STUDY OF IMMUNOMODULATORS FROM VARIOUS PLANT SPECIES

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Submitted by

A. SUMATHI, M.Pharm.,

Under the guidance of

Prof. Dr. R. SENTHAMARAI, M. Pharm., PhD.,

DEPARTMENT OF PHARMACEUTICS

PERIYAR COLLEGE OF PHARMACEUTICAL SCIENCES

K. SATHANOOR MAIN ROAD, TIRUCHIRAPPALLI – 620 021

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INTRODUCTION

An immune system is a system of biological structures and processes within an organism that protects against invading micro organisms by identifying and killing pathogens and tumor cells. It detects a wide variety of agents, from viruses to parasitic worms and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly. The immune mechanisms may vary from species to species. Unicellular organisms such as bacteria possess enzyme systems that protect against viral infections. Jawed vertebrates, the Gnathostomes including humans, have even more sophisticated defence mechanisms which consists many types of proteins, cells, organs, and tissues that interact in an elaborate and dynamic network.

Immune system is vulnerable to the free radical-induced oxidative stress. The cellular and humoral components of the immune system are particularly sensitive to increased levels of reactive oxygen species, which may cause premature immunosenescence. The immune response can be manipulated to suppress unwanted responses resulting from autoimmunity, allergy and transplant rejection and to stimulate protective responses against pathogens that largely elude the immune system[1].

The modulation of immune response with the aid of various bioactives in order to alleviate certain diseases is an active area of interest. Immunomodulators are substances that have been shown to modify the immune systems response to a threat upon it. They modulate and potentiate the weapons of our immune system keeping them in a highly prepared state for any threat it may encounter. They increase the immune responsiveness of the body against pathogens by activating primarily the non-specific immune system.

Immunomodulatory therapy is now being recognized as an alternative to conventional chemotherapy for a variety of disease conditions, involving the impaired immune response of the host. In clinical perspective immunomodulators can be classified as immunostimulants, immunosuppressants and immunoadjuvants.

- Immunostimulants are substances (drugs and nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components. Example: Granulocyte macrophage colony-stimulating factor. It enhances body's resistance against infections and may be against allergy, autoimmunity, and cancer as well.

- Immunosuppressants are the agents that inhibit or prevent activity of the immune system. They may be either exogenous, as immunosuppressive drugs, or endogenous as testosterone. When the immune system function is suppressed, there is an increased susceptibility to infectious diseases and cancers.

- Immunoadjuvants are the agents which are used for enhancing efficacy of vaccines and therefore, could be considered as specific immune stimulants. Example: Freund's adjuvant.

Disorders in the immune system can result in disease, including autoimmune diseases, inflammatory diseases and cancer. The immune system is a remarkably effective structure that incorporates specificity, inducibility and adaptation. Failures of host defence do occur, however, and fall into three broad categories such as immunodeficiencies, autoimmunity and hypersensitivities.

- Immunodeficiencies occur when the immune system is less active than normal, resulting in recurring and life-threatening infections. It can either be the result of a genetic disease, such as severe combined immunodeficiency, or be produced by pharmaceuticals or an infection, such as the Acquired Immune Deficiency Syndrome (AIDS) that is caused by the retrovirus HIV.

- Autoimmunity results from a hyperactive immune system attacking normal tissues as if they were foreign organisms. It includes Hashimoto's thyroiditis, rheumatoid arthritis, diabetes mellitus type 1 and lupus erythematosus. It arises from an abnormal immune response of the body against substances and tissues normally present in the body (autoimmunity).

- Hypersensitivity (also called hypersensitivity reaction or intolerance) refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. These reactions may be damaging, uncomfortable, or occasionally fatal. Hypersensitivity reactions require a pre-sensitized (immune) state of the host. They are classified in four groups after the proposal of P. G. H. Gell and Robin Coombs (Type I - Allergy or immediate; Type II – Cytotoxic or antibody-dependent; Type III – Immune complex disease and Type IV – Delayed type hypersentivity or antibody independent or cell-mediated immune memory response).

Each disorder will be treated according to the specific conditions it causes. For example, AIDS causes several different infections, including Kaposi’s sarcoma, which is treated with doxorubicin lipid complex, and cryptococcosis, which is treated with fluconazole. Treatment for immunodeficiency disorders commonly includes antibiotics and antibody replacement. A drug called interferon is often used to treat the viral infections caused by a disorder. If bone marrow is not producing enough lymphocytes, the person might undergo a bone marrow transplant.

Allopathic drugs are available for improving the immune system and immunity, but the side effects and prohibitive cost of these allopathic drugs makes it necessary to search for an alternative. The ayurvedic system of medicines not only provides that alternative, but also scores over the side effects and cost factor. In the past decade there has been a paradigm shift from single-target drugs to multi-target drugs. Multi-target approaches are directed towards the activation of defence, protective and repair mechanisms of the body rather than destruction of the damage-causing agent. This may be achieved by the use of a combination of herbal drugs.

Some of the plants with established immunomodulatory activity are *Asparagus racemosus, Azadirachta indica, Ocimum sanctum, Panax ginseng, Polygala senega* and *Viscum album*[2]. Also earlier studies indicate that *Emblica officinalis*, *Tinospora cordifolia*, *Terminalia arjuna* and *Piper longum* possess immunomodulatory and antioxidant activity[3,4]. Fresh fruits, vegetables, and foods rich in certain fatty acids may foster a healthy immune system. Some herbs such as Echinacea, Licorice, Ginseng, Astragalus, Sage, Garlic, Elderberry, and Hyssop, as well as Honey are believed to stimulate the immune system, although further research is needed to understand their mode of action. Medicinal mushrooms like Shiitake, Lingzhi mushrooms, the Turkey tail mushroom, *Agaricus blazei*, and Maitake have shown some evidence of immune system up-regulation in *in vitro* and *in vivo* studies, as well as in a limited number of clinical studies.

Novel drug delivery system is advantageous in delivering the herbal drug at predetermined rate and delivery of drugs at the site of action which minimizes the toxic effects with increase in bioavailability of drugs. In novel drug delivery technology, control of the distribution of drug is achieved by incorporating the drug carrier system or in changing the structure of the drug at molecular level. Several advancements resulted in the development of new techniques for drug delivery, which are capable of controlling the rate of drug delivery, sustaining the duration of therapeutic activity and targeting the delivery of drugs to a cell or tissue[5].

Herbal formulation means a dosage form consisting of one or more herbs or processed herb in specified quantities to provide specific nutritional, cosmetic benefits and other benefits meant for diagnosis, treatment, or to alter the structure or physiology of human beings or animals. Herbal preparations are obtained by subjecting whole plants, fragmented or cut plants, plant parts to treatment such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. Novel drug delivery systems of herbal drugs have a potential future for enhancing the activity and overcoming the problems associated with plant medicines. Also, there is a growing interest in identifying herbal immunomodulators and its applications in modern medicine[6,7].

The different novel drug delivery system carriers used for herbal drugs are phytosomes, liposomes, ethosomes, transferosomes, nanoparticles, microemulsions, etc. The drug delivery technology has certainly infused new interests in seemingly traditional old drugs by providing them new life specifically through their therapeutic targets. The concept of multi-targeted therapy exists in traditional medical treatments that employ multi-component extracts of natural products which simultaneously act on multiple targets. Polyherbal therapies involve the combination of various types of agents from different plant sources and thereby used to enhance efficacy. They have the synergistic, potentiative, agonistic/antagonistic pharmacological agents within themselves that work together in a dynamic way to produce therapeutic efficacy with minimum side effects. They also minimize the risk of development of drug resistance. Most of the biologically active constituents of plants are polar or water soluble molecules. However, water soluble phytoconstituents like flavonoids, tannins, terpenoids, etc., are poorly absorbed either due to their large molecular size which cannot absorb by passive diffusion or due to their poor lipid solubility; severely limiting their ability to pass across the lipid-rich biological membranes, resulting poor bioavailability. Phytosome is a patented technology, developed by a leading manufacturer of drugs and nutraceuticals used to incorporate plant extracts or water soluble phytoconsituents into phospholipids to produce lipid compatible molecular complexes and so vastly improve their absorption and bioavailability.

Phytosome, a type of vesicular drug delivery system or modified vesicular drug delivery of liposome. It exhibits better pharmacokinetic and pharmacodynamic profile than conventional herbal extracts. Most of the bioactive constituents of phytomedicines are water-soluble compounds like flavonoids, glycosides, terpenoids in which flavonoids are a major class of bioactive compounds possesses broad therapeutic activities. Because of water soluble herbal extract and lipophilic outer layer phytosomes shows better absorption and as a result produce better bioavailability and actions than the conventional herbal extracts containing dosage form. They are produced by a process whereby the standardised plant extract or its constituents are bound to phospholipids, mainly phosphatidylcholine, producing a lipid compatible molecular complex[8].

Phytosome technology has been effectively used to enhance the bioavailability of many popular herbal extracts or active molecules including *Ginkgo biloba* extract, bilobalide isolated from *Ginkgo biloba*, silybin isolated from milk thistle (*Silybum marianum*), curcumin isolated from turmeric, and green tea extract (*Camellia sinensis*) and can be developed for various therapeutic uses or dietary supplements. It represents a promising vesicular drug delivery system to deliver therapeutic compounds for a wide range of possible applications.

Thus the present study was undertaken to investigate and validate the immunomodulatory activity of two different plant species - *Nymphaea nouchali* and *Trichosanthes dioica* and to develop a novel drug delivery system for the most effective treatment of disease related patients.

AIM OF THE WORK

With the advancement of Science and Technology, the occurrence of diseases can be easily visualized by the health professionals. Our immune system detects a wide variety of agents, from viruses to parasitic worms, kills and disposes using various immune mechanisms. Disorders in the immune system can result in disease, including autoimmune diseases, inflammatory diseases and cancer. Although some studies have been successful, complete cure is still a major challenge. Herbal medicine has become an integral part of health care system, based on the combination of ayurvedic or traditional classics and ongoing scientific research.

To address this challenge, certain medicinal plants with immunomodulant activity, will be selected and studied. The aim of this study was to validate the immunomodulant potential of two different plant species using *in vitro* experimental models and to provide scientific evidence to the ethnomedicinal use of the plant species. Finally, they were incorporated into a novel carrier called phytosome to promote its therapeutic action significantly.

The principal goal was to conceptualize an ideal drug delivery system viz. phytosomes for the selected plant species using different types of phospholipids. The novel carriers loaded with bioactives not only deliver the drug(s) to specific organs within the body but also controls the delivery rate. Thus, using this novel drug delivery system, herbal drugs incorporated with phospholipids, it might be able to achieve constant and uniform concentration of the drug for longer period of time in the body.

OBJECTIVES

- ✓ To identify two different plant species which had been traditionally used to treat various ailments and extract the possible phytoconstituents using solvents of increasing polarity
- ✓ To perform systematic studies on the selected plants - *Nymphaea nouchali* (Nn) and *Trichosanthes dioica* (Td) for substantiating their therapeutic claims
- ✓ To investigate the immunomodulatory effects of different extracts of *Nymphaea nouchali* (Nn) and *Trichosanthes dioica* (Td) using *in vitro* assay methods
- ✓ To perform acute oral toxicity studies for the selected plant extracts of *Nymphaea nouchali* (Nn) and *Trichosanthes dioica* (Td) and thereby to identify the maximum tolerated dose (MTD)
- ✓ To identify the selected plant extracts by spectroscopic methods (UV and IR)
- ✓ To design and fabricate a novel drug delivery system using phytosomes, for effective delivery of herbal constituents
- ✓ To optimize the ratio of drug and phospholipids having an ideal spherical shape and an effective average particle size range
- ✓ To identify the best formulation which maintains steady state plasma drug concentration for prolonged period of time
- ✓ To summarize and finalize the obtained results by comparing with a standard using *in vivo* animal models for immunomodulant activity

LITERATURE REVIEW

- **Barzaghi *et al.*, (1990)** conducted a human study designed to assess the absorption of silybin when directly bound to phosphatidylcholine. Plasma silybin levels were determined after administration of single oral doses of silybin phytosome and a similar amount of silybin from milk thistle in healthy volunteers. The results indicated that the absorption of silybin from silybin phytosome is approximately seven times greater compared to the absorption of silybin from regular milkthistle extract (70-80 % silymarin content)[9].

- **Bombardelli *et al.*, (1991)** reported Silymarin phytosomes, in which Silymarin (A standardized mixture of flavanolignans extracted from the fruits of *S. marianum*) was complexed with phospholipids. Phytosomes showed much higher specific activity and a longer lasting action than the single components, with respect to percent reduction of odema, inhibition of myeloperoxidase activity, antioxidant and free radical scavenging properties[10].

- **Moscarella *et al.*, (1993)** investigated in one study of 232 patients with chronic hepatitis (viral, alcohol or drug induced) treated with silybin phytosome at a dose of 120 mg either twice daily or thrice daily for upto 120 days, liver function returned to normal faster in patients taking silybin phytosome compared to a group of controls (49 treated with commercially available silymarin, 117 untreated or given placebo)[11].

- **Ravarotto *et al.*, (2004)** reported silymarin phytosome show better antihepatotoxic activity than silymarin alone and can provide protection against the toxic effects of aflatoxin B1 on performance of broiler chicks[12].

- **Kidd P *et al.*, (2005)** reviewed the bioavailability and clinical efficacy of milk thistle phytosomes: a Silybin phosphatidylcholine complex. Certain of the water-soluble flavonoid molecules can be converted into lipid-compatible molecular complexes, aptly called phytosomes. Phytosomes are better able to transition from a hydrophilic environment into the lipid-friendly environment of the outer cell membrane, and from there into the cell, finally reaching the blood. The fruit of the milk thistle plant (*Silybum marianum*, Family Asteraceae) contains flavonoids that are proven liver protectants. The standardized extract known as silymarin contains three flavonoids of the flavonol subclass. Silybin predominates, followed by silydianin and silychristin. Although silybin is the most potent of the flavonoids in milk thistle, similar to other flavonoids it is not well-absorbed. Silybin-phosphatidylcholine complexed as a phytosome provides significant liver protection and enhanced bioavailability over conventional silymarin[13].

- **Maiti *et al.*, (2005)** developed the quercetin–phospholipids complex by a simple and reproducible method and also showed that the formulation exerted better therapeutic efficacy than the molecule in rat liver injury induced by carbon tetrachloride[14].

- **Yanyu X et al., (2005)** prepared of silybin-phospholipid complex and studied its pharmacokinetics in rats. The study results showed that silybin and phospholipids in the silybin-phospholipid complex were combined by non-covalent-bond, not forming a new compound and the solubility of silybin-phospholipid complex in water and in n-octanol was effectively enhanced. They found that mean plasma concentration-time curve of silybin after oral administration of silybin-phospholipid complex and silybin-N-methylglucamine in rats was both in accordance with open single-compartment model with first-order absorption. The bioavailability of silybin in rats was increased remarkably after oral administration of silybin-phospholipid complex comparing to silybin-N-methylglucamine. This was mainly due to an impressive improvement of the lipophilic property of silybin-phospholipid complex and improvement of the biological effect of silybin[15].

- **Li Y *et al.*, (2006)** formulated and evaluated the pharmacokinetic, tissue distribution and excretion of pueratin and pueratin-phospholipid complex in rats. They concluded that oral administration of pueratin phospholipid complex modified the pharmacokinetics and tissue distribution of pueratin and it could be an effective oral formulation for pueratin[16].

- **Maiti K *et al.*, (2006)** studied the enhanced therapeutic potential of naringenin-phospholipid complex in rats. Naringenin is a naturally occurring flavanone, possessing a variety of biological activity. Due to its rapid elimination, naringenin needs frequent administration to maintain an effective plasma concentration. Naringenin-phospholipid complex was prepared and assessed for antioxidant activity in carbon tetrachloride intoxicated rats at a dose level of 100 mg/kg/*p.o*. Liver function tests were studied by assessing serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase and total bilirubin. It was observed that the naringenin-phospholipid complex enhanced the antioxidant activity of the biomolecule and protected the liver significantly for a longer time as compared with free naringenin at the same dose level. Phospholipid complex of naringenin produced better antioxidant activity than the free compound with a prolonged duration of action, which might be helpful in reducing the fast elimination of the molecule from body[17].

- **Maiti *et al.*, (2007)** developed the phytosomes of curcumin (flavonoid from turmeric, *Curcuma longa* Linn.) to overcome the limitation of absorption and to investigate the protective effect of curcumin-phospholipid complex on carbon tetrachloride induced acute liver damage in rats. It provided better protection than free curcumin at same doses. Serum concentration of curcumin obtained from the complex was higher than pure curcumin and the complex maintained effective concentration of curcumin for a longer period of time in rat serum. The antioxidant activity of complex was significantly higher than pure curcumin in all dose levels tested. The result proved that curcumin-phospholipid complex has better hepatoprotective activity, owe to its superior antioxidant property, than free curcumin at the same dose level[18].

- **Mukerjee *et al.*, (2008)** developed a novel hesperetin phytosome by complexing hesperetin with hydrogenated phosphatidyl choline. This complex was then evaluated for antioxidant activity in CCl_4 intoxicated rats along with pharmacokinetic studies. It was found that the phytosome had a sustained release property for over 24h and enhanced antioxidant activity. Pharmacokinetic study revealed that the phytosome had higher relative bioavailability than that of parent molecule at the same dose level[19].

- **Nagavani V *et al.*, (2010)** investigated the antioxidant potential of *Nymphaea nouchali* Brum. flowers and also reported the qualitative analysis of major polyphenols by RP-HPLC. The flowers of *Nymphaea nouchali* Brum have a wide range of applications in ayurveda and traditional medicine. Studies have been taken up to evaluate the enzymatic, non-enzymatic and antioxidant potentials in ethanol, methanol and aqueous extracts of *N.nouchali* dry and fresh flowers. Obtained results showed that the high levels of non-enzymatic antioxidants like phenols, flavonoids, tannins etc. as well as antioxidant potential found to be more in methanolic extracts of *N.nouchali* dry flowers. Further, studies were also conducted for the identification of phenolic compounds with different solvents using RP-HPLC coupled with photo diode array detector[20].

- **Prashant Kumar Rai *et al.*, (2010)** reported the distribution of *Trichosanthes dioica,* pointed gourd (in English), parwal (in Hindi) and potol (in Oriya), in equatorial and subtropical regions and in northern India. The present study is a unique example of interdisciplinary research as it deals not only with the phytochemical investigation of *Trichosanthes dioica*, using natural product technology but also correlates the presence of certain trace elements with its biological activities using Laser induced break down Spectroscopy (LIBS). The study reveals the isolation of one known and two unknown flavonoids and the presence of certain glycemic elements, responsible for the observed antidiabetic activity of *Trichosanthes dioica*. Hence, the presence of isolated flavonoids and trace elements can be very well correlated with its medicinal value[21].

- **Choubey A *et al.*, (2011)** explained the improvement of bioavailability of some orally administered botanical extracts, whose biavailability is erratic and poor due to limited gastro-intestinal absorption. Bioavailability can be improved by using new delivery systems which can enhance the rate and the extent of solubilization into aqueous intestinal fluids and the capacity to cross biomembranes. Phospholipids based drug delivery system have been found promising for better and effective delivery of drug and providing much appropriate systematic drug delivery. Phytosomes exhibit better pharmacokinetic and pharmacodynamic profile than conventional herbal extracts. Phytosome technology has been effectively used to enhance the bioavailability of many popular herbal extracts including milk thistle, *Ginkgo biloba*, Grape seed, Green tea, Hawthorn, Ginseng etc., and can be developed for various therapeutic uses or dietary supplements This article reviews the current trends in phytosomes drug delivery[22].

- **Rajendran R *et al.*, (2011)** had focussed their view on production of herbal-based nanoparticles for medical textiles. The use of materials created through nanotechnology is expected to dramatically increase over the next few years. Nanotechnology can provide high durability for fabrics because nanoparticles have a large surface area to volume ratio and high surface energy, thus presenting better affinity for fabrics, leading to an increase in durability of the function. In this study herbal plants such as *Curcuma longa* and *Datura metal* were selected, and bioactive compounds were extracted and standardised. Nanoparticles of the medicinal plant extracts were prepared by coacervation method using bovine serum albumin, cross-linked with gluteraldehyde and finished on 100% pure cotton by pad-dry-cure method. The antimicrobial activities of the nanoparticles-treated cotton fabrics were found to be higher than that of the control fabrics in both AATCC 147 and Hohestein Challenge test[23].

- **Hugo J *et al.*, (2012)** reported that the snake gourd genus, *Trichosanthes*, is the largest genus in the Cucurbitaceae family, with over 90 species. Recent molecular phylogenetic data have indicated that the genus *Gymnopetalum* is to be merged with *Trichosanthes* to maintain monophyly. A revised infrageneric classification of *Trichosanthes* including *Gymnopetalum* is proposed with two subgenera, (I) subg. *Scotanthus* comb. nov. and (II) subg. *Trichosanthes*, eleven sections, (i) sect. *Asterospermae*, (ii) sect. *Cucumeroides*, (iii) sect. *Edulis*, (iv) sect. *Foliobracteola*, (v) sect. *Gymnopetalum*, (vi) sect. *Involucraria*, (vii) sect. *Pseudovariifera* sect. nov., (viii) sect. *Villosae* stat. nov., (ix) sect. *Trichosanthes*, (x) sect. *Tripodanthera*, and (xi) sect. *Truncata*. A synopsis of *Trichosanthes* with the 91 species recognized here is presented, including four new combinations, *Trichosanthes orientalis, Trichosanthes tubiflora, Trichosanthes scabra* var. *pectinata, Trichosanthes scabra* var. *penicaudii*, and a clarified nomenclature of *Trichosanthes costata* and *Trichosanthes scabra*[24].

- **Ishrat Jahan *et al.*, (2012)** studied the antioxidant, analgesic and anti-inflammatory activities of *Nymphaea nouchali* flowers. The methanolic extract of *Nymphaea nouchali* flowers (MNNF) was evaluated for anti-inflammatory activity using carrageenan induced hind paw edema model whereas hot plate, writhing and formalin tests was carried out for analgesic activity. Total phenolic and flavonoids content, scavenging of 1, 1-Diphenyl-2- Picrylhydrazyl (DPPH) radical, peroxynitrate (ONOO-) as well as inhibition of total ROS generation were used to evaluate antioxidant potential of MNNF. The same ranges of doses of MNNF caused significant ($p<0.05$) inhibition of carrageenan induced paw edema after 4 h in a dose dependent manner. In DPPH, ONOO- and total ROS scavenging method, MNNF showed good antioxidant potentiality with the IC50 value of 10.33±1.02, 20.16±0.61 and 31.72±0.48 μg mL-1, respectively. The findings of the study confirmed the traditional use of this plant for inflammatory pain alleviation to its antioxidant potentiality[25]

- **Rathore P *et al*., (2012)** reviewed the potential of phyto-phospholipid carriers for the bioavailability enhancement of herbal extracts. Planterosomes, term "PLANTERO" means plant while "SOME" means cell -like. A novel emerging technique applied to phytopharmaceutical for the enhancement of bioavailability of herbal extract for medicinal applications. Planterosomes are prepared by non conventional methods. Planterosomes absorption in GIT is greater resulting in increased plasma level than individual component. They act as a bridge between novel delivery system and conventional delivery system. Phospholipids molecule acting as vital carrier made up of water soluble head and two fat soluble tails, due to this nature they possess dual solubility and thus acting as an effective emulsifier. These drug-phospholipids complex can be formulated in the form of solutions, suspensions, emulsions, syrup, lotion, gel, cream, aqueous microdispersions, pill, capsule, powder, granules and chewable tablets[26].

- **Tawheed Amin *et al.*, (2012)** had reviewed the importance of phytosomes. The bioavailability and absorption of water soluble phytoconstituents is erratic due to poor solubility of these constituents in gastrointestinal tract. This can be overcome by a novel delivery system known as phytosome technology in which water soluble phytoconstituents are allowed to react with phospholipids. For better and improved bioavailability, natural phytoconstituents must have a good balance between hydrophilicity (helps in dissolution in gastro-intestinal fluids) and hydrophobicity (helps to cross lipid rich cell membranes). This is achieved through phytosome technology. Phospholipids have a dual solubility and acts as an emulsifier. Phytosome technology acts as a bridge between novel and conventional delivery systems. Many products are available in the market based on this phytosome technology which include popular herbal extracts such as *Ginkgo biloba, Silybum marianum*, grape seed, olive oil flavonoids etc[8]

- **Biplab Kumar Dash *et al.*, (2013)** studied the antibacterial activity of *Nymphaea nouchali* flower on human and plant pathogenic bacteria. Antibacterial potency of methanol, acetone, ethyl acetate and petroleum spirit extracts of *N.nouchali* flower has been tested against four human pathogenic bacteria *Bacillus subtilis* (FO 3026), *E. coli* (IFO 3007), *Klebsiella pneumonia* (ATTC 10031) and *Sarcina lutea* (IFO 3232) and one plant pathogenic bacterium *Xanthomonas campestris* (IAM 1671) by disc diffusion assay. The minimum inhibitory concentrations of various extracts were ranged between 128–2048 μgml^{-1}. *N.nouchali* flower could be a potential candidate for future development of novel broad spectrum antibacterial herbal formulation[27].

- **Patel Amit *et al.*, (2013)** reviewed and stated that the phytolipid drug delivery system has been used for improving bioavailability of herbal drugs. Novel drug delivery system in the field of medicine had taken a popular attention and makes the intake, bioavaibility and overall therapeutics of a drug easier and in short period of time. In current herbal drugs have been widely used because of their less side effects, cost effectiveness and easy availability. Phytosomes are herbal formulation which has enhanced the therapeutic effect of the plant extracts and herbal lead molecule by increasing bioavaibility in the target site compared to conventional herbal extract[28].

- **Raj Kumar Thapa *et al.*, (2013)** had presented the advancements in herbal treatment using herbal medicine incorporated nanoparticles. Use of herbal medicines dates back long history. These days the use of herbal medicines has increased because of their ability to treat different diseases with fewer side effects. Different scientific approaches are being developed these days to deliver herbal medicines. Novel formulations including nanoparticles have been developed for the effective delivery of herbal drugs. Nanoparticulate formulations such as polymeric nanoparticles, liposomes, proliposomes, solid lipid nanoparticles and microemulsions present potential to deliver herbal medicines effectively. This article summarises various nanoparticulate technologies that have been studied for the delivery of herbal medicines and which are gaining more attention for improved therapeutic response[29].

MATERIALS AND METHODS

The selected plant species *Nymphaea nauchali* (Nn) and *Trichosanthes dioica* (Td) were extracted using soxhlet apparatus by successive solvent extraction technique. Five different solvents of increasing polarity (n-Hexane, Chloroform, Ethyl acetate, Methanol and Distilled water) were used for the extraction process. All the extracts were subjected to preliminary phytochemical investigations and *in vitro* immunomodulatory studies such as Nitroblue tetrazolium (NBT) assay and cellular lysosomal enzyme activity. Then the selected plant extracts were identified by means of UV-Visible and Infra Red spectroscopic methods.

Acute toxicity studies were performed according to Organization for Economic Cooperation and Development (OECD) guidelines 425, received from CPCSEA, Ministry of Social Justice and Empowerment, Government of India[30]. Swiss albino mice weighing between 20-25 g in groups of six were used (n=6). The animals were fasted for 4 h with free access to water only. The both Nn and Td extracts were administered orally in doses of 2000 mg/kg to different groups of mice and observed for 14 days for mortality and physical/behavioural changes. The experiments were performed after the experimental protocols had been approved by the Institutional Animal Ethical Committee (183/SASTRA/IAEC/RPP).

The phytosomes were prepared by combining the two methanolic extracts (Nn and Td) in an equimolar amount with various ratios of phospholipids using solvent evaporation technique[31]. The ratio of drug and phospholipid employed was 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10. Thereby four batches of formulations each containing six formulae, totally 24 formulations were made using four different types of phospholipids such as phosphatidic acid (PA), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). Among the 24 phytosomal formulations, the one which produced better physicochemical characteristics, entrapment efficiency and sustained drug release for highest duration was selected for further *in vivo* studies.

The phospholipids and plant extracts were dissolved in tetrahydrofuran and dioxane:methanol (7:3) respectively. The above mixture was combined together and refluxed for 3 h at 100 rpm. Then the solvent was evaporated under reduced pressure at a temperature of about 60ºC using a rotary flash evaporator. This was resulting in the formation of thin film containing solid mixture deposited on the wall of the round bottom flask which was about 1.5 cm above the boiling water bath under reduced pressure. Then phosphate buffer saline (pH 7.4) solution was added to the flask heated to about 50ºC on a vortex, until a good dispersion of the mixture was obtained. The suspension was then sonicated for 10 min using 3 mm spindle in ultrasonicator.

Trial formulations were prepared by varying temperature, stirring speed and stirring time and thereby optimum conditions were selected, based on the physical characteristics, for formulating phytosomes.

The surface morphologies of the formulated phytosomes were examined by Scanning Electron Microscopy (SEM). The particle size distribution for each batch of phytosomes was determined with Nanosizer using Coulter Light Scattering Technique. All the phytosome formulations were sterilized by filtration technique using Millipore filter. Following sterilization, phytosome formulations were stored in desiccators at 20ºC until use.

Calibration curve was obtained by preparing a series of extract solutions using phosphate buffer saline (PBS) pH 7.4 of known concentration which ranges from 10 µg/ml to 50 µg/ml and their absorbances were measured spectrophotometrically at 278 nm. Each phytosome formulation was analyzed for drug content and the concentration of active principles in phytosomes and control samples were calculated from the obtained linear regression equation.

The *in vitro* release study was carried out by simple diffusion method using sigma dialysis membrane as a barrier. It consists of an open ended cylindrical glass tube with an inner diameter of 2.5 cm, open at both ends. One end of the tube was tied with sigma dialysis membrane which serves as a donor compartment, containing phytosomal suspension. This was placed in a beaker containing 400 mL of PBS pH 7.4, stirred at 200 rpm speed, maintaining the temperature at 37ºC, which serves as a receptor compartment. Periodically

5 mL of samples were withdrawn and after each withdrawal, same volume of medium was replaced. Then the samples were analyzed UV spectrophotometrically at 278 nm using PBS pH 7.4 as blank.

The selected phytosome formulation was finally fitted into various pharmacokinetic models (Zero order, First order, Higuchi and Peppas equation), by means of which we could able to find out the mechanism of release of the active constituents from the prepared formulations.

The efficacy of the selected phytosome formulation was compared with the plain extract and standard drug using *in vivo* animal models. The mice were treated with phytosome formulation containing Nn and Td extracts and plain extracts of the same for 21 days. The immunomodulant activity was measured in terms of their delayed-type hypersentivity (DTH) reaction and humoral antibody response in mice[32,33].

The animals were divided into eight different groups each containing twelve animals. Group-I was served as normal control receiving only vehicle (1% Tween 80) for 21 days; Group-II was served as disease control receiving Cyclophosphamide 100 mg/kg orally on day 9 and 16; Group-III was served as standard control receiving Levamisole (50 mg/kg orally) for 21 days; Group-IV was treated with Plain extract of Nn & Td for 21 days; Group-V was treated with Phytosome formulation containing Nn & Td for 21 days; Animals in Group-VI, VII and VIII were treated with Levamisole, Plain extract and Phytosome formulation for 21 days plus Cyclophosphamide on 9^{th} and 16^{th} day as a single dose respectively.

On day 7 and 14, six mice from all the groups were immunized and challenged respectively, with 0.1ml of 20% sheep red blood corpuscles (SRBCs) in normal saline, intraperitoneally. Blood was withdrawn from the animals on the 14^{th} and 21^{st} days, from the retro-orbital plexus, under mild ether anesthesia and centrifuged to obtain the serum. The collected serum was used to determine haemagglutination antibody titre value by 96 well microtitre plates.

The highest dilution giving haemagglutination was taken as the antibody titre. Antibody titre obtained on 14^{th} day after immunization (on 7^{th} day) and on 21^{st} day after challenge (on 14^{th} day) with SRBCs was considered as primary and secondary human immune responses respectively.

On day 7, the remaining six mice from all the groups were immunized with 0.1 mL of 20% SRBCs in normal saline intraperitoneally. Then they were challenged on 14^{th} day with 0.3 ml of 20% SRBCs in subplanatar region of right hind paw and their footpad reaction was assessed after 24 h with the help of Plethysmometer.

The purpose of stability testing is to provide evidence on how the quality of a drug substance varies with time under the influence of variety of environmental conditions such as temperature, humidity and light. The optimized batch of phytosome formulation containing Nn and Td was tested for its stability as per ICH guidelines (40±2ºC/RH 75% for 6 months).

OBSERVATIONS

The yield of the various extracts of the whole plant of *Nymphaea nouchali* (Nn) viz. n-Hexane extract (HNn), Chloroform extract (CNn), Ethyl acetate extract (ENn), Methanolic extract (MNn) and Aqueous extract (ANn) was found to be 0.7, 5.2, 3.5, 23.4 and 16.3% w/w respectively.

The yield of the various extracts of the whole plant of *Trichosanthes dioica* (Td) viz. n-Hexane extract (HTd), Chloroform extract (CTd), Ethyl acetate extract (ETd), Methanolic extract (MTd) and Aqueous extract (ATd) was found to be 0.6, 4.3, 5.9, 19.3 and 13.6% w/w respectively.

The preliminary phytochemical investigations revealed the presence of flavonoids, glycosides, polysaccharides, saponins, tannins & phenolic compounds and terpenoids in *Nymphaea nouchali*; and alkaloids, flavonoids, glycosides, proteins & free aminocids and saponins in *Trichosanthes dioica*.

From *in vitro* immunomodulatory studies, it was noted that HNn, CNn, ENn, MNn, ANn, HTd, CTd, ETd, MTd and ATd had shown 35.16%, 44.96%, 41.17%, 81.12%, 63.95%, 31.78%, 32.28%, 62.64%, 81.42% and 33.49% of NBT reduction respectively; 39%, 36.90%, 70.70%, 87.93%, 39.73%, 41.44%, 32.55%, 49.03%, 87.70% and 71% of lysosomal enzyme activity respectively. Thus it was concluded that the methanolic extract of both plants (Nn and Td) enhanced the NBT reduction and also activated the lysosomal enzyme activity. As described earlier by Rainard in 1986, the higher reduction in NBT assay represented higher activity of oxidase enzyme reflecting the stimulation of phagocytes in proportion to ingested foreign particles[34]. Suzuki *et al.,* 1990 stated that the enhanced transformation of *p*-nitrophenyl phosphate to a colour compound by the membrane associated acid phosphatase activity of the treated macrophages in the lysosomal enzyme activity assay is related to the stimulation effect[35]. Thus, methanolic extracts of both plants significantly activated the macrophages and enhanced their function as compared to all other extracts, suggesting that they can have immunostimulant effect.

The healthy albino mice of either sex weighing 25-30g were maintained under controlled conditions of temperature (20-25ºC) and humidity (55%) were used for acute toxicity study and the maximum tolerated dose (MTD) of methanolic extract of Nn and Td was found to be 2000 mg/kg body weight.

Preformulation studies are integral and important one for a stable formulation. It was carried out by using DSC and FT-IR.

The DSC thermograms of individual ingredients such as extracts of *Nymphaea nouchali* and *Trichosanthes dioica*, phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, polyvinyl alcohol and drug-excipients physical mixtures were obtained and compared for compatibility.

The IR spectra was also obtained and compared for the drug, excipients and drug-excipients physical mixtures. The results revealed that there was no interaction between the drug and other excipients used in the phytosome formulation.

The phytosomes were prepared by using solvent evaporation technique. Sonication brings about size reduction by breaking larger phytosomes into smaller ones. But increase in sonication time favours breakage of binding of drugs with phospholipids. Hence the sonication time was optimized to 10 min and further size reduction was not attempted.

The prepared phytosomes, containing both Nn and Td in varying ratios with phospholipids, were subjected to physicochemical characterization. The entrapment efficiency of Nn/Td phytosomes using various phospholipids like phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine was measured and found to be in the acceptable ranges from 32-89% w/v.

Microscopic examination in scanning electron microscope was done for characterising the shape and surface of the phytosomes. The results of microscopic examination revealed that the optimised phytosome formulation was found to consist of smooth and spherical vesicles showing discrete arrangement.

The particle size distributions of the selected batches of *Nymphaea nouchali*- and *Trichosanthes dioica*- extract loaded phytosomes demonstrated a Gaussian curve. The results of laser particle count analyzer had shown that the mean particle size of the phytosome formulations was ranged from 40-500 nm.

The *in vitro* release study was carried out by diffusion method using sigma dialysis membrane as a barrier. From this study, the percentage of drug diffused into the medium was evaluated. The maximum percentage of drug diffused from the phytosomes using various phospholipids like phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine were obtained and found that F5, F11, F17 and F23 phytosomes attained the maximum release of 97.34% after 9h, 98.45% after 11h, 99.82% after 17h and 66.45% after 13h respectively. Thus, F17 phytosomes formulated with phosphatidyl ethanolamine was found to attain maximum percentage of drug release with longer duration of time. The results revealed that F17 phytosomes loaded with phosphatidyl ethanolamine had highest entrapment efficiency and maximum longevity of release.

The results obtained from the *in vitro* studies were fitted into various pharmacokinetic models, by means of which it revealed the mechanism of release of the active constituents from the phytosome formulation.

Modulation of the immune system can be addressed through a variety of specific and non-specific approaches. For *in vivo* animal studies, Nn/Td loaded phosphatidyl ethanolamine phytosomes were prepared; evaluated for physicochemical properties; and haemagglutination antibody titre value and delayed type hypersensitivity reaction were assessed.

Haemagglutination antibody titre was determined to establish the humoral response against sheep red blood corpuscles (SRBC). Primary and secondary antibody titre values determined on day 14^{th} and 21^{st} respectively, for F17 phytosomes treated group with normal immune status showed significant increase ($p<0.01$) in haemagglutination antibody titre when compared with the control group.

Among the immunosuppressed groups (VI, VII & VIII), where the administration of cyclophosphamide on day 9^{th} and 16^{th} causing immunosupression Group VIII receiving F17 Phytosomes plus cyclophosphamide showed a significant rise ($p<0.01$) in haemagglutination antibody titre when compared with the cyclophosphamide group. The treatment with Nn/Td phytosomes improved the haemagglutination antibody titre reflecting an overall elevation of humoral immune response.

Delayed type hypersensitivity (DTH) reaction is characterized by large influxes of non-specific inflammatory cells. The mice treated with Nn/Td phytosomes had shown enhanced DTH reaction ($p<0.01$) due to increase in footpad thickness compared to control and other groups. This reflects the movement of more number of macrophages to the inflammatory site. This study had shown that Nn/Td phytosomes presents potent *in vivo* immunomodulatory activity in mouse immune system for both macrophage phagocytosis and splenocyte proliferation.

All the values were expressed as mean ± standard deviation (S.D.) and the results analyzed by using Student's t-test were statistically significant.

The stability study for the optimized phytosome formulation was carried out as per ICH guidelines (40±2ºC/ RH 75% for 6 months). The formulation showed no significant change with respect to physical appearance, drug content and diffusion pattern. Based on the above results it can be concluded that the optimized formulation, F17 was stable at the temperature of 40±2ºC and relative humidity of RH 75%.

INFERENCE

From the preliminary phase of this research work, it was observed that alkaloids, flavonoids, proteins & free aminocids, saponins, tannins & phenolic compounds and terpenoids were the phytoconstituents present in the methanolic extract of *Nymphaea nouchali* and *Trichosanthes dioica*. These phytoconstituents might play an effective role to bring out electron-transferring system and intracellular killing in *in vitro* immunomodulatory assays which gives them an immuno modulation property.

Also noted that phosphatidyl ethanolamine phytosomes was better than that of other phospholipids used (phosphatidic acid, phosphatidyl choline and phosphatidyl serine). Stability studies had shown that phosphatidyl ethanolamine phytosomes exhibited maximum drug content even after 6 months when stored at 40±2ºC / 75% RH. Also a maximum of 89% of entrapment efficiency was achieved by means of this research work.

The results of *in vitro* release of the phytosomes with various phospholipids have shown that the phytosomes formulated with phosphatidyl ethanolamine gave prolonged release with highest cumulative percentage of drug than other phospholipids.

In vivo characteristics of sterilized phytosomes containing *Nymphaea nouchali* and *Trichosanthes dioica* with phosphatidyl ethanolamine was administered intraperitonially to mice for 21 days produced excellent result in stimulating the immune system against the challenging agents.

Thus, the present study has shown the immunostimulatory activity of Nn and Td and suggested their therapeutic usefulness. They have stimulated both humoral as well as cellular arms of the immune system.

SUMMARY AND CONCLUSION

The methanolic extracts of two botanicals, *Nymphaea nouchali* and *Trichosanthes dioica*, were converted into phytosomes and evaluated for immunomodulatory activity. The therapeutic regimen of phytosomes containing both the plant extracts was able to produce significant synergistic immunostimulatory action.

Phytosome formulations containing combined plant extracts have produced effective immunomodulant activity as compared to plain extracts and standard drug.

These findings are very much promising that phytosome technology can be safely used to incorporate the phytoconstituents in various ratios using suitable phospholipids.

The results of this research work provide a scientific evidence for the traditional use of *Nymphaea nouchali* and *Trichosanthes dioica*. Also the phytosome technology can offer an alternative therapy for the treatment of many ailments including AIDS, TB, etc and might be used in immunocompromised patients for boosting their immune system.

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