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Original Article

In vitro and *In vivo* Immunomodulatory Activity of Hydro Ethanolic Extract of *Grewia hirsuta* Vahl.

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ABSTRACT

Background: The Advent of the COVID-19 pandemic has necessitated the quick exploration of new drugs. Synthetic approach is time consuming and expensive with uncertainty. The natural products can be explored as this approach is cost effective and takes less time to investigate. The ethnic significance of *Grewia hirsuta* has given clues of Immunomodulator potential. Hydro ethanolic extract of *Grewia hirsuta* was explored and compared with established levamisole. **Materials and Methods:** *In vitro*, immunomodulator models on human lymphocytes and *In vivo* carbon clearance model in Swiss albino mice were studied by lymphotoxicity and lymphocyte proliferation. **Results:** Hydro ethanolic extract of *Grewia hirsuta* has shown similar results of immunomodulation comparable with levamisole in statistically significant studies assessed by Dunnett's test. **Conclusion:** Hydro ethanolic extract has shown decreased lymphocyte toxicity and increased lymphocyte proliferation.

Keywords: *Grewia hirsuta*, Immunomodulatory, MTT assay, Carbon clearance, Phagocytic index.

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INTRODUCTION

The current trends indicate humans are susceptible to disease like SARS, HIV infections, Dengue fever, Zika virus etc. Changes in the environment has a negative impact on human well being which is reflected by weak immune response. The weak immune response is greatly influenced by nutritional aspects and as well humoral and cellular immunity. Existing immune modulatory drugs are acting by stimulating cellular hormones conducive for immune stimulation. The search for novel immune stimulant drugs is on high priority as available drugs are not matching the level of immuno stimulant and modulation activity.[1]

Advances in the histology have revealed the immunity can be broadly classified as cellular immunity and humoral immunity. Cellular immunity comprises of lymphocytes, basophiles, eosinophils, macrophages, natural killer cells, mast cells and T-cells, etc. which the selectively act on infectious bacteria and cancer cells.[2,3] Lymphocytes are vital components of immune system in normal conditions and indicative of immune health. Their role in combating infections and cancer cells is well established. They also have a role in tissue rejection and autoimmune disorders. Neutrophils and monocytes are mobile cells which migrate to tissues and ingest and destroy micro-organism and dead tissues.[4] In humoral immunity, molecular warfare which is stimulated by xenobiotics or foreign particles. The proteins which are not the part of the biological system are identified and antibodies specifically to combat the foreign molecule to synthesize in a customized manner. Hence, humoral immunity is highly specific and targeted.[5] *Grewia hirsuta* Vahl. is documented as Nagabala (Ayurvedic herb) and in other ethnic systems of medicine.[6]

MATERIALS AND METHODS

Chemicals and reagents

Levamisole (Vermisole-150, Khandelwal Laboratories Pvt. Ltd., Mumbai), chemicals and reagents used for experimental work were of analytical grade.

Carbon in suspension

Pelikan-4001(German black ink).

Procurement and Authentication

The title plant was collected from natural habitat of Chittoor, Andhra Pradesh, India. The taxonomic identification of the plant was confirmed by Dr. K. Madhava Chetty, Plant Taxonomist (IAAT:357), Assistant Professor, Department of Botany, Sri

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Venkateswara University, Tirupati, Andhra Pradesh with a voucher number 072.

Preparation of Extract

The dried leaves of *Grewia hirsuta* was subjected to continuous hot extraction using a soxhlet apparatus to obtain hydro ethanolic extract. The extract obtained was concentrated, dried and stored in airtight container at cool temperature.[7]

Animals

Swiss albino mice (25-30 g) of either sex were used. The animals were fed with standard pellet diet and water *ad libitum* and maintained under standard environmental conditions (22±5°C with 12 hr of light/dark cycle).

Fixation of doses

Based on the literature survey, the acute toxicity study was carried and the lethal dose is 2000 mg/kg. Based on this we have selected 1/8th (250 mg/kg) and 1/4th (500 mg/kg) of the dose.[8]

Evaluation of *in vitro* immunomodulator activity

Lymphotoxicity test by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) method

In vitro lypmhotoxicity and lymphocyte proliferation was evaluated by measuring the reduction of MTT by colorimetric assay. MTT was converted to formazan crystals (dark purple) in the mitochondria. Formazan crystals were formed gets entrapped in the mitochondria due to its insolubility. This reaction catalyzed by the enzyme succinate dehydrogenase present in the mitochondria. The water-insoluble formazan produced was solubilized by dissolving in a solvent Dimethylsulfoxide (DMSO) and measured colorimetrically. The viable cells are only capable of metabolic conversion. Hence, the extent of formazan formation is an indicative of viable cells as described by Mahshid Ghasemi *et al.*, 2021.[9]

Preparation of test dilutions

(HEEGH) 1 g was dissolved in 5 mL 70% ethanol and it was sterilized by filtration method using 0.22 µm syringe filter. HEEGH concentrations were prepared in dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) + 10% Fetal Bovine Serum (FBS) (complete medium) at different concentrations (w/v) by serial dilution (1 fold dilution). Whereas, 70% ethanol was used as vehicle control and Phytohemagglutinin (PHA) was used as standard (positive control).

Cells and controls

Heparinized human peripheral blood (10 mL) was collected from the diagnostic laboratory (Kripa Clinical Laboratory, Bengaluru) for this study. Lymphocytes were separated by Ficoll-Hypaque density gradient method, and cells were counted by haemocytometer method. Tissue culture complete medium (i.e. 90% DMEM/F12+10% FBS) was used as a control.

Cell culture

Lymphocytes are plated in triplicates to minimize the variability of the results. 100 µL of cell suspension (2000 cells/well) was used. Approximately 80,000 live cells with 97% viability were seeded into 96-well culture plate (100 µL each well), leaving the first-row empty (i.e., 35-wells total). The plate was observed under microscope and incubated for 24 hr.

Exposure of Lymphocytes to test compound

The HEEGH was added to the 96-well plate at 2 mg/100 µL, 1 mg/100 µL, 0.5 mg/100 µL, 0.25 mg/100 µL, 0.12 mg/100 µL, 0.06 mg/100 µL, 0.00 mg/100 µL (w/v) concentrations. Complete media (RPMI+10% FBS) were used as media control, 70% ethanol, 10 µL/100 µL (v/v) was used as vehicle control, whereas, 100 µg/100 µL, 80 µg/100 µL, 60 µg/100 µL, 40 µg/100 µL, 20 µg/100 µL, 10 µg/100 µL, 0.00 µg/100 µL Phytohemagglutinin (PHA) was used as standard (positive control) (Figure 1).

Incubation period

Lymphocytes were exposed to HEEGH by incubating for 72 hr to determine the proliferation of cells against each test compound in an incubator with 5% CO_2 at 37°C and 90% Rh.

MTT-incubation

After 72 hr of HEEGH exposure, added (10 µL MTT+90 µL plain/incomplete medium) MTT solution (5 mg/mL). The plates were placed in the plate-shaker and allowed for 5 min at 900 shakes/min. Later, the plates were incubated for 4 hr at 37°C in a CO_2 incubator.

Dissolution of formazan crystals

Formazan crystals were dissolved in 100 µL of DMSO, and each well resuspended crystal was dissolved (Figure 2).The solvent was mixed well using a multichannel pipette.[10-13]

Lymphocyte Proliferation Assay

Lymphocyte proliferation is an important property of an all immune stimulant drugs. Lymphocyte proliferation occurs by a complex protein synthesis cascade. Immunostimulant drug is going to stimulate in the cascade mechanism and thus manifesting immunostimulant activity. In this method, high throughput screen ELISA plate reader is applied through MTT formazan assay method it was used in immunotoxicity assay. Our results indicate dose dependent immunostimulant activity of lymphocyte proliferation see Table 1. This assay is compared with the standard immunostimulant proliferation with levamisole.[14-17]

Calculation

The percentage of cell viability is calculated using the following equation:

$$\text{Percentage Viability} = \frac{\text{Mean OD sample}}{\text{Mean OD blank}} \times 100$$

In vivo Carbon Clearance Assay

Phagocytosis is a property of white blood cells which engulf bacteria, virus and other foreign substances and offer prevention of harm by the organisms and substances. So, the capacity to engulf foreign organisms and substances is assessed by phagocytic index is the parameter which measures the average number of bacteria ingested per phagocyte in an incubated mixture of bacteria, phagocytes, and blood serum.

Swiss albino mice (25-30 g) were divided into four groups, consisting of six animals each.

Group I: Control-distilled water (10 mL/kg, p.o).

Group II: Standard-levamisole (50 mg/ kg, p.o).

Group III: Lower dose-HEEGH (250 mg/kg, p.o).

Group IV: Higher dose-HEEGH (500 mg/kg, p.o).

The treatment was given for 5 days and, after 48 hr of the last treatment, mice were injected via the tail vein, carbon ink suspension (0.1 mL). Blood samples were drawn (in EDTA solution 5 µl) from the retro-orbital sinus at 0 and 15 min, a 25 µL sample was mixed with 2 mL of 0.1% sodium carbonate solution and its absorbance was determined at 660 nm.

The phagocytic index was calculated using the following equation:

$$\text{Phagocytic index} = {}^{k}(\text{sample})/\,{}^{k}(\text{control})$$

Where,

$$K=(\text{Log}_e OD_1 - \text{Log}_e OD_2)/15$$

OD_1=Optical Densities at 0 min.

OD_2=Optical Densities at 15 min.

Leukocytes count was also carried for all the groups. Lymph node and spleen were isolated and histopathology studies were carried out in order to study qualitative lymphocyte proliferation property.[18-21]

Statistical Analysis

Experimental results were presented as mean±SEM (n=6) and the statistical significance between the groups was analyzed by means of one way Analysis of Variance (ANOVA) followed by Dunnet's multiple comparison test Where,*p<0.05, **p<0.01 ***p<0.001 as compared to control by using In Stat GraphPad Prism Version 6.00 (GraphPad Prism Software).

RESULTS

In vitro Lymphotoxicity Test by MTT Method

The optical density at 570 nm were presented in Table 1, from this value percentage of cell viability and cell death were calculated which is given in the Table 1. The results indicate dose dependent cell viability variations at zero concentration of HEEGH showed 96.74% and at 2mg/100 µL showed cell viability of 323.30% (see Table 1 and Figures 3, 4 and 5).

In vitro Lymphocyte Proliferation Test by MTT Method

Lymphocyte proliferation is an important index of immunostimulant activity. In our experiment, the lymphocyte proliferation showed a dose dependent lymphocyte proliferation see Table 1 and Figure 6. The lymphocyte proliferation activity of HEEGH was observed highest proliferation compared to media control 2000 cells/well, and levamisole 2725 cells/well and HEEGH 4962 cells/well shown in Figure 6. Hence, HEEGH at dose 2 mg/100 µL has statistically significant compared to levamisole and media control observed in a dose dependent manner which is shown Figure 6. Lymphocyte proliferation is an index of immunostimulant activity is remarkable a dose level 2mg/100 uL (Figures 5 and 7).

In vivo Immunomodulatory Activity Using Carbon Clearance Assay

The results indicate the phagocytic index of the test dose at 500 mg is comparable with standard levamisole (50 mg/kg) and showing dose dependent action (Table 2). An increase in the leukocyte number is an immunostimulant action of levamisole. In our experiment levamisole has boosted leuckocyte count

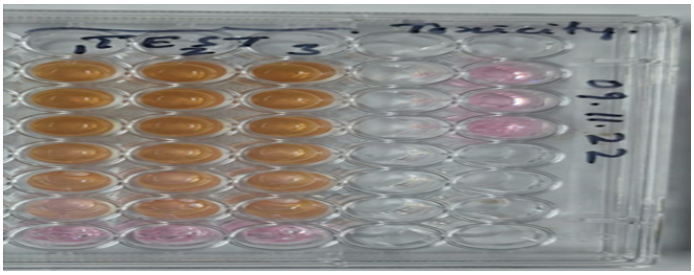

Figure 1: Exposure of Lymphocytes to HEEGH for 72 hr.

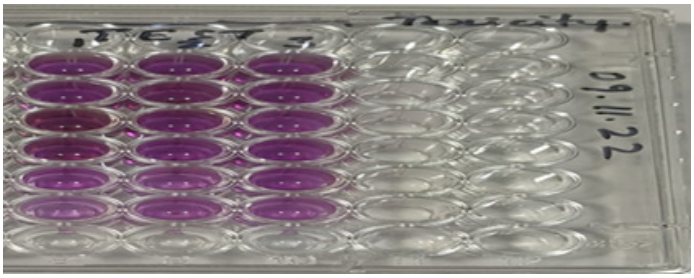

Figure 2: Plate shown after dissolving the formazan crystals with DMSO.

Table 1: Effect of HEEGH on cell viability and cell death by MTT method

Drug conc. in mg/100 µL	Average O.D at 570nm	Cell viability in %	Cell Death in %
Media control	0.369	100	00
Vehicle control	0.362	98.10	1.90
PHA	0.438	118.69	00 (-18.69)
HEEGH Conc. in W/V			
2	1.193	323.30	00 (-223.30)
1	1.153	312.46	00 (-212.46)
0.5	1.103	298.91	00 (-198.91)
0.25	0.902	244.44	00 (-144.44)
0.12	0.595	161.24	00 (-61.24)
0.06	0.460	124.66	00 (-24.66)
0	0.357	96.74	00 (3.26)

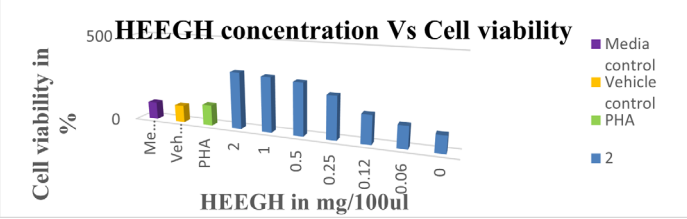

Figure 3: Effect of HEEGH on cell viability in lymphotoxicity test.

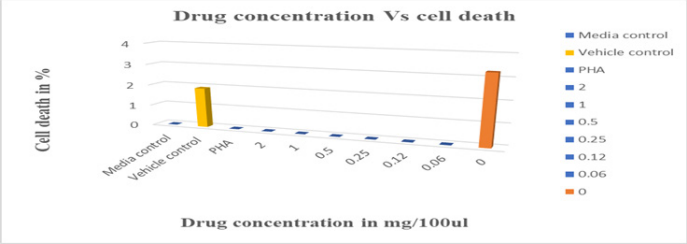

Figure 4: Effect of HEEGH on cell death in lymphotoxicity test.

11.25±1.23, whereas control is 7.76±1.05. The HEEGH has also shown increase in leuckocyte count on dose dependent manner at lower dose 8.68±1.49 and at higher dose 10.03±1.20 which is compare with levamisole activity. The results are presented in (Table 3) and (Figures 8 and 9).

Histopathology of Spleen of mice in Carbon clearance assay

Histopathology slides studied from the spleen showed white pulp and red pulp. Spleen anatomically divided into comprising of lymphoid arterioles. Red pulp consists of macrophages and reticular meshwork. The main function of the spleen is to pool and destroy the old and defective RBCs from the circulation. Whereas white pulp, is the source of macrophages which are processed in the white pulp. In our study, we observed mild to moderate action of levamisole and HEEGH which are administered as

Table 2: Effect of HEEGH on phagocytic index in carbon clearance assay.

Group	Dose (mg/kg)	Phagocytic Index
Control (Vehicle)	10 mL/kg p.	1.011±0.010
Standard (Levamisole)	50 mg/kg p.o	1.516±0.039***
Lower dose (HEEGH)	250 mg/kg p.o	1.194±0.061*
Higher dose (HEEGH)	500 mg/kg p.o	1.462±0.048***

The values are expressed as mean±SEM, (n=6), sex: male Where,*p<0.05, **p<0.01 ***p<0.001 as compared to control. **mL/g:** Milliliter per gram, **mg/kg:** Milligram per kilogram, **i.p.:** Intra peritoneal, **p.o.:** Per oral. One-way ANOVA followed by Dunnet's multiple comparison tests.

lower dose and higher dose. In the red pulp considerable increase in macrophages engulfing and destroying RBCs was clearly observed in at higher dose in levamisole treated and lower dose of HEEGH there is a marginal increase in the red pulp (Figures 10).

Histopathology of Lymph node of mice in Carbon clearance assay

Section studied from the lymph node showed intact architecture consisting of an intact capsule with cortex and medulla. The cortex consists of diffuse lymphoid cells and the medulla shows few congested blood vessels in control in albino mice. Section studied from the lymph node showed intact architecture consisting of an intact capsule with cortex and medulla. The cortex consists of diffuse lymphoid cells and the medulla shows mild congested blood vessels in standard (Levamisole 50 mg/kg) group in albino mice. Five days of pretreatment with HEEGH (250 mg/kg) showed the section studied from the lymph node showed intact architecture consisting of an intact capsule with cortex and medulla. The cortex consists of mild depletion of lymphoid cells and the medulla shows with mild congested blood

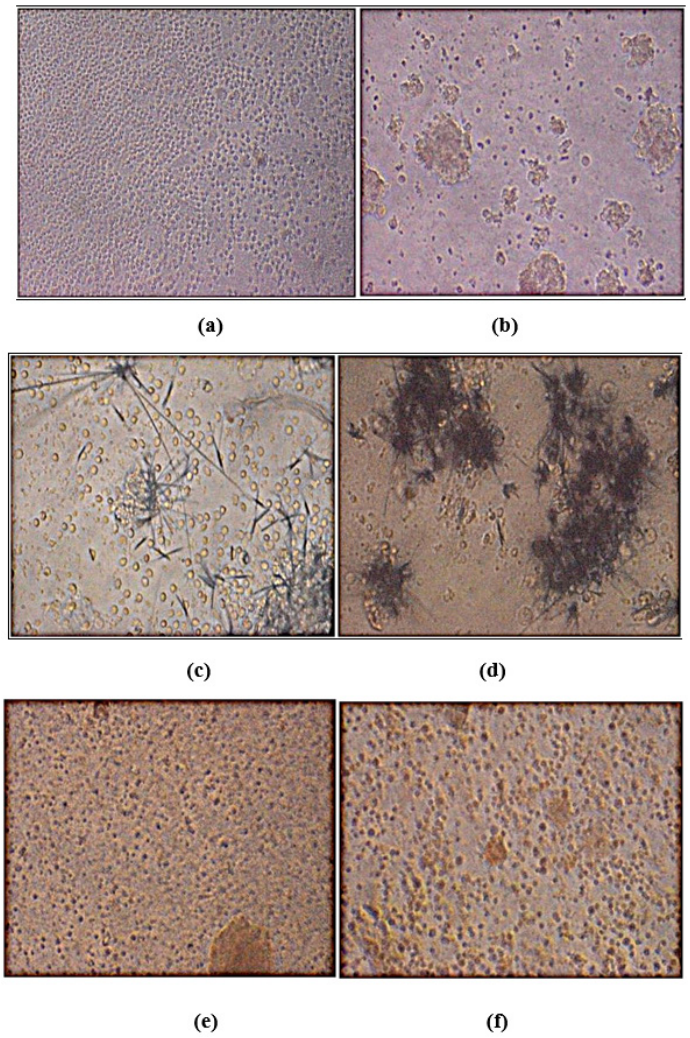

Figure 5: (a) Media control after 72hr drug expose. (b) PHA after 72hr drug expose. (c) Media control after crystal Formazen. (d) PHA after crystal formazen. (e) HEEGH 0.06 mg after 72 hr expose (Lymphocytes toxicity). (f) HEEGH 1 mg after 72 hr expose (Lymphocytes toxicity).

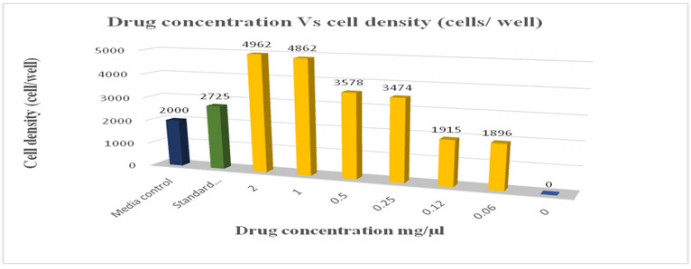

Figure 6: Effect of HEEGH on cell density in lymphocyte proliferation.

vessels on albino mice. Similarly, higher dose (500 mg/kg) had shown the section studied from the lymph node shows intact architecture consisting of intact capsule with cortex and medulla. The cortex consists of moderate depletion of lymphoid cells and the medulla shows mild congested blood vessels in albino mice (Figures 11).

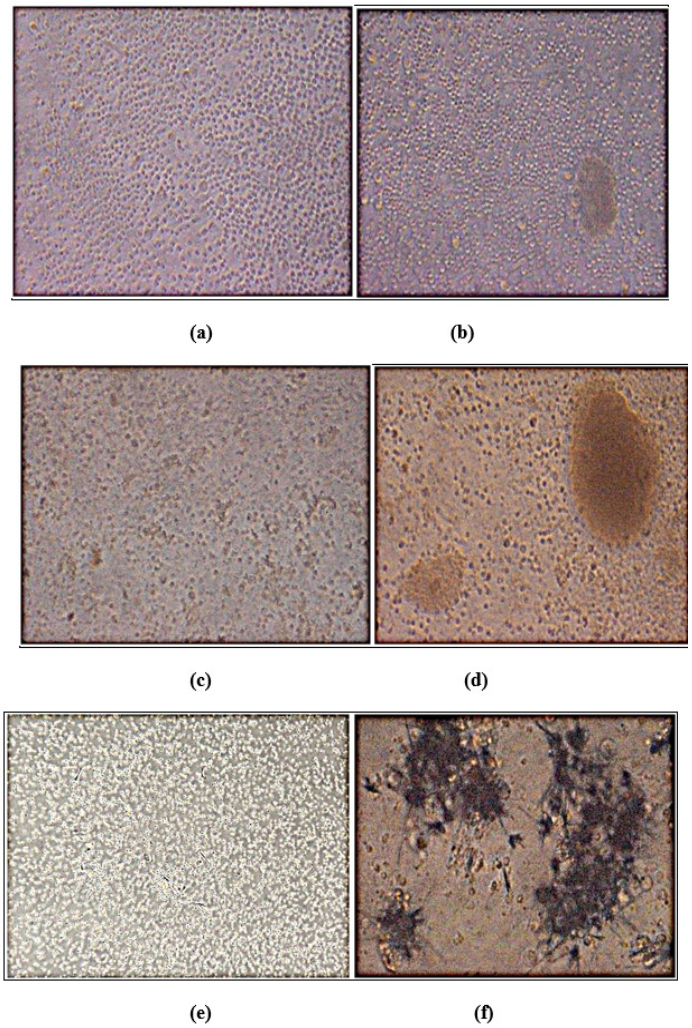

Figure 7: (a) STD (Levamisole, 10mg) after 72 hr expose. (b) STD (Levamisole, 100 mg) after 72 hr expose. (c) HEEGH 0.06 mg after 72 hr expose (Lymphocyte Proliferation). (d) HEEGH 2 mg after 72 hr expose (Lymphocyte Proliferation). (e) Lymphocytes under 10x magnification. (f) MTT metabolization by proliferating lymphocytes 40x magnification.

Table 3: Effect of HEEGH on number of leukocytes in carbon clearance assay.

Group	Dose (mg/kg)	Leukocytes (10^3/uL)
Control (Vehicle)	10 mL/kg p.o	8.093±0.25
Standard (Levamisole)	50 mg/kg p.o	11.25±0.30***
Lower dose (HEEGH)	250 mg/kg p.o	8.520±0.28*
Higher dose (HEEGH)	500 mg/kg p.o	9.846±0.2918***

The values are expressed as mean±SEM, (n=6). **mL/g:** Milliliter per gram, **mg/kg:** Milligram per kilogram, **i.p.:** Intra peritoneal, **p.o.:** Per oral. One-way ANOVA followed by Dunnet's multiple comparison tests.

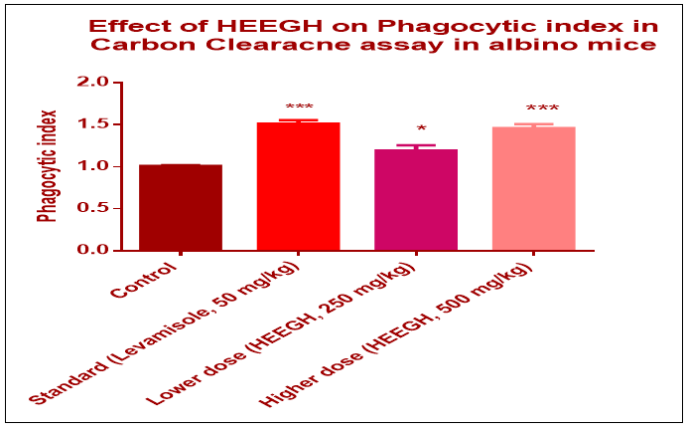

Figure 8: Effect of HEEGH on Phagocytic index in carbon clearance assay.

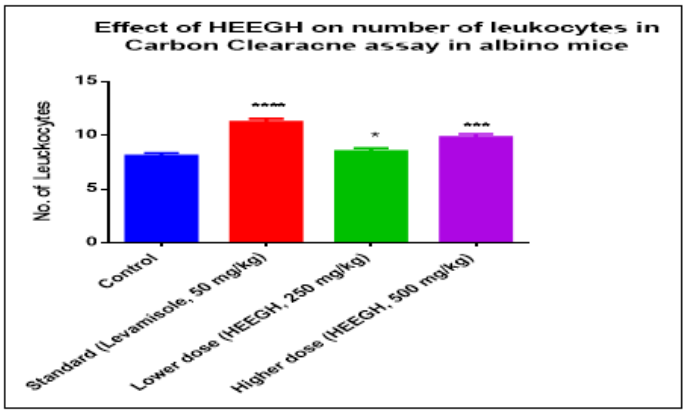

Figure 9: Effect of HEEGH on number of leuckocytes in carbon clearance assay.

DISCUSSION

Immunomodulator and immunostimulant activities are established for *Centella asciatica, Curcuma longa* L. *Panax ginseng, Withania somminfera* etc. Immunostimulant means in case of weak immune response immunostimulant drug is going to improve the immunity by cellular and humoral actions. Whereas, in the immunomodulation fine tuning of immune response is observed in case immune response is either dominant or weak. An immunomodulation happens by leveraging the cellular and humoral immunity. For example, *Withania somnifera* is a good example of immunostimulant and immunomodulatory.[22,23]

A battery of tests was conducted on human lymphocytes, *in vitro* and *in vivo* in albino mice. *In vitro* studies lymphocyte toxicity and proliferation were measured. The levamisole 50 mg/kg has shown reduced lymphocyte toxicity and enhanced lymphocyte proliferation compared to the control group. A similar observation of reduced lymphocyte toxicity and enhanced lymphocyte proliferation is observed comparable with levamisole effect. This indicates the HEEGH is having at least lympho toxicity and lympho proliferation similar to levamisole. It may be expected to be an immunomodulatory candidate like levamisole.

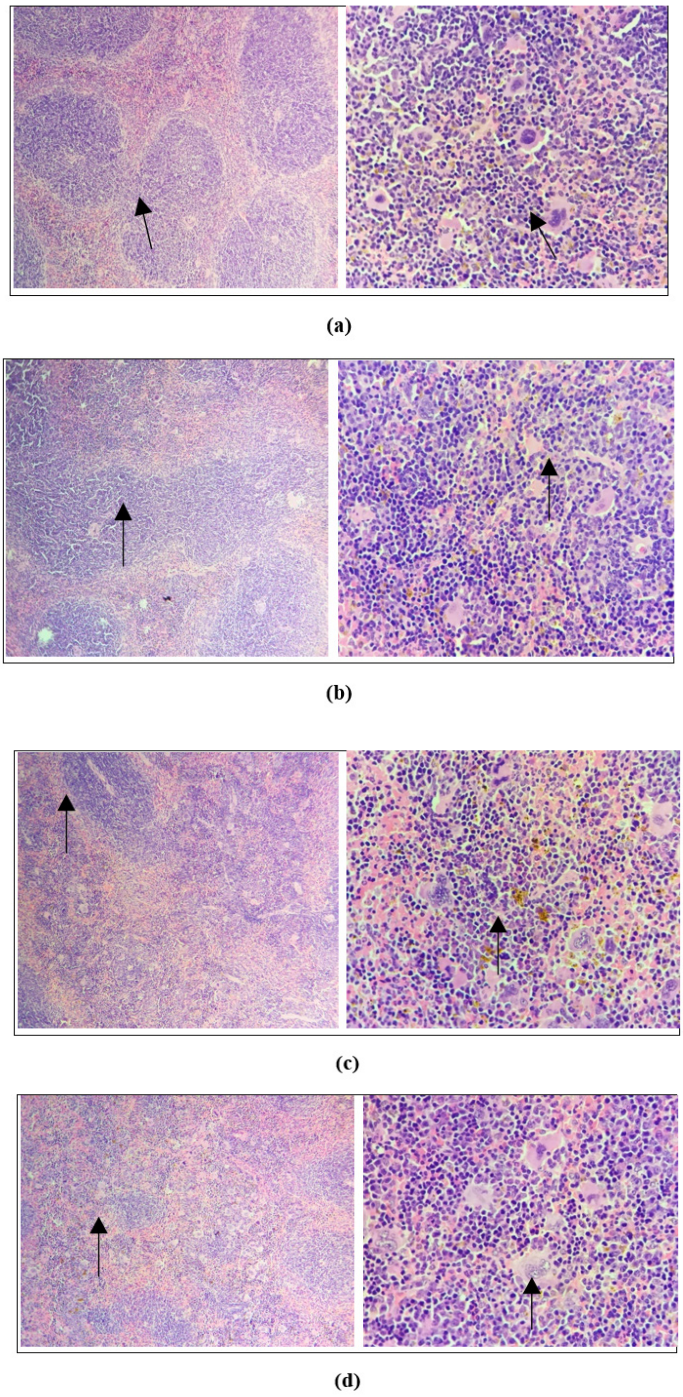

Figure 10: (a) Photomicrograph section of the spleen of Albino Swiss mice of control at 100 x and 400 x magnification Hematoxylene and Eosin stain [H&E]. (b) Photomicrograph section of the spleen of Albino Swiss mice of standard. (c) Photomicrograph section of the spleen of Albino Swiss mice of lower dose. (d) Photomicrograph section of the spleen of Albino Swiss mice of higher dose.

HEEGH is also showing increased phagocytic index and increased the total leukocyte count similar to levamisole effect. Histopathological studies on spleen and abdominal lymph nodes indicate enhanced viable cells similar to levamisole and supportive of the enhanced imunomodularoy effect of levamisole and HEEGH at different doses.

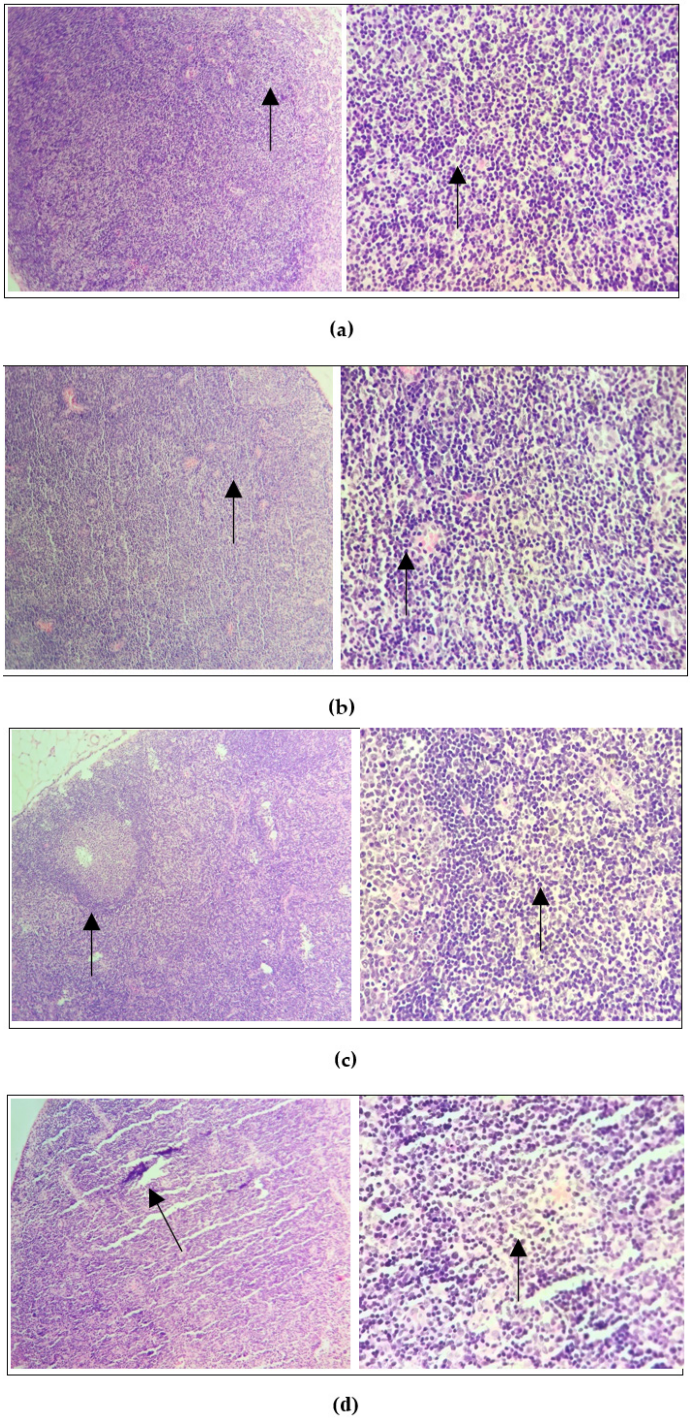

Figure 11: (a) Photomicrograph section of the lymph node of Albino Swiss mice of control at 100 x and 400 x magnification Hematoxylene and Eosin stain [H&E]. (b) Photomicrograph section of the lymph node of Albino Swiss mice of standard. (c) Photomicrograph section of the lymph node Albino Swiss mice of lower dose. (d) Photomicrograph section of the spleen of Albino Swiss mice of higher dose.

CONCLUSION

In our studies, the HEEGH has shown *in vitro* and *in vivo* experiments of decreased lymphocyte toxicity and increased lymphocyte proliferation. Further studies are required to establish active constituents responsible for immunomodulatory effect.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

COVID-19: Corona virus disease of 2019; **DMEM:** Dulbecco's Modified Eagle's Medium; **DMSO:** Dimethyl sulfoxide; **ELISA:** Enzyme-linked immunosorbent assay; **FBS:** Fetal bovine serum; **HEEGH:** Hydro ethanolic extract of *Grewia hirsuta*; **HIV:** Human immunodeficiency virus, **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **PHA:** Phytohemagglutinin; **RBC:** Red blood cells, **SARS:** Severe Acute Respiratory Syndrome.

ETHICAL APPROVAL

All experimental protocols were approved by Institutional Animal Ethical Committee Clearance (112/PO/Re/S/99/CPCSEA and dated on 21/02/2019), S.E.T's College of Pharmacy, S.R. Nagar, Dharwad-580002.

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