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Evaluation of immunomodulatory activity of hydroalcoholic extract of *Quisqualis indica* Linn. flower in wistar rats

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Abstract

The aim of the present context to evaluated to the immunomodulatory activity of hydroalcoholic extract of *Quisqualis Indica Linn* in wistar rats .it's also know as Rangoon Creeper or Madhumalati, Laal-chameli .The carbon clearances test cyclophosphamide induced myelosuppression, Total Leukocyte Count (TLC), and Differential Leukocyte Count (DLC), Delayed Type Hypersensitivity (DTH). The *Quisqualis Indica* flower extract was administered orally at a dose of 100 mg/kg and 150 mg/kg. Levamisol (50 mg/kg *p.o*) was used as standard drug. All treatment group is compared control group .Cyclophosphamide (50 mg/kg).higher dose of *Quisqualis Indica* flower extract is significantly exhibit immunomodulatory activity.

Key-Words: Immunomodulation, *Quisqualis Indica, Hydroalcoholic* DTH, Myelosuppression, immunity

Introduction

The term "immunomodulation" denotes a change, a strengthening of suppression, of the indicators of cellular and humoral immunity and nonspecific defense factors.[1] There is no effective drug for treatment of certain infections like AIDS, hepatitis, and other viral infections. For other infections the drug (mainly antibiotics) being used are becoming ineffective due to development of microbial resistance, necessitating the search for newer drugs. Modulation of immune responses to alle-viate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles[2]. Herbs that are rich in flavonoids, vitamin C, or the carotenoids may enhance immune function. Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health concept of strengthening host defences against different diseases. These plants, labelled as 'rasayana', have been endowed with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the psycho- neuro-immune axis[3].

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Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health promotive, disease preventive and rejuvenation approach available in the Indian systems of medicine like 'Ayurveda' is gaining greater attention and popularity in many regions of the world. A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the present day life, or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures (1-10) Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune

defense and lower the risk of cancer and degenerative diseases[4]. A number of plants used in Indian traditional system of medicines for upgrading therapy and chronic diseases have been shown to stimulate immune responses and several active substances have also been isolated. In recent years, immunostimulatory activity has been reported in a number of Ayurvedic plants like *Withania somnifera, Argyreia speciosa, Tridax procumbens, Ficus benghalensis, Tinidia macrosperma and Tinospora cordifolia*[5].

Quisqualis indica Linn. (Combreteceae) is a strong climber, ligneous vine that can reach from 2.5 meters to up to 8 meters. It is commonly known as Rangoon creeper. It is indigenous in Africa, Indo Malaysian region and cultivated all over India. Flower numerous, pendent, 7.5cm long, 3.8cm wide. At first they are white in color then they become deep red[6]. In Philippines the fruit is used as a vermifuge. The plant is also used as a cough cure. In Amboina the leaves are given in a compound decoction for flatulent distension of abdomen. In India the leaves are given in a compound decoction for flatulent distension of abdomen, seeds are given with honey as an electuary for the expulsion of entozoa in children .In China seed are used as vermifuge, In Indo-china region seeds are used as anthelmintic and for rickets of children .In Thailand seeds used as anthelmintic;. In Bangladesh seeds are used for diarrhea, fever, boils, ulcers and helminthiasis[7]. Leaves contains rutin, trigonelline, L-proline, laspargine and quisqualic acid whereas flower gum contains pelargonidin-3-glucoside. Seed Oil contains linoleic, oleic, palmitic, stearic and arachidic acids. ellagitannins, quisqualin A and quisqualin B is present in fruits of this plant (Ta Chen et al.,2004) and flower contains linalool oxides (furanoid and pyranoid), 2,2,6-trimethyl- 6-vinyl-3-oxo tetrahydropyran, (E,E)-alphafarnesene, (Z)-3-hexenyl benzoate and benzyl benzoate[8]. Four Diphhenyl propanoids were isolated from stem bark of *Quisqualis indica*.[9] There was no report on the extensive immunomodulatory study of the flower of this plant species.

Material and Methods

Selection and Collection of plant on the basis of ethno botanical survey, traditional use or literature survey. The mature flower of *Quisqualis indica* Linn were collected in the morning locally from Bhopal District, M.P, India, in the month of November 2009 The flower of *Quisqualis Indica* was collected from Bhopal, Madhya Pradesh, India. The powdered drug packed in a paper bags & stored in air tight container until use. Identification and Authentication of herb by Dr. Zia ul Hassan, Professor of Botany, Saifia College of Science, Bhopal, Madhya Pradesh, India (Voucher. No 138/Bot/Saifia/2010)

Extraction of plant material

About 180 gm of dry powder was taken in a closed bottle and it was deffatted with Petroleum ether. The deffating was continued for 9-10 days with occasional shaking. The Petroleum ether extract was filtered. The marc left after Petroleum ether deffating was taken out and dried under shade to get a dry mass, then extracted with Methanol and water (hydroalcoholic) by using cold maceration extraction. The extraction was continued for 9-10 days with occasional shaking. The hydroalcoholic extract was filtered, concentrated under reduced pressure to a semisolid mass and was made free from solvent. The final obtained extract was weighed; percentage yield was calculated and stored in a cool place

Preparation of dosage forms

For in vivo studies, the concentrated Hydroalcoholic extract of *Quisqualis indica (HEQI)* was administered orally after suspending in Distilled water The freshly prepared solution of *Quisqualis indica* extract was used in each experiment.100mg/kg and 150 mg/kg per ml test doses were selected on the basis oral acute toxicity study in rat The dose limits were selected on the basis of previously performed oral acute toxicity studies in rat, in accordance with the OECD guidelines.

Phytochemical analysis

Preliminary Phytochemical studies of the hydroalcoholic *extract of Quisqualis indica* was performed for major classes of constituents like alkaloids, carbohydrates, protein and amino acid ,Saponins, glycosides, steroids, tannins, flavonoid and phenolic compounds ac-cording to published standard methods[10] .

Experimental animals and feeds

Albino wistar rats (100-150gm) of either sex were used for this study and these animal were obtained from animal house [CPCSEA Reg. No.1283/c/09/CPCSEA], Pinnacle Biomedical Research Institute, Bhopal (M.P.). Throughout the experiment, the animals were housed, four animal per cage, maintained at ambient temperature of (25º±2); 30-60% humidity, under 12 hr. light-dark cycle. They were fed with standard pellet diet and water *ad libitum*. The animals were habituated to laboratory conditions for 48 hr. prior to the experimental protocol to minimize any non specific stress.

Acute toxicity study and dose selection

The hydroalcoholic extract of Quisqualis indica (HEQI) was investigated for its acute toxicity studies according to the OECD guidelines (420). The extract was given at different doses to the group of six animals at 200

mg/kg, 500 mg/kg, 1000 mg/kg, 1500 mg/kg and 2000 mg/kg, orally. Animals were observed for regular three hours after the dose administration and after 24 hours and 48 hours for the changes in behavior, changes in body weight and mortality. It was found that the extract has produced significant toxicity up to the dose of 1500 mg/kg. Thus the extract was highly tolerable up to 1500mg/kg.

Antigen

Fresh blood was collected from sheep's sacrificed in the local slaughter house in a sterile bottle containing Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride). Sheep red blood cells (SRBC) were washed three times in normal saline to adjust to a concentration of 0.1ml of $0.5x10^9$ cells and $0.025x\ 10^9$ cells for immunization and challenge respectively. Colloidal carbon (Indian ink, camel India Pvt. Ltd.).

Carbon Clearance Assay (Phagocytic activity)

To evaluate the phagocytic activity of the reciculo-endothelial system in-vivo, the Animals were divided in 4 groups each having 6 animals. Group 1-Vehicle (Distilled Water), Group-2, (HEQI) 100 mg/ kg, Treated, Group 3, (HEQI) 150mg/ kg, p.o Treated, Group 4 Standard (levamisol) 50mg/kg, Oral Treated a carbon clearance test was performed after completion of the drug pretreatment. On day 14, the treated rats received an intravenous injection (tail vain) of carbon suspension (1:50 dilution of Indian ink, Camel) in a dose of 0.5 mL/100 g body weight. Blood was withdrawn from the retro-orbital venous plexus before injection, immediately after injection and at 5 min and 15 min after injection of the carbon suspension. 0.05 mL of blood was lysed with 4 mL of 0.1% Na_2CO_3 and the optical density was measured spectrophotometrically at 650 nm wavelength. The phagocytic index K was calculated using the following equation[11] log (OD_0) - log (OD_0)/t Where OD_0 is the OD at 0 min and OD_t is the OD at t min

Delayed Type Hypersensitivity Responses (Cell Mediated Immunity)

Rats were divided into four groups of six each similarly to carbon clearances assay were immunized on day 0 by i.p. administration of $0.5x10^9$ SRBC/rat and challenged by a subcutaneous (subplantar region) administration of $0.025x\ 10^9$ SRBC/mL into right hind foot pad and on day +14. All treatment with respectively administered orally from day -14 until day +13. The thickness of the right hind footpad was measured (before challenge) using vernier caliper on the 14 th day Foot pad reaction was assessed after 24 hr. (on 15th day), in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to oedema, The footpad reaction was expressed as the difference in the thickness (m m.) between[12].

Cyclophosphamide-induced myelosuppression

Animals were divided into four groups of six animals each. Group I (Normal control group) and Group II (Cyclophosphamide-treated group) received the vehicle (distill water) for period of 13 days. Group III, IV were given dose of HEQI 100mg/kg and 150 mg/kg respectively, p.o., daily for 13 days. The animals of groups II-, IV were injected with Cyclophosphamide (30 mg/kg, i.p.) on the 11 th , 12 th , and 13 th day, 1 h after the administration of the respective drug treatments. Blood samples were collected from retro orbital plexus on the 14 th day of the experiment. Determination of total and differential white blood cells was carried out**.**

Total White Cell (Leukocyte) Count[13]

A sample of whole blood is mixed with a weak acid solution that lyses nonnucleated red blood cells. Following adequate mixing, the specimen is introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume are counted.White-count diluting fluid. Either of the following diluting fluids may be used: 2% acetic acid. Add 2 ml glacial acetic acid to a 100 ml volumetric flask. Dilute to the mark with distilled water.(1) Draw well-mixed capillary or venous blood exactly to the 0.5 mark in a white blood cell diluting pipet. This blood column must be free of air bubbles. (2) Wipe the excess blood from the outside of the pipet to avoid transfer of cells to the diluting fluid. Take care not to touch the tip of the pipet with the gauze. (3) Immediately draw diluting fluid to the "11" mark while rotating the pipet between the thumb and forefinger to mix the specimen and diluent. Hold the pipet upright to prevent air bubbles in the bulb. (4) Mix the contents of the pipet for 3-5 minutes to ensure even distribution of cells. Expel unmixed and relatively cell-free fluid from the capillary portion of the pipet (usually 4 drops). (5) Place the forefinger over the top (short end) of the pipet, hold the pipet at a 450 angle, and touch the pipet tip to the junction of the cover glass and the counting chamber.(6) Allow the mixture to flow under the cover glass until the chamber is completely charged. Similarly, fill the opposite chamber of the hemacytometer. Count the white cells in the four 1 sq mm corner areas and Calculated wbc count by following formula. The formula is as follows: WBCs per cu mm =avg no of chambers(2)WBCs counted ×diliution(20)/volume(0.4)

Differential Leukocyte Counts

A drop of blood drop was added on the centre line of the glass slide about 1 cm from one end and blood smear was prepared. Then smear was stained with

diluted Leishman's stain for 30 min and washed with distilled water and dried at room temperature. For counting of DLC the slide was examined under microscope at 100x using Cedar wood oil. Finally total number of Neutrophils, Lymphocytes, eosinophils and Monocytes in the 100 cells were counted and results were expressed in percentage[14].

Statistical analysis: The mean ± SEM values were calculated for each group. Significant difference between groups was determined using analysis of variance (ANOVA) followed by Dunnett's t test. P<0.05 was considered as significant

Results and Conclusion

Phytochemical investigation extract and toxicity

After extraction, the Physical analysis of hydroalcoholic extract of *Quisqualis Indica* (HEQI) was observed like Color-Dark blackish brown, Odor-Aromatic, Consistency-Slightly Sticky, State-Semisolid, Percentage yield-22.47% w/v and hydroalcoholic extract of *Quisqualis Indica* freely soluble in distilled water. Preliminary Phytochemical studies of the hydroalcoholic *extract of Quisqualis indica* was performed for major classes of constituents like alkaloids, carbohydrates Saponins, tannins, flavonoids and phenolic compounds are found positively in hydroalcoholic *extract of Quisqualis indica* whereas glycosides, steroids, protein and amino acid is negative The $1/10^{th}$ of maximum tolerable dose 1500 mg/kg was selected for the present studies. The dose selected was 100 mg/kg and 150 mg/kg.

Carbon clearance assay

Administration of HEQI (100 and 150 mg/kg p.o) increased the clearance of carbon particles from blood as indicated by a significant increase in phagocytic index (p<0.05) when compared with control group. The levamisol (50 mg/kg p.o) also show any significant effect on the phagocytic index in the carbon clearance assay (Table 1).

Delayed type hypersensitivity responses

Administration of HEQI (100 and 150 mg/kg p.o) increased the Delayed Type Hypersensitivity Responses significantly (p<0.05) in terms of increase in the mean difference of paw thickness when compared with control group. The levamisol (50 mg/kg p.o) also show any significant effect on the in Delayed Type Hypersensitivity Responses. (Table 1).

Cyclophosphamide-induced myelosuppression and Total Leukocyte Counts

A significant (P < 0.05) reduction in total white blood cell count was observed in rats treated with cyclophosphamide alone (group II) as compared to control group (group I). HEQI 100 and 150 mg/kg,p.o with cyclophosphamide increased the levels of total WBC count as compared to cyclophosphamide treated group. The rise in the total WBC count lowered by cyclophosphamide was observed at 100 mg/kg and 150 mg/kg of HEQI. The total WBC count was restored back to normal. (Table-2)

Differential leukocyte counts

There was a significant (P < 0.05) decrease in Neutrophils (N), lymphocytes (L) and eosinophil s(E) in animals and insignificantly decrease in monocytes(M) treated with cyclophosphamide (group II) as compared to control group (group I). HEQI with cyclophosphamide at 150 mg/kg dose significantly (P < 0.05) increased the Neutrophils (N), lymphocytes (L) and eosinophils(E) as compared to group I, but failed to significantly reduce the monocytes count as compared to group II in both HEQI with cyclophosphamide at 150 and 100 mg/kg dose. HEQI with cyclophosphamide 100 mg/kg also insignificantly increase of Neutrophils(N), lymphocytes(L) and eosinophils(E) (table3).

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of specific and non specific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immuno-suppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factor[15]. The immune responses through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy[16].

The results obtained in the present study indicate that *Quisqualis indica (QI)* flower extract is a potent Immunostimulants, stimulating specific and non-specific immune mechanisms. The role of phagocytosis is the removal of microorganisms and foreign bodies, dead or injured cells. The primary target of most of the immunomodulators is believed to be macrophages which play a major role by engulfing pathogens or foreign substances and initiating innate immune response .The increase in the carbon clearance index reflects the enhancement of the phagocytic function of mononuclear macrophage and nonspecific immunity. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by the opsonisation of parasites with antibodies and complementing C3b, leading to a more rapid clearance of parasites from the blood. The

phagocytic activity of the reticulo-endothelial system is generally measured by the rate of removal of carbon particles from the blood stream *Quisqualis indica* extract appeared to enhance the phagocytic function by exhibiting a clearance rate of carbon by the cells of the reticulo-endothelium system.the effect of *Quisqualis indica* flower extract. on the phagocytic activity by the carbon clearance test. In carbon clearance test, QI flower extract treated all groups, exhibited significantly high phagocytic index. The phagocytic index of (100 mg/kg) and QI flower extract (150 mg/kg) showed significant ($p<0.05$) increased in phagocytic index when compared to control group. This indicates stimulation of the reticuloendothelial system. Standard drug levomisol (50 mg/kg) is highest phagocytic index (highly significant) among the all group.

A high degree of cell proliferation renders the bone marrow a sensitive target particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and cyclophosphamide at the dose of 30 mg/kg, caused a significant reduction in total WBC count in rat as compared to control group. The rise in the total WBC count lowered by cyclophosphamide was observed at 100 mg/kg and 150 mg/kg of *Quisqualis indica* flower extract. The total WBC count was significant restoration of white blood cell count.

There was a significant decrease, Neutrophils lymphocytes, eosinophils and monocytes in animals treated with cyclophosphamide (group II) as compared to control group (group I) because cyclophosphamide showed that in rats lymphocytes decrease due to immunotoxic effect as well as decreases in the activity of lymphoid cells especially the CD4+ lymphocytes. Eosinophils attack parasites and phagocyte antigen-antibody complexes. Monocytes are the precursors of macrophages. They are larger blood cells, which after attaining maturity in the bone marrow, enter the blood circulation where they stay for 24-36 hours. Then they migrate into the connective tissue, where they become macrophages and move within the tissues. In the presence of an inflammation site, monocytes quickly migrate from the blood vessel and start an intense phagocytory activity. The role of these cells is not solely in phagocytosis because they have also an intense secretory activity. They produce substances which have defensive functions such as lysozime, interferons and other substances which modulate the functionality of other cells. Macrophages cooperate in the immune defense. *Quisqualis indica* flower extract 100mg/kg was insignificant restoration of differential WBC count but *Quisqualis indica* flower extract 150mg/kg significant ($p<0.05$) increase and restoration of lymphocytes, Neutrophils, eosinophils. But monocytes are insignificant increase by both 100 and 150 mg/kg dose of *Quisqualis indica* flower extract.

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase the vascular permeability, induce vasodilatation, macrophage accumulation, and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated TH1 cells encounter certain antigens, viz. SRBCs. They secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity. DTH comprises of two phases, an initial sensitization phase after the primary contact with SRBC antigen. A subsequent exposure to the SRBCs antigen induces the effectors phase of the DTH response, where TH1 cells secrete a variety of cytokines that recruits and activates macrophages and other non specific inflammatory mediators. The delay in the onset of the response reflects the time required for the cytokines to induce the recruitment and activation of macrophages. Therefore, increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of mega extract on T cells.DTH response was checked by increased footpad thickness using digital vernier caliper. The animals treated with levamisol and extracts showed a significant change in DTH response as compared to control animals (group I). As can be evident from (table), a significant ($P < 0.05$) increase in DTH response was observed at 150 mg/kg dose of *Quisqualis indica* flower extract and levamisol 50 mg/kg. *Quisqualis indica* flower extract 100 mg/kg showed a insignificant DTH responses.

The phytochemical screening revealed presence of flavonoids, Tanin ,Phenolics Compound, Terpinoids and saponin. Some phytoconstituent are able to regulated immune responses like polysaccharides, Polyphones, Flavonoids, alkaloids, Saponins and Omega 3 fatty acid. These types of components are reported to exhibit immunomodulatory activity in various experimental models. The phenolic com-pounds can stimulates or suppress the immune system due to the hydroxyl groups in the structure. These groups can affect the enzyme or electron transferring system regulating an immunomodulatory property

especially phagocytic activity. Administration of Quisqualis Indica was found to increase phagocytic activity by stimulation of macrophages, total WBC and differential leukocytes count. Delayed type hypersensitivity reaction also stimulated by *Quisqualis Indica higher dose* significantly indicating that the extract could stimulate the haemopoetic system

The present study reveals that, hydroalcoholic extract of *Quisqualis Indica* (HEQI*)* has Immunostimulants activity which strongly affected immune system seems to be bioactive fraction of this plant. However, the mechanism of action could be unfolded only after detailed investigations whereby the extract modulates the immune system however; the extract contains compounds which had Immunomodulatory activity. Besides, to isolate the active constituents and clarify its mechanism of action will be our auxiliary objective.

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Table 1: Carbon clearance assay of hydroalcoholic extract of *Quisqualis indica* Linn. flower

Group	Treatment (n=6)	Dose mg/kg	Phagocytic index Mean ±SEM	DTH responces Mean ±SEM
1	Vehicle(D.Water)	-	0.0521 ± 0.0061	0.350±0.028
2	HEQI	100	0.077 ± 0.0059*	0.401 ± 0.401
3	HEQI	150	0.082 ± 0.0029*	0.448 ± 0.026*
4	Standard (levamisol)	50	0.084 ± 0.0062*	0.455 ±0.083*

*p<0.05 as compared to vehicle treated group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multi comparis test

Table 2: Total wbc of hydroalcoholic extract of *Quisqualis indica* Linn. flower

Group	Treatment (n=6)	Dose mg/kg	Total wbc (mm^3) mean±sem
1	Vehicle(distil water)	-	7836±124.5
2	Cyclophosphamide	30	$5181.6±121.2^{b*}$
3	HEQI + Cyclophosphamide	100	$6571.5±128.2^{a*}$
4	HEQI + Cyclophosphamide	150	$8051.5±107.1^{a*}$

a=Group is compared with group 2, b = when group 2 is compared with group 1,
*= significantly different statistically analysed by one-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparis test

Table 3: Differential leukocyte counts of *Quisqualis indica* Linn. flower

Group	Treatment (n=6)	Dose mg/kg	N Mean±SEM	L Mean±SEM	E Mean ±SEM	M Mean ±SEM
1	Vehicle(D.W)	-	50.17±1.138	32.17±1.321	6 ±0.258	1.83 ±0.166
2	CYP	30	$40.83 ± 0.70^{b*}$	$26.5±0.76^{b*}$	$4.16 ±0.30^{b*}$	$1.167 ±0.166^{b\#}$
3	HEQI+CYP	100	$43 ± 0.632^{a\#}$	$27.17±0.79^{b\#}$	$4.5 ±0.22^{a\#}$	$1.5 ±0.223^{a\#}$
4	HEQI+ CYP	150	$49.17 ±1.60^{a*}$	$30.83±0.47^{b*}$	$5.167±0.30^{a*}$	$1.833 ±0.166^{a\#}$

a=Group is compared with group 2, **b** = when group 2 is compared with group1,*=significantly different ,# =insignificant. statistically analysed by one- way analysis of variance (ANOVA) followed by (Dunnett) multiple comparis test.

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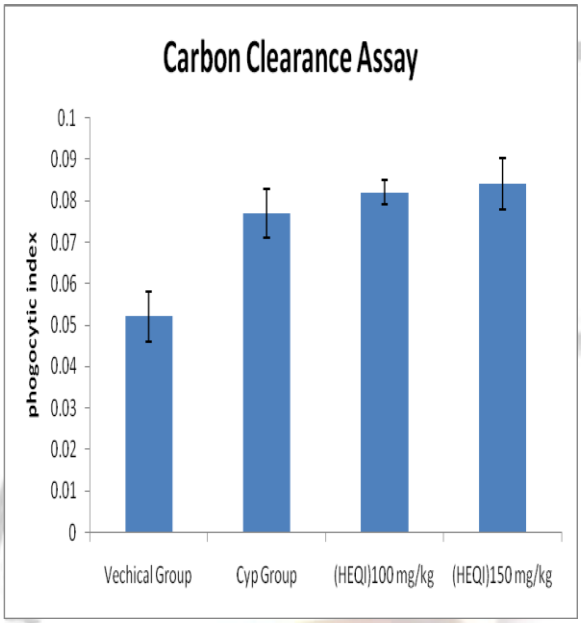

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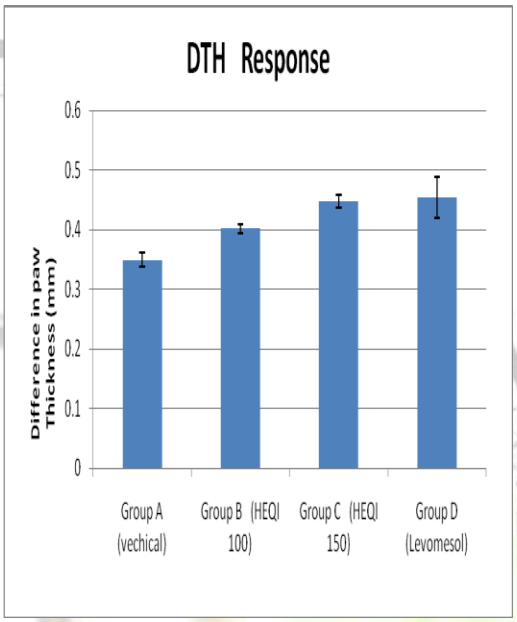

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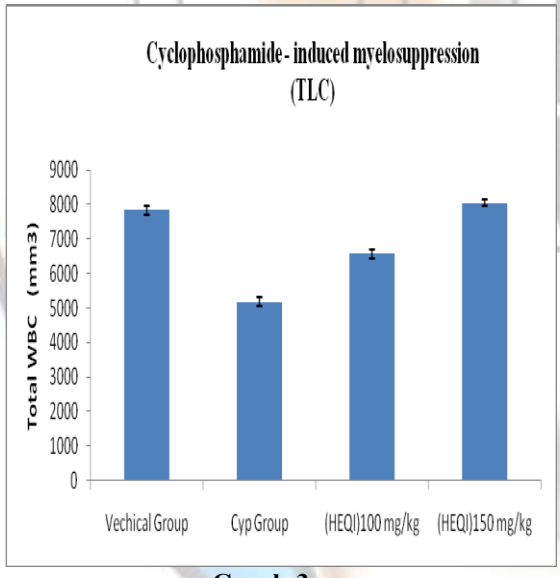

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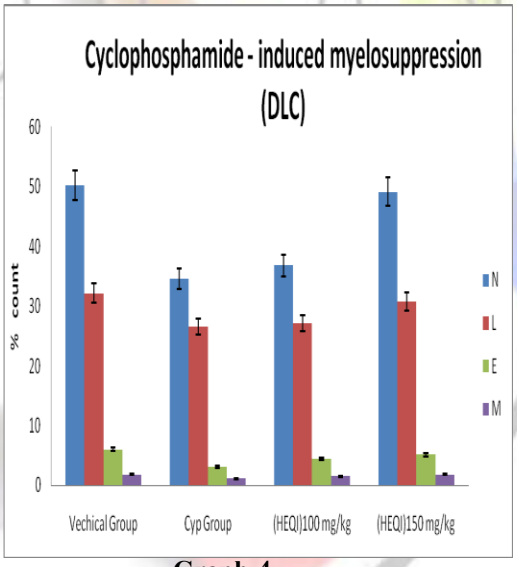

Graph-4