Herbal formulation includes dosage forms comprising of herbs or processed herbs to provide nutritional, cosmetic or other benefits viz. used to diagnose, treat or mitigate diseases altering structure and physiology of human beings or animals. Herbal formulations may also be adopted from traditional medicines and used either for externally or internally.

Indian medicinal systems including Ayurveda, Unani, Siddha, Yoga and Naturopathy have developed over an era but there are various limitations with traditional herbal formulations and their traditional preparation techniques such as *shodhana* that they are difficult to adopt by modern herbal industries. The prime objective of using modern herbal dosage form in herbal medicine was global acceptance as modern tools of quality control. Lack of scientific justification and processing difficulties including standardization, extraction and identification of individual components in polyherbal systems barred use of herbal medicine from ages. These difficulties can be overcome by employing the principles and outcomes of modern phytopharmaceutical research.

Immunomodulation deals with modifying immunity response using either natural or synthetic substances. This technique when employed as therapy using medicinal plants can be used to cure numerous disease and can be alternative to conventional chemotherapy particularly for activating immune response when it is impaired (HIV, immunodeficiency and infectious diseases) or suppressing it in autoimmune disease, inflammation and allergic diseases. The body regulates homeostasis and thus modulates immune system under the influence of factors synthesised by the immune cells which requires suitable interaction of numerous cells within particular microenvironment. Currently immunomodulation has emerged as one of the potential field in the chemotherapy and has become an alternative to conventional chemotherapy. Various substances purified from plants and microorganisms have been employed as immunomodulators.

Many clinical disorders are associated with the immune system. Suppression of the immune system is required in the management and treatment of inflammation and allergic diseases, while stimulation is highly desirable for the treatment of HIV, immunodeficiency and infectious diseases.

WHO in a number of resolutions has focused on ensuring the quality of herbs and herbal formulations by using modern techniques and has drafted various guidelines for evaluation of herbal medicines. These guidelines outline basic standards for

quality, safety and efficacy of herbal medicines assisting national regulatory authorities, scientific organizations and manufactures. This has enforced phytochemical standardization by analytical techniques along with evaluation of its activity to ensure quality and efficacy of herbal drugs which can be done at two levels.

- Standardization by chemical/biomarker compound analysis and standardization by fingerprinting techniques/chemo profiling.
- Standardization by assessment of anticipated activity.

Thus, current study was conceived for evaluating and standardization of the selected plant drugs such as *Glycyrrhiza glabra, Nelumbo nucifera, Prunella vulgaris* and *Zizyphus jujuba* for the claims made under traditional systems for their antioxidant and immunomodulatory activities and prepares standardized polyherbal formulations using GRAS level excipients.

Glycyrrhiza glabra is commonly known as *Yashtimadu,* which has been used worldwide in various systems of medicine viz, Ayurvedic, Allopathic and other traditional systems of medicine. Glycyrrhizin, a triterpenoid compound is the marker of *Glycyrrhiza glabra. Nelumbo nucifera gaertn* is a large aquatic herb commonly known as Indain lotus. Quercetin, a flavonoid is the marker of *Nelumbo nucifera. Prunella vulgaris* is also known as 'self-heal' or 'heal-all' is a long lived medicinal herb with a worldwide distribution. Rosmarinic acid is the marker of *Prunella vulgaris. Zizyphus jujuba* is commonly known as 'Ber'. Betulinic acid, a pentacyclic triterpenoid is the marker compound of *Zizyphus jujuba.*

The plant materials, *Glycyrrhiza glabra* roots were purchased from a local store of Baroda *and* leaves of *Nelumbo nucifera* were collected from the botanical garden of The M.S. University of Baroda, Vadodara.

The collected plant materials were dried and powdered. The plant materials were studied as per the WHO guidelines. The macroscopical and microscopical examination of the crude intact drugs and powdered drugs was done. The histological characters of the roots of *G. glabra* and leaves of *N. nucifera* were studied with reference to the standard monographs and recorded. The determination of ash values, extractive values, moisture content, volatile matter, pesticide residues, heavy metals, microbial content was also performed. All the parameters complied within the WHO guideline limits.

The physically standardized plant materials were isolated using petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and the aqueous extract. Subjecting the isolated extracts to preliminary phytochemical analysis using chemical tests and TLC studies showed presence of saponins, carbohydrates and glycosides, proteins and amino acids, phytosterols, flavonoid and phenolics in *G. glabra* root extract. The *N. nucifera* leaves extract revealed the presence of alkaloids, carbohydrates and glycosides, proteins and amino acids, phytosterols and flavonoids.

Phenolic and flavonoid contents present in different extracts of medicines containing radical scavengers show efficient antioxidant activity. Therefore the phenolic and flavonoid content was quantified in water extracts of *G. glabra, P. vulgaris and Z. jujuba* and hydro alcoholic extracts of *N. nucifera.* Total phenolic components in the water extract of *G. Glabra*, hydroalcoholic extract of *N. Nucifera,* aqueous extracts of *P. vulgaris* and *Z. jujuba* were $9.70 \pm 0.6\%$, $35.11 \pm 0.02\%$, 7.72 ± 0.34 and $6.72 \pm 0.08\%$ respectively. Total flavonoid content in the water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera,* aqueous extracts of *P. vulgaris* and *Z. jujuba* were $8.32 \pm 0.17\%$, $32.21 \pm 0.08\%$, $11.67 \pm 0.20\%$, $6.74 \pm 0.15\%$ respectively.

The primary *in-vitro* antioxidant activity was done by two methods, DPPH free radical scavenging activity and phosphomolybdanum complex method. The EC_{50} values of water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera,* water extracts of *P. vulgaris and Z. jujuba* were 55.82, 50.44, 100.52 and 62.32 % obtained by DPPH method. The values suggest that *N. nucifera* has highest antioxidant activity compare to other three extracts. Total antioxidant capacity of extracts was indicated as the number of gram equivalents of ascorbic acid by phosphomolybdanum complex method. *Nelumbo nucifera* extract showed better antioxidant capacity when compared to other extracts.

Different analytical methods were developed depending on the number of markers and revels of sensitivity and selectivity e.g. HPLC, HPLTC and spectrofluorimetry. All the methods were validated by determining parameters including linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), system suitability and robustness. HPLC method (Method I) was developed for simultaneous estimation of glycyrrhizin and betulinic acid in polyherbal formulation. The analysis was performed on Oyster C_8 column using acetonitrile and water (90:10 v/v), (0.03%) orthophosphoric (pH 3.1) at 1ml/min with detection wavelength of 230nm. The glycyrrhizin and betulinic acid standards were prepared in methanol within

concentration range of 10-100μg/ml. Accurately weighed 1gm of developed polyherbal formulation was extracted in 25 ml methanol by sonication for 20 minutes at room temperature and filtered through 0.2μ filter paper. Precision, accuracy, sensitivity and robustness of the developed method were confirmed by the validation parameters.

A polyherbal formulation contains multi component preparation with a number of active chemical constituents. In such formulations, method development for simultaneous estimation of different components is immensely important.

An HPLC method for simultaneous estimation of glycyrrhizin, quercetin, rosmarinic acid and betulinic acid in polyherbal formulation was developed and validated. Separation of such markers was accomplished on RP phenomenex- C_{18} column (250mm×4.6mm i.d., 5μ particle size) in gradient system at 1.0 ml/min. A gradient mobile phase comprised of (A) water (pH 3.1 adjusted with 85% Ortho phosphoric acid) and (B) acetonitrile at 1.0 ml/min flow rate with PDA detection at 239nm. The calibration curves showed linearity with regression coefficient of 0.9973, 0.9992, 0.9981 and 0.9992 for glycyrrhizin, quercetin, betulinic acid and rosmarinic acid respectively. % RSD values were less than 2% in the concentration range of 20 - 60μg/ml for glycyrrhizin, quercetin and betulinic acid and concentration range of 5-15μg/ml for rosmarinic acid. The developed analytical method was employed for simultaneous estimation of glycyrrhizin, quercetin, betulinic acid and rosmarinic acid in polyherbal formulation. Validation parameters indicated that the developed method was reliable, reproducible and accurate.

HPTLC method was developed for quercetin analysis in hydroalcoholic extract of *N. nucifera* leaves. Methanolic solution (1 mg/ml) of quercetin was used for calibration curve. Different volumes from 1-25μl of stock solution were spotted on the plate developed using toluene: methanol: ethyl acetate: formic acid, 5: 1: 4: 0.2 v/v as solvent system. The plate was scanned at 366nm and quantified. The plate was sprayed with 10% $AlCl_3$, immediately scanned and quantified using the Camag TLC Scanner-3. The developed method was precise, accurate, sensitive and robust.

HPTLC analysis was developed for simultaneous estimation glycyrrhizin, quercetin, rosmarinic acid and betulinic acid in the developed polyherbal formulation. 1 μg/ml stock solutions of each four markers were prepared in methanol. It was further diluted to 100μg/ml in methanol. Then each standard solution was mixed in equal proportion to make final working standard solution. A 10mg/ml methanolic solution of

formulation was prepared. The methanol extract of the powdered formulation was prepared by sonication for 20 minutes. The extract was filtered through Whatman paper no.1. Different volumes from 4-19μl of the solution were applied, which gave different concentration 400-1900ng per spot respectively. The solvent system comprised of ethyl acetate: toluene: methanol: formic acid (7: 1: 0.5: 0.5 v/v). The plate was analysed after spraying it with AAS reagent, scanned and quantitated at 546 nm using the Camag TLC Scanner-3. The results suggested that the method was unaffected by presence of other components present in the formulation indicating suitability of this method for routine analysis of all four compounds in the herbal dosage form. The method was precise, accurate, sensitive and robust.

A spectrofluorimetric method for analysis of quercetin in *N. nucifera* extract as well as in the polyherbal formulation was also developed. Quercetin showed strong fluorescence having excitation and emission wavelength of 242 nm and 515 nm respectively in methanol. The linearity range was 10-70ng/ml. Validation parameters suggested that the developed method was precise, accurate, sensitive and robust. It was suitable for estimation of quercetin in the *N. nucifera* extract and also in the developed polyherbal formulation.

A spectrofluorimetric method for the analysis of glycyrrhizin in *G. glabra* extract as well as in the polyherbal formulation was also developed. Glycyrrhizin showed strong fluorescence having excitation and emission wavelength of 272 nm and 545 nm in methanol. The linearity range was 100-600pg/ml. Validation parameters suggested that the developed method was precise, accurate, sensitive and robust. It was also suitable for estimation of glycyrrhizin in the *G. glabra* extract and also in the developed polyherbal formulation.

Factorial design is used to estimate the effects of multiple factors on response parameters. Factorial design can be used to estimate important interactions between variables and whether every selected variable contributes significantly. In order to develop a polyherbal formulation dose is the main factor to influence the process. The formulations were optimized for *in- vitro* antioxidant activity by applying factorial design. Factorial design was applied so that optimization of the formulation can be done employing lesser number of experiments.

In the present study, four-factor two level full factorial designs (2^4 runs) and three-factor two level full factorial designs (2^3 runs) were used for the optimization of

formulations. Factors were plant extracts of *Glycyrrhiza glabra* root (A), *Nelumbo nucifera* leaves (B), aerial parts of *Prunella vulgaris (C)* and fruits of *Zizyphus jujuba (D)*. For 2^3 runs, the factor *Prunella vulgaris* was omitted. The chosen dependent variable was EC_{50} (50% effective concentration). The level -1 indicates the dose 30mg and +1 indicates the dose 100mg.

From the results of 2^4 runs, it can be concluded that dose of the all four factors A, B, C and D were the influencing factors affecting positively to achieve desired EC_{50} value. The lowest EC_{50} value 25.2 ± 1.27 of the batch indicates that 100mg dose of the all four plants give the best batch for the combination of all four plant extracts (Form B). The another batch having EC_{50} value 32.4 ± 1.45 was also selected which indicates that 100mg dose of *Glycyrrhiza glabra* root (A), *Nelumbo nucifera* leaves (B), aerial parts of *Prunella vulgaris (C)* and 30mg dose of fruits of *Zizyphus jujuba (D)*(Form A).

From the results of 2^3 runs, it could be concluded that dose of the all three factors *Glycyrrhiza glabra* (A), *Nelumbo nucifera* (B), *Zizyphus jujuba* (C) were the influencing factors affecting positively to achieve desired EC_{50} value. The optimized batch having EC_{50} value 20.16 ± 1.17indicates that 100mg dose of *Glycyrrhiza glabra* (A), 100 mg of *Nelumbo nucifera* (B) and 30mg of *Zizyphus jujuba* (C) plants give the best batch (Form C).

Using above three optimized batches i.e. From A, Form B and Form C, prepared a tablet dosage form by direct compression method. Due to its hygroscopic nature of two of the extracts, direct compression was selected as the suitable method for the preparation of polyherbal tablet dosage form. The developed polyherbal tablet dosage form was subjected to physical testing as per the WHO guidelines in order to set the standards for the analysis of the formulations.

The physical standardization of the formulations was done and compared by the methods prescribed in the pharmacopoeia. The uniformity of weight, disintegration test, hardness, diameter, thickness, friability, ash values, extractive values, pesticide residues, heavy metal analysis, microbial contamination studies were performed. All the results were within limits and complied with the standard limits.

The formulations were extracted in methanol. Preliminary phytochemical analysis was performed on the extracts using chemical tests and TLC studies which concluded presence of alkaloids, steroids, saponins, volatile oils, phenolics and flavonoids in the extracts of the formulations.

Biological screening of medicinal plants/herbal formulations is of vital importance, not only to provide a scientific basis for their continued usage but also to validate their traditional utilization. Additionally these studies aid to correlate the activity with some components of the plant. Thus, biological screening provides additional means of standardization of the herbal formulations.

The biological standardization was performed so as to compare the effects of the three optimized 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.

The immune system protects the body from adverse invasion by foreign immunogens and thus helps in biological adaptation maintaining homeostasis and body integrity. Hence, current experimental work related to immunomodulatory explores the improvement of defence mechanism of the host.

Toxicity study of formulations A, B and C was carried as per the OECD (Organisation for Economic Co-operation and Development) 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. Hence 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 (FORM A, FORM B and FORM C) 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. Reticulo Endothelial 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.

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. 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.

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.

From the *in-vivo* and *in-vitro* data, it can be concluded that the Form B is having more immunostimulant activity as compared to Form A and Form C.

The developed polyherbal formulations were stable at specified conditions of temperature and relative humidity. There is no significant change in colour, odour, texture, hardness, and friability and disintegration time. The developed formulation was also compatible with the GRAS category of excipients.

The results of complete standardization of plants and formulations studies showed that the authentication of genuine plant drugs from other varieties of the same species. From the above study it can be can concluded that for the development of a new polyherbal formulation, standardized traditional methods and raw materials should be used. Further, the concentration of the markers should be assayed which have been observed to have direct effect on the safety and efficacy. The biological activity of a developed polyherbal formulation helps in justifying the traditional claims endowed upon these drugs in scientifically accepted manner.

So, it is highly recommended that the standardization of this herbal medicine must be performed regularly which may lead to patient safety and good manufacture practices. This has aroused the need for developing dependable standardization tools for optimal usage of such traditional medicines.

To demonstrate the applicability of herbal medicines, society requirements must be met for which intensive research is required to be conducted on various herbal disciplines. This can be achieved by standardization, methods and measures for preparation, preservation, presentation and administration of herbal drugs. Thus, it can be justified that investigating modern scientific methods finally affect the development of herbals.