Original Article

Murraya koenigii Extract Loaded Phytosomes Prepared using Antisolvent Precipitation Technique for Improved Antidiabetic and Hypolidemic Activity

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ABSTRACT

Background: Phytosome is a novel technique introduced by Indena that combines standardised herbal extract and phospholipid in preferably equal ratio to provide better absorption enhancing bioavailability. **Purpose:** The objective of proposed study is to prepare, optimise and characterize phytosomes of *Murraya koenigii* (Linn.) Spreng extract to improve its antidiabetic properties. **Materials and Methods:** Antisolvent precipitation technique was used to prepare phytosomes. Design expert software was used to evaluate the impact of soya lecithin and cholesterol concentration on various dependent variables such as particle size, span value and entrapment efficiency. Streptozotocin-nicotinamide induced diabetes model was used to evaluate *in vivo* antidiabetic activity in rats for extract as well as prepared phytosomes. **Results:** The phytosomes were successfully designed and optimised for particle size, entrapment efficiency, span value, and zeta potential of 236 nm, 75.1%, 0.395 and -16.85 mV respectively. Streptozotocin-nicotinamide induced diabetes model was used to study antidiabetic potential of plant extracts and its optimized phytosomal formulations in male Wistar rats. Optimized phytosomal formulation showed significant reduction in serum glucose concentration at lower dose, suggesting enhancement in its therapeutic efficacy. **Conclusion:** We were successful in formulation, optimization and characterization of phytosomes for *Murraya koenigii* extract. The tested phytosomes showed better antidiabetic and hypolipidemic activities as compared to crude extract in male Wistar rats.

Key words: *Murraya koenigii*, Phytosomes, Antidiabetics, Herbal formulation, Herbal extract.

INTRODUCTION

Murraya koenigii (Linne.) Spreng normally called curry leaf or kari patta belongs to family Rutaceae. It has a special place in Indian cuisine for its characteristic aroma and potential health promoting properties. A variety of phytochemicals are isolated from its each part especially leaves. It is a rich source of carbazole alkaloid, bioactive coumarins and acridine alkaloid.[1] Additionally, it also contains phytochemicals such as girinimbin, iso-mahanimbin and koenimbin. It has been reported that the leaves also contain cyclomahanimbin, tetrahydromahanmbine, Murrayastine and murrayalin. Variety of phytochemicals found in *M. koenigii* (curry leaves) have been reported its antidiabetic, stimulant, antidysentery,[2] antioxidant,[3,4] lipid-lowering,[5] anti-nociceptive, antiaging, anticancer,[6] hepatoprotective,[7,8] cardio protective, antifungal, antibacterial properties.[8] It could also be useful in treating various gastrointestinal disorders, to improve craving and metabolism. Despite of having potential antidiabetic activities, formulations with *M. koenigii* are inadequately absorbed through biological membranes potentially causing low bioavailability. Therefore, to

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provide better absorption and bioavailability, there is a need for better, efficient delivery systems. Such delivery systems will not only enhance the absorption by altering release characteristics but also can provide sustained release of phytochemicals.

Phytosome is a novel drug delivery system introduced by Indena. It incorporates standardised herbal extracts into suitable phospholipids in various ratios to provide better release characteristics and absorption to enhance bioavailability.[9,10] To the best of our knowledge, phytosomes for *M. koenigii* extract have not been prepared yet. Therefore, the main objective for this study was to design a new delivery system for *M. koenigii* using soya lecithin and cholesterol. Antisolvent precipitation technique[11] was used to formulate phytosomes and to determine the impact of different formulation parameters (independent variables viz. soya lecithin and cholesterol concentration) on dependent variables (*in vitro* release, particle dimension and encapsulation efficiency). Streptozotocin (STZ)-nicotinamide (NIC) induced diabetic model is a well-studied animal model to test antidiabetic potential of test compounds. Diabetes was induced with STZ-NIC in male Wistar rats and used to study antidiabetic potential of plant extracts and its optimized phytosomal formulation and a comparative profile was drawn.

MATERIALS AND METHODS

M. koenigii leaves were collected from the Campus of University campus in August, 2016 and submitted in Botany Department for authentication (Deposition number IPS/KUK/MK/16/01). We received a gift sample for glibenclamide from Tirupati Medicare Ltd., Paonta Sahib, Himachal Pradesh, India. It was used as a standard for *in vivo* antidiabetic model. All other ingredients used were of analytical grade.

Preparation and Standardization of Extract

500 gm of *M. koenigii* dried powdered leaves were taken. Maceration process was opted to prepare hydroalcoholic extract (10:90) by using water in 1 part and alcohol in 9 parts. Mass spectroscopic analysis was performed to standardize the extract. Mass spectrometer used in this study employs hybrid quadrupole time of flight. Experiment was performed with positive electrospray ionization (ESI +) under general conditions by dissolving in 50% (v/v) of acetonitrile (containing 0.1% (v/v) of trifluoroacetic acid) at 3 kV voltage and at desolvation gas temperature 80°C. A spectra was obtained by scanning from 100 to 1500 m/z.[12]

Formulation of Phytosomes

Known quantities of Hydroalcoholic extract and lecithin (obtained from soya) were solubilised in alcohol, while another lipid (i.e. cholesterol) was solubilised in dichloromethane. The mixture was heated at not more than 60°C and concentrated to almost 1/4th amount. 20 ml of hexane (acts as an antisolvent by reducing the solubility of solute and aids in precipitation) was mixed with frequent agitation to obtain the precipitate that was filtered and placed in a vacuum desiccator.[11,13-15] The dried powder was crushed and stored in amber colour glass bottle at -20°C.

Factorial Design

Factorial design offers a measurably precise methodology for the design, development and optimisation of phytosomes. A 3^2 factorial design was created utilising design expert software to obtain nine different formulations. The studied factors soya lecithin concentration (X1) and cholesterol concentration (X2) were set as independent variables. For each variable, three levels were set. The main purpose behind this study was to check the combined impact of different independent variables upon the dependent one. Mathematical calculations were accustomed connecting each parameter to the response obtained. On the basis of counterplots and response surface plots, an optimised formula was selected.[16-18]

Physicochemical Characterization of Phytosomes

Phytosomes were characterized using multiple methods for their size, form and framework. Criteria such as least particle size and highest entrapment efficiency were used for the physicochemical characterization of phytosomes.

Entrapment Efficiency

Entrapment efficiency of phytosomes was calculated by diluting them with methanol and then centrifuging them with high-speed cooling centrifuge machine at 10,000 revolutions per minute at -40°C for 30 min. The supernatant was collected and quantity of plant extract entrapped was determined by measuring absorbance of extract against UV spectrophotometer according to following formula:

$$\frac{[\text{Total amount of drug} - \text{Amount of free drug}]}{\text{Total amount of drug}} \times 100 \quad \text{Eq (1)}$$

Total amount of drug = Amount of extract taken

Amount of free drug = Amount of extract left free from entrapment

Particle Size and Size Distribution

Zeta sizer Nano ZS90 version 7.11 was used to determine particle size and span value. 20 µl sample was placed in a cuvette and automatic measurement mode was selected. Limited size distribution is expressed by lesser value of span.[19]

$$\text{Span} = [D(90\%) - D(10\%)]/[D(50\%)] \qquad \text{Equation (2)}$$

Where D (90) corresponds to 90% cumulative volume
D (50) corresponds to 50% cumulative volume
D (10) corresponds to 10% cumulative volume

Choice of Optimized Formula

An optimized formulation was chosen depending upon the parameters, particle size and maximum encapsulation efficiency.

Visualisation

Scanning electron microscopy (Hitachi-S 3400N) was used to study visualisation that provides surface morphology and basic composition of the sample by emitting electrons.[20] Phytosomes were placed on metal (aluminium) stubs by fracturing with razor blade whereas extract was placed on double sided tape. An acceleration voltage of 15.0 kV was used.[21]

Zeta Potential

Zeta potential was used to measure electrokinetic potential for colloidal dispersions. It is a critical factor for the stability of phytosomes. The higher the electrostatic repulsion amid particles, more is the stability.[22] Diluted samples were placed in cuvette in Zeta sizer Nano ZS90 and value of zeta potential was estimated.[23]

Differential Scanning Calorimetry (DSC Analysis)

DSC studies are generally performed for removal of certain peaks, evolution of unique peaks, difference in top height and its origin. It likewise gives facts concerning interactions of drug with other formulation ingredients and evolution of latest molecules. Dried nitrogen gas was used as fuel and waft was set at 5ml/min. As given in the literature, the gadget was provided with indium for flow of heat. Each sample turned into heated within the variety of temperature 25°C to 300°C at a heating speed of 5°C per minute.[24]

Fourier Transform Infrared (FTIR) Analysis

FTIR is used to study the molecular interaction between various components of phytosomes. The infrared spectra of previously dried sample of crude plant extracts as well as other individual formulation components, physical mixture and prepared phytosomes was obtained by using FTIR spectrophotometer (Bruker Alpha FTIR) in the wavelength 3500-1000 cm^{-1}.[25]

XRD Study

XRD study for phyto-phospholipid complex is described either by total elimination or reduced intensity of large diffraction peaks concerned to its structure. The analysis was carried out at 35 mA current and 40 kV voltages using x-ray diffractometer from 3° to 60° in the scan timings of 32.8sec at the individual stage.[26,27]

Functional Characterization

In vitro Release Study

In vitro release was estimated utilizing dialysis method. Due to the ease of set-up and sampling with the dialysis membrane, it is a highly adaptable and well-known strategy among the methods used to study release characteristics of various types of formulations. Dialysis membrane-70 (HiMedia) with pore size of 2.4 nm and molecular weight cut off (MWCO) value near 10KDa was used. The donor compartment had extract or phytosomes whereas double distilled water was placed in receiving compartment. The entire assembly was kept at proper conditions. At a consistent time interval, samples were withdrawn and replaced with fresh solvent to maintain sink condition.[28-30] UV/Vis spectrophotometry at 366nm was used for the analysis of withdrawn samples and kinetics model was used to analyse the release characteristics. A comparison was made between release characteristics of optimised formulation and the *M. koenigii extract*.

In vivo Antidiabetic Effect

The *in vivo* antidiabetic potential study was performed after getting approval from Institutional Animal Ethical Committee (protocol no.: IPS/IAEC/2017/296) by using the method given in the literature.[31-35] The animals (male Wistar rats) were given standard diet and water *ad libitum* and maintained under normal laboratory condition (room temperature 30±2°C and 60-65% relative humidity).

Induction of Diabetes

Diabetes was induced in overnight fasted rats by streptozotocin (60 mg/kg) (i.p), 15 min after the intraperitoneal (i.p.) administration of nicotinamide (120 mg/kg). Citrate buffer (0.1 M, pH 4.5) was used as vehicle to dissolve streptozotocin and nicotinamide was solublised in normal saline. Diabetic animals were divided into 6 groups (G1-G6) where each group consisted of 6 animals.

G1: Treated with vehicle (Normal saline)

G2: Treated with *M. koenigii* extract (200 mg/kg)
G3: Treated with *M. koenigii* extract (400 mg/kg)
G4: Treated with phytosomes of *M. koenigii* extract (100 mg/kg)
G5: Treated with phytosomes of *M. koenigii* extract (200 mg/kg)
G6: Treated with standard drug (glibenclamide) (10 mg/kg)

Treatment

Animals were fasted for overnight and used for the experiment. Oral weight-based dosing for vehicle, test extracts and phytosomes was done once a day for 21 days. The response of all groups (i.e. G1 to G6) for blood glucose levels as well as the effects on body weight of fasted animals was calculated at an interval of 7 days from first day to last day (i.e. day 21). On the last day animals were anaesthetized and euthanized by decapitation method; blood samples were collected and used for histopathological studies.

Oral Glucose Tolerance Test (OGTT)

OGTT is an important research tool in preclinical and clinical studies that elucidate the vital role of insulin release and insulin unresponsiveness in the development of glucose intolerance.[34] The plant extracts and phytosomes were given 60 min before oral administration of glucose. (2.0 g / kg). Animals were randomly divided into 6 groups (G1-G6) with 6 animals in each group.

G1: Glucose treatment given at a dose of 2 g/kg.
G2: Treatment given [Glucose +*M. koenigii* extract (200 mg/kg)]
G3: Treatment given [Glucose + *M. koenigii* extract (400 mg/kg)]
G4: Treatment given [Glucose + Phytosomes of *M. koenigii* extract (100 mg/kg)]
G5: Treatment given [Glucose + Phytosomes of *M. koenigii* extract (200 mg/kg)]
G6: Treatment given [Glucose + Glibenclamide (10 mg/kg)]

At an interval of 30 min, from the beginning of experiment, blood sample were taken from animals after glucose administration at an interval of 30 min. Glucometer was used for analysis of glucose level.

Histopathological Studies

After completion of the experiment, animals from all groups were sacrificed and dissected. Kidney and pancreas were collected and stored in formalin solution till further experiments. The slides were prepared from excised organs, pictures were taken with advanced camera fitted to microscope and analysed.

Stability Studies

A six-month stability study was designed under three different conditions: refrigerated condition by keeping the same at (2-8°C), room temperature and at accelerated condition (40±2°C/75±5% RH). Samples were withdrawn and stability of prepared phytosomal preparation was estimated at 0, 1, 3 and 6 month for particle size, span value and percent encapsulation efficiency.

Statistical Analysis

Graph pad Prism software was used to calculate statistical significance. The values were represented as Mean ± SD. For a result to be significant, value of p is set at $p < 0.05$.

RESULTS

Mass Spectra Analysis

The results for mass spectroscopic analysis are described in Figure 1. A molecular ion peak of 479 m/z for quercitin-3-glucoside and 301 m/z for quercetin is obtained. A peak at 504.24 m/z relates to Quercetin-3-acetylhexoside. A peak at 489 m/z correlating to kaempferol-acetylglucoside is obtained that gives a fragment ion peak at 285 m/z. Another peak at 301 m/z is obtained that gives fragment ion peak of 151 m/z value. All peaks are in accordance with the previously reported literature,[36] and suggesting the quality of extract.

Optimisation

Factorial design technique was utilised in order to achieve an optimised formulation exhibiting properties such as minimum particle dimension and maximum efficiency of entrapment. The values of particle dimension, Span and percent entrapment efficiency obtained from

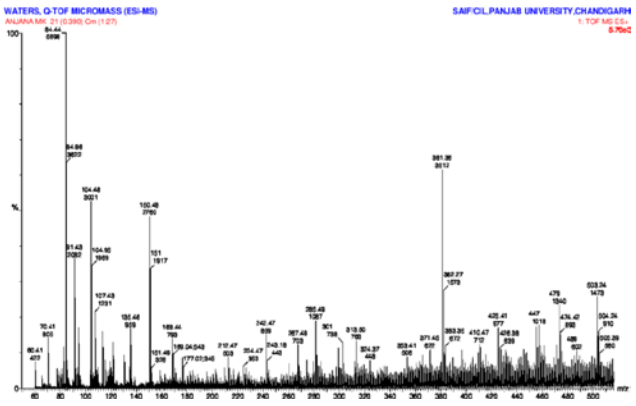

Figure 1: MS spectra of *M. koenigii* extract.

Table 1: The values of Span, Entrapment efficiency (%) and Particle size (nm).

Batch	Particle size in nm	Span	Entrapment efficiency (%)
F1	235.3±0.43	0.34±0.04	55.48±1.32
F2	212.2±1.45	0.27±0.05	31.62±0.85
F3	225.6±1.51	0.16±0.65	49.22±0.35
F4	258.8±2.31	0.43±0.23	57.72±1.53
F5	249.2±0.32	0.38±0.32	52.69±1.82
F6	222.4±1.37	0.23±0.53	37.53±1.43
F7	250.8±1.02	0.57±0.58	76.58±1.51
F8	236.7±0.56	0.42±0.74	60.27±1.98
F9	222.1±0.63	0.27±0.14	68.43±1.65

(Values are designated as mean±SD, $n = 3$).

the trial experiment are summarised in Table 1. Nine different formulations were prepared, and comparison was done to obtain an optimised formulation. An increase in mean particle size was obtained with the increase in phospholipid concentration. This is because of increment in number of polymeric chains/ solvent volume that conducts to encounter and development of bigger nanoparticles.

Percent entrapment efficiency also enhanced as the extract to soya lecithin ratio is enlarged, that could be because of the greater polymer content that was presumed to enhance the % entrapment efficiency by giving extra area to encapsulate the extract.[11]

The impact of concentration of the independent variables on the dependent ones is also represented by various plots Figure 2(A-F). The analysis of variance of the responses and Model summary statistics for the selected significant models are shown in Table 2 and Table 3 respectively. The mathematical modelling of prepared phytosomes was carried out and the following equations were obtained.

Particle size (Y1) $=231.52-2.86X1+0.7346X2+17.62X1X2-5.78X1^2+8.23X2^2$

Span (Y2) $= 0.3050+0.0524X1+0.0223X2+0.1189X1X2-0.0725X1^2+0.1277X2^2$

Entrapment efficiency (Y3) $= 57.67+5.61X1+7.12X2+15.91X1X2+0.0650X1^2-6.70X2^2$

After analysing the effect of the amount of soya lecithin and cholesterol on dependent variables such as particle size, span and percent entrapment efficiency, the level of factors was specified using computation method and the presumed values of Y1, Y2 and Y3 were 231.86 nm, 0.365 and 71.71%, respectively. For confirmation of the predicted values, a fresh formulation of phytosome was prepared using the optimized concentration value (i.e.1 for soya lecithin and 0.41 for cholesterol) that yielded a new formulation with a particle size of 236 nm, span value of 0.393 and entrapment efficiency of 75.1%. Experimental and predicted values were close confirming the accuracy of the optimisation process.

The optimum formulation was selected on the criteria of attaining maximum for %EE and minimum for

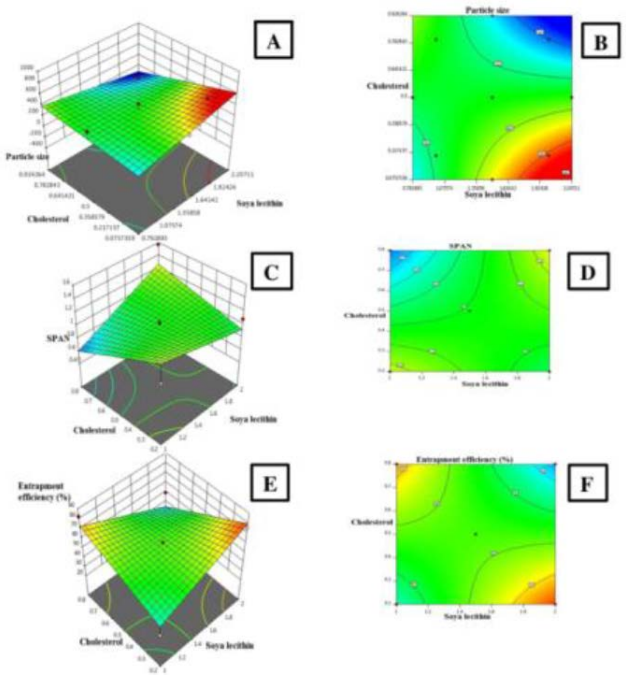

Figure 2: (A-F) The response surface plot and contour plots as a function of concentration of soya lecithin and cholesterol (A, B) Particle size (C, D) SPAN (E, F) Entrapment efficiency.

Table 2: ANOVA for response surface quadratic model for *Murraya koenigii.*

Response factor	Model *F*-value	*P* value Prob>F	Lack of fit	
			F-value	*p*-value
Particle Size	12.06	0.0335	**8.365**	**0.04356**
Span	14.36	0.0263	**10.347**	**0.02345**
%EE	12.33	0.0325	**12.259**	**0.01678**

Table 3: Model summary statistics-Influence of formulation variables on the response factors for *Murraya koenigii.*

Response factor	Source	Standard deviation	R^2	Adjusted R^2	Predicted R^2	Adequacy Precision
Particle size	Quadratic	5.56	0.9526	0.8736	0.8692	9.0314
Span	Quadratic	0.0406	0.9599	0.8931	0.7605	11.2735
%EE	Quadratic	4.93	0.9536	0.8762	0.8279	11.4487

Table 4: Solution provided by the factorial design for *Murraya koenigii*.

Constraints							
Name			**Goal**		**Lower Limit**		**Upper Limit**
X1: Soya lecithin			Is in range		1		2
X2: Cholesterol			Is in range		0.2		0.8
Particle size (nm)			Minimize		212.2		258.8
Span			Less than 1		0.16		0.57
Entrapment efficiency (%)			Maximize		31.62		76.58
Solution							
Batch	**Soya lecithin (g)**	**Cholesterol (g)**	**Particle size**	**SPAN**	**Entrapment efficiency**	**Desirability**	**Solution**
Optimized	1	0.41	231.863	0.365	71.71	0.802	Selected

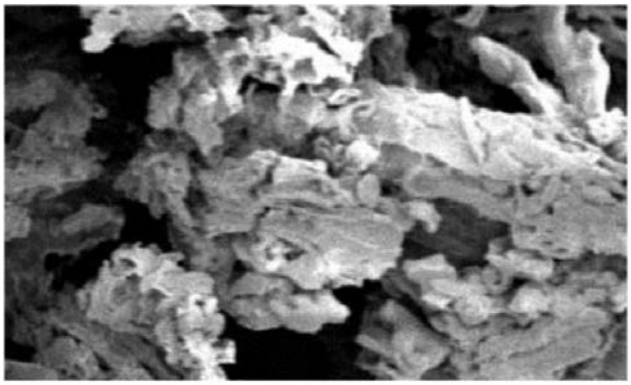

Figure 3: SEM of optimized Formulation of *Murraya koenigii.*

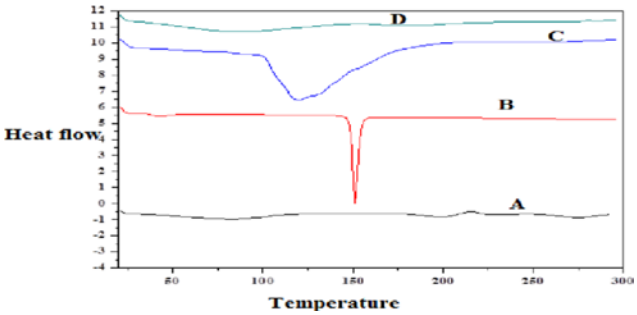

Figure 4: Comparative DSC thermogram of (A) Soya lecithin (B) Cholesterol (C) *Murraya* extract (D) Optimised formulation.

particle size. Table 4 depicts the constraints set and the solution provided by the software.

Visualization

SEM was utilised for representation of surface appearance of optimised phytosomes and the results showed irregular shape particles (Figure 3).

Zeta Potential

Zeta potential is identified as a charge on the surface of the molecule, thus impacts an extensive range of properties of colloidal materials, for example, their stability, interaction with electrolytes, and suspension rheology. Zeta potential value -16.85 mv indicated the formation of stable formulation.

DSC

In Figure 4A, two different peaks at 86.71°C and 202.91°C were obtained that corresponds to melting and transition temperatures respectively. Figure 4B shows three peaks at 41.87°C, 103.76°C and at 150.90°C (sharp peak that designates melting point of cholesterol). A single peak was obtained at 119.73°C for *Murraya* extract (Figure 4C). Two new peaks at 85.92°C and 184.67°C were observed for optimized formulation as shown in Figure 4D representing the emergence of novel phytosomal complex. In the case of optimized formulation, new peaks were obtained, confirming that a formulation was produced by some molecular cooperation that can be hydrogen bonding or van der waal's force of attraction between formulation ingredients that distributed the extract evenly in phospholipid matrix.[37] The investigation revealed phase transition along with isomerization of phospholipid structure due to the kinetics of hydrophilic part of phospholipid at enhanced temperature.[38]

In vitro Drug Release Study

We determined *in vitro* drug release for optimised formulation. It was noticed that a fixed amount of drug was released at certain interval. Mathematical modelling was performed by using kinetic models to determine regression values. Maximum regression was found to be 0.9907 by Korsmeyer–Peppas model suggesting that the model used was best suited. The phytochemical was found to be released by passive diffusion process. Comparative profiles (Figure 5) revealed that percent phytochemical release from phytosome (represented

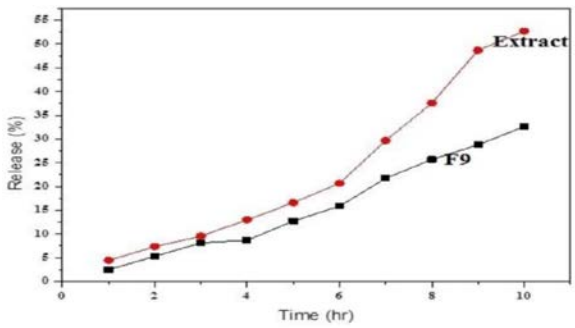

Figure 5: Comparative release profile of optimized formulation (F9) and *Murraya koenigii* extract.

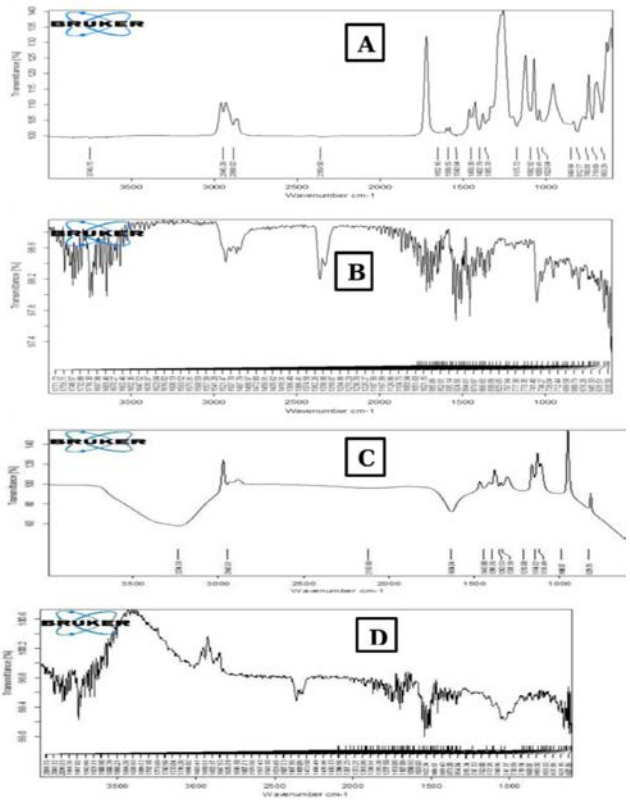

Figure 6: (A-D) FTIR spectra of (A) Soya Lecithin (B) Cholesterol (C) *Murraya* extract (D) Optimised formulation.

by red colour) was much higher than *Murraya* extract (represented by blue colour).

FTIR Analysis

The spectra for soya lecithin (Figure 6A), cholesterol (Figure 6B), extract (Figure 6C) and optimised formulation (Figure 6D) showed characteristic signals at different wave numbers. Optimised formulation spectra (Figure 6D) showed different peaks such as at 1952 cm^{-1}, 156 cm^{-1}, 922 cm^{-1} and 74 cm^{-1}, possibly due to some molecular interaction between different ingredients of optimized formulation.

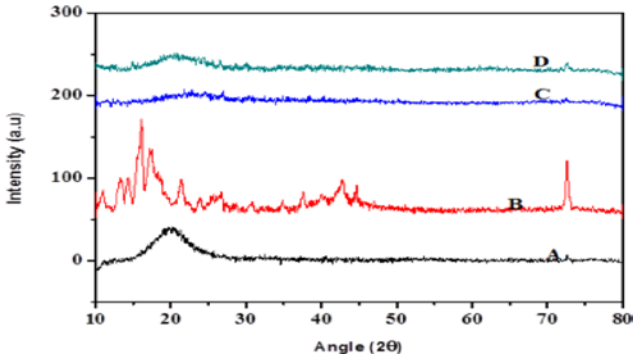

Figure 7: XRD spectra of (A) Soya Lecithin (B) Cholesterol (C) *Murraya* extract (D) Optimised formulation.

Table 5: The effect of 21 days treatment on glucose concentration in STZ+NIC Induced Diabetic Model.

Groups	Initial day	7th day	14th day	21st day
G1	275.20±5.80	275.40±7.30	274.40±8.75	275.00±11.49
G2	343.20±3.51*	287.40±5.08*	274.75±2.15*	227.50±2.14*
G3	340.36±2.16**	269.02±1.02**	230.04±0.25**	207.32±1.85**
G4	327.35±2.02*	258.06±1.37*	211.06±0.96*	201.36±3.02*
G5	302.27±1.05*	246.27±5.21*	183.42±2.56*	173.25±2.98*
G6	214.40±1.82*	197.06±0.60*	178.35±2.95*	151.70±4.30*

Values corresponds to Mean±SD (n=6), *p<0.05; **p <0.01

XRD Analysis

XRD analysis was performed to study the physical structure of soya lecithin, cholesterol and *Murraya* extract in optimized formulation as shown in Figure 7. The plant extract (*Murraya*) showed peaks at 19.6628, 22.1509, 24.5703, 26.8419, 31.0299, 34.1507, 38.5274, and 72.5498 indicating less intense as well as broader peaks. Phytosomes showed peaks at 14.9416, 19.8794, 23.4928, 26.5943, 29.9260 and 72.5070 indicating amorphous form. In the case of the optimised formulation, no peaks for soya lecithin, cholesterol and crude extract were visible, suggesting phase transformation.

Antidiabetic Effect

The effect of treatment on blood glucose level and glucose tolerance is depicted in Table 5 and Table 6. A relative decline in glucose levels was observed as shown in Figure 8. The standard drug likewise hindered the rise in blood glucose concentration after 30 min.

Effect of 21 Days Treatment on Body Weight

The effect of 21 days treatment on body weight is depicted in Table 7. G1 undergoing no treatment

Table 6: Hypoglycemic effect of G1, G2, G3, G4, G5 and G6 on glucose tolerance.					
Groups/ Treatment	Time (min)				
	0	30	60	120	180
G1	72.55±2.13	178.11±1.43	192.13±1.32*	183.24±1.32*	173.13 ±0.34*
G2	75.23±1.27	171.47±4.52	168.32±2.74*	163.32±4.72*	160.33± 4.34*
G3	82.43±0.15	168.43±4.21	166.5 ± 3.54*	161.44±5.85*	151.53± 5.23*
G4	75.8 ± 1.32	167.34±4.72	164.50±3.71*	162.5 ± 2.52*	146.25± 3.83*
G5	84.12±1.31	160.54±4.74	158.72±4.32*	154.5 ± 4.72*	139.3 ± 2.81*
G6	87.12±0.72	133.32±1.55	125.21±1.32*	117.01 ± 0.2*	101.14 ± 0.5*

The values designates Mean±SD (n=6), *p<0.05

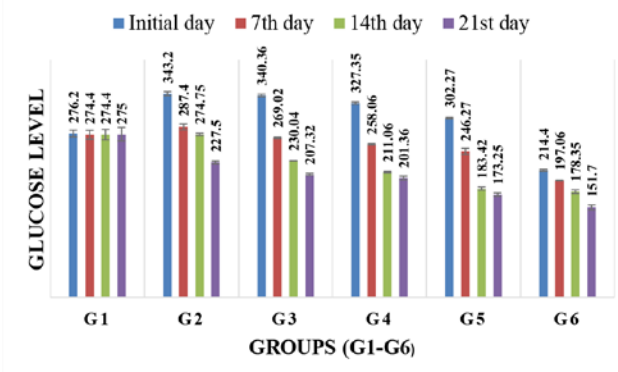

Figure 8: The effect of 21 days treatment on glucose level in groups (G1-G6) after Streptozotocin nicotinamide (STZ+NIC) induced diabetic rats.

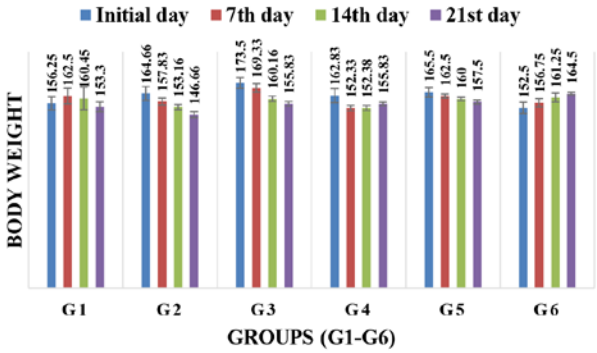

Figure 9: Effect on body weight in groups (G1-G6) after Streptozotocin nicotinamide induced diabetic rats.

Table 7: Effect of G1, G2, G3, G4, G5 and G6 on body weight in STZ+NIC Induced Diabetic Model				
Groups	0 day	7 day	14 day	21 day
G1	156.25±5.54	152.5±6.61	149.45±9.57	143.3±4.21
G2	164.66±5.84*	166.83±2.91*	167.16±2.22*	169.66±2.47*
G3	163.5±4.53*	169.33±3.66*	170.16±2.27*	174.83±2.00*
G4	162.83±6.00*	164.33±2.34*	168.38±2.23*	173.83±1.54*
G5	165.5±4.03*	168.5±1.70*	170.0±1.39*	175.5±1.70*
G6	152.5±4.78*	156.75±3.49*	161.25±3.75*	167.5±1.12*

showed decrease in body weight, whereas the other treated groups showed a significant (p<0.05) increase in body weight as compared to first day of treatment as depicted in Figure 9.

Effect on Level of HDL, LDL, VLDL, Cholesterol and Triglycerides

As shown in Table 8 and Figure 10, significant reduction in the levels of total cholesterol, triglycerides, LDL and VLDL for test groups in comparison to diabetic control group was observed.[39] However, HDL levels were found to be increased as compared with untreated diabetic rats.

Effect on Urea, Protein and Creatinine Levels

Urea, protein and creatinine levels for all test groups are shown in Figure 11. A significant dose dependent reduction in urea and creatinine concentration whereas increase in the protein concentration was observed as depicted in Table 9.[40]

Histopathological Examination

Histology of Pancreatic Sections

Histologically, the β cells of islets of Langerhans of pancreas showed architectural disarrangement, increase in intercellular space as well as peripheral widening between exocrine tissue and islet cells in control group (Figure 12A). The size and dimension of β cells were found to be decreased. The group receiving *M. koenigii* extract somehow exhibited upgradation in the architectural disarray of β cells, but to a lesser extent (Figure 12B and 12C). In contrast to the *M. koenigii* extract, phytosomes showed comparatively more development in the structure of pancreatic islets, reduced intercellular space. In addition, dimensions of pancreatic islets were found to be increased as

Table 8: Effect of Effect of G1, G2, G3, G4, G5 and G6 on serum cholesterol, triglycerides, HDL-C, VLDL-C and LDL-C level in STZ+NIC Induced Diabetic Model.

Groups	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)
G1	257±1.2	298±2.43	42.1±0.27	156.2±1.12	58.7±1.16
G2	200±0.27*	211±1.50*	45±0.13*	115.2±0.52*	50.2±2.17*
G3	197±2.16*	198±0.66*	46±1.82*	102.3±0.35*	49.5±1.64*
G4	191±2.3*	195±0.84*	47.5±1.04*	101.2±0.26*	44.6±1.32*
G5	189±2.49*	189±3.16*	48±1.74*	86.4±0.21*	43.8±1.85*
G6	179±1.53*	194±0.31*	49.3±2.52*	102.1±0.18*	41.9±1.39*

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

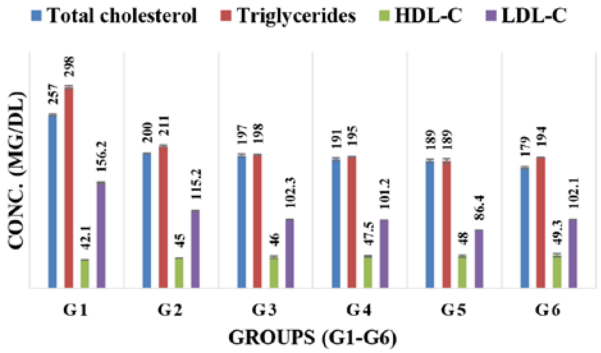

Figure 10: Effect on serum cholesterol, triglycerides, HDL, VLDL and LDL level in groups (G1-G6) after Streptozotocin nicotinamide induced diabetic rats.

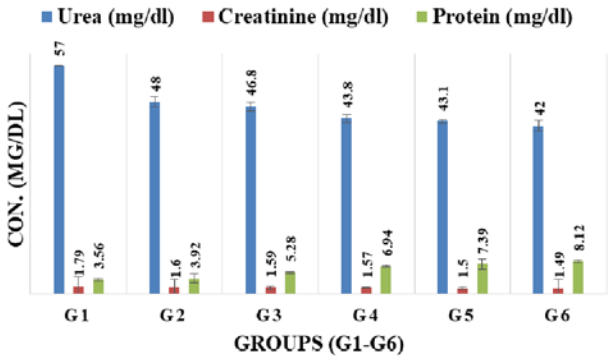

Figure 11: Effect on Urea, Creatinine and Protein level in groups (G1-G6) after Streptozotocin nicotinamide induced diabetic rats.

represented in Figure 12D and 12E. G6 treated with glibenclamide exhibited momentous development in the histology of islets (Figure 12F). Reduced peripheral widening between acinar cells (exocrine tissue) and islets indicated the hypoglycaemic effect of the drug.

Histology of Kidney Section

Kidney sections from diabetic rats showed improvement in thickness of basal membrane of glomerulus

Table 9: Effect of Effect of G1, G2, G3, G4, G5 and G6 on Urea, Creatinine and Protein in STZ+NIC Induced Diabetic Model.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Protein (mg/dl)
G1	57±0.11	1.79±2.46	3.56±0.27
G2	48±1.26*	1.6±2.05*	3.92±1.08*
G3	46.8±1.12*	1.59±0.42*	5.28±0.22*
G4	43.8±1.03*	1.57±0.11*	6.94±0.15*
G5	43.1±0.42*	1.5±0.32*	7.39±1.21*
G6	42±1.21*	1.49±2.11*	8.12±0.41*

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

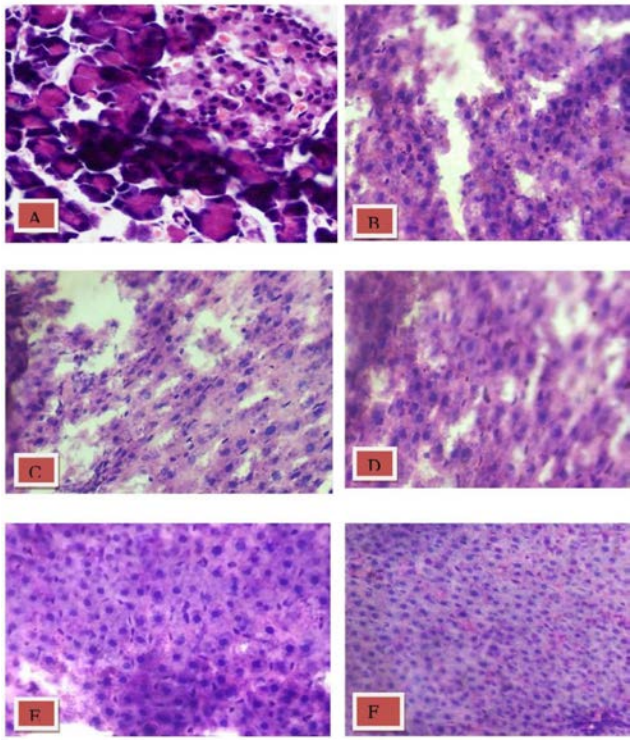

Figure 12: (A-F) Photomicrograph of Pancreatic section in groups (G1-G6) (A) Diabetic control (B) *Murraya* extract dose 200mg/kg (C) *Murraya* extract dose 400 mg/kg (D) Optimised formulation dose 100mg/kg (E) Optimised formulation dose 200mg/kg (F) Standard drug.

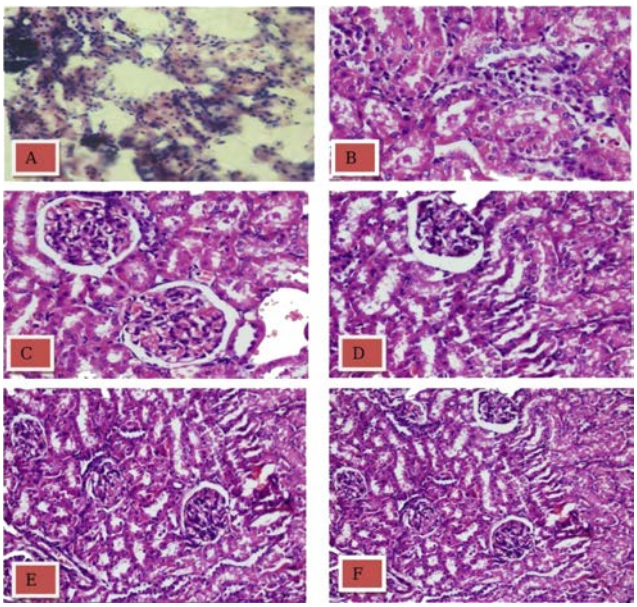

Figure 13: (A-F) Photomicrograph of kidney section in groups (G1-G6) (A) Diabetic control (B) *Murraya* extract dose 200mg/kg (C) *Murraya* extract dose 400 mg/kg (D) Optimised formulation dose 100mg/kg (E) Optimised formulation dose 200mg/kg (F) Standard drug.

along with a significant increase in mesangial density (Figure 13A). When treated, all the groups exhibited improvement towards normal condition[40] as shown in Figure 13(B-F).

Stability Study

Stability of the formulation must be maintained. Previous studies have shown that cholesterol plays a vital role in maintaining the physical stability of phytosome by providing flexibility to lipid bilayer.[41] An interaction exists amid used phospholipids that increases the electrostatic repulsive forces between phospholipid bilayer and ultimately ensued in enhanced stability. A stability study conducted at 0, 1, 3 and 6 months for particle size, span and percent entrapment efficiency[42] has been summarised in Table 10, Table 11 and Table 12 showing that formulation is consistent throughout the stability studies. Even after 6 months no significant difference was observed in physicochemical characteristics at refrigerated condition. However, at room temperature and accelerated temperature conditions, slight deviation in particle dimension, span and percent entrapment efficiency values was observed.

Table 10: Effect on Mean particle size.

Stability condition	Mean particle size (nm)			
	At t=0	1 month	3 months	6 months
2-8°C	231± 0.63	231± 0.65	231.45± 0.32	231.87± 0.52
Room temperature	231± 0.63	231.87± 0.11	232.85± 0.35	233.32± 0.59
40±2°C/75±5% RH	231± 0.63	231.36± 0.53	232± 0.56	235.54± 0.68

Data are expressed as mean ± SD, n=3

Table 11: Effect on Span.

Stability condition	Span			
	At t=0	1 month	3 months	6 months
2-8°C	0.36±0.11	0.36±0.21	0.36±0.24	0.36±0.28
Room temperature	0.36±0.11	0.36±0.14	0.37±0.21	0.38±0.35
40±2°C/75±5% RH	0.36±0.11	0.372±0.15	0.38±0.45	0.39±0.47

Data are expressed as mean ± SD, *n*=3

Table 12: Effect of storage period on Entrapment efficiency.

Stability condition	Entrapment efficiency			
	At t=0	1 month	3 months	6 months
2-8°C	71.7±0.23	71.7±1.05	71.7±1.12	71.7±1.26
Room temperature	71.7±0.23	71.7±1.34	71±1.21	70±1.72
40±2°C/75±5% RH	71.7±0.23	71±0.35	69.8±0.32	68±0.36

Data are expressed as mean ± SD, *n*=3

DISCUSSION

M. koenigii is recognised by its bioactive ingredients, having significant potential for its use in pharmaceutical preparations.[1-8,43] Antisolvent precipitation technique was used for the preparation of phytosomes.[12-15] For optimisation of phytosomes, 3^2 factorial design was chosen.[44] The phytosomes formulated in the present study showed negative zeta potential. Generally, Both high positive or high negative zeta potential values might cause stronger repulsion, while particles having alike charges might cause repulsion between them, preventing accumulation of the particles.[45] Physicochemical characterisation such as SEM, DSC, FTIR and powder XRD analysis was done to evaluate the optimised formulation. SEM study revealed that resulting particles were polydispersed and have an irregular shape. DSC thermogram was plotted for extract, soya lecithin, cholesterol and optimised formulation. The respective peaks for soya lecithin, cholesterol and leaves extract was found to be disappeared and new peaks were obtained

suggesting phase transformation.[38] Soya lecithin was used in the formulation owing to its property of regulation of cellular membrane permeability whereas cholesterol was used to provide stability[41] to the formulation. X-ray diffraction study revealed that the sharp peaks obtained were associated with crystalline structure of substance whereas; less intense and broad peaks suggested the formation of amorphous state. Functional characterisation was performed using *in vitro* release study and *in vivo* diabetes study. Comparative release profile suggested that in case of optimised formulation about 30% of phytochemicals were released in 6 hr in comparison to crude extract that showed about more than 50% release. Hence suggesting sustained drug release. The result demonstrated that the phytosomes not only maintained the basic property of the extract, augmented its antidiabetic effect also. In comparison to the results obtained with *M. koenigii* extract, optimized phytosomal formulation showed 39% and 42% reduction in serum glucose concentration at lower dose, suggesting enhancement in its therapeutic efficacy.

The mechanisms associated with decline in glucose level in blood could be amplification of insulin effect by inducing either the insulin secretion from the remainder β-cells islets or its receptiveness. A marked reduction in body weight was observed in diabetic control group and reason associated could be fat breakdown and protein decay and also may be due to unavailability of glucose for energy usage.[38,46] In treated groups significant increase in body weight due to increased glucose metabolism was observed. The diabetic hyperglycaemia leads to increased urea and creatinine levels, important markers for renal disorder. The levels of these parameters reverted to near normal in cases of the treated groups, which suggested antidiabetic activity of phytosomes. A sound impact on lipid level was also seen suggesting its hypolipidemic effect. Hence, the present study suggesting the *in vivo* safety of this phytosomal preparation and the significant clinical use so as to deliver the bioactive compounds.

CONCLUSION

Current study indicates for the first time, the potential of phytosomal formulation of *M. koenigii* extract for enhancing the bioavailability. The investigation also underscores the utilization of factorial design for development of optimized formula. The result obtained from animal studies shows the potential of phytosomal formulation for antidiabetic as well as hypolipidemic uses. Moreover, the results demonstrated that the phytosome based drug delivery approach could be a valuable tool to improve the therapeutic efficacy of phytochemicals by improving their absorption, and bioavailability via altering their physicochemical and release properties. To the best knowledge of the authors, there is no document of any composition with *M. koenigii* involving phytosomes. Hence, the authors could suggest that phytosomal preparations might be regarded as potential candidates for the future delivery of bioactive ingredients of the *M. koenigii* extracts. Although, further integrated research is still required to enhance meticulousness, deepen the mode of action, and its release pattern.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

STZ: Streptozotocin; **NIC:** Nicotinamide; **FTIR:** Fourier Transform Infrared; **DSC:** Differential Scanning Calorimetry; **XRD:** X-ray Diffraction; **SEM:** Scanning Electron Microscopy; **OGTT:** Oral Glucose Tolerance Test.

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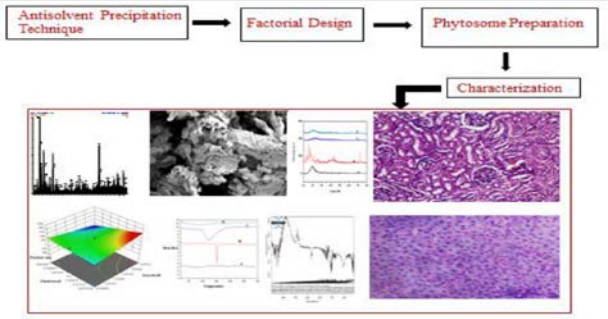
PICTORIAL ABSTRACT



SUMMARY

Antisolvent Precipitation technique was used to prepare phytosomes and for optimisation full factorial design was opted. Physico-chemical characterisation i.e Scanning electron microscopy (SEM), Differential scanning calorimetry (DSC), Fourier transform Infrared (FTIR) spectrometry and powder X ray diffraction (PXRD) analysis was done to evaluate an optimised formulation. *In vivo* antidiabetic activity was studied by using streptozotocin nicotinamide induced model. A sound effect on lipid profile was also observed i.e decrease in serum concentration of cholesterol, triglycerides, LDL, VLDL and increase in HDL level in Streptozotocin nicotinamide induced diabetic rats, when treated, suggesting its hypolipidemic effect. An improvement towards normal condition was shown in the photomicrograph of pancreatic and kidney section treated with optimised formulation in comparison to the hydroalcoholic extract.

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