RESEARCH ARTICLE

Formulation, Development, Characterization & *In-vivo* Anti-diabetic Evaluation of Hesperidin Phytosome

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ABSTRACT

The aim of the present investigation is to develop formulation, characterization & In-vivo Anti-diabetic Evaluation of Hesperidin Phytosome. The formulation was prepared using Lecithin 45 mg and cholesterol 15 mg, precisely weighed, were dissolved in 10 ml of chloroform in a round-bottom flask (RBF) and subjected to a 10-minute bath sonication. Organic solvents are removed using a rotary evaporator set to 45–50 degrees Celsius. A thin layer of phospholipid mixture formed after the solvent was completely removed. Hesperidine rotary evaporator was used to hydrate this film for one hour at 37–40°C. Transmission electron microscopy was used to examine the morphology of Phytosomes. After being applied to 400 mesh carbon-coated copper grids, the phytosome dispersions were negatively stained using 1% w/v phosphotungstic acid. The Malvern mastersizer S laser diffraction size analyzer (Malvern Instruments Ltd., UK) was used to examine the phytosomes' size distribution. Using the methodology previously described in the literature, the in vivo anti-diabetic activity was assessed. Wistar rats weighing 150–200 g were purchased and kept in the Animal House Facility with 12-hour cycles of light and dark. The diagnostic kit (ERBA Diagnostic Mannheim, Germany) in Autoanalyser was used to estimate the biochemical parameters. The F1 and F2 batches were chosen as the optimal formulation and subsequently assessed further based on morphology (digital photos and TEM), particle size, and encapsulation efficiency. Vesicles with sizes ranging from 100 nm to 500 nm were seen under a scanning electron microscope. The F1 and F2 phytosomes had an average size of 109.71 and 133.24 nm, respectively. There was less peripheral widening between the islets and acinar cells (exocrine tissue) in some areas; both cells now appear close to one another, indicating a return to normal. In summary, a formulation based on Phytosomes may be a useful strategy to increase therapeutic efficacy, lower dosage, and enhance dosage regimen. For their anti-diabetic properties to be claimed, more research involving human subjects must be confirmed.

Keywords: Formulation, Characterization, In-vivo, Anti-diabetic Evaluation, Hesperidin, Phytosome

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INTRODUCTION

Whereas "some" means cell-like, "phyto" refers to the plant.1 Phytosomes are vesicular drug delivery systems that improve low-soluble drug absorption and bioavailability.[1,2] Plant extracts and phosphatidylcholine (or any hydrophilic polar head group) react to form phytosomes, which are complexes of phospholipids and naturally occurring active phytochemicals bound in their structures.[3,4] When compared to common preparations, these formulations show better pharmacological and pharmacokinetic characteristics. The hydrophilic phytoconstituent-choline complexes are entirely covered by the lipid-soluble phosphatidyl portion. High drug encapsulation, a better stability profile (chemical bonds are formed between the phytoconstituent and the polar head of the amphiphile molecule,[5] and improved bioavailability[6] are just a few of the impressive advantages of Phytosomes. The only phytochemicals that can be incorporated into a Phytosomes structure are those that contain an active hydrogen atom (-COOH, -OH, -NH2, -NH, etc.), such as polyphenols. The hydrophilic portions of amphiphile molecules and the herbal derivatives can establish a hydrogen bond with an active hydrogen atom.[7,8] However, the road from product development to successful commercialization is a long one. Once a successful formulation has been created, proving safety is the main obstacle to Phytosomes' commercialization. Because of their biologically neutral structures, Phytosomes can be introduced into the human body without raising any concerns about immunological reactions or safety.[9] However, before they are marketed, certain factors like bioaccumulation, biocompatibility, metabolism, and excretion should be established in relation to their nano size.[10] A curcumin Phytosomes that was successfully prepared for intravenous administration in rats demonstrated a high accumulation in the tissues of the spleen and bone marrow.[11] The capacity of Phytosomes to bind with biological membranes and passively target healthy cells should also be taken into account. Therefore, both clinical trials and carefully planned animal models should be used to ascertain their true biological effects. Various studies have demonstrated the biological safety of Phytosomes in this regard.[12,13] The goal of the current study is to prepare and assess Phytosomes of bioactive flavonoids,

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Table 1: Formulation composition of Phytosomes formulation of Hesperidine.

S No	Formulation	Cholesterol	Lecithin	Chloroform	Hesperidin	Cholesterol: lipid ratio
1	F1	15 mg	40 mg	10 ml	10 ml	1.5:4
2	F2	15 mg	45 mg	10 ml	10 ml	1.5:4.5
3	F3	15 mg	50 mg	10 ml	10 ml	1.5:5
4	F4	15 mg	55 mg	10 ml	10 ml	1.5:5.5
5	F5	15 mg	60 mg	10 ml	10 ml	1.5:6
6	F6	15 mg	65 mg	10 ml	10 ml	1.5:6.5
7	F7	15 mg	70 mg	10 ml	10 ml	1.5:7

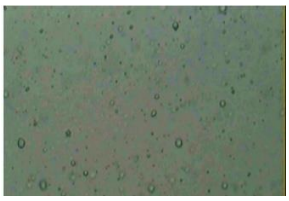

Figure 1: Digital microscopy of F1 & F2

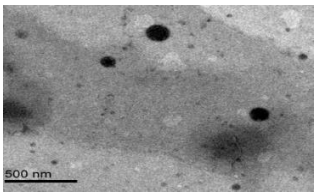

Figure 2: TEM of Phytosomes of F1

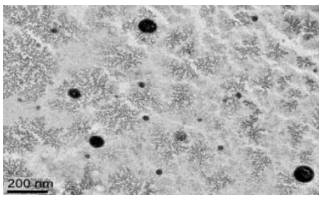

Figure 3: TEM of Phytosomes of F2

Table 2: Optimized formulation composition of Phytosomes.

Formulation	Cholesterol	Lecithin	Chloroform	Hesperidin	Cholesterol: lipid ratio
F1	15 mg	45 mg	10 ml	10 ml	1.5:4.5
F2	15 mg	45 mg	10 ml	10 ml	1.5:4.5

Table 3: The effect of 21 days treatment of Phytosomes F1 and F2 on serum glucose level in STZ-nicotinamide induced diabetic rats.

Groups/ Treatment	Time (Days)			
	0	7	14	21
Diabetic control (Normal saline)	276.20 ± 5.80	274.40 ± 7.30	274.40 ± 8.75	275.00 ± 11.49
STZ+NIC + Glibenclaimide 10 mg/kg	214.40 ± 1.82*	197.06 ± 0.60*	178.35 ± 2.95	151.70 ± 4.30*
STZ+NIC + F2 Phytosomes 100mg/kg	263.83 ± 1.01*	261.16 ± 0.83*	259.33 ± 1.02*	253.60 ± 1.43*
STZ+NIC + F2 Phytosomes 200mg/kg	240.40 ± 4.23**	225.08 ± 2.36**	201.08 ± 1.95**	199.04 ± 1.32**

such as hesperidine, based on the aforementioned viewpoints.

MATERIALS AND METHODS

Chemicals

Sigma-Aldrich Chemicals (St. Louis, MO, USA) supplied the 30% soy lecithin, HiMedia Laboratories Pvt. Ltd., the cholesterol, Kem Light Laboratories Pvt. Ltd., the glibenclamide, nicotinamide, and streptozotocin, which were kept between 2 and 4°C for later use. The remaining chemicals were all analytical grade and were acquired from well-known commercial vendors.

Method of preparation of phytosomes

As shown in Table 1 Lecithin 45 mg and cholesterol 15 mg, precisely weighed, were dissolved in 10 ml of chloroform in a round-bottom flask (RBF) and subjected to a 10-minute bath sonication. Organic solvents are removed using a rotary evaporator set to 45–50 degrees Celsius. A thin layer of phospholipid mixture formed after the solvent was completely removed. Hesperidine rotary evaporator was used to hydrate this film for one hour at 37–40°C. Following hydration, the lipid and plant extract mixture was sonicated for 20 minutes using a PCI Analytics Ultrasonic Probe Sonicator while an ice bath was present to dissipate heat. After that, the prepared phytosomes were kept in a freezer between 2 and 8 °C until they were needed.[14]

Characterization of phytosomes

Morphology of phytosomes

Transmission electron microscopy (TEM) was used to examine the morphology of Phytosomes. After being applied to 400 mesh carbon-coated copper grids, the phytosome dispersions were negatively stained using 1% w/v phosphotungstic acid. A Philps CM 120 BioTwin transmission electron microscope (Philips Electron Optics BV, The Netherlands) was used to view the morphology of the phytosomes.[14,15]

Size analysis of phytosomes

The Malvern mastersizer S laser diffraction size analyzer (Malvern Instruments Ltd., UK) was used to examine the phytosomes' size distribution. To maintain the phytosomes' dispersion throughout the size measurement process, a medium-speed stirring magnetically stirred cell dispersion unit (Malvern Instruments Ltd., UK) was used. The polydisperse mode of analysis was selected, and the measurement position was 1.05 mm. These setup parameters allowed for precise measurement of particles with sizes ranging from 0.1 to 10,000 d.nm. The device measured the size and size distribution as ZAve.[15]

In-vivo anti diabetic activity

Using the methodology previously described in the literature, the in vivo anti-diabetic activity was assessed.[16,17] Wistar rats weighing 150–200 g were purchased and kept in

the Animal House Facility with 12-hour cycles of light and dark. Under typical laboratory conditions, the animals were housed in standard polypropylene cages with a standard diet and unlimited access to water, at a room temperature of 30 ± 2°C and 60–65% relative humidity. The institutional Animal Ethical Committee approved the experimental protocol, and the in-house ethical committee has granted written consent for the study to be completed.

Streptozotocin- nicotinamide induced diabetic model

The chemical name of streptozotocin is 2-Deoxy-2-[[(methylnitrosoamino) - carbonyl] amino]-D-glucopyranose is a naturally occurring substance that is especially harmful to the β-cells in the mammalian pancreas that produce insulin.[18,19] When administered intraperitoneally 15 minutes prior to streptozotocin administration (60 mg/kg i.v.) to 2-month-old Wistar rats, a dose of 200–230 mg/kg b.w. nicotinamide results in a maximum of 40% reduction in pancreatic-cell mass and moderate stable non-fasting hyperglycemia (150–180 mg/dl). With reduced β-cell mass and maintained insulin responsiveness to glucose and tolbutamide, STZ-NA-induced diabetic syndrome may offer a particularly useful tool for pharmacological studies of both novel insulinotropic agents[20] and factors promoting β-cell growth.[21] Several characteristics of human type 2 diabetes are shared by the streptozotocin-nicotinamide type 2 model, including glucose intolerance, moderate stable hyperglycemia, and an altered.[22] Streptozotocin (60 mg/kg) was administered intraperitoneally (i.p.) 15 minutes after Nicotinamide (120 mg/kg) was administered to overnight-fasted rats to induce diabetes. Nicotinamide was dissolved in regular saline, and streptozotocin was dissolved in citrate buffer (0.1 M, pH 4.5).

Experimental protocol

The following groups of six animals each were randomly selected from among the diabetic animals.

Group I: Normal saline, the diabetic control vehicle

Group II: Glibenclaimide (10 mg/kg) was given to diabetic animals.

Group III: Hesperidine Phytosomes (F1-100 mg/kg) were given to diabetic animals.

Group IV: Hesperidine Phytosomes (F2-200 mg/kg) were given to diabetic animals.

Treatment

For 21 days, the medication solution and vehicle were administered orally once daily. In fasted animals, the effects of the vehicle, Phytosomes, and standard medication on body weight and blood glucose were assessed at 0, 7, 14, and 21 days following oral administration. Under mild anesthesia, a blood sample was obtained from the retro orbital plexus and cardiac puncture of every animal on the twenty-first day. The diagnostic kit (ERBA Diagnostic Mannheim, Germany) in Autoanalyser was used to estimate the biochemical parameters.

Determination of biochemical parameters

All animals' blood was drawn using the retro-orbital plexus technique, placed in tubes with EDTA sprinkled on top, and centrifuged for 20 minutes at 3000 rpm. Prior to analysis, the serum was separated and kept at -20°C. Cholesterol, HDL, LDL, VLDL, and triglycerides were measured in serum samples using an auto analyzer and a diagnostic kit from ERBA Diagnostics in Mannheim, Germany.

Histopathological examination

All groups of animals underwent anesthesia and dissection. After the animal was sacrificed, the kidney and pancreas were removed, and the tissues were preserved in 10% formalin and rinsed in ice-cold normal saline. To prevent decomposition, the tissues were preserved in 10% formal saline for a full day. Following cleaning, the tissues were embedded in paraffin wax, which has a melting point of 58 to 60° Celsius. The paraffin-embedded pancreas was sectioned using a semi-automated microtome at a thickness of 7 µm. A pathologist used a light microscope (BX43, Olympus, Japan) with a digital camera to examine the slides under a 400× magnification.

Statistical analysis

The mean ± SEM for each group of six rats is used to present the results. A minimum of three replicates were used in each triplicate experiment. Mean ± S.E.M. is used to express values. One-way analysis of variance (ANOVA) and Dunnet's multiple comparison test were used to analyze the data using Graph Pad Prism.

RESULTS AND DISCUSSION

Phytosomes characterization

An organized, structured approach to figuring out the relationship between a process's output and the factors influencing it is experiment design.[23] Designing a

Table 4: The effect of Phytosomes F1 and F2 and glibenclaimide on serum cholesterol, triglycerides, HDL-C, VLDL-C and LDL-C level in diabetic rats.

Groups/ Treatment	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)
Diabetic control (Normal saline)	256.32± 1.2	297.55± 2.43	43.12± 0.27	156.22 ± 1.12	58.72 ± 1.16
STZ+NIC + Glibenclaimide 10 mg/kg	178.22± 1.53*	186.33 ± 0.31*	48.34 ± 2.52*	103.14 ± 0.18*	41.94 ± 1.39*
STZ+NIC + F1 Phytosomes 100mg/kg	200.45± 2.27*	211.12± 1.50*	44.97± 2.13*	114.22 ± 0.52*	50.28 ± 2.17*
STZ+NIC + F2 Phytosomes 200mg/kg	197.78 ± 2.16**	197.96± 1.66**	45.33± 1.82**	103.34 ± 0.35**	49.52 ± 1.64**

All values represent means ± S.D of the mean (n=6), *p<0.05, vs diabetic control group

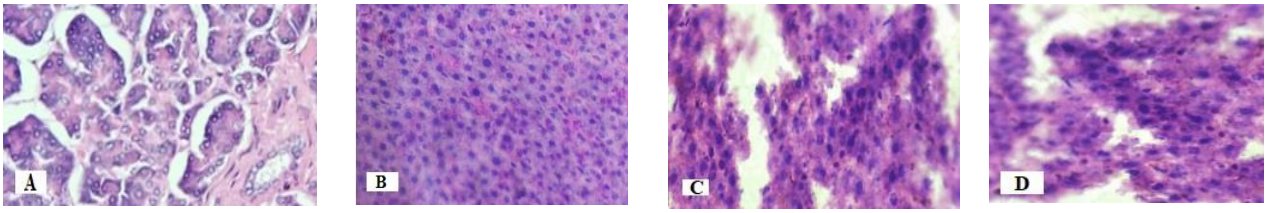

Figure 4: Photomicrograph of pancreatic section (A) Diabetic control (B) Standard drug (C) F1 Phytosomes 100mg/kg (D) F2 Phytosomes 200mg/kg.

formulation with optimal quality in a short amount of time and with the fewest number of trials and at a lower cost than traditional methods of formulating dosage forms is a crucial issue in the development of any pharmaceutical product.[24] Seven batches of bioactive flavonoid formulations with varying lipid and lecithin ratios were chosen based on the formation of phytosomes, the effectiveness of encapsulation, and the required particle size (Table 2). The F1 and F2 batches were chosen as the optimal formulation and subsequently assessed further based on morphology digital photos (Figure 1) and TEM (Figure 2 and Figure 3), particle size, and encapsulation efficiency. Under a digital microscope, uniform, regular, and rigid vesicles were visible. The formation of vesicles was demonstrated by TEM, and an analysis of their morphology revealed tiny, spherical, unilamellar vesicles. Vesicles with sizes ranging from 100 nm to 500 nm were seen under a scanning electron microscope.

Size analysis of phytosomes

The Malvern mastersizer S laser diffraction size analyzer (Malvern Instruments Ltd., UK) was used to examine the phytosomes' size distribution. The F1 and F2 phytosomes had an average size of 109.71 and 133.24 nm, respectively.

Streptozotocin- nicotinamide induced diabetic model

Streptozotocin (60 mg/kg) was administered intraperitoneally (i.p.) 15 minutes after nicotinamide (120 mg/kg) was administered to overnight-fasted rats to induce diabetes. Nicotinamide was dissolved in regular saline, and streptozotocin was dissolved in citrate buffer (0.1 M, pH 4.5). as shown in Table 3. All values represent means ± S.D of the mean (n=6), *p<0.05,**p<0.01 vs diabetic control group For 21 days, the animals were given the dose once a day. Following 21 days, blood samples were taken under mild anesthesia using the retroorbital plexus. The fasting serum glucose level in the diabetic control group, which was not receiving medication, did not significantly change after 21 days of treatment in comparison to the first day of treatment. Serum glucose levels in treated diabetic groups, however, decreased gradually and steadily. After 21 days of dosing, the Phytosomes F1 and F2 treated groups showed a steady decrease in serum glucose levels, and the results were significant when compared to the untreated diabetic control group.

Effect of different lipid levels

As shown in Table 4, diabetic rats treated with Phytosomes F1 and F2 for 21 days demonstrated a significant decrease in total cholesterol, triglycerides, HDL-C, LDL-C, and VLDL-C levels at a dose of 200 mg/kg. In comparison to the control group, the standard medication glibenclaimide (10 mg/kg) also demonstrated a decrease in total cholesterol, triglycerides, HDL-C, LDL-C, and VLDL-C levels.

Histopathological examination

Microscopic analysis of the diabetic control group's pancreatic sections revealed severe vacuolation in the islet, necrotic changes, β-cell degranulation, pycnotic β-cell nuclei, and a breakdown of the architecture of the pancreatic β-cells. The anatomical structure of the islets was significantly improved in group II, which received glibenclaimide treatment. The groups III and IV, which received the optimized phytosome formulation at doses of 100 mg/kg and 200 mg/kg, demonstrated greater improvement, with β-cell recovery beginning, cell proliferation occurring, vacuolation being decreased, and an increase in the number and size of pancreatic islets. There was no vacuolation and the formation of new β-cells occurred. Histologically, the pancreatic β cells' islets of Langerhans displayed peripheral widening between exocrine tissue and islet cells, architectural disarray, and an increase in intercellular space. There was less peripheral widening between the islets and acinar cells (exocrine tissue) in some areas; both cells now appear close to one another, indicating a return to normal as shown in Figure 4.

CONCLUSION

Lipids were discovered to be compatible with phytoconstituents. Large molecules may be able to cross the lipophilic biological membrane and enter the systemic circulation thanks to this value-added herbal drug carrier system. In summary, a formulation based on Phytosomes may be a useful strategy to increase therapeutic efficacy, lower dosage, and enhance dosage regimen. For their anti-diabetic properties to be claimed, more research involving human subjects must be confirmed.

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