Research Article

Development and characterization of solid lipid nanoparticles containing *Murraya koenigii* leaves extract for management of diabetes mellitus

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Abstract

Objective: Formulation and characterization of solid lipid nanoparticles containing murrayanol for better management of diabetes mellitus. **Material and methods:** Successive solvent extraction of crude drug of *Murraya koenigii* plant was done. Active constituent of leaves that is murrayanol was isolated and identified through HPLC and FTIR. SLN was selected as carrier system and formulated by solvent diffusion method. Characterization and performance evaluation of particulate system loaded with herbal plant extract of the *Murraya koenigii* leaves was done. TEM, In-vitro drug release profile, entrapment efficiency and particle size was determined. **Results:** Solid lipid nanoparticles have enormous effect in loading high amount or loading dose concentration in body and also maintain the same over prolonged period of time. SLN was formulated and characterized for the particle size, shape and its distribution, percentage drug entrapment and *In-vitro* drug release profile along with the stability studies. *In-vivo* bio distribution studies on animals suggested the accumulation of formulations in the different organs. Solid lipid nanoparticles also show good stability as compared to other novel carrier systems. **Conclusion:** Prolonged release of natural drug from carrier system, decrease the dosing frequency and also decrease the dose size. Better results than marketed synthetic anti-diabetic drugs.

Keywords: Particulate system, plant extract, *Murraya koenigii*, diabetes mellitus

Introduction

Diabetes mellitus is a heterogeneous metabolic disorder characterized by common feature of chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism. The main pathophysiology of diabetes mellitus is decrease in number of the beta cells of pancreas. The reduction in number of beta cells corresponds to the lack of insulin which results in a peripheral underuse and hepatic overproduction of glucose which results hyperglycemia (Deepthi et al., 2017). Two major types of diabetes mellitus are: Type-I Insulin dependent diabetes mellitus (IDDM), characterized by the loss of insulin-producing beta cells of the islets of langerhans in the pancreas leading to a deficiency of insulin. Type-II Non insulin dependent diabetes mellitus (NIDDM), there is no loss or moderate reduction in beta cell mass; insulin in circulation is low (Olokoba et al., 2012).

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Conventional drug delivery systems utilized in the treatment of diabetes extend a number of drawbacks such as transient leucopenia, agranulocytosis, allergy, edema, weight gain, lipodystrophy occurs at injection site etc (Piero et al., 2014).

Solid Lipid Nanoparticles (SLNs) are submicron sized (50–1000 nm) lipid particles comprising of lipid core made from high melting lipid and coated by surfactant. Physiological and nontoxic lipids are basically used for the preparation of SLN which makes them widely acceptable for topical drug delivery. SLNs are formed by dissolving or dispersing the hydrophobic drug into the lipid matrix or by dispersing the drug into the outer coating around the solid lipid core (Dubey et al., 2017). Colloidal particles ranging in size between 10 and 1000 nm are also known as nanoparticles. They are manufactured from synthetic/natural polymers and ideally suited to optimize drug delivery and reduce toxicity. Over the years, they have emerged as a variable substitute to liposomes as drug carriers. The successful implementation of nanoparticles for drug delivery depends on their ability to penetrate through several anatomical barriers, sustained release of their contents and

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their stability in the nanometer size.

Current study aimed at the modified release of drug from the novel carrier system for which solid lipid nanoparticles were selected as a carrier system this will ultimately decrease the dosing frequency and dose size of active constituent. After that in case of diabetes treatment generally 3-4 drugs at a time patient takes, synthetic drugs also have much more side effects if they take regularly for which we selected, natural source for the drug (Waghmare et al., 2015). Curry leaves (*Murraya koenigii*) are another Indian medicinal plant, which has enormous traditional values against various diseases and many bioactive compounds have been isolated from this plant (Igara et al., 2016). *Murraya koenigii* commonly known as curry leaves belongs to family Rutaceae. By combining natural constituent with the novel carrier system is much better concept in case of diabetes; in which daily dosing regimen is approx of 3-4 drugs (Chauhan et al., 2010).

Materials and methods

Collection and authentication of plant material

Crude drug or plant part of *Murraya koenigii* was procured from agriculture college, Indore. Their identification and authentication was confirmed by Department of Botany, Holkar Science College, Indore by correlating their morphological and microscopic characters with those given in literature. The leaves were collected, cleaned well to remove all the dirty material and were shade dried and then powdered kept into airtight containers with proper labelling for further use. Chloroform, ethanol, methanol, petroleum ether and ethyl acetate were purchased from S.K. Traders, Indore. All solvents were analytical grade.

Preparation of extracts

The coarsely powdered, dried leaves (50 g) were extracted with 300-500 ml petroleum ether by hot extraction process (soxhlet) for 4 hours. After completion of extraction the solvent was removed by distillation and concentrated in vacuo. The marc left after petroleum ether extraction was dried and extracted with 300 ml -500 ml chloroform by hot extraction process (soxhlet) for 4 hours. After completion of extraction the solvent was removed by distillation and concentrated in vacuo. Likewise extraction was performed with ethyl acetate, Methanol and ethanol. The above extracts were used for phytochemical studies.

Table 1. Successive extractive values of the powdered leaves of *Murraya koenigii*

S. No.	Extracts	Yield (% W/W)
1.	Petroleum Ether Extract	2.41
2.	Chloroform Extract	2.72
3.	Ethyl acetate Extract	2..05
4.	Methanol Extract	4.11
5.	Ethanol Extract	3.90

Isolation and identification of murrayanol from *Murraya koenigii*

50gm of ethanolic extract obtained was dispersed in distilled water (200ml) in small amounts. It was extracted successively and exhaustively with solvents by considering polarity viz. petroleum ether (60-80 C) (200ml X 5), solvent ether (200 X 5) and ethyl acetate (200X 3). Each fraction was treated with distilled water (5ml), dried over anhydrous sodium sulphate and freed from solvent by distillation. The aqueous part was freed of organic solvent by distillation under reduced pressure and then evaporated to dryness on a water bath. The yield of each extract was recorded and subjected to chemical investigation. Murrayanol was confirmed by analytical techniques, FTIR and HPTLC.

Isolated murrayanol from *murraya koenigii* showed a melting point at 188^0C which was in agreement with the standard range of 180-189^0C, as reported.

The Rf values of isolated and standard murrayanol in several mobile phases was determined.

Spectrophotometric analysis

UV double beam spectrophotometer – Shimadzu model no 1800

Infrared spectrum of the isolated murrayanol was determined using KBr disk methodology

FT-IR spectrophotometer (ABB, FTLA-2000, Japan)

HPLC analysis: The isolated murrayanol was identified by HPLC technique and compared with standard murrayanol using column and a combination of methanol: water (1:1 ratio) as a mobile phase with a flow rate of 1 ml min^{-1} and detected 200-210 nm.

The isolated murrayanol (in ethanol) was filtered through membrane filter (Millipore, USA) and were injected (10µL) through the Sunfire – C18 column column (5µm, 250 X 4.6 mm) at column temperature 25°C. The mobile phase composed of methanol, and water (1:1) was eluted at a flow rate of 1mL/min and the effluent was monitored at 210nm by UV-PDA detector. The peaks were detected and compared with the standard.

Acute toxicity study

All experiments were in agreement with ethical guidelines for investigations of experimental plant in conscious animal. Research protocol was approved by the Institutional Animal Ethics Committee.

The acute toxicity study is use to establish the therapeutic

index, i.e. the ratio between the pharmacologically effective dose and lethal dose on the same strain and species (LD_{50}/ED_{50}). Greater is the index; safer is the compound and vice versa. The acute toxicity study was done according to OECD (Organization of Economic Co-operation and Development) guidelines 425- Fixed Dose Procedure (FDP).

Antidiabetic activity

Nicotinamide solution was freshly prepared by dissolving 480 mg of nicotinamide in 8 ml of 0.9 % NaCl solution, and the volume of the solution was made upto 10 ml with the same solution. A solution of STZ (streptozotocin) was prepared by dissolving the weighed quantity of streptozotocin in 0.1 M freshly prepared ice cold citrate buffer (pH 4.5) solution. The solution of STZ so prepared was administered in the volume of 0.5- 1ml.

The selected animals were fasted overnight and administered with Nicotinamide 120 mg/kg i.p route and after 15 minutes, Streptozotocin 60 mg/kg IP. Fasting blood sugar levels were determined on 12th day after induction to confirm stable hyperglycemia.

Oral Glucose Tolerance Test

Animals were divided in nine groups and each group consisted of six rats. Overnight fasted rats were used for study.

Group I: Normal control rats administered saline (0.9% w/v);

Group II: Diabetic rats administered standard drug Glibenclamide (2.5 mg / kg) daily

Group III: Diabetic rats administered test sample (50 mg/kg);

Group IV: Diabetic rats administered test sample (100 mg/kg);

FBS (Fasting blood glucose level)

Fasting blood sugar level was determined by using glucose oxidase peroxidase reactive strips. In which lipid Profile, liver glycogen content was determined, animal sacrificed by decapitation and the livers were excised out and glycogen content was estimated.

Assessment of changes in Body Weight

The changes in the body weight were calculated by checking the weights of individual animals on 0th, 5th, 10th and 15th day.

Serum Insulin Estimation

On day 15 before the animals were sacrificed 2ml of blood was withdrawn from each animal. The blood was then centrifuged at 3000rpm for 10 minutes to obtain the serum. The serum was analyzed for insulin levels by Radio Immuno Assay at Choithram hospital, Indore, Madhya Pradesh.

Insulin concentration in the serum was estimated by using RIA technique. This technique used a RIA kit provided by the Board of Radiation and Isotope Research (BRITS), Mumbai.

Preparation of solid lipid nanoparticles

Solid lipid nanoparticles were optimized on the basis of % entrapment, drug content, stirring time and no. of particles formed. Optimized formula used for further work. Solvent diffusion method has been used in which Glyceryl monostearate was dissolved in acetone and ethanol (1:1 v/v) in water bath at 60ºC and this was added to aqueous phase (distilled water) under mechanical agitation for 45 minutes. This was subjected to centrifugation at 4000 rpm for 10 minutes, re-suspended in water which results in formation of solid particles. Finally the nanoparticles were collected by filtration and are washed with demineralized water.

Characterization of microparticles

Particle size and shape

SLN were visualized under Philips Morgani 268 Transmission Electron Microscope. A drop of the different formulations was placed on different carbon coated copper grids to leave a thin film on the grids. Then, the film was negatively stained with 1% phosphotungastic acid (PTA) by placing a drop of the staining solution on to the film and the excess of the solution was drained off with a filter paper. The grid was allowed to dry thoroughly and formulations were viewed under a transmission electron microscope and photographs were taken at suitable magnification.

Particle size and distribution

The size and size distribution of particles was determined using laser diffraction particle size analyzer (Cilas, 1064 L, France). The particulate suspension was dispersed in distilled water and then it was put into the sample chamber of particle size analyzer and measurement of particle size was carried out.

Entrapment efficiency

One g of sephadex G-75 was allowed to swell in 10 ml of 0.9% NaCl solution in distilled water in a glass screw capped bottle for 5 hours at room temperature. The hydrated gel was filled to the top in the barrel of 1ml disposable syringe plugged with whatman filter pad. The barrel was then placed in the centrifuge tubes. The tubes were centrifuged at 2000 rpm for 3 minutes to remove excess saline solution. Eluted saline was removed from the centrifuge tubes and exactly 0.2 ml of suspension (undiluted) was applied dropwise on the top of the gel bed in the center. Columns were again centrifuged at 2000 rpm for 3 minutes to expel and remove void volume containing nanoparticles in to the centrifuge tubes. Elute was removed and 0.25 ml saline was applied to each column, and centrifuged as previously. The amount of drug entrapped in the particles was then determined by disrupting the particles

followed by filtration and subsequent determination of the drug content using spectrophotometric method.

In-vitro drug release

One ml of pure suspension was placed in dialysis tube, which in turn was placed in a beaker containing 20 ml of PBS (7.4 pH). The solution containing the dialysis tube was stirred on a magnetic stirrer while keeping the temperature constant at 371°C throughout the study. Samples were withdrawn at different time intervals with subsequent analyzed for drug using Shimadzu 1601 UV spectrophotometer at 210 nm.

Statistical analysis

All the means are presented with their standard error of mean (mean ± S.E.M.). The consolidated mean data of OGTT, fasting blood glucose level, bodyweight changes, serum lipid profile were analyzed by one-way ANOVA followed by Dunnet t test using INSTAT-V3 computer software program.

Results and discussion

Isolation and identification of murrayanol

Isolated Murrayanol from *Murraya koenigii* leaves was verified by Rf value 0.38 (TLC), 0.56 (paper chromatography) and spectrophotometrically shown by spectra (UV spectrophotometer, IR and HPLC) (Figure 1, 2 and 3).

Acute toxicity study and antidiabetic activity

Murrayanol shows effective decrease in blood glucose level as compared the standard glibenclamide drug which is tested by animal studies. Acute toxicity of alcoholic and aqueous extract was performed after 14 days 4 animals recovered by 5. Results of OGTT shows that murrayanol (100 mg/kg) initially shows 103.09±1.67 mg/dl and after 2 hrs. gives 94.83±2.11 mg/dl. Fasting blood glucose level in rats initially 179.67±0.65 mg/dl, after 15 days 116.52±1.08

Table 2. Result of acute toxicity study of Murrayanol

Groups	No. of animal used	Treatment Dose (mg/kg) body wt.	No. of animals recovered after study		
			24 hrs.	72 hrs.	14 days
Group A (Alcoholic Extract)	5	2000	5	4	4
Group B (Aqueous Extract)	5	2000	5	5	4

Table 3. Effect of Murrayanol from *Murraya koenigii* on oral glucose tolerance test in rats

Treatment (n=6)	Fasting blood glucose level (mg / dl)			
	0 min	30 min	60 min	120 min
Normal	91.42± 0.92	132.33± 1.12	117.29± 1.11	111.03± 1.17
Standard (Glibenclamide, 2.5mg/kg)	94.01± 0.73	110.33±0.56*	83.09 ± 0.97*	79.39± 0.05*
Murrayanol (50mg/kg)	95.01± 1.32	123.33±1.48*	104.67±0.92*	92.01± 0.37*
Murrayanol (100mg/kg)	103.09±1.67	129.04±1.46	108.31±1.87*	94.83± 2.11*

Normal Control- Vehicle 10 ml/kg, Reading are values ± S.E.M; n = Numbers of animals in each group; *P < 0.05 v/s Normal control; One-way ANOVA followed by Dunnet t test

Table 4. Effect of Murrayanol on fasting blood glucose levels in rats

Treatment	Fasting blood glucose level (mg / dl)			
(n=6)	Day 0	Day 5	Day 10	Day 15
Normal	97.14±1.53*	94.17±1.25 *	91.83± 1.01*	88.67±1.15 *
Diabetic control	181.67±1.12	189.11± 0.88	196.83± 1.08	199.18± 1.31
Standard (Glibenclamide, 2.5mg/kg)	184.33±1.45	127.55±0.76*	116.51±1.01*	107.67±1.14*
Murrayanol (50mg/kg)	180.11±1.83	137.33±1.33	125.83±1.34*	119.18±0.97*
Murrayanol (100mg/kg)	179.67±0.65	135.65±1.50*	126.33±1.03*	116.52±1.08*

Values expressed as mean ±S. E. M.; n = no. of animals in each group. *p< 0.05 significant Vs diabetic control. One-way ANOVA followed by Dunnet t test

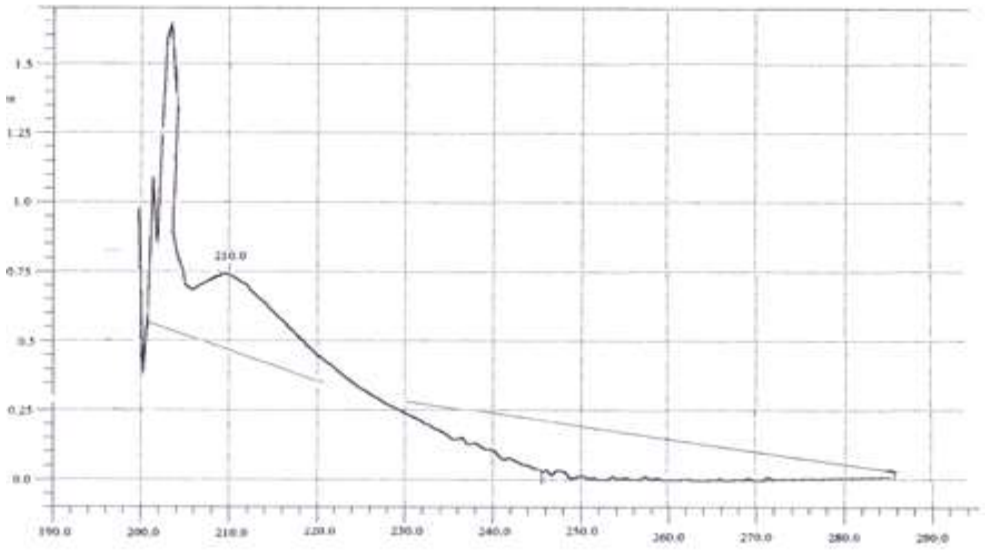

Figure 1. UV Spectra of murrayanol

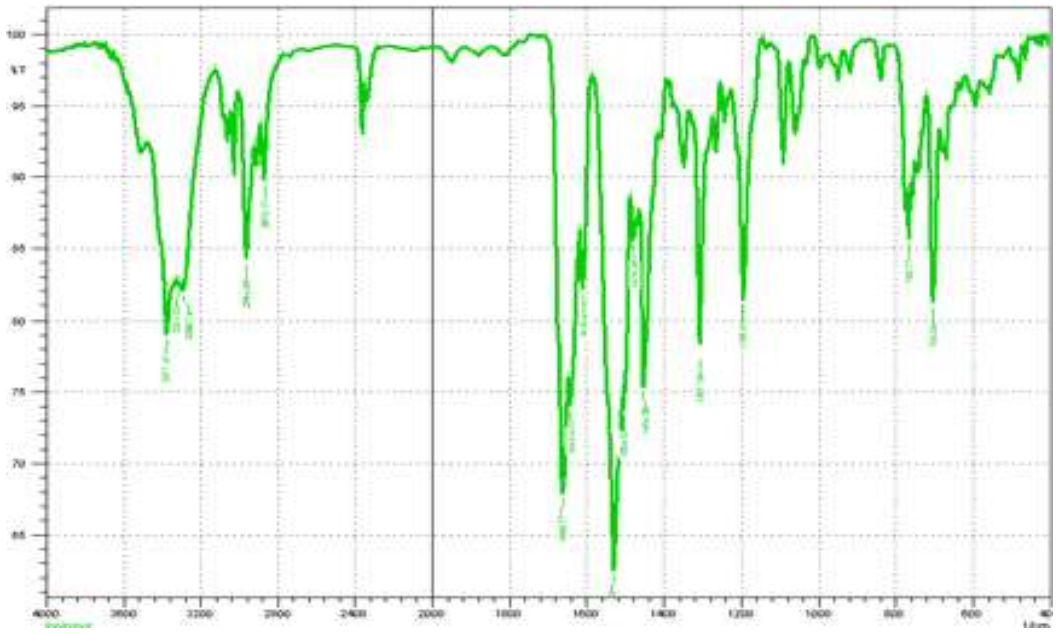

Figure 2. IR Spectra of Isolated murrayanol

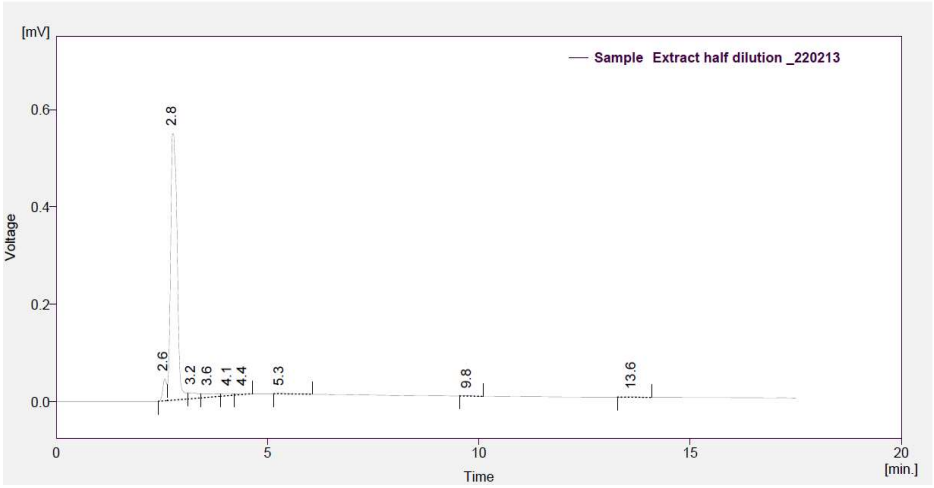

Figure 3. HPLC spectra of isolated Murrayanol

Table 5. Effect of murrayanol on serum lipid profile in rats

Treatment (n=6)	TG	TC	HDL	LDL	VLDL
Normal	53.86±1.04*	55.52±0.94*	23.88±0.73	13.45±0.87*	18.72±0.20
Diabetic control	110.52±1.54	96.56± 1.03	15.55±0.83	53.73± 0.41	27.29± 0.33
Standard (Glibenclamide, 2.5mg/kg)	56.26±1.07*	57.21±1.08*	19.20±0.52	17.35±0.44*	20.85±0.21
Murrayanol (50mg/kg)	77.87±1.14*	75.27±1.20*	14.26± 0.39	37.84±0.86*	23.17±0.23
Murrayanol (100mg/kg)	67.06±1.04*	65.19±1.24*	18.59±0.38	25.23±1.40*	21.37±0.30

Values expressed as mean ± S. E. M.; n = no. of animals in each group. *p < 0.05 significant Vs diabetic control. One-way ANOVA followed by Dunnet t test

Table 6. Effect of Murayanol on body weight changes in rats

Treatment (n=6)	Body weight (gm)			
	0^{th} Day	5^{th} Day	10^{th} Day	15^{th} Day
Normal	145.10±1.89*	145.70±2.93*	146.85±1.36*	147.66±2.69*
Diabetic control	235.12± 1.42	231.56± 2.65	223.14 ± 2.11	210.25 ± 1.54
Standard (Glibenclamide, 2.5mg/kg)	225.12± 1.31	192.0± 2.94*	218.32 ± 1.45	224.35 ± 0.65*
Murayanol (50mg/kg)	166.56±2.69*	161.43±1.37*	155.44 ± 3.28*	153.37 ± 1.83*
Murayanol (100mg/kg)	158.35±3.72*	153.46±1.28*	149.37 ± 3.93*	142.21 ± 1.27*

Values expressed as mean ± S. E. M.; n = no. of animals in each group. * p < 0.05 significant Vs diabetic control. One-way ANOVA followed by Dunnet t test.

Table 7. Effect of Murrayanol on serum insulin and liver glycogen levels in rats

Treatment (n=6)	Serum Insulin Level (µU/ml)	Liver glycogen level (mg/g)
Normal	13.19 ± 0.33*	23.25 ± 1.24*
Diabetic control	42.70 ± 0.15	9.24 ± 1.47
Standard (Glibenclamide, 2.5mg/kg)	12.47 ± 0.56*	24.65 ± 2.25*
Murrayanol (50mg/kg)	7.11 ± 0.38*	19.31 ± 2.47
Murrayanol (100mg/kg)	8.12 ± 1.21	20.63 ± 045

Values expressed as mean ± S. E. M.; n = no. of animals in each group. *P< 0.05 significant Vs diabetic control. One-way ANOVA followed by Dunnet t test.

Table 8. Optimization of Drug: polymer ratio

Formulation code	Ratio (Murrayanol: Polymer)	Average size(µm)	No. of particles per mm^3x1000	% Entrapment
MPL-1	9:1	2.23±0.35	27±2.5	64.4±1.2
MPL-2	8:2	2.34±0.54	28±2.2	68.8±0.98
MPL-3*	7:3	2.69±0.57	36±1.9	70.5±1.10
MPL-4	6:4	2.75±0.15	28±1.6	65.4±1.43
MPL-5	5:5	2.77±0.24	23±1.5	64.8±0.85

*Data are shown as mean SD (n= 3)

mg/dl shows effective decrease in glucose level. Change in body weight was compared with standard glibenclamide which shows less change in body weight as compared to murrayanol (100mg/kg), initial weight was 158.35±3.72 g and after 15 days of dosing weight was found 142.21 ± 1.27 g. Serum insulin and liver glycogen level in normal rats was 13.19 ± 0.33 µU/ml and 23.25 ± 1.24 mg/g respectively. In treated animals it was 8.12 ± 1.21µU/ml and 20.63 ± 045 mg/g respectively.

Preparation and characterization of solid lipid nanoparticles

By optimizing the formula on the basis of drug and polymer ratio and process on the basis of stirring speed and time

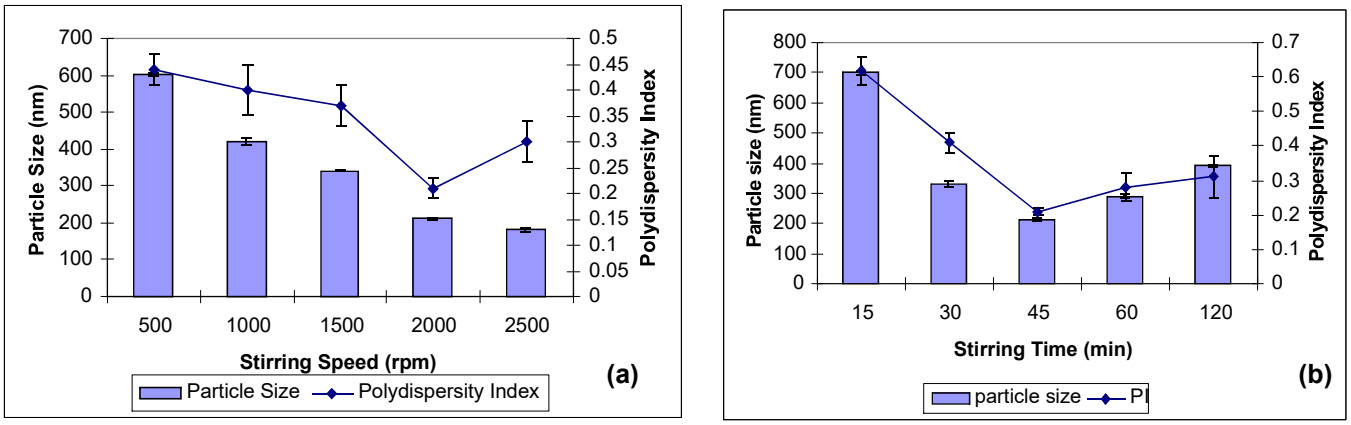

Figure 4. (a) Effect of stirring speed on SLN (b) Effect of stirring time on SLN

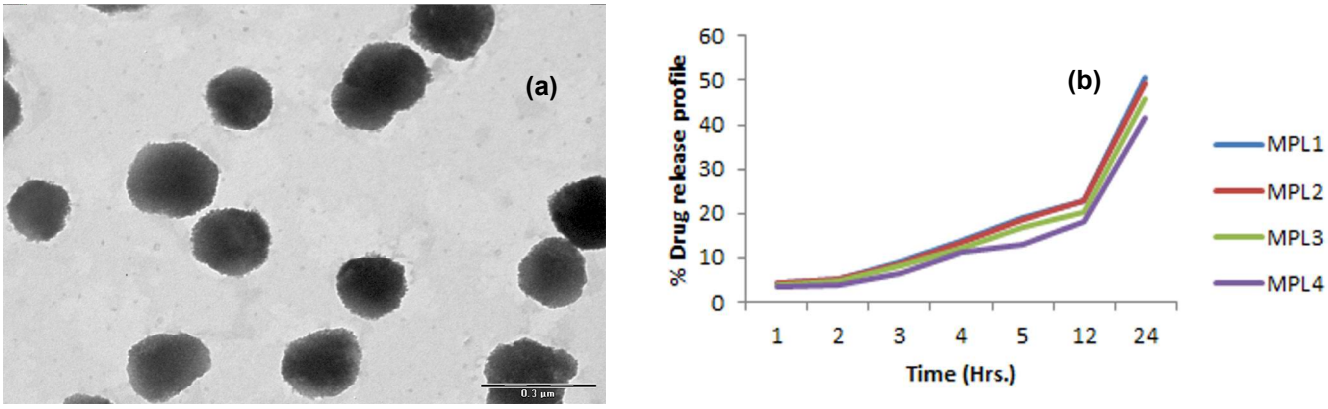

Figure 5. (a) TEM Photomicrograph of SLNs (b) *In-vitro* drug release profile

(Figure 4a and b), formulation MPL-3 (7:3) shows average size 2.69µm with % entrapment of 70.5±1.10 % was selected for further experiment. Particles size and shape was observed by transmission electron microscope (Figure 5a). Drug release pattern performed and after different time interval drug release pattern continuously increased in sustained manner. Release also altered with the solid lipid nanoparticles prepared by solvent diffusion method; shows improved drug release profile (Figure 5b). In-vitro drug release studies shows that drug release controlled over prolong period of time i.e. after 24hrs. 45.7% drug was released; this will also decrease the dosing frequency of active constituent.

Conclusion

After development of solid lipid nanoparticles containing murrayanol shows effective management of diabetes in animals and release also modified because of carrier system which results in zero order drug delivery in body. We used drug of herbal origin which is again a benefit to get rid of continuous use of synthetic drugs daily, which ultimately gives side effects on body.

Conflict of interest: The authors declare no conflicts of interest.

References

Chauhan A, Sharma PK, Srivastava P, Kumar N, Dudhe R. 2010. Plants having potential antidiabetic activity: A review. Der Pharmacia Lettre, 2(3): 369-387.

Deepthi B, Sowjanya K, Lidiya B, Bhargavi RS, Babu PS. 2017. A modern review of diabetes mellitus: an annihilatory metabolic disorder. Journal of in silico and in vitro pharmacology, 1(14): 1-5.

Dixit M, Kini AG, Kulkarni PK. 2010. Preparation and characterization of microparticles of piroxicam by spray drying and spray chilling methods. Research in Pharmaceutical Sciences, 5(2): 89-97.

Dubey S, Sharma R, Mody N, Vyas SP. 2017. Novel carriers and approaches: insight for psoriasis management. Nanostructures for novel therapy, 657-684.

Igara CE, Omoboyowa DA, Ahuchaogu AA, Orji NU, Nduknve MK. 2016. Phytochemical and nutritional profile of *murraya koenigii* (L.) leaf. Journal of Pharmacognosy and phytochemistry, 5(5): 07-09.

Josepine LJ, Yathish M, Wilson B, Premakumari KB. 2012. Formulation and evaluation of microparticles contain

curcumin for colorectal cancer. Journal of Drug Delivery and Therapeutics, 2(3): 125-128.

Mukherjee S, Ray S, Thakur RS. 2009. Solid lipid nanoparticles: A modern formulation approach in drug delivery system. Indian Journal of Pharmaceutical Sciences, 71(4): 349-358.

Nagendra R, Charan CS, Joshi KH, Jayanthi C. 2015. Preparation and evaluation of microparticles containing oxybutynin chloride for controlled release. Asian Journal of Biomedical and Pharmaceutical Sciences, 5(41): 15-17.

Nishan M, Subramanian P. 2015. Murraya koenigii (curry leaves)- A review on its potential. International Journal of Pharm Tech Research, 7(4): 566-572.

Olokoba AB, Obateru OA, Olokoba LB. 2012. Type II diabetes mellitus: A review of current trends. Oman Medical Journal, 27(4): 269-273.

Parida, RK, Panda, SK, Ravanan, P, Roy, H, Manickam, M, Talwar, P. 2013. Microparticle based drug delivery systems: Preparation and applications in cancer therapeutics. International Archive of Applied Sciences and Technology, 4: 68-75.

Parikh R, Dalwadi S. 2014. Preparation and characterization of controlled release poly-E-caprolactone microparticles of isoniazid for drug delivery through pulmonary route. Powder Technology, 264: 158-165.

Patel DK, Prasad SK, Kumar R, Hemlatha S. 2012. An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pacific Journal of Tropical Biomedicine, 27(4): 897-912.

Piero MN, Nzaro GM, Njagi JM. 2014. Diabetes mellitus- a devastating metabolic disorder. Asian Journal of Biomedical and Pharmaceutical Sciences, 04(40): 1-7.

Taylor RN, John MR, Taylor J, Kruger MC. 2015. Sorghum condensed tannins encapsulated in kafirin microparticles as a nutraceutical for inhibiton of amylases during digestion to attenuate hyperglycaemia. Journal of Functional Foods, 12: 55-63.

Waghmare AN, Tembhurne SV, Sakarkar DM. 2015. Phytochemical analysis and in-vitro antioxidant properties of *murraya koenigii* (L.). American Journal of Phytomedicine and clinical therapeutics, 3:(5), 403-416.