacyclus pyrethrum*.<sup>31</sup>

In an in vitro study, the findings of V.K. Singh *et al* 2015 explains that presence of active compounds, Gymnemic acid in methanolic extract of *Gymnema sylvestre* leaf stimulates both myeloid and lymphoid components of immune system and therefore can restore the innate immune function. Thus our in vivo experiment is also in accordance to V K Singh *et al*.<sup>11</sup>

This is important to note that enhancing immune response is one of the best strategies to reverse the host defence system in immunosuppressed individuals. In the present study, the restoration of immunity is characterized by rise in DTH response, HA titer and percentage neutrophil adhesion, phagocytic index and lymphoid organ weights in CP induced immunosuppressed rats. In this experiment, the preliminary screening for identifying the phytoconstituents of hydroalcoholic extract of *Gymnema sylvestre* leaves revealed the presence of alkaloids, triterpenoids, flavonoids, steroids, tannins and phenolic compounds. In many researches, scientifically it has been proved that, the alkaloid, terpenoid and flavonoid components of plant extracts possess immunostimulatory property.<sup>12,29,32</sup> In an in vitro test, the immunostimulatory property of *Gymnema sylvestre* plant has been explained due to presence of gymnemic acid.<sup>11</sup> In the present study, probably this immunostimulatory activity possessed by *Gymnema* could be due to the presence of the above phytoconstituents.

## CONCLUSION

The present investigation suggests that, orally administered *Gymnema sylvestre* may accelerate the recovery of cyclophosphamide-

caused immunosuppression, without evident side effects. By potentiating the humoral and cellular immunity and phagocytic activity, it may provide a mechanistic basis for using *Gymnema* as an alternative means in lessening chemotherapy induced immunosuppression in cancer patients. Isolation of the phytoconstituents and their role on immunity needs further research.

## REFERENCES

1. Kumar VP, Venkatesh YP. Alleviation of cyclophosphamide-induced immunosuppression in Wistar rats by onion lectin (*Allium cepa* agglutinin) *Ethnopharmacol.*; **186**: 280-288 (2016).
2. Goldberg M. A., Antin J. H., Guinan E.C., Rapoport J.M. Cyclophosphamide Cardiotoxicity: an analysis of dosing as a risk factor. *Blood.*; **68**:1114–1118 (1986).
3. Perini P., Calabrese M, Rinaldi L, Gallo P. The safety profile of cyclophosphamide in multiple sclerosis therapy. *Expert opinion drug safety.*; **6**: 183-190 (2007).
4. Mahsa Zarei, T Shivanandappa. Amelioration of cyclophosphamide-induced hepatotoxicity by the root extract of *Decalepis hamiltonii* in mice. *Food and chemical toxicology.*; **57**:179-184 (2013)
5. Qiang Yua, Shao-Ping Niea, Jun-Qiao Wang, Xiao-Zhen Liua, Peng-Fei Yina, Dan-Fei Huang, Wen-Juan Lia, De-Ming Gong, Ming-Yong Xie. Chemoprotective effects of *Ganoderma atrum* polysaccharide in cyclophosphamide-induced mice. *International Journal of Biological Macromolecules.*; **64**: 395– 401 (2014).
6. Sheweita, S. A., El-Hosseiny, L. S., Nashashibi, M. A. Protective effects of essential oils as natural antioxidants against hepatotoxicity induced by cyclophosphamide in mice. *PLoS One.*; **11**(11), e165667 (2016).
7. Huang Y, Li L. DNA cross linking damage and cancer 'a tale of friend and foe'. *Transi. Cancer research.* **2**: 144-154 (2013).
8. Ju-qing Huang, Mei-rong Pang, Guang-yu Li, Nan Wang, Lu Jin & Ying Zhang. Alleviation of cyclophosphamide-induced immunosuppression in mice by naturally acetylated hemicellulose from bamboo shavings. *Food and Agricultural Immunology.*; **28**(2): 328-342 (2017).
9. C. Huang, K. Song, W. Ma, J. Ding, Z. Chen, and M Zhang, "Immunomodulatory mechanism of Bushen Huoxue Recipe alleviates cyclophosphamide-induced diminished ovarian reserve in mouse model," *Journal of Ethnopharmacology.*; **208**:44–56 (2017).
10. Y. Gong, J. Wu, and S.-T. Li, "Immuno-enhancement effects of *Lycium ruthenicum* Murr. polysaccharide on cyclophosphamide-induced immunosuppression in mice," *International Journal of Clinical and Experimental Medicine.*; **8**(11): 20631–20637 (2015).
11. Vineet Kumar Singh, Padmanabh Dwivedi, B. R. Chaudhary, Ramesh Singh. Immunomodulatory Effect of *Gymnema sylvestre* (R.Br.) Leaf Extract: An In Vitro Study in Rat Model. <https://doi.org/10.1371/journal.pone.0139631> 2015. Oct.16.
12. Nagarathna PKM, Reena K, Reddy S and Wesley J. Evaluation of immunomodulatory activity of the Flavonoid of *Kigelia africana*. *Int J Pharm Sci Res.*; **5**(10): 4359-65 (2014).
13. Zarei M, Shivanandappa T. Amelioration of cyclophosphamide-induced hepatotoxicity by the root extract of *Decalepis hamiltonii* in mice. *Food chem. Toxicol.*; **57**: 179-84 (2013).
14. Zarei M, Shivanandappa T. Neuroprotective effect of *Decalepis hamiltonii* on cyclophosphamide-induced oxidative stress in the mouse brain. *Journal of basic and clinical physiology and pharmacology.*; **27**(4): 341-348 (2016).
15. Mahesh S Sahu, Prasant Y Mali, Sekhar B Walkar, Vinod D Rengari. Evaluation of immunomodulatory potential of ethanolic extract of *Roscoea procera* rhizomes in mice. *J Pharm Bio allied Sci.*; **2**(4): 346-349 (2010).
16. Leach MJ. *Gymnema sylvestre* for diabetes mellitus: a systematic review. *J Altern Complem Med.*; **13**: 977–983 (2007).
17. Kokate C K Pharmacognosy. Edn12, Nirali Prakasan, Mumbai, 210-211 (1999)
18. Al-Romaiyan A, King AJ, Persaud SJ, Jones PM. A novel extract of *Gymnema sylvestre* improves glucose tolerance in vivo and stimulates insulin secretion and synthesis in vitro. *Phytother Res.* **27**:1006–1011 (2013).
19. OECD guidelines for testing of chemicals.423, Adopted, 17<sup>th</sup> Dec, 2001
20. N. L. Dashputre, N. S. Naikwade. Immunomodulatory Activity of *Abutilon indicum* linn on Albino Mice. *International Journal of Pharma Sciences and Research.* **3**: 178-184 (2010).
21. S. Dash, L.K. Nath, S. Bhise, P. Kar, S. Bhattacharya. Stimulation of immune function activity by the alcoholic root extract of *Heracleum nepalense* D. Don. *Indian J Pharmacology.*; **38**(5): 336-40 (2006).
22. Lagrange PH, Mackaness GB, Miller TE. Potentiation of T-cell-mediated immunity by selective suppression of antibody formation with

- cyclophosphamide. *J Exp Med.*; **139**: 1529–39 (1974).
23. Fulzele SV, Satturwar PM, Joshi SB, Doric AK. Study of the immunomodulatory activity of Haridradi Ghrita in rats. *Indian J Pharmacol.*; **35**: 51–54 (2003).
24. V. Gayathri, V.V. Asha, A. Subramoniam. Preliminary studies on the immuno-modulatory and antioxidant properties of *Selaginella species*. *Indian J Pharmacol.*; **37**: 381-5 (2005).
25. B.V. Ghule, G. Muruganathan, P.D. Nakhat, P.G. Yeole. Immunostimulant effects of *Capparis zeylanica* Linn. Leaves. *J Ethnopharmacology.*; **108**: 311–15 (2006).
26. J.B. Harborne. *Phytochemical methods. A guide to modern technique of plant analysis.* 3rd ed. Springer;; 40-96, 170 (2005).
27. Ramanatha KR, Lakshminaryana R, Gopal T. Potency test of Duck Pasteurella vaccine in mice. *Mysore J Agri Sci.*, **29**: 155-157 (1995).
28. Catharina Hagerling, Amy-Jo Casbon and Zena Werb. Balancing the innate immune system in tumor development. *Trends Cell Biol.* **25**(4): 214–220 (2015).
29. R. S. Bachhav and R. Sambathkumar. Evaluation of immunomodulatory activity of the alkaloid fraction of *Trichopus zeylanicus gaertn* on experimental animals. *Indian J Pharm Sci.*; **78**(1): 161-166 (2016).
30. Jayathirtha MG, Mishra SH. Preliminary immunomodulatory activities of methanol extracts of *Eclipta Alba* and *Centella asiatica*. *Phytomedicine*; **11**: 361-365 (2004).
31. Sharma V Thakur, Chauhan NS, Dixit VK. Immunomodulatory activity of petroleum ether extract of *Anacyclus pyrethrum*. *Pharmaceutical Biol.*; **48**(11):1247-54 (2010).
32. Fariba Sharififar, Shirin P., M Arabnejad. Immunomodulatory activity of aqueous extract of *Achillea wilhelmsii* C. Koch in mice. *Ind J of Exp Biol.*; **47**: 668-671 (2009).