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Research Article

DEVELOPMENT AND CHARACTERIZATION OF NOVEL PHOSPHOLIPID-HERBAL COMPLEX OF A POLYHERBAL FORMULATION

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ABSTRACT

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia that may cause several complications like peripheral arterial disease, coronary artery disease, stroke, neuropathy, retinopathy, nephropathy, etc. Herbal drugs are commonly used in the traditional system of medicine in the management of diabetes. *Momordica charantia*, *Andrographis paniculata* and *Withania somnifera* are well-known plants for their therapeutical potential like antidiabetic, antihyperlipidemic, hepatoprotective action, cardioprotective, immunomodulatory effects etc. Active phytoconstituents from plant extracts showed excellent *in-vitro* activities but less efficacious *in-vivo* activities, which could be because of their macromolecular size and poor lipid solubility resulting in reduced bioavailability. The phospholipid-based delivery system has been developed to improve the oral availability of compounds in the plant extract. Hence, the present study was conducted to develop a novel phospholipid-herbal complex (Phytosome) of an optimized polyherbal formulation containing three herbs *Momordica charantia*, *Andrographis paniculata* and *Withania somnifera*. The phospholipid-herbal complex was prepared by solvent evaporation technique using methanol. Complex thus formed was characterized by shape, morphology, SEM, entrapment efficiency, *in-vitro* release profile and Fourier Transform Infrared spectroscopy (FTIR) analysis. Efficacy of phosholipid-herbal complex and polyherbal formulation was evaluated by using Oral Glucose Tolerance Test (OGTT) in normal rats. The particle size of formulation showed size in range of 758.0 nm with polydispersity index of 0.140 and possessed good stability, indicated by Zeta potential of -11.9 mV. FTIR analysis of formulation revealed formation of a complex and presence of hydrogen bonding. In OGTT, the phosholipid-herbal complex showed significant reduction in glucose levels in rats as compared to the polyherbal formulation.

Keywords: Phospholipid, *Momordica charantia, Andrographis paniculata, Withania somnifera*, FTIR.

1. INTRODUCTION

Diabetes mellitus is a term for heterogeneous disturbances of metabolism and characterized by high blood glucose levels and long term complications include chronic kidney disease, cardiovascular disease, damage to nerves and eyes [1]. Active constituents derived from plants have been used for health maintenance and to treat various diseases since ancient time [2]. Medicinal plants used in ancient systems of medicine are used for the development of safer and effective oral hypoglycemic agents for diabetes management [3]. However, many active constituents extracted from plants are poorly absorbed when administered orally, which limits their therapeutic applications [4]. Most of the bioactive constituents of plants like flavonoids, tannins, glycosidal aglycones, etc., are polar or water soluble and they are poorly absorbed either due to their large molecular size or due to their poor lipid solubility, thus severely limiting their ability to transport across lipid-rich biological membranes resulting in their poor bioavailability [5, 6].

Phytosome is a novel drug delivery system in which hydrophilic choline moiety (head) of phospholipids binds to phytoconstituents (polar) and its lipophilic phosphatidyl moiety surrounds choline bound phytoconstituents or form outer layer, hence water soluble phytoconstituents become lipid soluble [7]. It is reported that hydrogen bond forms between the entrapped phytochemicals and polar head group of phospholipids [8]. It is a new patented technology developed to incorporate standardized plant extracts or water-soluble phytoconstituents into phospholipids to produce lipid compatible molecular complexes, which improves their

gastrointestinal absorption, entrapment efficiency, stability profile and bioavailability [9, 10]. Moreover, phospholipids are used in all known life forms to make cell membranes. In humans and animals, phospholipids are also employed as natural digestive aids and as a carrier for both fat and water soluble phytonutrients [11]. Recent studies revealed the significance of this technology for standardized extracts of *Ginkgo biloba* [12], grape seed, green tea [13] and ginseng [14]. Phytosomes of boswellic acid, curcumin [15], silymarin [16], naringenin and polyphenols improved the clinical efficacy without compromising the safety for various therapeutic activities [17, 18].

Momordica charantia (Karela) is a member of the cucumber family (Cucurbitaceae), also known as bitter melon. The fruit of *Momordica charantia* contains glycosides, saponins [19], alkaloids, triterpenoids [20], flavonoids [21], polypeptides [22] and sterols [23]. It has been reported that the fruit of *Momordica charantia* have antidiabetic [24], antihyperlipidemic, anticarcinogenic [25], anti-inflammatory, hepatoprotective, antiviral, antipyretic and antimalarial activities [26]. Aqueous and ethanolic extracts of *Momordica charantia* showed antidiabetic potential by increasing glucose uptake by inhibiting enzymes involved in the glycolysis pathways including glucose-6-phosphatase and fructose 1, 6-diphosphatase [27] and stimulate glucose-6-phosphatase dehydrogenase in liver [28]. Moreover, aqueous and chloroform extracts of *Momordica charantia* have showed significantly increased insulin sensitivity and glucose uptake by up-regulation of Glut-4, peroxisome proliferator-activated receptor (PPAR-gamma) and PI3K [29]. A study revealed that bitter melon has several components like D-(+)-trehalose, saponin analogs that have α-glucosidase inhibitory activity that contribute to the suppression of post-prandial hyperglycemia and hyperinsulinemia [30].

Andrographis paniculata (Kalmegh) belongs to the family Acanthaceae, commonly known as king of bitters, is an annual, branched, erect herb running half up to one meter in height [31]. Phytochemical studies reported the presence of various secondary metabolites like flavornoids, quinones, terpenoids [32], alkaloids, glycosides, steroids, tannins, saponins and phenolic compounds [33]. It has shown to possess a wide spectrum of pharmacological activities like antihyperglycemic, antihyperlipidemic [34], hepatoprotective [35], cytotoxicity [36], anti-inflammatory [37], immunomodulatory and anti-viral [38]. A study revealed that ethanolic extract of *Andrographis paniculata* and its active compound and rographolide showed glucose lowering and hypolipidemic effects in high-fat-fructosed fed rats [39]. In another study, and rographolide showed significant decrease in blood glucose level and improved morphology of pancreatic islet, beta cells density and pancreatic insulin contents in Streptozotocin (STZ)-induced diabetic rats [40]. A study showed that the ethanolic extract of *Andrographis paniculata* exhibits a significant anti-hyperglycaemic, antioxidant and islet cell regenerative effect in STZ-induced diabetic rats [41].

Withania somnifera (Ashwagandha) is an erect, evergreen, branched shrub from Solanaceae family [42]. The plant is known for its therapeutical uses in Ayurvedic and Unani practices in India [43]. The extract of roots of *Withania somnifera* contains secondary metabolites like alkaloids, saponins, flavonoids, steroids, tannins and coumarins [44, 45]. Numerous studies demonstrated that roots of *Withania somnifera* have been shown to exhibit various pharmacological activities like anticancer [46], anti-microbial [47], hepatoprotective [48], anti-diabetic, antioxidant [49], anti-inflammatory [50], cardio-protective [51], neuroprotective activities [52] and immunomodulator potential [53]. A study reported that the reduction in number and size of pancreatic beta cells were preserved to near normal in morphology by treatment with an aqueous root extract of *Withania somnifera* in STZ induced diabetic rat [54]. According to a recent study, aqueous extract of *Withania somnifera* significantly attenuated the production of pro inflammatory cytokines (IL-6 and TNF-α) through increase in secretion of IL-10 and inhibition of NF-kB activity at 300 mg/kg dose in rats [55].

Hence, the present study was designed to encapsulate optimized polyherbal formulation prepared from lyophilized hydroalcoholic (80%) extracts of *Momordica charantia, Andrographis paniculata and Withania somnifera,* with phospholipid to form a novel phospholipid-herbal complex formulation (PHP) known as Phytosome to enhance the bioavailability of herbal extracts.

2. MATERIAL AND METHODS

2.1. Drug, Chemicals and Instruments

The dried fruit of *Momordica charantia*, the whole plant of *Andrographis paniculata* and roots of *Withania somnifera* were procured from crude drug supplier Herb Heal Consortium Pvt. Ltd., Ramtirath road, Amritsar, Punjab. Crude drugs were authenticated by Dr. Bikarma Singh, Herbarium & Crude Drug Repository Division,

CSIR-IIIM, Canal Road, Jammu (Ref. No: CSIR-IIIM/JAH/2020/07). Phospholipids, other chemicals and reagents used in the study were of analytical grade. Instruments such as Cooling Centrifuge, Rotary evaporator (Buchi Rotavapor R-210, Switzerland), UV/Visible Spectrophotometer (UV-1800, Shimadzu), Lyophilizer, and Scanning Electron Microscope were used.

2.2. Extraction and Lyophilization

All the three herbal drugs were dried under the shade, coarsely powdered and stored in an airtight container. Each drug was extracted with hydro-alcohol (80% v/v) by soxhlation [56]. The prepared extracts were concentrated under vacuum using rotary evaporator at 45°C. The concentrated extracts were freeze dried at -20°C for 12 hrs and then lyophilized using lyophilizer. The lyophilized extracts' powders were stored in an airtight container and kept in the desiccators till used.

2.3. Preparation of Phospholipid-herbal complex (PHP)

Lyophilized extracts of *Momordica charantia*, *Andrographis paniculata* and *Withania somnifera* were combined in different ratios. The specific amount of optimized polyherbal formulation was taken along with 2 times the amount of Phospholipid (soya lecithin) and dissolve in methanol. Particles size of complex was reduced by ultra sonication and solvent was evaporated by rota-evaporator at 40°C to get semisolid mass, which was further dried. The dried complex was placed in an amber colored glass bottle and was stored at room temperature.

2.4. Characterization of Phospholipid-herbal complex

2.4.1. Particle size and Zeta potential determination

Particle size and zeta potential of prepared PHP were determined by the dynamic light scattering (DLS) technique (Malvern Zetasizer, Malvern instruments, Malvern, UK), at a scattering angle of 90° at 25°C [57]. The particle size of prepared PHP was analyzed by photon correlation spectroscopy using Shimadzu particle size analyzer (SALD, Japan). Diluted PHP suspension was placed into the sample dispersion unit and was stirred at room temperature (in order to reduce inter particle aggregation). All the analysis has been performed in triplicate. The surface charge of PHP was determined by using the Malvern Zetasizer (Nano-ZS, UK). Samples were diluted (50 times) using distilled water and analysis was performed at 25°C and 149 watt. The average of three zeta potential values of complex was calculated.

2.4.2. Entrapment efficiency of Phospholipids-herbal complex

The proportion of encapsulated polyherbal complex was determined by centrifuging 0.5mL of the formulation at 18000 rpm for 60 minutes at room temperature. Supernatant was collected and dissolved in methanol, then appropriate dilution was made to measure herbal content based on total phenolic (Gallic acid equivalent) at 765 nm and total flavonoid contents (Rutin equivalent) at 415 nm using UV-Visible spectrophotometer.

2.4.3. In- vitro drug release studies

Phosphate buffer (pH 7.4), as simulated intestinal fluid, was used for the study. Samples were incubated at 37°C using 100mL beaker glass at 100 rpm and the formulation was passed through a membrane. After a time interval of 0, 15, 30, 60, 120, 180 and 240 minutes, aliquots from simulated solutions were withdrawn to determine the polyherbal extract combination based on total phenolic (Gallic acid equivalent) and total flavonoid contents (Rutin equivalent) using UV-Visible spectrophotometer [58].

2.4.4. Scanning Electron Microscopy (SEM)

SEM was used to determine particle size distribution and surface morphology using JSM-6360 scanning microscope (Japan). Dried samples were placed on brass stub and were coated with gold in an ion sputter. Digital images of complex were taken by random scanning of the stub [59].

2.4.5. Fourier Transform Infrared spectroscopy (FTIR) analysis

The interaction studies between herbal drugs and excipients have been carried out by FTIR. Lyophilized powders of *Momordica charantia*, *Andrographis paniculata* and *Withania somnifera,* their optimized combination ratios, soya lecithin along with their physical mixture and phosholipid-herbal complex were analyzed. The samples were mixed with dry crystalline KBr powder in the ratio of 1:100 and examined by FTIR (PerkinElmer FT-IR Spectrometer). The scanning range was 4000-400 cm^{-1} with 1 cm^{-1} resolution [60].

2.5. Estimation of efficacy of phosholipid-herbal complex and polyherbal formulation using OGTT in normal rats

2.5.1. Experimental Animals

Albino wistar rats weighing 180-240 g were housed at Central animal house, Panjab University, Chandigarh.

The rats were housed in clean, sterile, polypropylene cages under standard laboratory conditions (25±2°C, 60-70% humidity) and 12 h light/dark cycle with standard chow (Aashirwad Industries, Mohali, India) and water provided *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Panjab University (PU/45/99/CPCSEA/IAEC/2020/424). The experiment was conducted according to CPCSEA guidelines for the use and care of experimental animals.

2.5.2. Oral Glucose Tolerance Test (OGTT) Study

Rats were fasted overnight to conduct OGTT study. Hyperglycemia was induced by administration of glucose (2 g/kg) to normal rats. The rats were divided into four groups each containing 6 animals. Group I: Normal control rats were administered 0.5% w/v carboxy methyl cellulose solution, Group II: Positive control were administered glucose (2 g/kg), group III and group IV rats received polyherbal formulation and phospholipid -herbal complex (PHP) respectively at single dose of 500 mg/kg per oral prior to one hour of glucose load. The blood was obtained from tail vain at time -60, 0, 60 and 120 minutes after glucose administration. The serum glucose level (SGL) was estimated by glucose oxidase-peroxidase method [61].

2.6. Stability Study

Stability study was carried out as per ICH guidelines to monitor any physical or chemical changes in the phytosomal preparation. The phytosomal complex was stored in sealed glass vial at different temperature ranges, 4±2°C and 25±2°C for a period of 3 months [62]. After 3 months, effect of temperature on physical and chemical stability of phytosomal formulation was determined by FTIR.

3. RESULTS

3.1. Particle size and Zeta potential determination

The particle size of formulation showed size in average range of 758.0 nm (fig. 1) with zeta potential (-11.9 mV) (fig. 2) and polydispersity index of value 0.140.

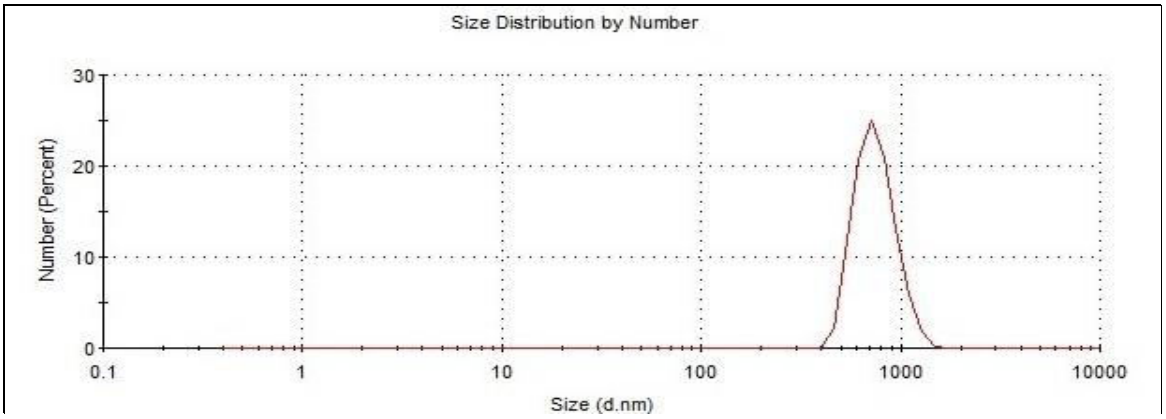

Fig. 1: Partical size distribution of phosholipid-herbal complex

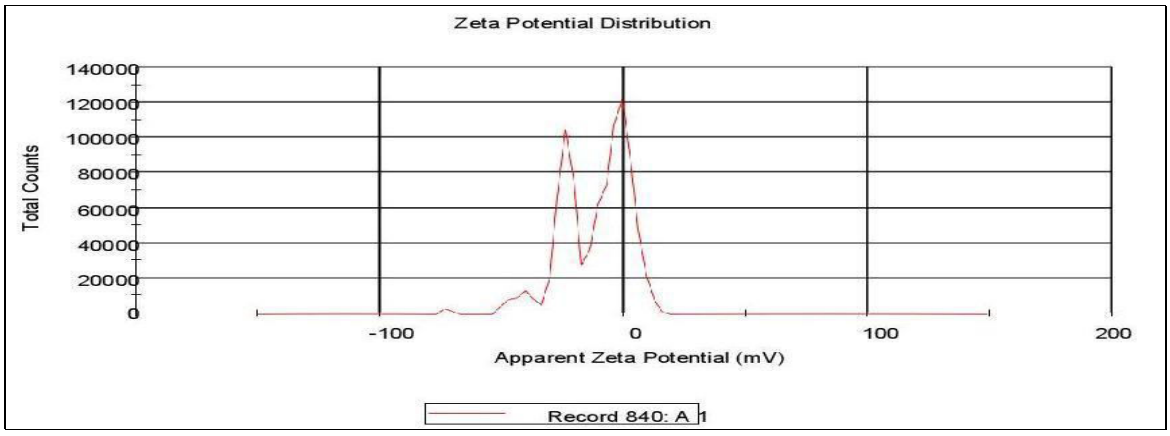

Fig. 2: Zeta Potential (mV) of phosholipid-herbal complex

3.2. Entrapment efficiency of phospholipids herbal complex and *In-vitro* drug release studies

It was found that phospholipid-herbal complex showed 96.79 % entrapment efficacy and *in-vitro* drug release found to be 85.47±5.33% at 150 minutes.

3.3. Scanning Electron Microscopy (SEM)

The scanning electron micrograph showed the abundance of spherical nano-vesicles in the suspension bulk. The structural appearance confirmed the complexation of drugs with phospholipids observed by denser excellent outline that seals the drug perfectly. No disruptions of vesicular structure have been observed which further confirmed that vesicle integrity have been retained even after application of various mechanical stresses (fig. 3).

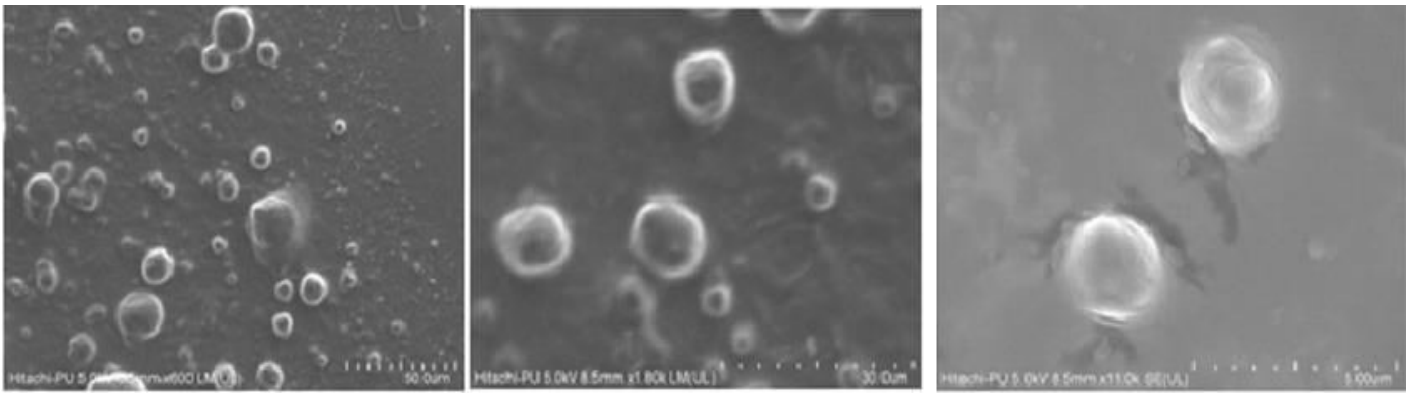

Fig. 3: SEM of phosholipid-herbal complex at different magnification

3.4. Fourier Transform Infrared spectroscopy (FTIR) analysis

The interaction and incompatibility studies between herbal drugs and excipient have been studied using FTIR. FTIR spectroscopy of lyophilized extracts of herbal drugs, soya lecithin, its physical mixture and phospholipid-herbal complex were analyzed. The FTIR spectra of *Momordica charantia* showed its peaks at 3381.62 cm^{-1}, which indicated the presence of amine (N-H) stretching and at 1732.19 cm^{-1} attributed to carbonyl (C=O) stretching. Spectral analysis of *Andrographis paniculata* displayed its peaks at 3392.32 cm^{-1} and 1618.19 cm^{-1} showed the presence of amine (N-H) stretching and (α, β unsaturated) group stretching respectively. The FTIR bands of *Withania somnifera* displayed peaks at 3397.62 cm^{-1} and 1137.9 cm^{-1} which indicated the presence of amine (N-H) stretching and alcohol group with (C-O) stretching respectively. Physical mixture showed peak at 3375.03 cm^{-1} which indicated negligible incompatibility between the drug and excipients. Another peak at 1625.23 cm^{-1} was also observed indicating no shifting in peak has been occurred. Overall, analysis of drugs and excipients compatibility showed that there was neither significant shifting, nor the absence of the peak was reported as a sign of incompatibility. The spectrum of phospholipid-herbal complex showed a new peak at 2036.49 cm^{-1} which did not appear in individual drugs and phospholipid. The spectrum also showed a decreased intensity in the peak at 2925 cm^{-1}. These results suggested that hydrogen-bonding between components of individual drugs and phospholipid play a leading role during the formation of the phospholipid-herbal complex, since hydrogen bonding caused significant differences such as intensity changes of vibration band of functional group involved in interaction (fig. 4).

3.5. Estimation of efficacy of phosholipid-herbal complex and polyherbal formulation using OGTT in normal rats

The normal vehicle administered rats have shown non-significant changes in serum glucose level (SGL). The administration of glucose showed significant rise in SGL after 60 minutes in positive control rats, when compared with normal control group. Polyherbal formulation and phospholipid-herbal complex treated groups at a dose of 500 mg/kg have shown significant lowering of SGL after 60 min of glucose administration when compared with positive control (table 1). Increase in SGL level were found to be significantly higher at 60 minutes, when compared with phospho-lipid-herbal complex, which indicated that phospho-lipid-herbal complex are more efficacious in lowering the blood glucose level than polyherbal formulation.

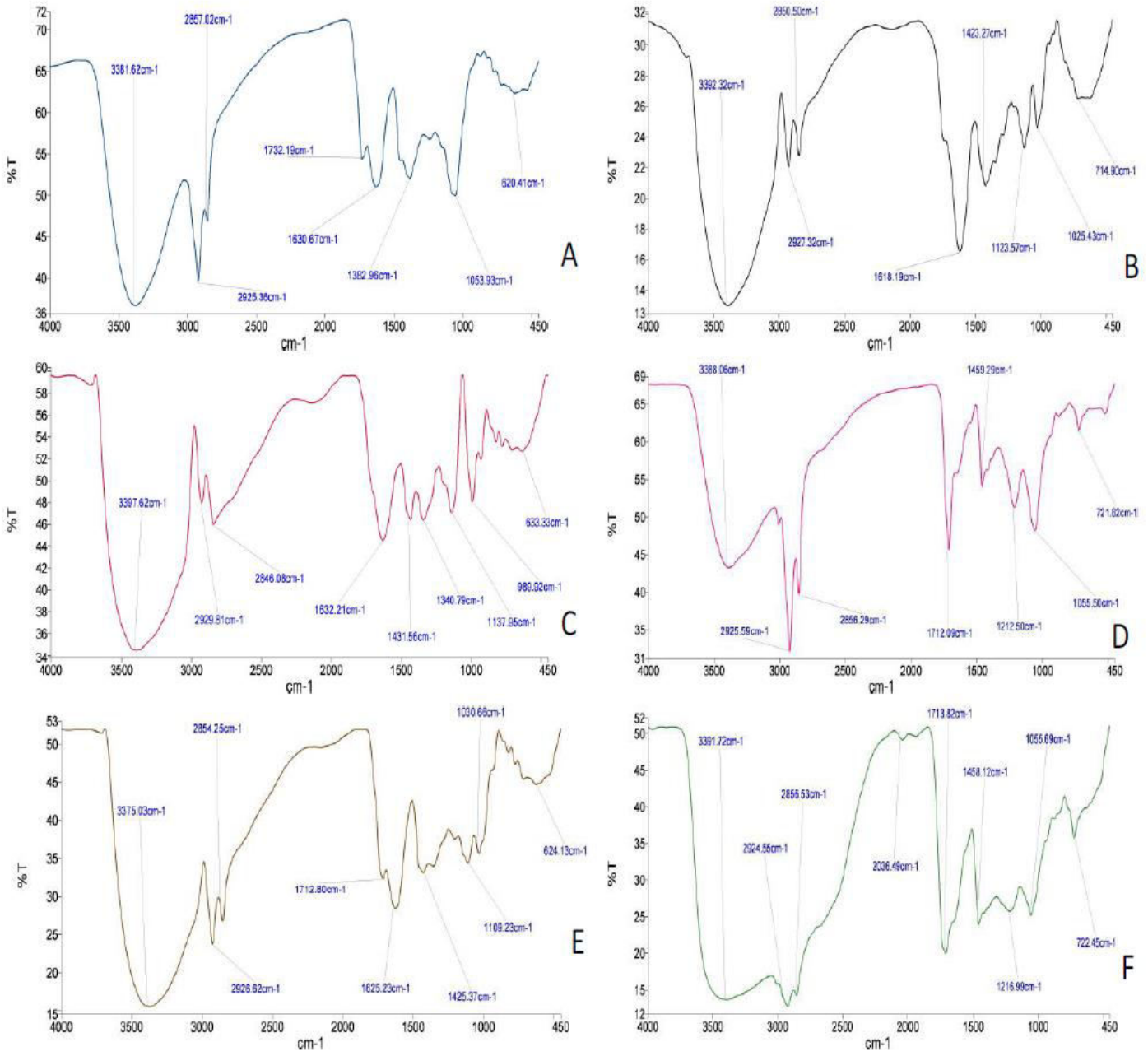

Fig. 4: FTIR Data of *Momordica charantia* (A), *Andrographis paniculata* (B), *Withania somnifera* (C), Phospholipids (D), Physical mixture (E), Phospholipid-herbal complex (F)

Table 1: Effect of polyherbal formulation and phosholipid-herbal complex at the dose of 500 mg/kg body weight on serum glucose level of glucose loaded normal rats

Groups	Serum Glucose levels (mg/dL)			
	-60 mins	0 min	60 min	120 min
Normal Control	91.3±9.18	91± 7.51	93.2±8.13	92.7±7.23
Positive Control	91.7±7.61	92.2±5.98	139±8.56 (49.14%) ↑	105±7.46
Group A Polyherbal Formulation (500 mg/kg b.wt)	92±7.67	88.5±6.80	114±4.23 (22.31%)[a] ↑	95.2±3.76
Group B PHP complex equivalent to (500 mg/kg b.wt)	91.70±8.55	87.7±6.02	102±3.44 (09.44%)[a] ↑	91.7±5.47

([a] = P< 0.05 vs positive control)

3.6. Stability Study

3.6.1. Fourier Transform Infrared Spectrometry (FTIR)

After three months of storage of the phytosomal complex at different temperature ranges, 4±2°C and 25±2°C, effect of temperature on physical and chemical stability of formulation was determined by FTIR. As per the results of FTIR, both conditions of storage are appropriate to keep the formulation.

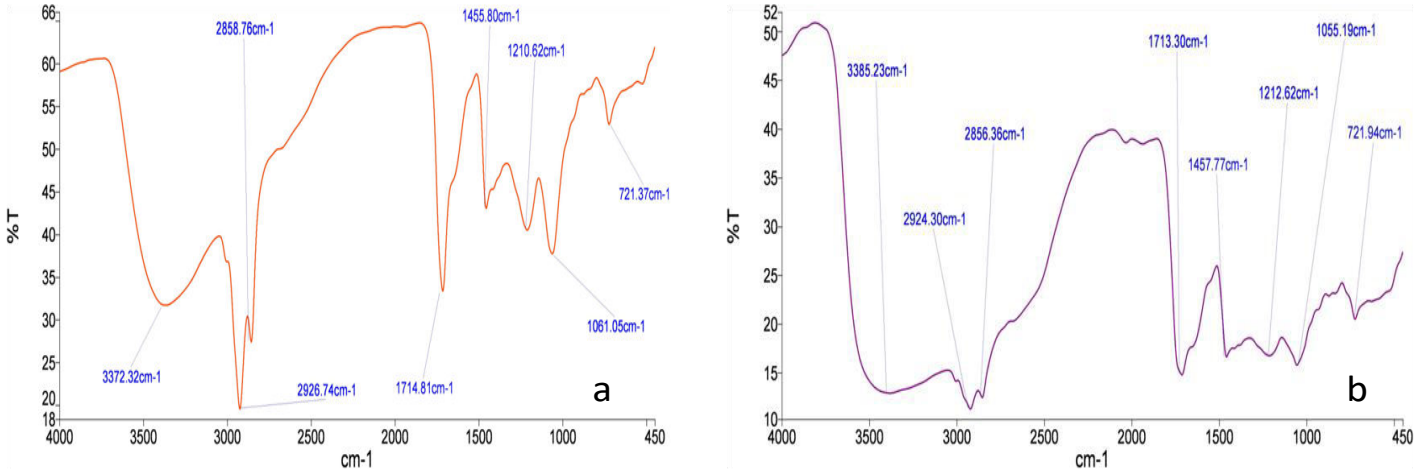

Fig. 5: FTIR data of (a) Phospholipid-herbal complex at 25±2°C, (b) Phospholipid-herbal complex at 4±2°C

4. DISCUSSION

Phytosome, a novel drug delivery strategy, is a technique developed to incorporate standardized herbal extracts or phytoconstituents into phospholipids to produce lipid compatible molecular complexes, which improves their entrapment efficiency, stability profile and bioavailability [63]. In general, the encapsulation of herbal formulations in phospholipids process can improve the absorption and improve their ability to cross lipid-rich biomembranes [64]. The phytosome technology has been successfully applied for the formulation enhancement of many popular herbal extracts including *Ginkgo biloba*, grape seed, *Silybum marianum, Thea sinensis* and *Panax ginseng* [65]. Phytosomal formulations of these herbal extracts shows more efficacy than their non-complexed form in the treatment of various diseases like cardiovascular, anti-inflammatory [66], hepatoprotective, anticancer [67] and as cosmetics against skin-aging [68]. The narrow size distribution of particles ensures the uniformity and anticipation of delivery of bioactive compounds [69]. Our results showed that PHP was successfully prepared with average size of 758 nm, given that small particle size is an important factor for oral absorption and formulation stability. PDI is a parameter used to describe the degree of non-uniformity of a size distribution of particles. PDI showed whether the population of particles is homogenized (PI value<0.3) or heterogenizly (PI value>0.3) distributed [70]. Our results showed the value of PDI (0.147) that indicated the homogenous population.

Zeta potential is an important parameter for physical stability of the phytosomes. In general, particles with similar electric charges may cause repulsion between them that prevent their aggregation and made easy to re-disperse. For a physically stable system desired zeta potential range is of ±20 mV [71]. PHP complex prepared in our work exhibited negative zeta potential (-11.9 mV) indicating good stability of the formulation. A study has demonstrated that negatively charged particles are remaining within the bloodstream for longer periods of time when compared to positively charged particles [72]. This contention supported by suggest the potential of this formulation was also related to entrapment efficacy calculated, which is 97.85 %.

FTIR spectroscopy is an important method for structural analysis and yield different functional groups that show distinct characteristics in band numbers, position, shape and intensity. The phytosome complex can be verified by comparing the spectrum of the complex with individual components and their physical mixture [73]. Our results showed a decrease in intensity in OH peak of phopholipid-herbal extract complex, compared with individual extracts and phospholipid

alone. This proves that there was a complex formed through the formation of hydrogen bonds between extracts and phopholipid. However, in the physical mixture spectrum there was no decrease in the intensity of OH peak, this indicated the absence of chemical bonds between the compounds.

SEM photographs showed important insight into the solid state properties and surface morphology of the phospholipids-herbal extract complex [74]. Our results showed that herbal drug particles are associated with the phospholipids forming complexes with spherical shaped, uniform and rigid vesicles.

In OGTT study, the results obtained for blood glucose concentration of positive control group rats showed high values as compared to normal control rats. In response to a glucose load, the excessive amount of glucose in the blood induces insulin secretion that stimulates the peripheral glucose utilization [75]. In polyherbal drug and phytosome treated groups the blood glucose concentrated was lower than positive control at each time interval. This may be due to the supportive action of glucose utilization by these drugs.

5. CONCLUSION

A novel synergistic drug system, phospholipid-herbal extract (PHP) complex has been successfully prepared for polyherbal formulation. In the OGTT studies, after oral administration of the novel complex formulation, rats showed enhanced antihyperglycaemic effect, indicating that the complex could produce a significantly improved oral bioavailability of the drug. Moreover, based on the current work, it is suggested that novel complex has potential to be further investigated for anti-diabetic studies.

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Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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