6.1) Development of polyherbal formulation

Traditional medicine systems including Ayurveda, Unani, Siddha, Yoga and Naturopathy have developed over a long period of time but there are many limitations with the traditional herbal formulations and their traditional preparation techniques such as *shodhana* that they are difficult to adopt by modern herbal industries. So efforts were always there to prepare these formulations by modern technique. Now a day, it has also been considered that if the modern herbal dosage form can be used along with modern tools in herbal medicine, it may help in global acceptance of these formulations. Formerly, lack of scientific justification and processing difficulties development of herbal medicines as modern formulations was not preferred. This was overcome by phytopharmaceutical research which can solve the various scientific needs related to herbal medicines. [1, 2]

6.2) Materials and Methods

6.2.1) Optimization of formulations by *in-vitro* antioxidant activity using full factorial designs

Factorial design is effective tool to estimate the effects of several variables on response. Conventionally, experiments were designed such that effect of one variable could be determined at a time. This was later revolutionized by R.A. Fisher who first conceptualized factorial design experiments and showed advantages by conjoining the study of multiple variables in the same experiment. This concept reduces actual number of experiments to be performed to get a significant piece of information. Furthermore, it can be applied to derive main effects as well as interaction effects. Although, conversion of relative values, obtained by factorial design, to actual values is bit difficult as it requires regression analysis, the factorial design is important tool to plan experiments in laboratory as well as industrial settings. Factorial design considers all the possible conditions and settings as variables and thus can become expensive and time-consuming for large number of variables. Even though, accompanied with several limitations factorial design provides best alternative to determine interactions between variables and whether every variable contributes significantly. [3, 4]

6.2.1.1) 2x4 full factorial design

To develop polyherbal formulation dose is the main factor to influence the process. The formulations are optimized for *in- vitro* antioxidant activity by applying factorial design. Factorial design, used to determine process variations, is applied for optimizing process parameters with reduced number of experiments. Commonly, it involves variables set at 2 levels i.e. 'high' and 'low' or '+1' and '-1' respectively. For experiments with *k* factors at two-levels, a full factorial design has 2^k runs. [5]

In current work, a four factor system consisting of the four herbal drugs such as *Glycyrrhiza glabra* root, *Nelumbo nucifera* leaves, aerial parts of *Prunella vulgaris* and fruits of *Zizyphus jujuba* and 2^4 runs were used for the optimization of formulations. The chosen dependent variable was EC_{50} (50% effective concentration). The coded and actual levels are depicted in Table 6.1.

Table 6.1: Formulation variables and their levels for 2x4 full factorial desgin

Factors	Coded levels	Actual levels
A: *G. glabra*	-1	30
	1	100
B: *N. nucifera*	-1	30
	1	100
C: *P. vulgaris*	-1	30
	1	100
D: *Z.jujuba*	-1	30
	1	100

6.2.1.2) 2x3 full factorial design

Another factorial design was also performed to develop a three drug formulation. For this, 2^3 full factorial design was employed to optimize factors. This implies the need of 8 runs (excluding replications or center points). Factors are plant extracts viz. *Glycyrrhiza glabra* root, *Nelumbo nucifera* leaves and fruits of *Zizyphus jujuba.* The chosen dependent variable was EC_{50} (50% effective concentration). The coded and actual levels are depicted in Table 6.2.

Table 6.2: Formulation variables and their levels for 2x3 full factorial desgin

Factors	Coded levels	Actual levels
A: *G. Glabra*	-1	30
	1	100
B: *N. nucifera*	-1	30
	1	100
C: *Z. jujuba*	-1	30
	1	100

6.2.2) Development of Dosage form

- Method of preparation of formulation into vati: [6, 7]

All the four extracts were mixed in equal quantity. Sufficient quantity of tragacanth solution was added to it and then a small size vati was rolled manually. But the vati was found to be sticky formulation is somewhat sticky so a tablet dosage form was prepared and evaluation was done.

- Method of preparation of formulation into tablet dosage form: [6, 8]

Three methods i.e. wet granulation; dry granulation and direct compression are normally used for formulating tablet dosage form. Due to hygroscopic nature of two of the extracts, direct compression was selected as the suitable method for the preparation of polyherbal tablets.

6.2.2.1) Selection of Excipients [9]

The primary aim of current work was to develop a stable polyherbal tablet. Common excipients of generally regarded as safe (GRAS) category e.g. microcrystalline cellulose (MCC/Avicel), dibasic calcium phosphate (DCP), polyethylene glycol (PEG) 4000, magnesium stearate and methyl paraben were used during tablet formulation.

6.2.2.2) Method for the preparation of tablets (Direct Compression method) [10]

The most effective combination of extracts derived after factorial optimization and all other excipients mention above were used to prepare a tablet dosage form. Microcrystalline cellulose (MCC/Avicel) and dibasic calcium phosphate (DCP) are commonly used for direct compression method as diluents. Polyethylene glycol (PEG) 4000 acts as a lubricant as it reduces friction between tablet and die during ejection. It has solubility enhancing activity also. All the ingredients were weighed

accurately on Shimadzu precision Balance. Mixing of the above ingredients was done by sieving (45#).

Pre-sieved magnesium stearate (85#), weighed and mixed to the above blend powder which act as an anti-adherent. Methyl paraben (1%) was added as a preservative. The mixed powder was compressed into a tablets using Rotary tablet machine with flat punches having 9.9 mm punch size.

6.2.3) Physical Standardization of Formulation [8, 11]

The prepared tablet formulations were subjected to physical testing as per the WHO guidelines in order to set the standards for the analysis of the formulations. Although the methods for chemical standardization of the formulations have been developed and are much more sophisticated, the physical standardization proves to be equally important. The appearance of the formulation, the amount of the dose administered, and the time required for the disintegration and release of active constituents are all important factors which affect the physiological action of the formulation. The marketed formulations are therefore supplied in the form of tablet to ascertain that a fixed dose is administered. Thus, the developed formulations (tablets) should be analysed as per the IP monographs for the following parameters:

6.2.3.1) Evaluation parameters for extracts

6.2.3.1.1) Angle of repose

The funnel was positioned at a height of 2 cm. Accurately weighed powder sample was poured and retained in funnel and later allowed to pass through the funnel. Powder heap diameter formed was measured using a ruler and replicated thrice. The average angle of repose was derived using following formula.

Angle of repose (Θ) = $\tan^{-1}$ (h/r)

Where, Θ = angle of repose; h = height (cm); r = radius (cm)

Table: 6.3 Flow property and Angle of repose

Flow property	Angle of repose (degrees)
Excellent	25-30
Good	31-35
Fair	36-40
Passable	41-45

Poor	46-55
Very poor	56-65
Very, very poor	>66

6.2.3.1.2) Bulk density/ Tapped density

For apparent bulk density, accurately weighed powder blend (25 gm) was poured into 100 ml graduated cylinder and volume occupied was noted. Later, measuring cylinder was tapped from 2 inch height and tapped volume was recorded. The bulk density and tapped density were calculated using following formula:

$$\text{Bulk density} = \text{Bulk density} / \text{Bulk volume}$$

$$\text{Tapped density} = \text{Tapped density} / \text{Tapped volume}$$

6.2.3.1.3) Carr's index (Compressibility index)

Carr's index is the simplest method to determine the flow property of powder which indicates the ease by which powder can be induced to flow was calculated as follows:

$$\text{Carr's index} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100$$

6.2.3.1.4) Hausner's ratio

This is indirect index for powder flow. It can be calculated as follows,

$$\text{Hausner's Ratio} = \frac{\text{Tapped density}}{\text{Bulk density}}$$

6.2.3.2) Evaluation of dosage form

6.2.3.2.1) Uniformity of Weight:

The weight variation of the formulations was studied according to IP. 20 tablets were weighed and average weights and maximum deviation of average weights were calculated. The uniformity in weight is important parameter for quality control of herbal formulations because a number of small scale manufacturers use the traditional manual method of tablet preparation. Thus, weight variation is bound to occur and may lead to variation in the amount of formulation administered.

6.2.3.2.2) Disintegration test:

The disintegration test of the formulations was studied as per the IP. The disintegration of a number of herbal tablets has been questionable in the past as their

hardness is very high. Therefore, in order to ascertain that the tablet has been disintegrated for the release of active constituent, the disintegration and the duration of disintegration were determined.

6.2.3.2.3) Hardness:

Hardness is an important parameter which was determined for tablets as per the IP. The official limit of hardness of a tablet is 3-5 kg/cm^2. If the tablet is below the limit then the tablet may be prone to breakage or loss of the uniformity in weight. If the hardness is above the limit then the tablet will not disintegrate in the required period of time. Thus the tablets were subjected to hardness test using Pfizer hardness tester as per the IP.

6.2.3.2.4) Diameter:

Diameter of tablets was estimated by Vernier callipers as per IP method.

6.2.3.2.5) Thickness:

Thickness of tablets was determined using Vernier callipers as per IP method.

6.2.3.2.6) Friability:

The friability test was performed using the instrument named 'Roche friabilator' as per IP method.

Following quality parameters were determined as per WHO guidelines.

6.2.3.2.7) Determination of Ash:

The procedure prescribed in WHO guidelines was used for the determination of ash value for formulation.

6.2.3.2.8) Determination of extractable matter:

The procedure prescribed in WHO guidelines was used for the determination of extractable matter of the formulation.

6.2.3.2.9) Determination of pesticide residues:

The formulation was subjected to tests for presence of organo-phosphorus and organo-chlorinated pesticides using TLC studies.

6.2.3.2.10) Determination of heavy metals:

The concentration of heavy metals in the formulation was analyzed using Atomic absorption spectroscopy.

6.2.3.2.11) Determination of microorganisms:

The formulation was analyzed for bio-burden present as per the reported culture media described in WHO guidelines.

6.2.3.2.12) Chemical tests:

The qualitative phytochemical screening of water extract of the formulation was performed.

6.2.3.2.13) TLC studies:

The qualitative screening of water extract derived from the formulation was performed.

6.2.4) Accelerated stability studies of developed formulations according to WHO guidelines

The optimized herbal formulations (Form A, B and C) of the drug were subjected to accelerated stability studies at specified conditions of temperature and relative humidity of 25ºC/60% RH, 30ºC/60% RH and 40ºC/75% RH for 3 months [12].

6.2.5) BIOLOGICAL EVALUATION OF FORMULATION

6.2.5.1) Acute toxicity study [14]

The acute toxicity studies for the Polyherbal formulations were conducted in healthy rats and mice with body weight 180-200g and 25-30g respectively, as per the Organisation for Economic Co-operation and Development (OECD) guidelines. All experiments were performed according to the guidelines of Institutional ethics committee, Pharmacy department/The M. S. University, Baroda//CPCSEA. The protocol number is MSU/PHARM/IAEC/2011/06. The age of the selected animals was between 8 to 12 weeks.

The temperature of the animal room was maintained 22°C (±3 °C) with relative humidity 50-60% throughout the study. The lighting, provided artificially, was set such that it resulted 12 hours light, 12 hours dark. Feeding to animals was provided by conventional laboratory diets ad-libitum with unlimited supply of drinking water. In rodent, dose volume was not exceeded 1ml/100g body weight.

6.2.5.1.1) Number of animals and dose levels

The test was performed using three animals in a group. The study was carried by fixed dose procedure using starting dose from one of four fixed levels- 500, 1000, 1500, 2000 mg/kg body weight.

From the reference literature it was found that even the highest starting dose i.e. 2000 mg/kg would not lead to mortality thus limit test was performed.

6.2.5.1.2) Limit test

1. Limit test is mainly performed when test substance is non-toxic i.e. possess toxicity above regulatory limit doses.
2. This test was carried out using dose of 2000 mg/kg using six animals (3 animals per step).

6.2.5.1.3) Observations

Animals were observed for any signs of morbidity or mortality for 14 days with special observation for 30 min after dosing, periodically during 24 hr and daily thereafter.

In addition to signs for morbidity or mortality they were also observed for signs including changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems and somatomotor activity and behaviour pattern. A keen watch should be kept for adverse effects like tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Animals found moribund or suffering severe pain or distress should be euthanized. The time of death or euthanasia should be recorded accurately.

6.2.5.1.4) Body Weight

The body weight of all the animals used for the study was determined before initiation and termination of the test. Additionally, weight change of animals was tracked during the test periodically. Any significant change in weight is reported.

6.2.5.2) *In-Vivo* Immunomodulatory Activity

The formulations showing best antioxidant activity will be further evaluated for *in-vivo* immunomodulatory activity. Immunity has significant contribution in biological adaptation resulting in sustaining body integrity and thus homeostasis.

Immunomodulatory activity using following models and parameters

- Cyclophosphamide induced myelosupression assay.
- Carbon clearance assay in mice.
- Delayed hypersensitivity reaction in rats.
- Haemagglutination antibody titre value.

Animals

Mice of Swiss albino strain (25-30 g) and wistar rats (180-200 g) were used for this study. The animals were housed according to the guidelines maintain surrounding environment w.r.t. temperature, humidity and light. The animals were fed with rodent diet ad-libitum and free access to water.

Chemicals

Carbon ink suspension (Pelican AG, Germany), diluted 8 times using saline, was used in carbon clearance test at dose of 10µl/gm body weight. Cyclophosphamide injection (Endoxan 200mg vial of Zydus Biogen, Cadila Healthcare Ltd). Levamisol (Himedia), dextrose, EDTA, sodium chloride, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate 96 well micro-titre plates, micropipette, Vernier callipers, Neubaurs chamber, microscope, cylindrical vessel (50 x 40cm) etc.

Preparation of 20% v/v SRBC suspension [14]

The blood, from healthy sheep, was collected in 0.49% EDTA and 0.9 % of sodium chloride solution. It was preserved at a 2-8 ºC till use. Before immunization, plasma was separated from blood by centrifugation and washed with 0.9% sodium chloride. Later SRBC (20% v/v) suspension was made using 0.9% sodium chloride.

Collect sheep blood in 0.49%EDTA in saline (Equal volume)

⇩

Centrifuge blood to get thick pellet of SRBC

⇩

Wash the pellet continuously with saline till u get clear supernatant.

⇩

Prepare 20% SRBC (2ml SRBC in 10 ml of saline)

6.2.5.2.1) Cyclophosphamide induced myelosupression assay [15, 16]

The test animals were allocated into three groups (6 per group). (1) Four test groups (Form A, Form B, Form C and Levamisol) (2) Positive control (3) Negative control. Blood samples were collected on 0 day and hemogram was done. Polyherbal formulations (PHF) were administered orally for 13 days to test group. 1% SCMC orally was administered to positive and negative control group for 13 days. Blood was collected on 10th day and hemogram was done with ERMA PC 607 cell counter. Cyclophosphamide (30mg/kg) was administered in all animals except negative

control group on 11th, 12th, and 13th day, 1hr after the administration of HF. Blood was collected on 14th day and hemogram was performed. (Fig. 6.1)

6.2.5.2.2) Carbon clearance assay in mice [17-19]

0.2 ml carbon suspension was administered intravenously via tail vein on seventh day in all animals of treatment groups. Blood was collected by retro-orbital sinus puncture before and at interval of 5 min for 20 min after injecting carbon suspension. 25 µl blood was mixed with 2 ml 0.1% acetic acid and absorbance was noted at 675 nm. The graph of absorbance vs. time was plotted and phagocytic index was determined from its slope. (Fig. 6.2)

6.2.5.2.3) Delayed hypersensitivity response in rats [20-22]

The test animals were allocated into three groups (6 per group). On day 0 intra-peritoneal administration of 0.1 ml 20 % fresh sheep RBCs lead to immunization of animals. Herbal formulation was administered per orally at dose of 200 mg/kg in treatment groups using 1.0 % sodium carboxy methyl cellulose dispersion daily for 7 days. In positive control, cyclophosphamide 50mg/kg was administered from day 4 to 6. The foot pad thickness of hind paw was determined using digital Vernier caliper. Later, the mice were challenged by injecting 20 µl 1% SRBCs in right hind foot pad and foot thickness was measured. The difference between foot thickness was considered as measure of DTH. (Fig. 6.3)

6.2.5.2.4) Humoral Antibody titre [17, 20]

The test animals were allocated into three groups (6 per group). On day 0 intra-peritoneal administration of 0.1 ml 20 % fresh sheep RBCs lead to immunization of animals. Herbal formulation was administered perorally at dose of 200 mg/kg in treatment groups using 1.0 % sodium carboxy methyl cellulose dispersion daily for 7 days. In positive control, cyclophosphamide 50mg/kg was administered from day 4 to 6. Blood was collected in micro centrifuge tubes by retro-orbital sinus puncture on day 7 and serum was separated by centrifugation. Haemagglutination technique was used to estimate antibody levels and the reciprocal of highest dilution of test serum causing agglutination was considered as antibody titre. (Fig. 6.4)

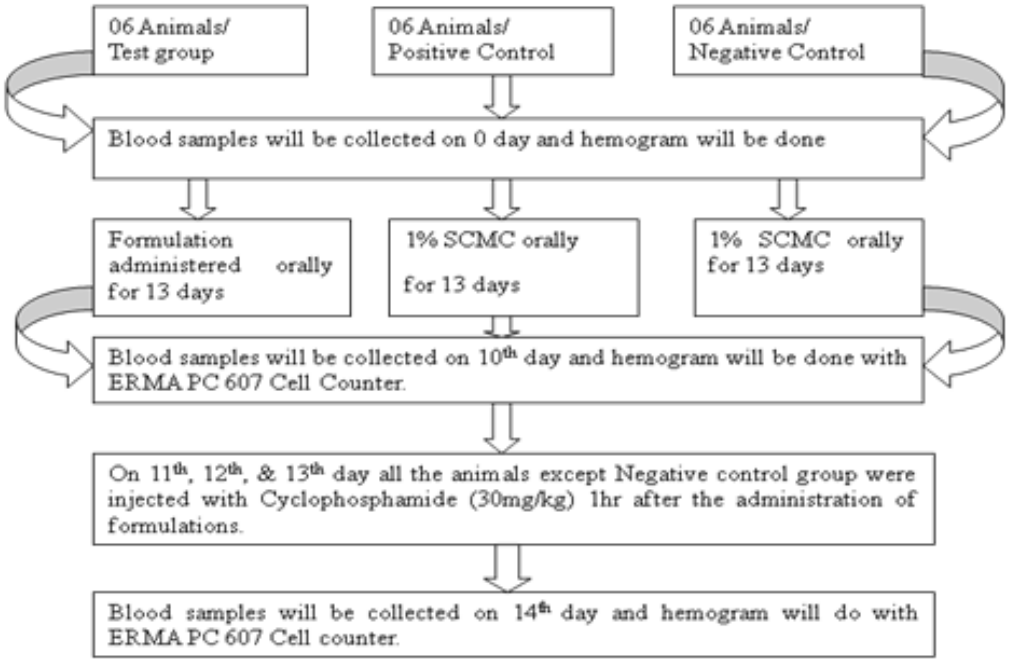


Fig. 6.1 Cyclophosphamide induced myelosupression assay

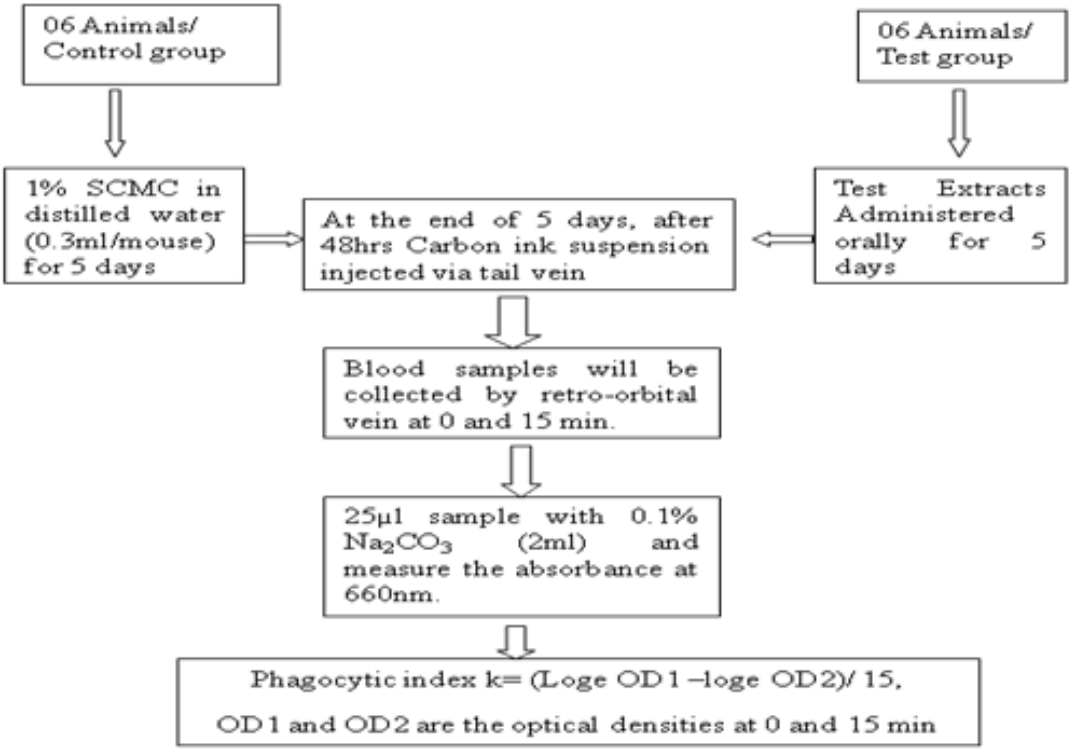


Fig. 6.2 Carbon clearance assay in mice

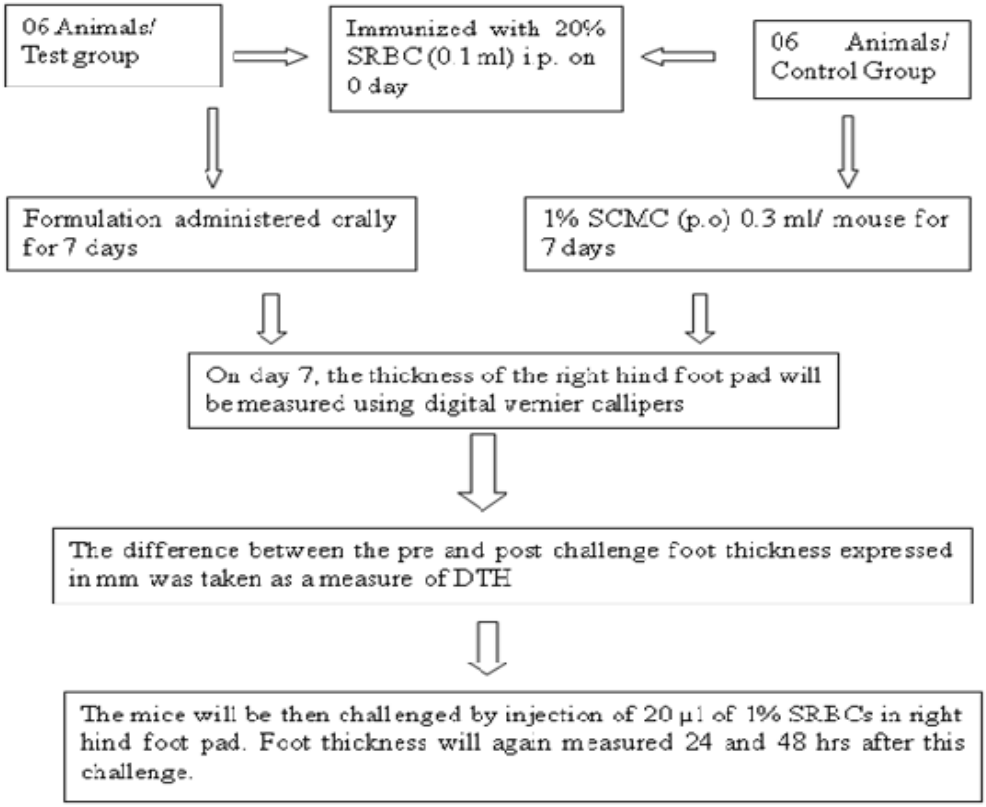


Fig. 6.3 Delayed hypersensitivity response in rats.

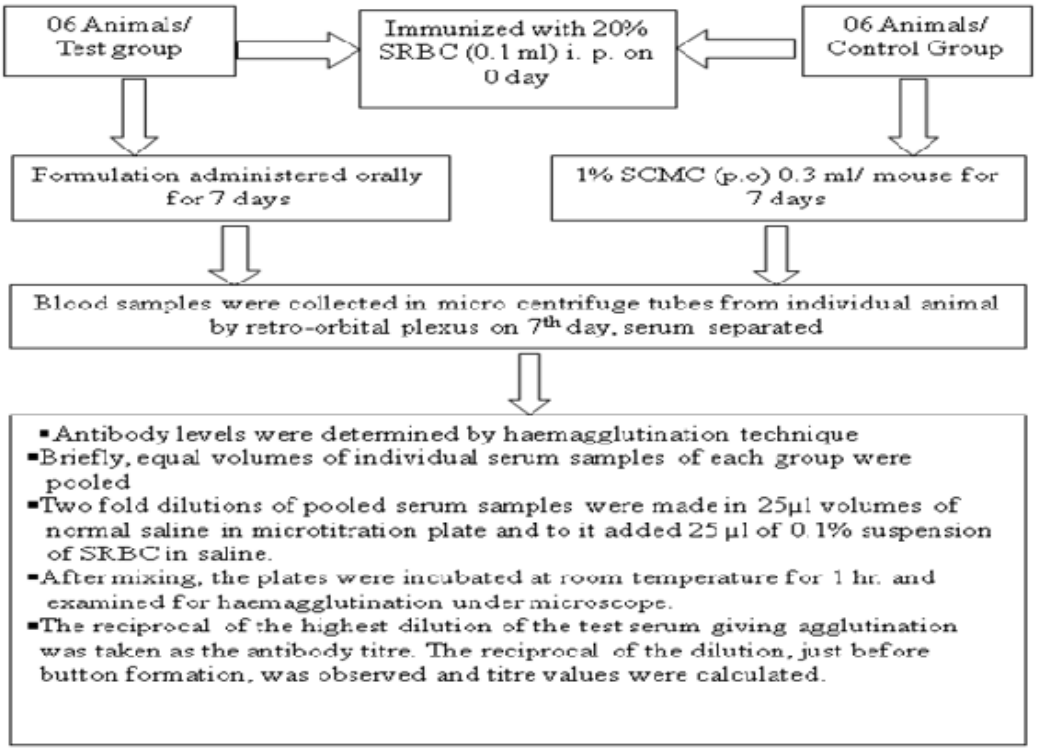


Fig. 6.4 Humoral Antibody titre

6.2.5.3*) IN-VITRO* STUDIES

6.2.5.3.1) Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from GIBCO, 3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Histopaque-1119 and Histopaque-1077 were purchased from Sigma Aldrich, USA. Levamisol was purchased from Himedia. All other chemicals used were of the highest grade available.

6.2.5.3.2) Isolation of PMN (Polymorpho nuclear) cells from healthy human blood [23]

Reagents: Histopaque-1119 (Polysucrose, 6.0 g/dl and sodium diatrizoate, 16.7 g/dl), Histopaque-1077 (Polysucrose, 5.7 g/dl, and sodium diatrizoate, 9.0 g/dl), healthy human blood, phosphate buffer saline

Procedure:

1. Histopaque -1119 (3 ml) was added to 15 ml centrifuge tube.
2. 3 ml of Histopaque E-1077 was carefully layered on Histopaque -1119.
3. Later, 6 ml whole blood was carefully layered on upper gradient of tube from step 2.
4. The mixture was centrifuged at 700 g for 30 minutes at room temperature. Cell clumping was observed at lower temperatures resulting in poor recovery.
5. Two distinct opaque layers, labelled as layer A and B in Fig 6.5, were observed in centrifuge tubes.
6. The fluid was aspirated and discarded within 0.5 cm of layer A. The cells were transferred from this layer to a tube marked “mononuclear”.
7. The fluid was aspirated and discarded within 0.5 cm of layer B. The cells were transferred from this layer to a tube marked “granulocytes”.
8. 10 ml isotonic PBS was used to wash the cells thrice and supernatant was removed after centrifugation.
9. The resultant cell pellet was re-suspended in isotonic phosphate buffered saline.

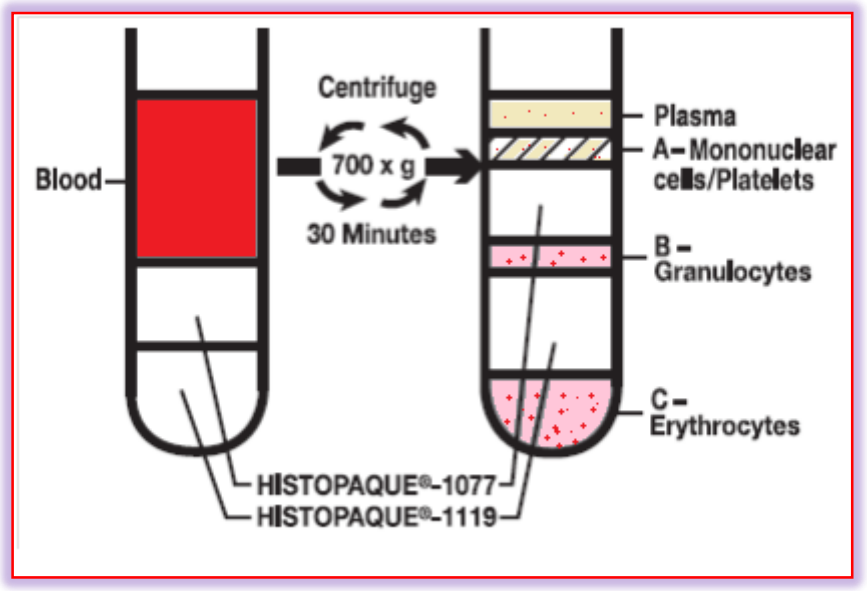


Fig. 6.5 Isolation of PMN cells from healthy human blood

6.2.5.3.3) Cell culture

Neutrophils isolated from healthy human blood were cultured in DMEM media with 10% FCS, 1% antibiotic-antimycotic solution (100 U/ml of penicillin and 100 µg/ml of streptomycin) in 25 cm^2 tissue culture flasks incubated at 37°C in a humidified atmosphere with 5% CO_2. Media was changed every 2–3 days, and cells were sub-cultured when confluence is about 70 – 80%. Dimethyl sulfoxide (DMSO) was used as a solvent to dissolve the formulations such that its final concentration was less than 0.2 %.

6.2.5.3.4) Analysis of cell viability (MTT assay) [24, 25]

MTT test was employed to determine the cell viability after their treatment with herbal formulations at different doses. Briefly, cells were seeded in 96-wellplate at density of 5×10^3 cells per well, and incubated overnight for proper cell adherence. The formulations in concentration range of 10-800 µg/ml were added in triplicate. Later, 10µl 5 mM MTT solution was added, and incubated in the dark for 4hrs. Subsequently, media was removed, and 100 µl DMSO was added and the absorbance was measured at 570 nm using ELISA plate reader (Biorad, USA). Cell viability of each group was expressed as a relative percentage to that of control.

6.2.5.3.5) *In-vitro* immunomodulatory activity

6.2.5.3.5.1) Preparation of neutrophils

Neutrophils were isolated from blood as described earlier. The cells were washed with HBS solution after hypotonic lysis to exclude the erythrocytes. Neutrophils were suspended in HBS solution, such that final concentration of 5×10^6 neutrophils/ml was achieved for NBT reduction test and 10^7 neutrophils/ml for *in-vitro* candidacidal assay. Neutrophils' viability was determined by trypan blue test and the results confirmed more than 92% cell viability.

6.2.5.3.5.2) Pretreatment of neutrophils (activation) with formulations

Herbal formulations with concentration of 50, 100 and 200 µg/ml were prepared using 5.0 % v/v dimethysulphoxide in 0.15 M phosphate buffer saline (PBS). Later, neutrophils (5×10^6cells/ ml) were mixed with above formulated solutions and incubated at 37^{o}C for 4 hours.

6.2.5.3.5.3) Nitroblue tetrazolium (NBT) test [26-28]

0.1% NBT solution was prepared in 0.9 % NaCl and 0.15M phosphate buffer saline (PBS) and 0.1 ml was added into 4 wells of microtitre plate. Unstimulated neutrophils were added to control well while neutrophils pretreated with formulations were added to the remaining wells respectively. The plates were incubated at 37^{o}C for 10 min such that it provided moist atmosphere. The incubation was further continued for 15 min at room temperature and the contents were mixed thoroughly after which smear was prepared and air dried on microscopic slide. The smear was stained with safranin for 1 min and later treated with Sorenson's buffer (0.067 M) for 10 min. Subsequently, the slides were observed under microscope using oil immersion objective (100x) and 100 neutrophils were counted. Then recording both total neutrophils and numbers which contain deposits of black formazan (reduced NBT dye) i.e. cells containing black material greater than the granules normally showing in neutrophils.

6.2.5.3.5.4) Neutrophils candidacidal assay [28-30]

6.2.5.3.5.4.1) Preparation of Candida albicans cells suspensions

Candida albicans were grown in stationary phase by inoculating it in 50 ml Saubouraud's 2% dextrose broth for 72 h at 33 ^{o}C. The test organisms were washed twice in hanks balance salt solution (HBSS) and its viability was checked using

trypan blue staining and observing using hemacytometer. Spherical cells or attached doublets were observed under microscope, while filamentous forms were absent. The viability of cells was more than 95%. For Candidacidal assay the cells were diluted such that final concentration of the cellular dispersion obtained was 10^7 cells/ml.

6.2.5.3.5.4.2) Serum preparation

Blood (AB Donor) was collected from Divine pathological Laboratory, Fatehgunj, Vadodara. Blood was collected in a sterile centrifuge tube and allowed to clot for 1 h. The supernatant layer after centrifugation was collected and stored in refrigerator (4–7 °C) until use.

6.2.5.3.5.4.3) *In vitro* neutrophils candidacidal assay

Neutrophil suspension (10^7 cells/ml) pretreated with polyherbal formulation and HBSS was taken in sterile centrifuge tube and 0.25 ml AB serum was added to it. The neutrophils were excluded from the tube used as a control. After incubating the tubes for 10 min at 37 ºC, 0.25 ml *Candida albicans* (10^7yeast phage cells/ml) was added, with stirring at 30 RPM maintaining temperature at 37 ◦C for 60 min. Later, 0.25 ml 2.5% sodium deoxycholate (pH: 8.7) was added to each tube (Note: deoxycholate causes instant blood cell lysis without affecting *Candida* cells). The resultant mixture of cell suspension was treated with 0.01% methylene blue and the suspension was centrifuged at 1100×g for 15 min. The pellet was washed and resuspended in 0.5 ml residual supernatant fluid. The tubes were kept in ice bath till observed microscopically. 300 *Candida* cells were examined and fraction of stained cells, representing non-viable organisms, was determined. To derive the Candidacidal activity due to the action of neutrophils, the percentage of stained yeast cells in the control tubes, usually 2.5% was substracted from that in the experimental tubes. Viable *Candida* cells, which were unstained, visibly differ from the non-viable organism which attained a uniform and dark blue colour cytoplasm stain.

6.3) Statistical Analysis of Data

Results of all the *in vivo* and *in vitro* experiments are expressed as mean ± SEM. Stastically significance was estimated by analysis of variance (ANOVA) followed by Bonferroni post-test and Dunnett multiple comparisons tests.

6.4) RESULTS AND DISCUSSIONS

6.4.1) Optimization of formulations by *in-vitro* antioxidant activity using full factorial designs

6.4.1.1) 2x4 full factorial design

The matrix of the experiments and results of the measured response are depicted in the Table 6.4. Results of the measured response EC_{50} is depicted in Table 6.4. ANOVA results and the regression coefficients of response variables and results of ANOVA are shown in Table 6.5.

Concentration of *N. nucifera* plays an important role in the case of EC_{50}. Other parameters like *G. glabra, P. vulgaris* and *Z. jujube* also affected the antioxidant activity significantly.

The results were analyzed using the software Design Expert 8.0. The coded mathematical model and observed EC_{50} values for 2x4 factorial designs can be given as:

$$R = 47.20-6.10*A-6.11*B-4.99*C-5.82*D-2.18*B*C+ 1.61*C*D$$

Where R (%) is the effective concentration (EC_{50})

The results indicated that dose of the factors A, B, C and D were the influencing factors affecting positively (Table 6.5) to achieve desired EC_{50} value. The same can be inferred from the pareto chart, half normal chart and predicted Vs. actual chart. (Fig. 6.6) When a change in the level of a factor, it produced change in response is called a effect of a factor. It refers to the primary factors of interest in the experiments so it is called a main effect. The main effects plot and interaction between two factors are shown in the Fig 6.7 and 6.8 respectively.

To predict the optimum levels of independent variables, desirability function was applied which merged the multi criteria responses in one single criterion measurement. The desirability value one reveals the response is on target/optimum, in case of totally unexpected value it reaches zero. The desirability charts are given in the Figure 6.9.

The overlay plots shown the optimized area (Fig. 6.10) and contour plots for the response EC_{50} (Fig. 6.11)

From 2x4 full factorial designs, we selected two batches (batch no. 8 and 16) which had low EC_{50} values for further studies.

Table 6.4: Matrix of the experiments for 2x4 full factorial design and results for the measured response

Run no.	Coded values of independent variables				*EC_{50} Observed
	A	B	C	D	
1	-1	-1	-1	-1	70.71 ± 1.09
2	1	-1	-1	-1	55.31 ± 1.37
3	-1	1	-1	-1	63.6 ± 1.55
4	1	1	-1	-1	48.8 ± 0.93
5	-1	-1	1	-1	61.11 ± 1.72
6	1	-1	1	-1	49.9 ± 1.38
7	-1	1	1	-1	41.97 ± 1.06
8	**1**	**1**	**1**	**-1**	**32.4 ± 1.45**
9	-1	-1	-1	1	54.2 ± 1.14
10	1	-1	-1	1	44.22 ± 1.28
11	-1	1	-1	1	48.21 ± 1.31
12	1	1	-1	1	32.7 ± 0.89
13	-1	-1	1	1	50.8 ± 1.15
14	1	-1	1	1	40.2 ± 1.08
15	-1	1	1	1	35.8 ± 1.63
16	**1**	**1**	**1**	**1**	**25.2 ± 1.27**

*Mean ± SD (n=6)
Regression coefficients are in coded value.

Table 6.5: Regression analysis results

Source	sum of squares	Mean sum of squares	Coefficients	F value	P value*
A	1	596.21	-6.10	167.47	< 0.0001
B	1	597.44	-6.11	167.81	< 0.0001
C	1	397.70	-4.99	111.71	< 0.0001
D	1	541.38	-5.82	152.07	< 0.0001
BC	1	76.17	-2.18	21.39	0.0012
CD	1	41.31	1.61	11.60	0.0078

Regression coefficients are in coded value.
* Statistically significant ($p < 0.05$)

Table 6.6: ANOVA results showing the effect of independent variables on the measured responses

Model	Full model
sum of squares	2250.21
Mean sum of squares	375.04
F value	105.34
P value	< 0.0001
R-squared	0.9860
Adj R-squared	0.9766
Pred R-squared	0.9556

Table 6.7: Comparison of responses between predicted and experimental values for the cross validation set

Response	**Test**	**Factors/Levels**				**Experimental values**	**Predicted Values**	**Residuals**
		A	B	C	D			
EC50	1	-0.6	-1	0.4	-0.4	58.24	57.68	0.56
	2	0.4	0	-0.4	0.5	46.52	47.88	1.36
	3	0.6	-0.5	0	-0.4	60.27	59.51	0.76

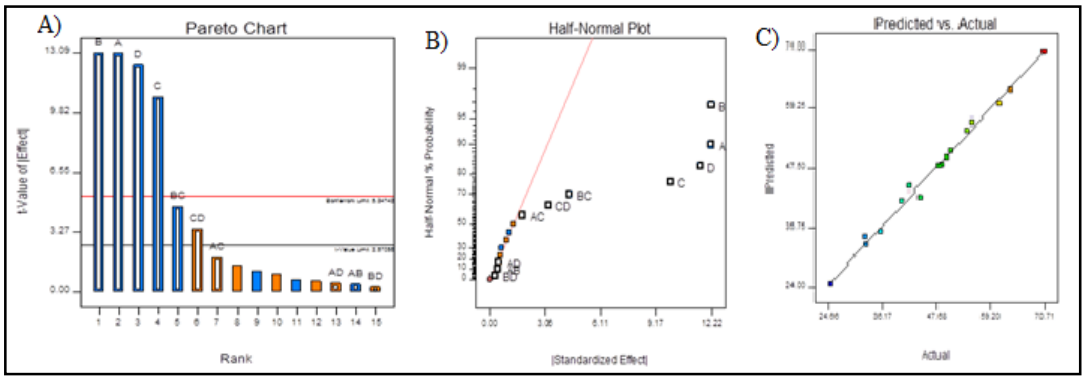


Fig. 6.6 A) Pareto chart B) Half Normal plot C) Predicted Vs Actual plot

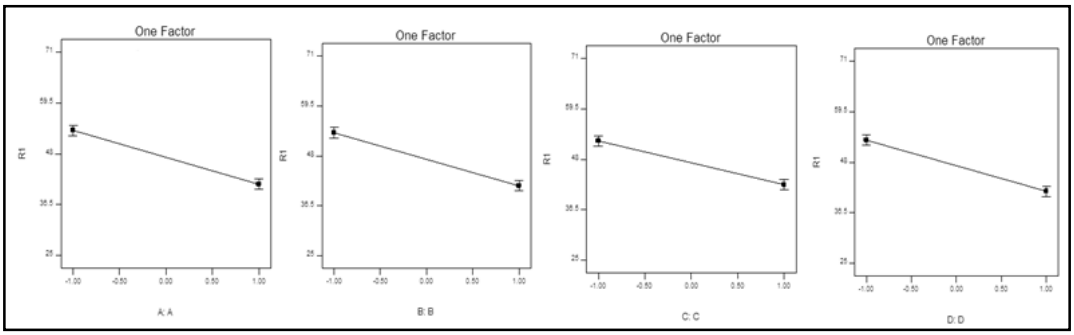


Fig. 6.7 Main effect plots of the response EC_{50}

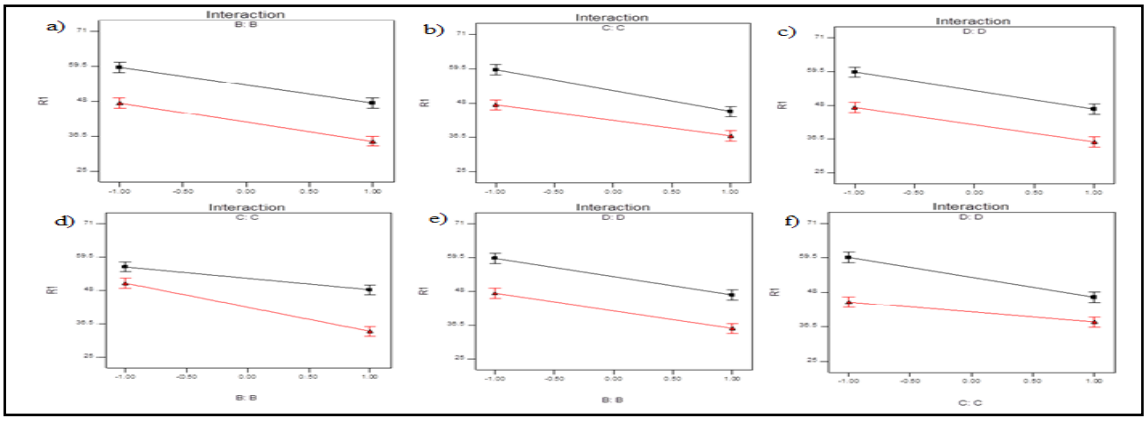


Fig. 6.8 Interactions plots of the response EC_{50}

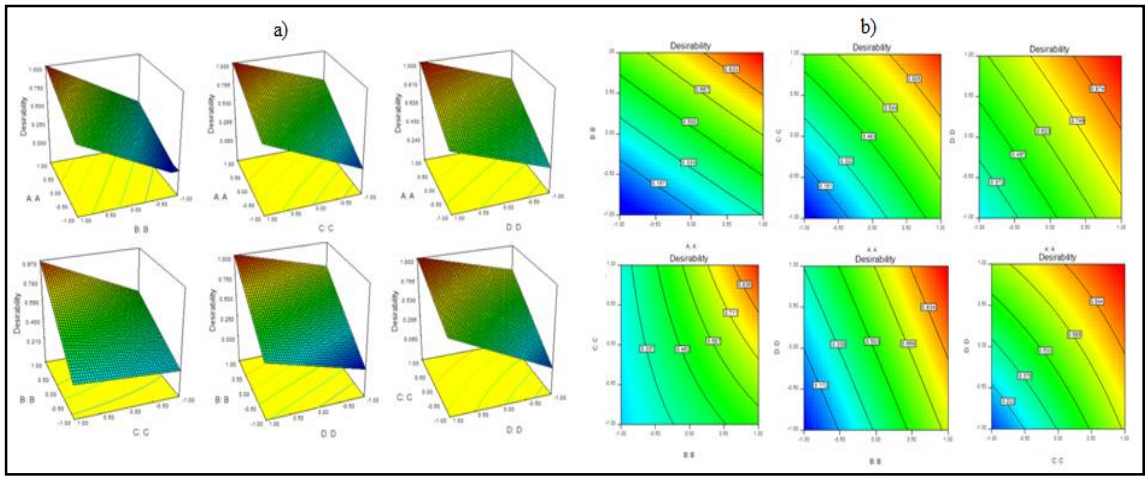


Fig.6.9. a) 3D Desirability plots b) Desirability contour plots

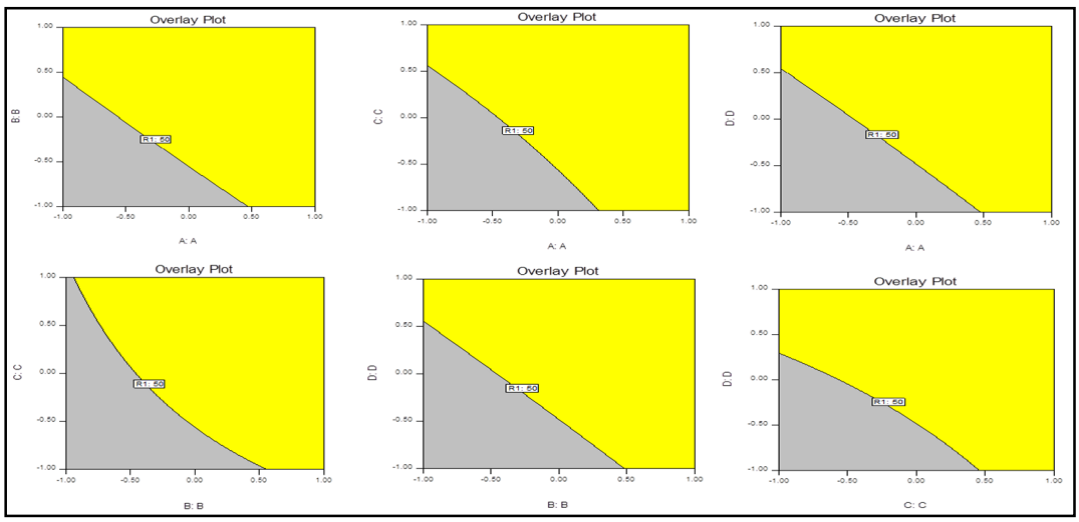


Fig. 6.10 Overlay plots showing optimized area (yellow colour)

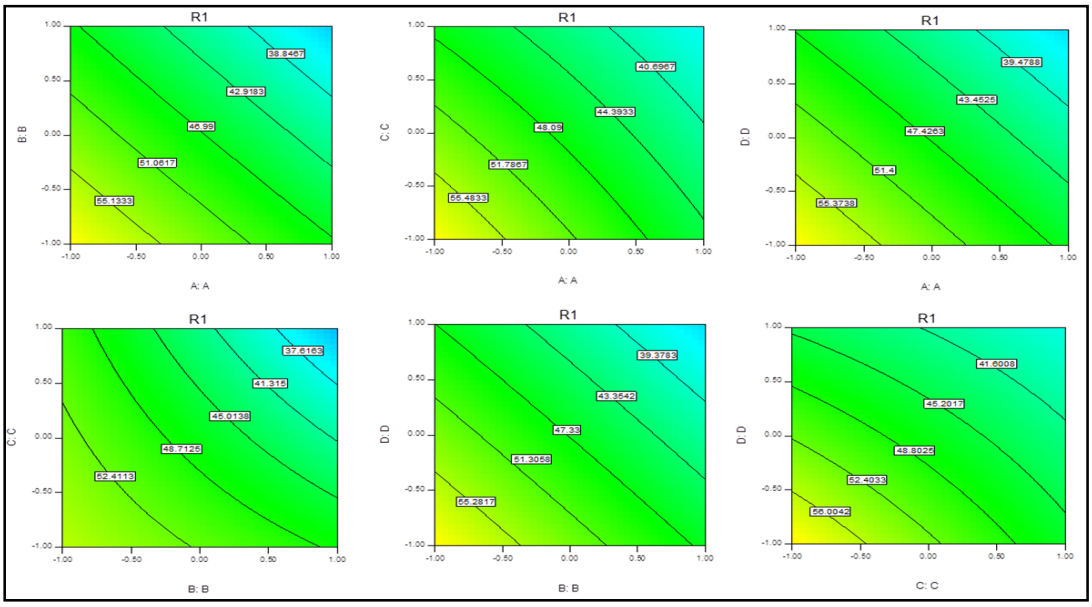


Fig. 6.11 Contour plots of response (R1) EC_{50}

6.4.1.2) 2x3 full factorial design

The matrix of the experiments and results of the measured response are depicted in the table 6.8. Results of the measured response EC_{50} is depicted in Table 6.9. ANOVA results and regression coefficients of response variables are shown in Table 6.10.

The results were analyzed using the software Design Expert 8.0. The coded mathematical model and observed EC_{50} values for 2x3 factorial designs can be given as:

$$EC_{50} = 45.64 – 9.12*A – 11.71*B + 7.07*C \quad + 0.21*A*B – 0.27*A*C – 1.63*B*C$$

Where R (%) is the effective concentration (EC_{50})

The results indicated that dose of the factors A, B and C were the influencing factors affecting positively to achieve desired EC_{50} value. The same can be inferred from the pareto chart, half normal chart and predicted vs. actual chart. (Fig. 6.12) The main effects plot and interaction between two factors are shown in the Fig 6.13 and 6.14 respectively. The desirability charts are given in the figure 6.15.

The overlay plots shown the optimized area (Fig. 6.16) and contour plots for the response EC_{50} (Fig. 6.17)

From 2x3 full factorial designs, we selected one batch (no. 8) which had lowest EC_{50} value for further studies

Table 6.8: Matrix of the experiments for 2x3 full factorial design and results for the measured response

Run no.	Coded values of independent variables			EC_{50}* (Observed)
	A	B	C	
1	-1	1	1	48.86 ± 1.2
2	-1	1	-1	36.81 ± 1.34
3	1	-1	-1	39.28 ± 1.5
4	-1	-1	1	75.34 ± 1.26
5	1	-1	1	56.75 ± 1.61
6	-1	-1	-1	58.01 ± 1.33
7	1	1	1	29.88 ± 0.97
8	**1**	**1**	**-1**	**20.16 ± 1.17**

*Mean ± SD (n=6)

Regression coefficients are in coded value.

Table 6.9: Regression analysis results

Source	sum of squares	Mean sum of squares	Coefficients	F value	P value
A	1	872.28	-9.12	872.28	0.0215*
B	1	1438.16	-11.71	1438.16	0.0168 *
C	1	524.54	7.07	524.54	0.0278*
AB	1	0.47	0.21	0.47	0.618
AC	1	0.79	-0.27	0.79	0.5382
BC	1	27.83	-1.63	27.83	0.1193

Regression coefficients are in coded value.

a Statistically significant ($p < 0.05$)

Table 6.10: ANOVA results showing the effect of independent variables on the measured responses

Model	Full model
sum of squares	2184.17
Mean sum of squares	364.03
F value	477.34
P value	0.035
R-squared	0.9997
Adj R-squared	0.9976
Pred R-squared	0.9777

Table 6.11: Comparison of responses between predicted and experimental values for the cross validation set

Response	Test	Factors/Levels			Experimental values	Predicted Values	Residuals
		A	B	C			
EC_{50}	1	-1	-0.6	-0.6	38.41	39.6	1.19
	2	-0.6	0.0	0.4	52.19	53.05	0.86
	3	-0.4	0.6	0.0	57.08	56.2	0.88
	4	0.0	-0.4	0.6	67.12	67.69	0.57
	5	0.5	0.5	-0.5	50.87	51.43	0.56

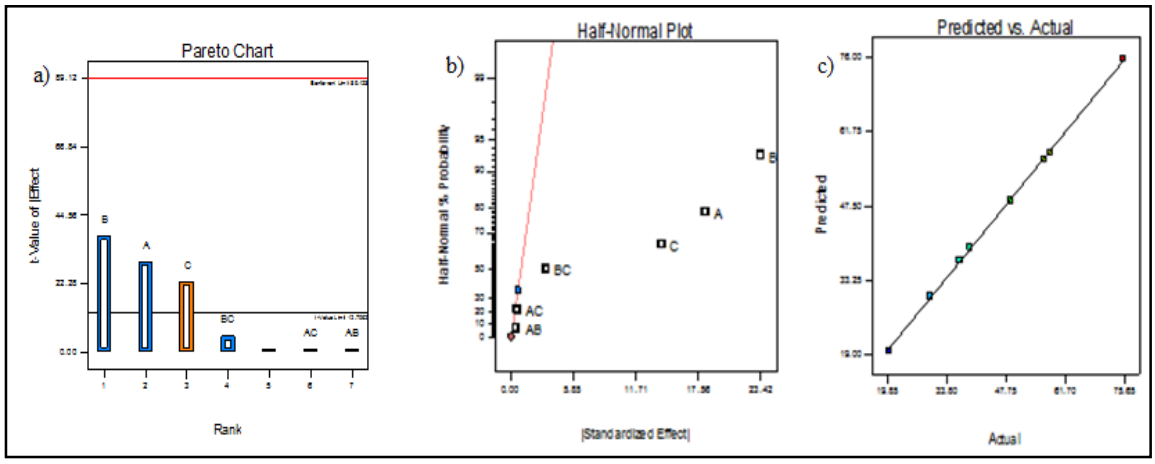


Fig. 6.12 (a) Pareto chart (b) Half normal plot (c) Predicted Vs Actual plot

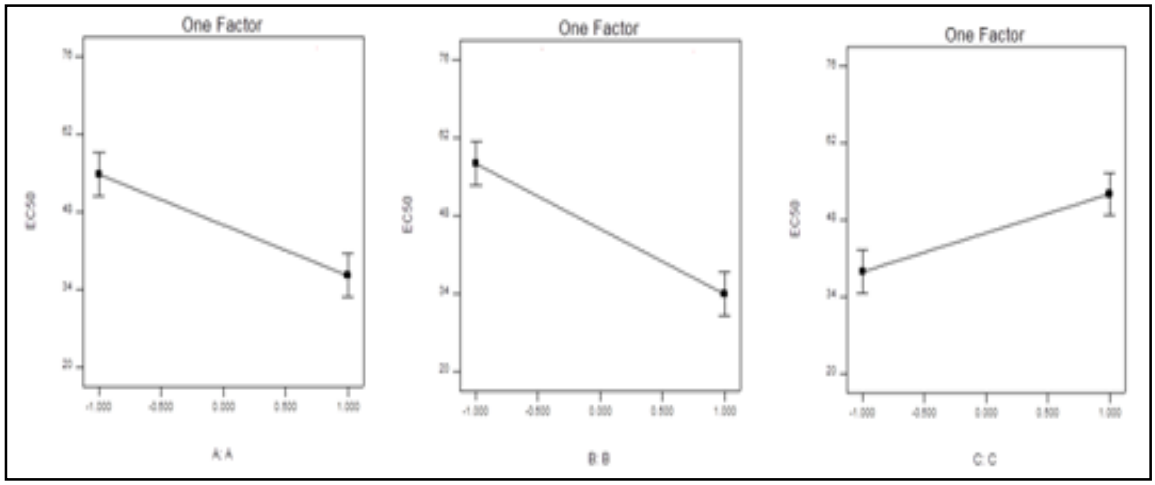


Fig. 6.13 Main effect plots of the response EC_{50}

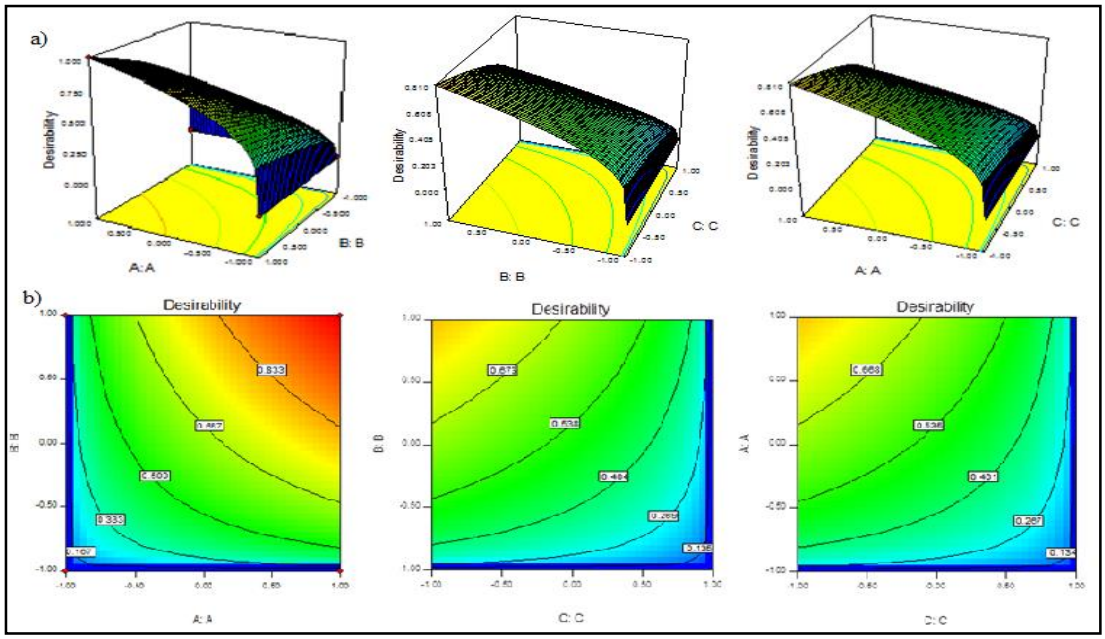


Fig.6.14. a) 3D Desirability plots b) Desirability contour plots

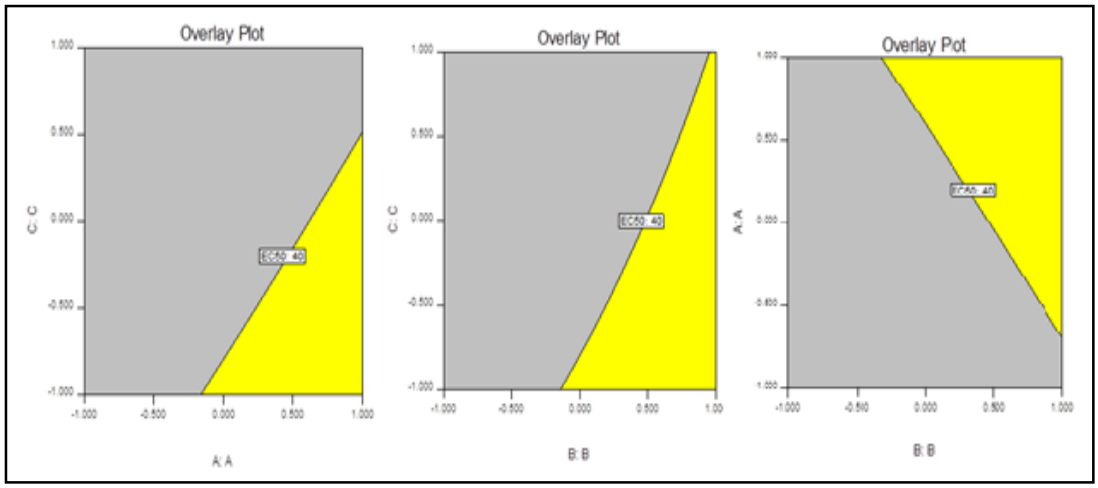


Fig. 6.15 Overlay plots showing optimized area (yellow colour)

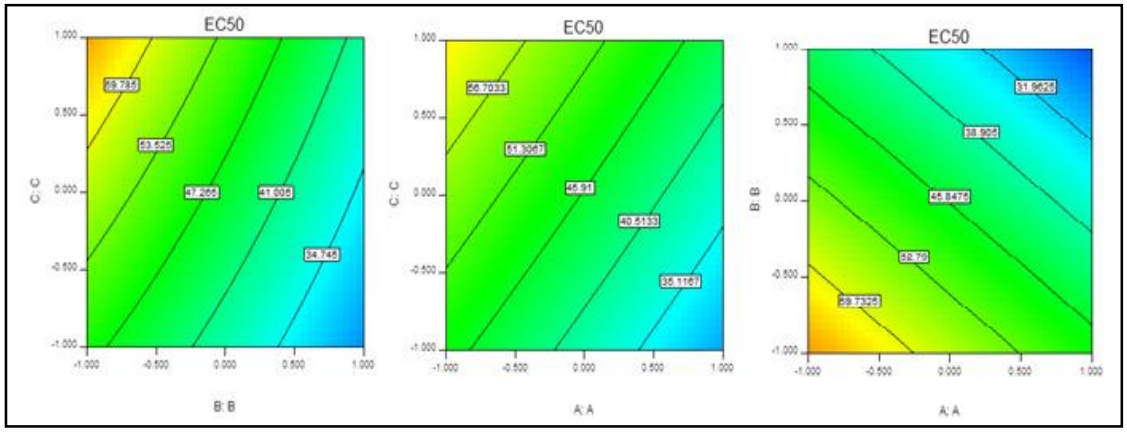


Fig. 6.16 Contour plots of response (R1) EC_{50}

6.4.2) Development of Dosage form

Considering the results of factorial studies, three formulations (Form A, B and C) were selected for further studies. The ratio of all three herbal formulations is shown in Table 6.12.

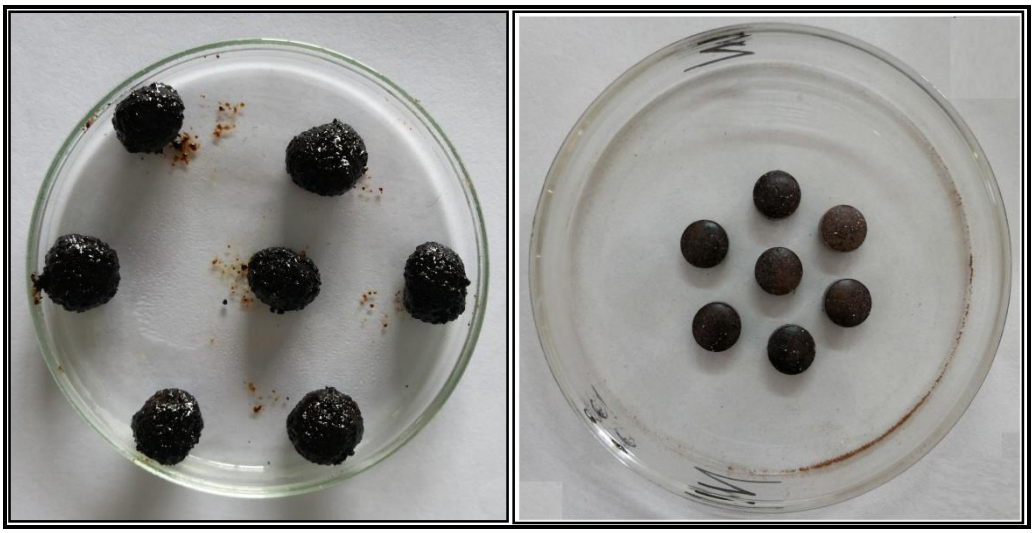
a) Vati: The manually prepared vati are round in shape, brownish black in colour and texture was somewhat sticky. The photo of vati is shown in the fig. 6.17(a)

b) Tablets: Tablets were prepared by using direct compression method. The colour of the tablets is dark brown in colour and texture of the tablet was very smooth. The photo of tablets is shown in the fig 6.18 (b). The plant dose of Form A, Form B and Form C are shown in Table 6.12. The selected excipients such as dibasic calcium phosphate (DCP) - 50mg, micro crystalline cellulose (MCC) - 40mg, polyethylene glycol (PEG) 4000 - 8mg, magnesium stearate - 2mg and methyl paraben-1% were used in all three formulations.

Table 6.12 Plant dose of different formulations

Formulation	Dose (mg)			
	G. glabra	***N. nucifera***	***P. vulgaris***	***Z. jujuba***
FORM A	100	100	30	100
FORM B	100	100	100	100
FORM C	100	100	-	30

The evaluation parameters of all three tablet formulations are mentioned below.



(a) Vati **(b) Tablets**

Fig. 6.17 (a) Photograph of vati (b) Photograph of tablets

6.4.3) Physical Standardization of the developed polyherbal tablet formulation

6.4.3.1) Determination of Physical properties as per IP

The developed polyherbal tablets fulfilled the official tests for uniformity of weight. Other physiochemical properties such as, hardness, thickness, diameter, and friability results were determined and presented in Table 6.13.

Table 6.13 Results of Official tests

Sr. No.	Parameters	Form A	Form B	Form C
1.	Uniformity of Weight (mg ± SD)	430 ± 2.41	500 ±3.78	330 ± 2.63
2.	Disintegration test, minutes (n=10)	10 ± 0.5	7.6 ± 0.3	8.1 ± 0.2
3.	Hardness, kg/cm^2 (n=10)	7.3 ± 0.4	7.1 ± 0.2	6.5 ± 0.2
4.	Diameter, mm (n=10)	8.0 ± 0.1	8.2 ± 0.07	7.95 ± 0.04
5.	Thickness, mm (n=10)	3.95 ± 0.5	4.1 ± 0.05	3.5 ± 0.3
6.	Friability, %	0.69 ± 0.7	1.1 ± 0.28	0.75 ± 0.4

The results obtained for the official tests showed that the physical properties of the formulation were within the limit according to I.P.

6.4.3.2) Determination of Ash

The Table 6.14 shows the ash values of the tablet formulations.

Table 6.14 Ash values of the tablet formulation (n=3)

Sample	Total ash	Acid insoluble ash	Water soluble ash
Form A	10.5 ± 0.55	7.1 ± 0.72	6.5 ± 1.09
Form B	12.4 ± 0.64	7.2 ± 0.87	6.8 ± 1.21
Form C	9.6 ± 0.41	6.97 ± 0.46	6.4 ± 1.05

The low values signify presence of lower quantities of inorganic contents. High ash values prove to be toxic in nature. Thus, the ash value should be determined to perform the preliminary standardization. **The ash values of all three selected herbal formulations are within the limit in the tablet formulation.**

6.5.3.3) Determination of extractable matter

Coarse powder of the formulations was extracted with methanol and water in a soxhlet extractor; separately, the extract was concentrated and dried under vacuum. The extractive values of the formulations in the methanol and water have been given in Table 6.15.

Table 6.15 Extractive values of formulation (n=3)

Sample	Water soluble extractive value	Alcohol soluble extractive Value
Form A	23.67 ± 01.56	24.97 ± 1.67
Form B	26.45 ± 1.90	31.07 ± 1.34
Form C	21.76 ± 1.77	21.55 ± 1.08

The extractive values of all three herbal formulations were found to be higher depicting the presence of phytochemicals in the particular solvents in higher quantity.

6.4.3.4) Determination of water and volatile matter

The % loss on drying of the formulations is given in Table 6.16. The values comply with those of the limits prescribed in the standard monographs.

Table 6.16 % Loss on drying of formulation (n=3)

Sample	% Loss on Drying
Form A	1.89 ± 0.08
Form B	2.15 ± 0.17
Form C	1.74 ± 0.14

6.4.3.5) Determination of pesticide residues

The TLC studies were performed for detection of possible organochlorinated pesticides and organophosphorus pesticides. The results of the analysis of pesticide residues are shown in Table 6.17.

Table 6.17 Analysis for Pesticide residues in formulations

Samples	Organochlorinated Pesticides		Organophosphorus pesticides	
	A	B	C	D
Form A	-ve	-ve	-ve	-ve
Form B	-ve	-ve	-ve	-ve
Form C	-ve	-ve	-ve	-ve

A- Solvent system: DMF: Ether (4:6), Detecting Reagent: Tetrabromophenolphthalein.

B- Solvent system: Methylcyclohexane, Detecting Reagent: Tetrabromophenolphthalein .

C- Solvent system: DMF: Ether (4:6), Detecting Reagent: 0.5 % Silver nitrate in water an acetone.

D- Solvent system: DMF: Ether (4:6), Detecting Reagent: 0.5 %Silver nitrate in water and acetone.

It should become mandatory for the manufacturers to perform the tests for pesticides residues.

The three developed herbal formulations did not show the presence of any pesticide residues.

6.4.3.6) Determination of heavy metals

Elemental analysis of all three herbal formulations was done by Atomic absorption spectrophotometer where cadmium, arsenic, lead were found to be within the limit and the mercury is absent. (Table.6.18)

Table 6.18 Heavy metal analysis of formulation

Sample	*Cadmium (not more than 0.30 ppm)*	*Arsenic (not more than 10 ppm)*	*Lead (not more than 10 ppm)*	*Mercury (not more than 1 ppm)*
Form A	0.185	0.55	4.10	Absent
Form B	0.217	0.78	4.12	Absent
Form C	0.156	0.49	3.76	Absent

The heavy metal content of all three herbal formulations was within acceptable limits metals as per the WHO guidelines.

6.4.3.7) Determination of microorganisms

The analysis of bio-burden present in the formulations was performed and all the results were within the limits prescribed in the WHO guidelines (Table 6.19).

Table 6.19 Results of the tests performed for the microbial contamination in the formulations

Parameters	**Form A**	**Form B**	**Form C**
Total bacterial count	> 10,000 cfu/gm	> 10,000 cfu/gm	> 10,000 cfu/gm
E.coli	Absent	Absent	Absent
Salmonella. Typhii	Absent	Absent	Absent
S,aureus	Absent	Absent	Absent
Pseudomonas aeruginosa	Absent	Absent	Absent
Yeast and mould	Absent	Absent	Absent

Microbial bioburden should be tested to determine the safety of the formulations. The natural organic components present in the formulation lead to presence of high microbial contamination. Thus, the test becomes important for the quality control of the formulation.

6.4.3.8) Qualitative Phytochemical Screening

The phytochemical screening of the formulation was done by qualitative chemical tests and TLC studies. Qualitative chemical tests were performed for water extract of the formulation. The water extract of tablet formulations showed the presence of alkaloids, anthranol glycosides, saponins, phenolics, tannins, proteins and amino acids (Table 6.20).

Table 6.20 Qualitative chemical tests of Methanol extract of formulation

Sr. No.	Class of Compounds	Form A	Form B	Form C
1	Carbohydrates	+ve	+ve	+ve
2.	Alkaloids	+ve	+ve	+ve
3.	Glycosides			
	Anthranol	+ve	+ve	+ve
	Cardiac	-ve	-ve	-ve
4.	Saponins			
	Triterpenoids	+ve	+ve	+ve
	Steroidal	+ve	+ve	+ve
5.	Phytosterols	-ve	-ve	-ve
6.	Fixed oils and fats	-ve	-ve	-ve
7.	Phenolics and tannins	+ve	+ve	+ve
8.	Proteins and amino acid	+ve	+ve	+ve
9.	Flavonoids	+ve	+ve	+ve

6.4.3.9) TLC studies of water extract of tablet formulation

Glycosides, alkaloids, saponins, flavonoids and essential oils were found to be present in the water extracts. The results along with the inferences have been shown in Table 6.21.

Table 6.21 TLC studies of Methanol extract of formulation

Sr. No.	Class of Compounds	Form A	Form B	Form C	Inferences
1.	Anthraglycosides	black	black	black	Present
2.	Alkaloids	Dark reddish orange	Dark reddish orange	Dark orange	Present
3.	Favonoids	yellow	yellow	yellow	Present
4.	Bitter principles	No spot	No spot	No spot	Absent
5.	Saponins	violet	violet	violet	Present
6.	Essential oils	violet	violet	violet	Present

The qualitative tests showed the presence of anthranol glycosides, saponins, alkaloids, flavonoids, tannins, amino acids, proteins and essential oils. The qualitative tests are important to determine the presence of the active ingredients in the polyherbal formulations. They form an important standardization parameter for the poly herbal formulations where a large number of ingredients are present.

6.4.4) Accelerated stability studies of developed formulation according to WHO guidelines

All three optimized formulations (Form A, B and C) of the drug were stable for 3 months when it is subjected to accelerated stability studies at specified conditions of temperature and relative humidity. The data of three months for different parameters are summarized in table 6.22.

Table 6.22 Accelerated stability studies of developed polyherbal formulations

Sr. No.	Parameters	Formulation	Observations						
			Initial	I st month		II nd month		III rd month	
				RT	40ºC	RT	40ºC	RT	40ºC
1.	Nature	A	Compact solid	Compact solid	Compact solid	Compact solid	Compact solid	Compact solid	Compact solid
		B							
		C							
2.	Colour	A	Dark blackish	Dark black	Dark blackis	Dark blackis	Dark blackis	Dark blackis	Dark blackis
		B							

		C	brown	ish brow n	h brown	h brown	h brown	h brown	h brown
3.	Odour	A	Charact eristic	Char acteri stic	Charac teristic	Charac teristic	Charac teristic	Charac teristic	Charac teristic
		B							
		C							
4.	Textur e	A	Smooth	Smo oth	Smoot h	Smoot h	Smoot h	Smoot h	Smoot h
		B							
		C							
5.	Hardne ss (kg/cm ^{2})	A	7.3	7.2	7.2	7.2	7.1	7.2	7.1
		B	7.1	7.0	7.2	6.9	7.2	7.1	7.0
		C	6.5	6.5	6.4	6.4	6.6	6.5	6.6
6.	Friabili ty (%)	A	0.69	0.69	0.70	0.68	0.69	0.71	0.70
		B	1.1	1.0	1.1	1.0	0.9	1.2	1.0
		C	0.75	0.75	0.74	0.75	0.76	0.75	0.76
7.	Disinte gration Time (D.T) in min.	A	10.0	9.8	9.9	10.0	9.8	9.9	9.8
		B	7.6	7.8	7.5	7.8	7.4	7.7	7.6
		C	8.1	8.1	8.0	8.2	8.2	8.1	8.2

The developed herbal formulations A, B and C were found to be stable up to three months.

6.4.5) BIOLOGICAL EVALUATION OF FORMULATION

6.4.5.1) Acute oral toxicity study

Toxicity was performed on the individual extracts of all four plants and also in developed polyherbal formulations (FORM A, FORM B and FORM C) as per OECD guidelines in albino rats. Animals when administered with single oral dose of 2000mg/kg body weight did not show any toxicity or mortality. There were also no abnormal behavioural changes, , salivation or food aversion and diarrhoea. There was no major change in gross weight of animals. Hence considering this the safe dose, the formulations were screened for immunomodulatory activity.

6.4.5.2) *In-vivo* Immunomodulatory Activity

In-vivo immunomodulatory activity of developed polyherbal formulations were performed using four different animal models as described below. Immunomodulatory activity was proved with DTH response, humoral antibody titre, phagocytic function and cyclophosphamide induced myelosupression models.

6.4.5.2.1) Cyclophosphamide induced myelosupression assay

Cyclophosphamide induced myelosupression assay was performed using albino rats. The total leucocyte count of FORM A, FORM B and FORM C were increased from 7.93 ± 0.52 to 9.8 ± 1.2 on pretreatment for 10 days and 6.0 ± 0.2 on 14th day of FORM B and 6.3 ± 0.5 to 9.7 ± 1.3 and 4.63 ± 0.84 of FORM A and 9.66 ± 1.17 to 11.83 ± 1.2 and 7.33 ± 0.64 of FORM C on 0, 10th and 14th days respectively. No significant changes are observed with respect to RBC and hemoglobin values.

Thus, it can be established from the study that developed Polyherbal formulations possess the ability to counteract the myeosupressive effects of cytotoxic drug, cyclophosphamide by stimulating the bone marrow activity. Among three formulations, Form B showed better activity compared to Form A and C.

Table 6.23 Effect of polyherbal formulations on leucocyte (WBC), RBC and HB count in cyclophosphamide induced immunosupression

GROUP	TOTAL LEUCOCYTE 10^3 cells/mm^3 MEAN ± SEM			RBC COUNT 10^6 cells/mm^3 MEAN ± SEM			HAEMOGLOBIN (g%) MEAN ± SEM		
	0 DAY	10th DAY	14th DAY	0 DAY	10th DAY	14th DAY	0 DAY	10th DAY	14th DAY
Control	7.50 ± 1.03	8.1 ± 1.02	8.3 ± 1.05	8.81 ± 0.12	9.11 ± 0.17	9.23 ± 0.52	15.1 ± 0.45	14.3 ± 0.42	14.8 ± 1.2
CPM	7.46 ± 0.27	7.7 ± 0.35	1.53 ± 0.23	8.99 ± 0.31	8.39n ± 0.48	7.95 ± 0.42	15.75 ± 0.29	15.36 ± 0.48	14.43 ± 0.65

Form A	6.3 ± 0.5	9.7 ± 1.3	4.63 ± 0.84	9.28 ± 0.51	8.52 ± 0.36	8.79 ± 0.31	15.73 ± 0.78	14.56 ± 0.24	13.2 ± 1.65
Form B	**7.93 ± 0.52**	**9.8 ± 1.2**	**6.0 ± 0.2**	9.94 ± 0.3	9.43 ± 0.22	7.61 ± 0.19	16.73 ± 0.81	16.26 ± 0.44	13.26 ± 0.24
Form C	9.66 ± 1.17	11.83 ± 1.2	7.33 ± 0.64	9.78 ± 0.29	9.84 ± 0.37	7.70 ± 0.31	15.73 ± 0.35	16.23 ± 0.38	14.06 ± 0.60
Levamisol	7.53 ± 0.35	8.67 ± 1.10	8.30 ± 1.73	8.56 ± 0.66	9.17 ± 0.84	9.07 ± 0.70	14.67 ± 0.78	15.58 ± 0.78	15.93 ± 1.37

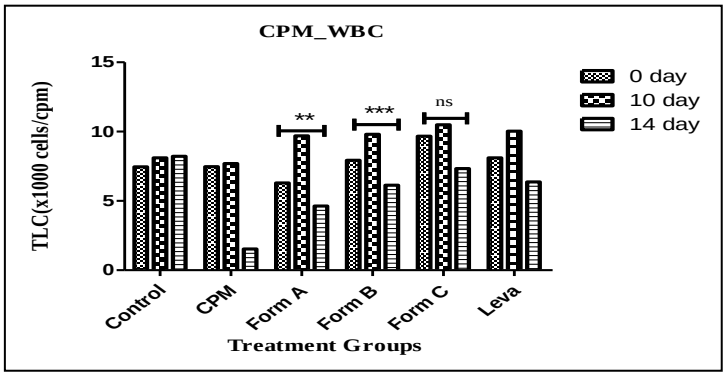


Fig 6.18 Comparison of treatment groups for cyclophosphamide induced myelosupressive effect

6.4.5.2.2) Carbon clearance assay in mice

The developed Polyherbal formulations were found to stimulate the phagocytic activity of the macrophage as evidenced by an increase in the rate of carbon clearance 0.423 ± .0066, 0.713 ± 0.0049 and 0.402 ± 0.0078 of FORM A, FORM B and FORM C respectively. The result shows the stimulatory effect of the developed polyherbal formulation on the mononuclear cells of the phagocytic system (Table 6.24 and Fig 6.20). **The Form B is having high rate of carbon clearance as compared to Form A and C. So, the Form B is better than other two.**

6.4.5.2.3) Delayed hypersensitivity response in rats

DTH response of normal control group was 0.0015 ± 0.16 while that of FORM A, FORM B and FORM C 0.96 ± 0.014, 0.17 ± 0.37 and 0.083 ± 0.45 respectively.

Increased in DTH response as evidenced by increased in paw thickness in rats revealed stimulatory effect of developed PHF on T cells as well as accessory cell types which required for the expression of reaction (Table 6.24 and Fig 6.21). **The Form B is having higher DTH response as compared to Form A and Form C. So, the Form B is better than other two.**

6.4.5.2.4) Humoral Antibody titre

The index of humoral response is the increase in antibody titer value due to increase in immune response. Further, antibody functions as the effectors of the humoral response. They produced the response by binding to antigen by neutralizations. It also facilitating its exclusions by cross linking to form clusters which are more easily ingested by phagocytic cells. HA titre of FORM A, FORM B and FORM C are 238.00 ± 0.17, 460.00 ± 0.87 and 324 ± 1.12 respectively was found effective in increasing HA titre value indicating the increased responsiveness of macrophages T and lymphocytes B that are associated with the antibody production. (Table 6.24 and Fig 6.22)

FORM B showed more agglutination compared to that of FORM A and FORM C.

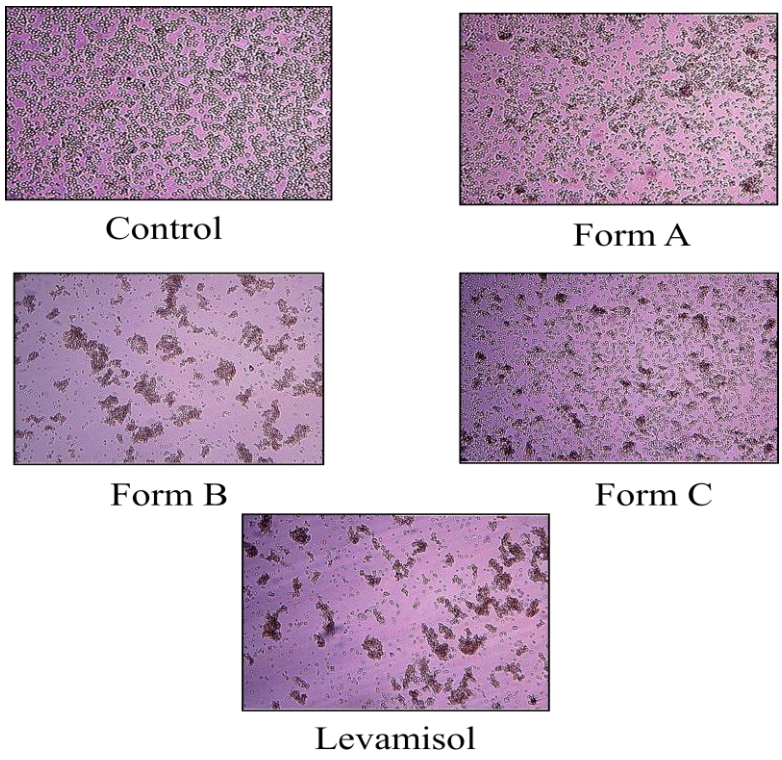


Fig. 6.19 Agglutination in different treatment groups

Table 6.24 Effect of polyherbal formulations on HA titres, phagocytic index and DTH response

Group	HA titres	Phagocytic Index	DTH Response	
	Mean ± SEM	Mean ± SEM	Mean ± SEM 24 hr	Mean ± SEM 48 hr
Control	85.33 ± 1.18	-	0.12 ± 0.009	0.0015 ± 0.16
CPM	62.00 ± 1.92	0.0293 ± 0.007	0.15 ± .005	0.0033 ± 0.012
Form A	238.00 ± 0.17	0.0423 ± 0.0066	0.22 ± 0.008	0.096 ± 0.014
Form B	**460.00 ± 0.87**	**0.0713 ± 0.0049**	**0.34 ± 0.017**	**0.17 ± 0.037**
Form C	324.00 ± 1.12	0.0402 ± 0.0078	0.17 ± 0.29	0.083 ± 0.045
Levamisol	480.67 ± 1.08	0.078 ± 0.007	0.95 ± 0.012	0.77 ± 0.033

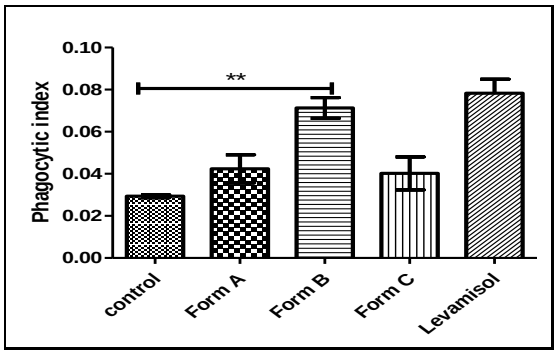


Fig. 6.20 Carbon clearance assay in mice

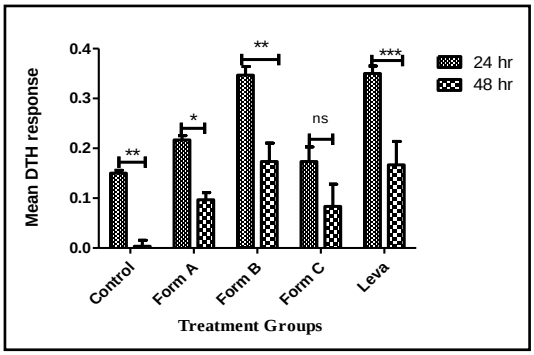


Fig.6.21 DTH response in rats

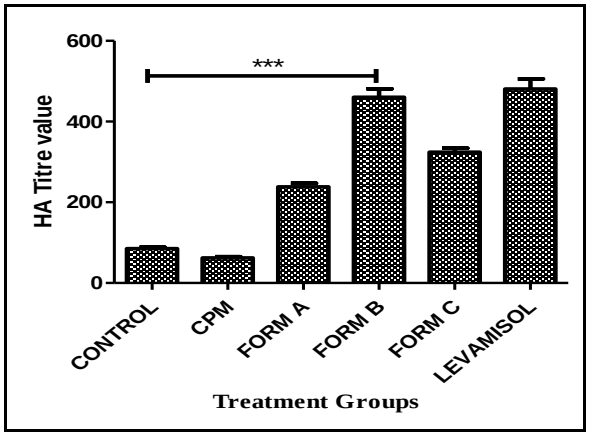


Fig.6.22 Humoral antibody titre in rats

6.4.5.3) *IN-VITRO* STUDIES:

6.4.5.3.1) Analysis of cell viability (MTT assay)

In recent times *in vitro* cytotoxicity of plant extracts and herbal products has gained importance for primary level screening. Prior to the therapeutic use of any herbal products or herbal extracts, it is imperative to perform a cytotoxicity assay. This is because crude herbs or extracts of many herbs have been shown to be non toxic but some of its bioassay of guided fractions may show toxicity. Isolated healthy human neutrophils from blood are used to check the cell viability. Cytotoxicity evaluation on all three formulations (FORM A, FORM B and FORM C) was performed in the dose range of 10-2000 µg/ml. The dose up to 200 µg/ml showed more than 95 % cell viability after that cell viability is decreases. So, selected dose for *in vitro* immunomodulatory activity are 50, 100 and 200µg/ml. The results of all three formulations are shown below.

Table 6.25 Effect of Formulation A on percentage cell viability

FORMULATION A (µg/ml)	*MEAN ABS.± SEM	*% CELL VIABILITY ± SEM
A 10	0.130 ± 0.0023	97.67 ± 4.09
A 30	0.137 ± 0.0012	100.55 ± 0.85
A 50	0.136 ± 0.0023	99.82 ± 1.69
A 60	0.132 ± 0.0035	97.13 ± 2.55
A 80	0.132 ± 0.0029	97.13 ± 2.13
A 100	0.136 ± 0.0003	100.06 ± 0.24
A 200	0.133 ± 0.002	97.37 ± 2.00
A 400	0.125 ± 0.0027	91.99 ± 1.60
A 600	0.127 ± 0.0022	92.97 ± 2.54
A 800	0.128 ± 0.0018	93.94 ± 2.54
A 1000	0.127 ± 0.0015	93.21 ± 2.78
A 1500	0.116 ± 0.0026	85.38 ± 1.91
A 2000	0.120 ± 0.0035	87.83 ± 2.59

Table 6.26 Effect of Formulation B on percentage cell viability

FORMULATION B (µg/ml)	*MEAN ABS.± SEM	*% CELL VIABILITY ± SEM
B 10	0.134 ± 0.0026	98.35 ± 1.94
B 30	0.134 ± 0.0029	98.59 ± 2.13
B 50	0.133 ± 0.0035	97.61 ± 2.58
B 60	0.133 ± 0.0041	97.37 ± 2.98
B 80	0.135 ± 0.0035	98.84 ± 2.55
B 100	0.135 ± 0.0026	99.33 ± 1.91
B 200	0.132 ± 0.0049	97.13 ± 3.60
B 400	0.119 ± 0.0027	87.07 ± 1.96
B 600	0.124 ± 0.0033	90.76 ± 2.45
B 800	0.126 ± 0.0043	92.23 ± 3.18
B 1000	0.125 ± 0.0021	91.74 ± 1.53
B 1500	0.122 ± 0.0046	89.54 ± 3.36
B 2000	0.115 ± 0.004	84.40 ± 2.97

Table 6.27 Effect of Formulation C on percentage cell viability

FORMULATION (µg/ml)	*MEAN ABS.± SEM	*% CELL VIABILITY ± SEM
C 10	0.136 ± 0.0062	99.57 ± 4.53
C 30	0.134 ± 0.0032	98.59 ± 2.33
C 50	0.133 ± 0.0024	97.37 ± 1.76
C 60	0.133 ± 0.0029	97.61 ± 2.12
C 80	0.133 ± 0.0024	97.37 ± 1.76
C 100	0.135 ± 0.0029	99.33 ± 2.13
C 200	0.136 ± 0.0029	100.06 ± 2.13
C 400	0.133 ± 0.0019	97.37 ± 1.36
C 600	0.131 ± 0.0044	95.90 ± 3.24
C 800	0.124 ± 0.0036	91.01 ± 2.65
C 1000	0.124 ± 0.0027	90.76 ± 2.00
C 1500	0.120 ± 0.0044	88.07 ± 3.20
C 2000	0.115 ± 0.0058	84.65 ± 4.24

*Values are expressed as mean ± SEM of % cell viability and are average of three determinations.

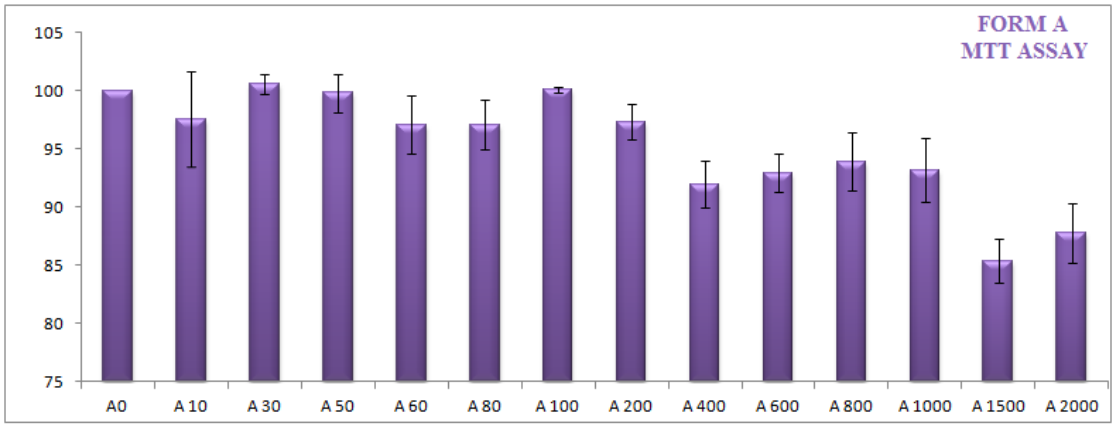


Fig. 6.23 Cytotoxicity assessment of Formulation A

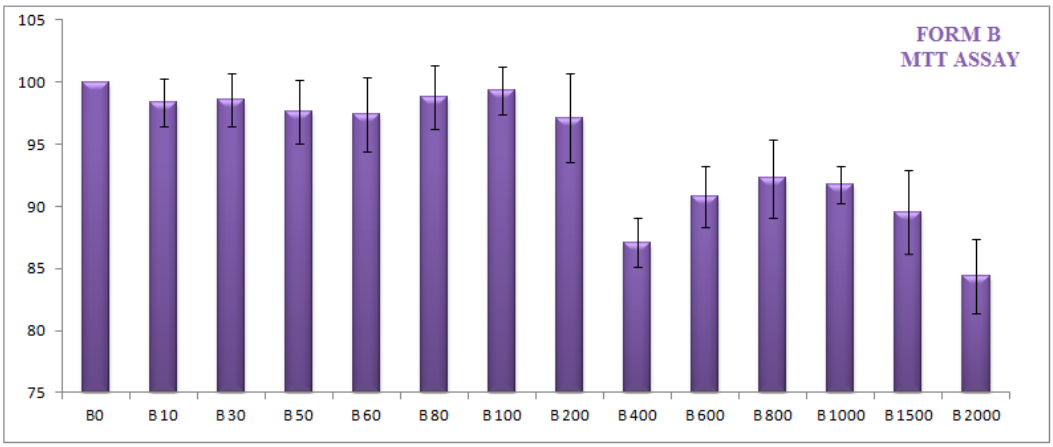


Fig. 6.24 Cytotoxicity assessment of Formulation B

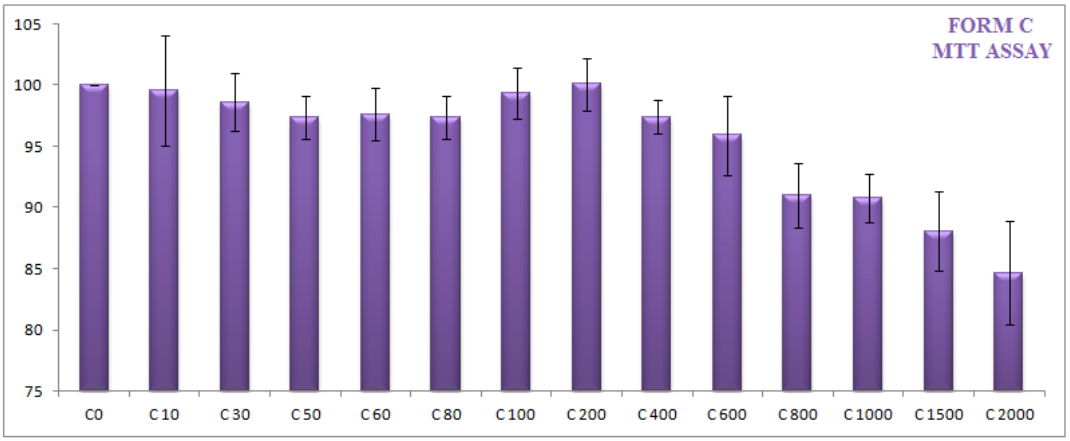


Fig. 6.25 Cytotoxicity assessment of Formulation C

6.4.5.3.2) Nitroblue tetrazolium (NBT) test

The NBT dye reduction test was performed to determine the phagocytic activity and neutrophil functionality necessary for microbicidal activity. Neutrophils taken up the dye by phagocytosis which is then reduced by stimulation of the hexose monophosphate shunt pathway (HMP) of glucose oxidation and associated modifications in oxidative metabolism. Developed polyherbal formulations (FORM A, FORM B and FORM C) significantly enhanced the intrcellar killing property of stimulated human neutrophils. So, it can be concluded that developed polyherbal formulations may contain some chemical constituents which are liable to intracellar killing of human neutrophils greater than degranulation (Table 6.28 and Fig 6.26, 6.27).

The Form B contains more deposits of black formazon i.e. reduced NBT dye compared to Form A and C. The cells contain black colour material more than granules are normally showing in neutrophils. So, the Form B is better compared to Form A and C.

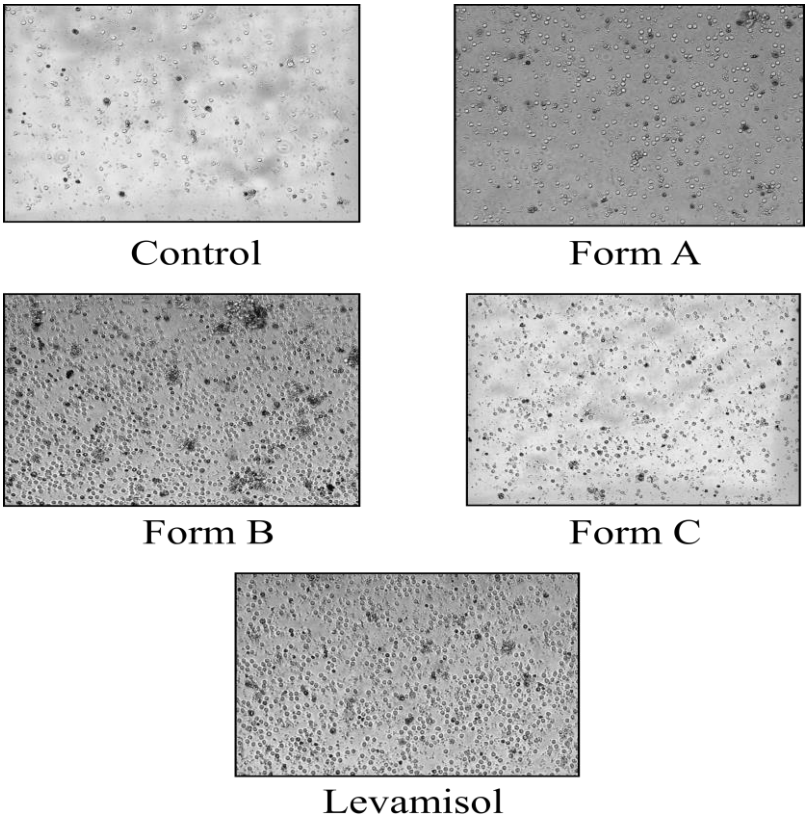


Fig 6.26 Black formazon in different treatment groups

Table 6.28 Effect of different treatment groups on NBT positive neutrophils (% and range)

Groups	Treatment (µg/ml)	Mean (and range) NBT-positive neutrophils per cmm	
		NBT-positive neutrophils (%)	NBT-positive neutrophils (range)
I	Control	26.5 ± 1.73	25-31
II	FORM A 50	40.00 ± 3.79	37-50
III	FORM A 100	56.67 ± 5.93	44-64
IV	FORM A 200	75.83 ± 2.60	71-80
V	FORM B 50	58.50 ± 4.04	48-61
VI	FORM B 100	98.33 ± 5.21	94-112
VII	FORM B 200	83.00 ± 8.39	76-105
VIII	FORM C 50	50.33 ± 2.91	48-58
IX	FORM C 100	65.17 ± 2.33	59-67
X	FORM C 200	88.50 ± 3.79	85-98
XI	Levamisol 50	106.33 ± 5.78	97-117

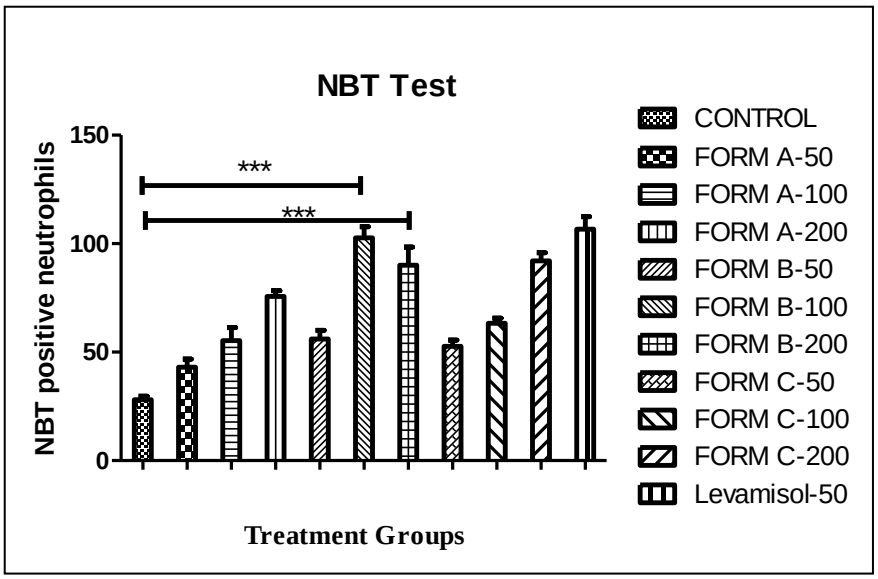


Fig 6.27 Comparison of different treatment groups for NBT positive neutrophils

6.4.5.3.3) Neutrophils candidacidal assay

In the *in vitro* neutrophils candidacidal assay, *C. albicans*, foreign organisms or particles actively engulfed by polymorpho nuclear (PMN) cells which are present in the assay medium. Intensity of phagocytic activity is related to the average count of

candida cells ingested and associated with each PMN cells. The results concluded the increase in candidacidal activity of all three developed polyherbal formulations.

The Form B showed more unstained i.e. viable *candida* cells and they are visibly differ from the non-viable organisms which attained a uniform, dark blue colour cytoplasm when compared to Form A and Form C. So, the Form B is better compared to Form A and C.

Table 6.29 Effect of different treatment groups on percent *Candida albicans* cells killed in 60 mins by stimulated normal neutrophils

Groups	Treatment (µg/ml)	Total Candida albicans cells counted (300)	Candidacidal activity (%)	
		viable cells	Non-viable cells	
I	Control	284.67 ± 4.91	15.33 ± 4.91	5.11 ± 1.64
II	FORM A 50	244.67 ± 7.54	55.33 ± 7.54	18.44 ± 2.51
III	FORM A 100	219.00 ± 3.21	81.00 ± 3.21	27.00 ± 1.07
IV	FORM A 200	197.33 ± 8.76	102.67 ± 8.76	34.22 ± 2.92
V	FORM B 50	243.33 ± 4.63	56.67 ± 4.63	18.89 ± 1.54
VI	FORM B 100	191.67 ± 4.41	108.33 ± 4.41	36.11 ± 1.47
VII	FORM B 200	204.33 ± 4.91	95.67 ± 4.91	31.89 ± 1.64
VIII	FORM C 50	274.67 ± 7.54	25.33 ± 7.54	8.44 ± 2.51
IX	FORM C 100	272.67 ± 5.46	27.33 ± 5.46	9.11 ± 1.82
X	FORM C 200	223.67 ± 4.41	76.33 ± 4.41	25.44 ± 1.47
XI	Levamisol 50	186.00 ± 5.13	114.00 ± 5.13	38.0 ± 1.71

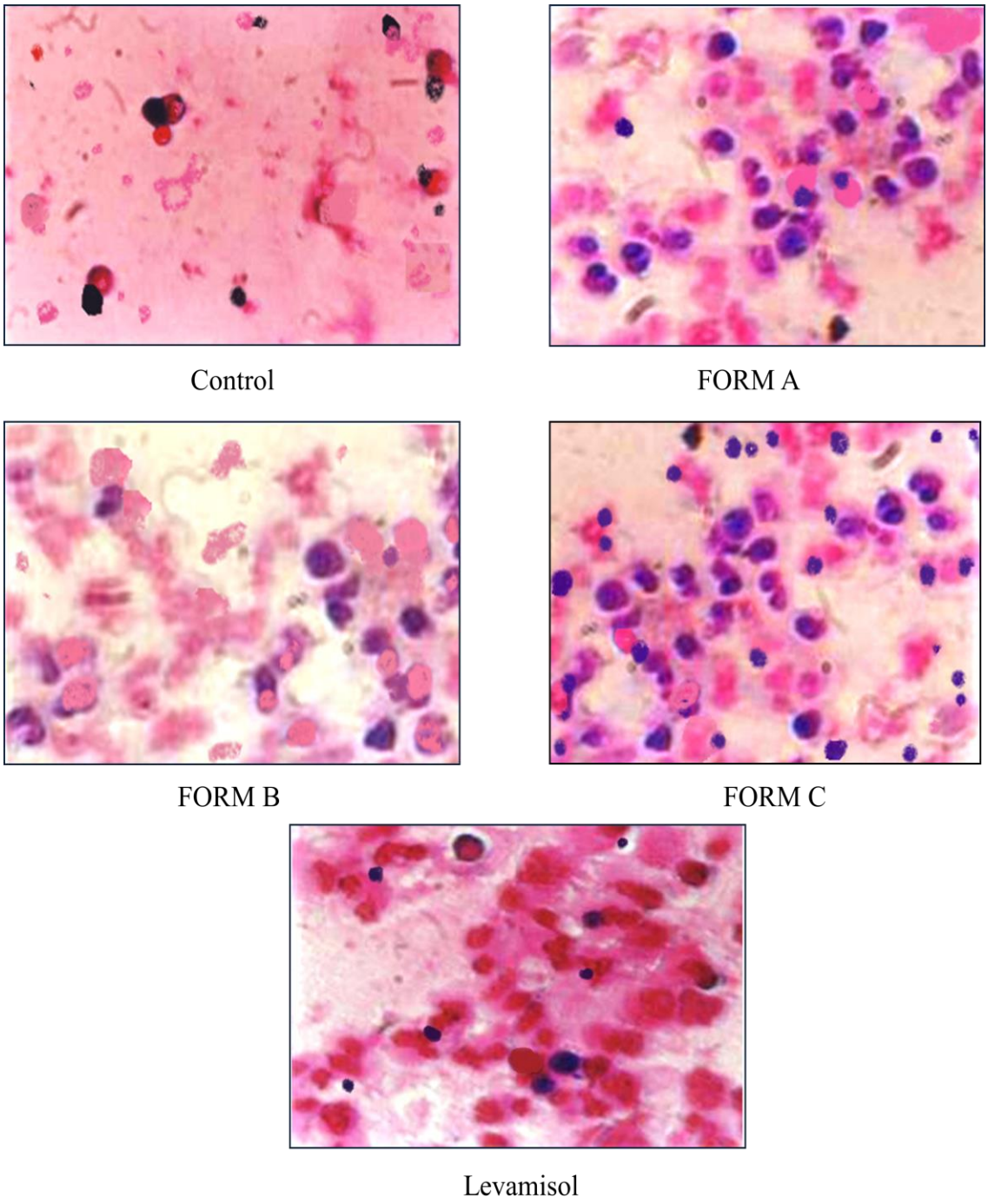


Fig 6.28 Viable (unstained) and non-viable cells (blue stained) in different treatment groups by Neutrophils candidacidal assay

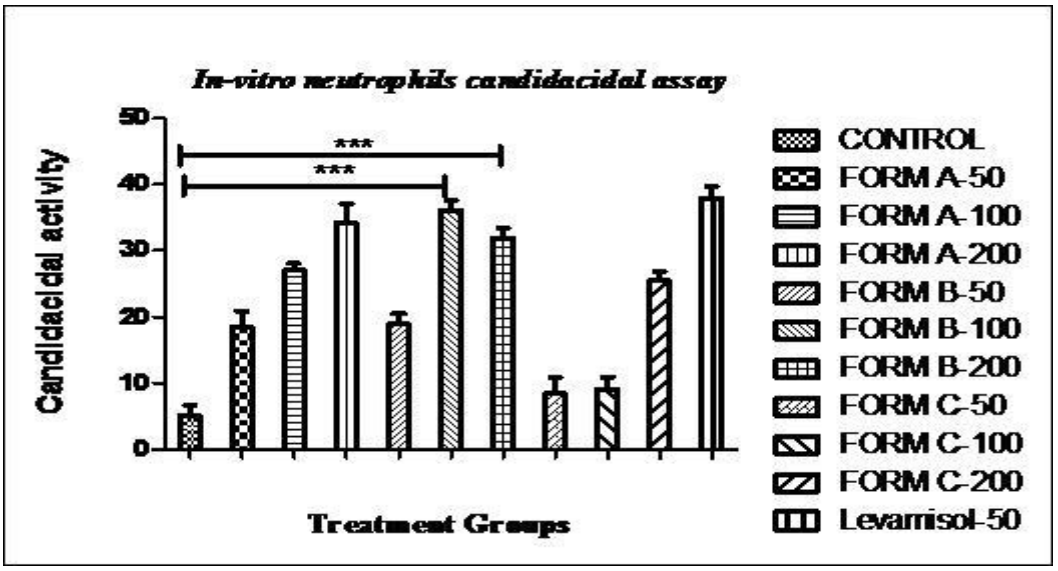


Fig. 6.29 Comparison of different treatment groups for neutrophils candidacidal assay

6.5) COMPARISONS OF DEVELOPED POLYHERBAL FORMULATION WITH REPORTED/MARKETED POLYHERBAL FORMULATIONS FOR IMMUNOMODULATORY ACTIVITY

There are many herbal formulations available commercially for immunomodulatory activity. Though not much published data is available, we have tried to compare immunomodulatory activity of some reported polyherbal formulations with presently developed polyherbal formulation (Form B). Septilin (The Himalaya Drug Company, Bangalore, India) is a polyherbal preparation, claimed to have immunostimulatory activity in low dose (1-1.5kg/kg) and immunosuppressive activity in high dose (2-3g/kg) [31]. Comparing the *in-vivo* carbon clearance data, the developed polyherbal formulation (200mg/kg) had higher phagocytic index compared to septilin.

Guard Sansar (GS) is a polyherbal formulation, developed by Pradhan herbal company, Bangalore [32]. Comparing the *in-vivo* carbon clearance data, the developed polyherbal formulation (200mg/kg) had 0.071 phagocytic index. The doses of Guard Sansar are 390mg/kg and 780mg/kg and the phagocytic index are 1.345 and 1.498 respectively.

Bharangyadi is a polyherbal preparation described in Ayurvedic system of medicine [33]. By comparing the *in-vivo* cyclophosphamide induced myelosupression assay data, the total WBCs counts of the developed polyherbal formulation (200mg/kg) was

increased on pretreatment for 10 and 14 days as compared to Bharangyadi compound (500mg/kg).

Shrishadi is a polyherbal preparation developed by Ayurvedic system of medicine [34]. By comparing the *in-vivo* Cyclophosphamide induced myelosupression assay data, there are no significant change in WBCs counts of both formulations but the dose of the developed polyherbal formulation was 200mg/kg while the dose of Shrishadi was 500mg/kg. So, one can conclude that the developed polyherbal formulation may better compared to above mentioned polyherbal formulations.

6.6) CONCLUSION

The present study was undertaken with the objective to design, develop and standardize a polyherbal formulation according to WHO guidelines. To optimize the ratio of the plant extracts in the formulation, 2x4 and 2x3 full factorial design were used by design expert 8.0 software. We selected three optimized batches from the factorial design data i.e. FORM A, FORM B and FORM C and prepared traditional (vati) and modern (tablet) dosage form. But evaluation of only tablets was done in detail due to sticky nature of vatis. We observed significant weight variation in vatis which was manually prepared. Physical standardization was done as per the WHO guidelines in order to set the standards for the analysis of the formulations. All the physiochemical properties such as, hardness, thickness, diameter, and friability are within the limit according to I.P. Other parameters like determination of ash, extractive values, water and volatile matter, pesticide residues and heavy metal analysis comply with those of the limits prescribed in the standard monographs.

Qualitative phytochemical screening and TLC studies of the tablet formulation reveals the presence of carbohydrate, alkaloids, saponins, glycosides, phenolics and tannins, amino acids and proteins and flavonoids.

The developed polyherbal formulations were stable at specified conditions of temperature and relative humidity. There was no significant change in colour, odour, texture, hardness disintegration time and friability.

The biological standardization was performed so as to compare the effects of the three formulations in the biological system. The biological evaluation of the developed polyherbal formulations was performed by *in-vivo* acute toxicity studies, *in-vitro* cell viability studies and the therapeutic potential was studied using *in-vivo* as well as *in-vitro* immunomodulatory activity.

Toxicity study was performed on all three developed polyherbal formulations according to the OECD guidelines in female albino mice. In acute toxicity studies the developed polyherbal formulations did not show any toxicity or mortality when the formulations are administered up to the dose of 2000 mg/kg maximum. Hence the 100mg/kg dose of polyherbal formulations was screened for their *in-vivo* immunomodulatory activity using phagocytic function, DTH response, antibody titre and cyclophosphamide induced myelosupression models.

Myelosupression i.e. bone marrow suppression by cyclophosphamide was significant and was accompanied by lowered total WBC counts. The total WBCs counts of all three formulations were increased on pretreatment for 10 and 14 days. No significant changes were observed with respect to RBC and hemoglobin values. Thus, it can be established from the study that all the three formulations possess the ability to counteract the myelosupressive effects of cytotoxic drug, cyclophosphamide by stimulating the bone marrow activity.

Macrophages are important in nonspecific and specific immunity. The phagocytosis via. Reticuloendothelial System (RES) was measured as removal rate of carbon particles from the blood. Developed polyherbal formulations stimulated macrophage's phagocytic activity supported by increased carbon clearance. The result shows that the stimulatory effect of all three developed polyherbal formulations on the mononuclear cells of phagocytic system.

In hypersensitivity reactions, antigen-antibody complex induce local inflammation and increased edema, vascular permeability and infiltration of PMN leukocytes. It is hypothesized that increase in the arthus reaction could be due to increased immune complex formation as a result of elevated IgM levels. Increased DTH reaction in rats concluded stimulatory effect of the polyherbal formulations on T cells.

Humoral immune response was assessed by HA titre values. Increased antibody titres in rat blood by drugs in turn indicates the augmentation of the humoral immune response to SRBCs. Increase in HA titres in developed polyherbal formulations indicating the improved sensitivity of macrophages and T and B lymphocytes involved in the antibody production.

In order to derive scientific evidences to the reported traditional claims, *in-vitro* cytotoxicity assay (MTT assay) was performed for developed polyherbal formulations (FORM A, FORM B and FORM C) at the dose range from 10-2000 µg/ml. The

polyherbal formulations showed more than 80% cell viability but up 200µg/ml it showed more than 97% viability. So, 50, 100 and 200 µg/ml doses are selected for further *in–vitro* immunomodulatory activity.

The NBT dye reduction test was performed to determine the phagocytic activity and neutrophil functionality necessary for microbicidal activity. Neutrophils take up the dye by phagocytosis which is then reduced by stimulation of the hexose monophosphate shunt pathway (HMP) of glucose oxidation and associated modifications in oxidative metabolism. Developed polyherbal formulations effectively increased the neutrophils' functionality i.e. intracellular killing assayed by *in-vitro* NBT reduction test. Thus, developed polyherbal formulations are hypothesized to contain constituents responsible for intracellular killing.

In the *in vitro* neutrophils candidacidal assay, *C. albicans*, foreign organisms or particles actively engulfed by PMN cells, are present in the assay medium. Intensity of phagocytic activity is related to the average count of candida cells ingested and associated with each PMN cells. The results concluded the Candidacidal activity of developed polyherbal formulations.

It is evident from the results of the study that developed polyherbal formulations have significant immunomodulatory activity when compared with the control groups.

Table 6.30: Results of the Standardization of the three developed formulations

Sr. No.	Standardization Parameters	FORM A	FORM B	FORM C
1.	Uniformity of Weight, mg	430 ± 2.41	500 ±3.78	330 ± 2.63
2.	Disintegration test, minutes	10 ± 0.5	7.6 ± 0.3	8.1 ± 0.2
3.	Hardness, kg/cm2	7.3 ± 0.4	7.1 ± 0.2	6.5 ± 0.2
4.	Diameter, mm	8.0 ± 0.1	8.2 ± 0.07	7.95 ± 0.04
5.	Thickness, mm	3.95 ± 0.5	4.1 ± 0.05	3.5 ± 0.3
6.	Friability, %	0.69 ± 0.7	1.1 ± 0.28	0.75 ± 0.4
7.	Total ash, %	10.5 ± 0.55	12.4 ± 0.64	9.6 ± 0.41
8.	Acid insoluble ash, %	7.1 ± 0.72	7.2 ± 0.87	6.97 ± 0.46
9.	Water soluble ash, %	6.5 ± 1.09	6.8 ± 1.21	6.4 ± 1.05

10.	Water soluble extractive value, %	23.67 ± 01.56	26.45 ± 1.90	21.76 ± 1.77
11.	Alcohol soluble extractive value, %	24.97 ± 1.67	31.07 ± 1.34	21.55 ± 1.08
12.	Loss on Drying, %	1.89 ± 0.08	2.15 ± 0.17	1.74 ± 0.14
13.	Pesticide Residue	Absent	Absent	Absent
14.	Heavy metals	Within limits	Within limits	Within limits
15.	Microbial Contamination	Absent	Absent	Absent
16.	Qualitative tests (Active constituents)	Present	Present	Present
17.	Acute Toxicity Studies	No toxicity	No toxicity	No toxicity
18.	Cell viabilty Studies (up to 200 µg/ml)	>98%	>98%	>98%
19.	*In-vivo* immunomodulatory activity			
A.	Cyclophosphamide induced myelosupression assay.	Significant increase in total leucocytes count	Significant increase in total leucocytes count	Increase in total leucocytes count
B.	Carbon clearance assay in mice.	Higher phagocytic index (more than Form C)	Higher phagocytic index (more than Form A and C)	Higher phagocytic index (but lower than Form A and C)
C.	Delayed type hypersensitivity response in rats. (24hr	Significant increase in DTH response	Significant increase in DTH response	Increase in DTH response

	and 48 hr)			
D.	Haemagglutination antibody titre value.	More agglutination, high HA titre (more than Form C)	More agglutination, high HA titre (more than Form A and C)	Agglutination (but lower than Form A and C)
20.	*In-vitro* immunomodulatory activity			
A.	Nitroblue tetrazolium (NBT) test	more black formazon, high range of NBT positive neutrophils (more than Form C)	more black formazon, high range of NBT positive neutrophils (more than Form A and C)	more black formazon, high range of NBT positive neutrophils (but lower than Form A and B)
B.	Neutrophils candidacidal assay	More viable cells, higher candidacidal activity (more than Form C)	More viable cells, higher candidacidal activity (more than Form A and C)	viable cells, higher candidacidal activity (but lower than Form A and B)

6.7) REFERENCES

1. Kulkarni GT. Herbal formulation development. Abstract GL12, National seminar on emerging trends in ethnopharmacology, Bagalkot. 12th – 13th Nov. 2005.
2. Rao VE. Modern Approach to Herbal Medicine. The Eastern Pharmacist, 2000 May; 35-7.
3. Box GEP, Hunter WG, Hunter JS, Statistics for Experimenters- An introduction to Design, Data Analysis and Model Building, John Wiley and Sons, New York, 1978.
4. Brasil JL, Martins LC, Ev RR, Dupont J, Dias SLP, Sales JAA, Airoldi C, Lima EC. Factorial design for optimization of flow injection preconcentration Procedure Copper (II) determination in natural waters, using 2-aminomephylpyridine grapfted silica gel as adsorbant and spectrophotometric detection. Int. J. Environ. Anal. Chem. 2005: 15: 475-491.
5. Gottipati R, Mishra S. Process optimization of adsorption of Cr (VI) on activated carbons prepared from plant precursors by a two-level full factorial design. Chemical Engineering Journal. 2010:160:99-107.
6. Agrawal SS, Paridhavi M, Herbal Drug Technology, University Press (India) Pvt. Ltd. 2007: 663-672.
7. The Ayurvedic Formulary of India, Anonymous, 1978, Government of India, Delhi.
8. Indian Pharmacopoeia, Vol I and II. New Delhi: Controller of publication, Ministry of Health and Family Welfare, Govt of India.
9. Aulton ME, Cellulose Powdered, in Handbook of Pharmaceutical Excipients, Rowe R C., Weller P J., Eds., The Pharmaceutical Press, UK., 2003;112-114.
10. Lachman L, Schwarts JB. Pharmaceutical dosage forms, Tablets, 2nd Edition, vol.2, Marcel Dekker, Inc. USA. 1990, 25-201, 246-313, 349-570.
11. Alton ME. (2002) Pharmaceutics, The science of dosage forms design, 2nd Edition, Harcoust publishers Ltd., London, 18-21.
12. ICH Harmonised Tripartite Guideline. Stability testing of new drug substances and products Q1A (R2). Geneva: International Conference of Harmonization; 2009.

13. OECD 'Guidelines for the testing of chemicals', Revised draft guidelines 423: Acute oral toxicity – Acute toxic class method, Revised Document, Organization for economic co-operation and development, Paris: October 2000.
14. Ghule BV, Nakhat MG, Yeole PG, Immunostimulant effects of Capparis zeylanica Linn. leaves. Journal of Ethnopharmacology. 2006:108:311–315.
15. Manjarekar PN, Jolly CI, Narayanan S. Comparative studies of the immunomodulatory activity of *Tinospora cordifolia* and *Tinospora sinensis*. Fitoterapia. 2000: 71: 254-257.
16. Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B, 1996. Studies on the immunomodulatory effects of Ashwagandha. Journal of Ethnopharmacology. 50, 69–76.
17. Hudson L, Hay P, Chapter 5: Antibody interaction with antigen. In: Thomas, A. (Ed.), Practical Immunology. Blackwell, Edinburgh, 1980. pp. 73–92.
18. Biozzi G, Benacerraf B, Halpern BN, Quantitative study of the granulopectic activity of the reticulo-endothelial system I: the effect of ingredient present in India ink and of substances affecting blood clotting in vivo on fate of carbon particles administration intravenously in rats mice and rabbits. British Journal of Experimental Pathology. 1953: 34, 426–457.
19. Gonda R, Tomoda M, Shimizu N, Kanari M. Characterization of an acidic polysaccharide from the seeds of *Malva verticillata* stimulating the phagocytic activity of cells of the RES. Planta Medica. 1990: 56:73-76.
20. Puri A, Saxena RP, Saxena KC, Srivastava V, Tandon JS. Immunostimulant activity of *Nyctanthes arbortristis* L. Journal of Ethanopharmacology. 1994: 42: 31-37.
21. Lagrange PH, Mackaness GB, Miller TE. Influence of dose and route of antigen injection on the immunological induction of T cells. Journal of Experimental Medicine. 1974:139, 528.
22. Doherty NS, Selective effects of immunosuppressive agents against the delayed hypersensitivity response and humoral response to sheep red blood cells in mice. Agents and Actions. 1981:11: 237–242.

23. English D, Andersen BR: Single-step separation of red blood cells. Gran leukocytes and mononuclear leukocytes on discontinuous density gradient of ficoll-hypaque. J Immunol Methods 5:249, 1974.
24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferstion and cytotoxicity assays. Journal of Immunological methods. 1983:65:55-63.
25. Monks A. Feasibility of high flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. Journal of National Cancer Institute. 1991:83:757-766.
26. Miller LE, Ludke HR, Peacock JE, Tomar RH, 1991. Manual of Laboratory Immunology, second ed. Lea and Febiger, Philadelphia, London.
27. Park BH, Fikrig SM, Smithwick EM, 1968. Infection and nitroblue tetrazolium reduction by neutrophils. Lancet 2, 532–534.
28. Ghule BV, Yeole PG. *In-vitro* and *in-vivo* immunomodulatory activities of iridoids fraction from *Barleria prionitis* Linn. Journal of Ethnopharmacology. 2012: 141: 424-431.
29. Lehrer RI, Cline MJ. Interaction of Candida albicans with human leukocytes and serum. Journal of Bacteriology. 1969: 98, 996–1004.
30. Schmid L, Brune K. Assessment of phagocytic and antimicrobial activity of human granulocytes. Infection and Immunity. 1974:10, 1120–1123.
31. Daswani BR, Radha Yegnanarayan, Immunomodulatory Activity of Septilin, a Polyherbal Preparation. Phytotherapy Research 2002: 16, 162-165.
32. Meera S, Atyam VG, Kumar NS. Immunomodulatory and antioxidant activity of a polyherbal formulation. Int. J. Pharmacol. 2008: 4(4); 287-291.
33. Kajaria D, Tripathi JS, Tiwari SK, Pandey BL. *In-vitro* Evaluation of Immunomodulatory Effect of Polyherbal Compound – Bharangyadi. Journal of Drug Deliver and Therapeutics. 2013: 3(1), 36-39.
34. Kajaria D, Tripathi JS, Tiwari SK, Pandey BL. Immunomodulatory effect of ethanolic extract of Shirishadi compound. AYU. 2012:33(2):322-326.
35. Akbay P, Calis I, Undeger U, Basaran N, Basaran AA. *In-vitro* immunomodulatory activity of verbascoside from *Nepeta ucrainica* Linn. Phytotherapy Research. 2002:16, 593–595.