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Research Article

Pharmaceutics for effective drug dosage

Development and Characterization of Metformin Nanoparticles for the Effective Treatment of Diabetes Mellitus

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Abstract: Metformin hydrochloride is a BCS class III drug used in the management of non-insulin-dependent diabetes mellitus, as a single or in combination therapy. The aim of the present work was to develop starch nanoparticles for the delivery of Metformin hydrochloride that could reduce its dose related side effects and may prolong its release for the treatment of diabetes mellitus. Nanoparticles were prepared by the solvent evaporation technique method using starch as polymer, tween 80 as a stearic barrier and citric acid to increase the stability. The present work was aimed to develop metformin nanoparticles incorporated with starch for the effective treatment of diabetes mellitus. Framed nanoparticles were exposed to in-vitro characterization procedures for compatibility concentrates among drug and polymer, size, surface morphology, encapsulation efficacy and delivery attributes. Fourier Transform Infrared Spectroscopy results showed the compatibility nature of selected excipients for the synthesis of metformin nanoparticles. The X-Ray Diffraction Analysis (2θ values (12)) results showed developed metformin nanoparticles were non crystalline in nature. The selected developed metformin nanoparticles were in cubic phase with average particle size of 60.7± 22.54 to 118.9 ± 15.96 nm with charge ranging from 18.9 ± 0.81 to 160.7 ± 6.81mV. The encapsulation efficiency for metformin within metformin nanoparticles was about 67.84 ± 1.50 to 79.97 ± 0.9 %. The *in vitro* drug release studies of MN3 showed controlled drug release profile as compared to pure metformin. Further, the results of *in vivo* studies in terms of blood glucose profile showed significant effect for the developed metformin nanoparticles for the treatment of diabetes mellitus.

Keywords: Metformin, Diabetes, Nanoparticles Starch, Citric acid, controlled release

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1. INTRODUCTION

Diabetes mellitus (DM) is a condition which occurs due to the disorders of carbohydrate metabolism with characteristic features of chronic hyperglycemia associated with defects in insulin secretion, insulin action, or a combination of those. Worldwide, the quantity of individuals with diabetes mellitus has quadrupled in the previous thirty years, and diabetes mellitus is the significant reason for death .Asia is a significant zone of the quickly arising T2DM worldwide epidemic, with China and India the best two focal points[1]. DM is a metabolic disorder of numerous aetiologies, portrayed by hyperglycaemia coming about because of imperfections in insulin emission, insulin activity or both, and related with aggravation of carbohydrate, fat and protein digestion. The three commonest sorts of diabetes are Type 1 Diabetes Mellitus (T1DM), Type 2 Diabetes Mellitus (T2DM) and Gestational Diabetes Mellitus (GDM)[2]. Attenuation of chronic complications in type 1 diabetes mellitus was accomplished by tight glycemic control by numerous daily bolus injections of insulin by subcutaneous route[3]. The adverse effects associated with insulin therapy are hyperinsulinemia, insulin resistance, glucose intolerance, weight gain and cardiovascular complications[3]. T2DM is strongly associated with insulin resistance, due to obesity, caused by poor eating habits and lifestyle. Insulin sensitivity changes by eating a high -carbohydrate diet, decreased Physical activity, and stress. The obese people mass contains a high tier of adipose tissue, which is associated with high resistance and other substances that can increase insulin sensibility[4].Designing novel delivery systems to deliver insulin by oral, nasal, buccal, pulmonary, rectal, ocular and transdermal routes has conveniently and effectively led to greater output. The changes in the metabolism of carbohydrates, lipids, and proteins results in the changes in insulin (an anabolic hormone) release. The metabolic abnormalities occur due to low levels of insulin to achieve adequate response and/or insulin resistance of target tissues (skeletal muscles, adipose tissue) and liver, at the level of insulin receptors, signal transduction system, and/or effector enzymes or genes[5]. The interaction between genetic and non-genetic factors creates the involvement towards the development of DM are Free radicals, oxidative stress, and many metabolic stressors serves as the pathogenesis and complications for DM. The development and maintenance of pancreatic β cell function were achieved by the female hormone 17-β estradiol, which acts through the oestrogen receptor-α (ER-α). Several environmental factors have been also implicated in the etiology of the DM development. Several environmental factors have been also implicated in the aetiology of the DM development. The increased risk associated with rapid development of atherosclerosis has been reported in diabetic patients with early development of atherosclerosis in adolescents and children with type I DM[6]. Epidemiological studies also emphasized that the effect of type I DM and type II DM is in correlation with the metabolic changes in atherosclerosis development. The symptoms associated with DM include excessive urine production (polyuria), excessive thirst or increased fluid intake (polydipsia), excessive food intake (polyphagia), blurred vision, hypertension etc. For Type 2 DM metformin has been considered as one of the most widely used drugs in the United Kingdom and in the United States. Metformin provides its role in DM patients by decreasing intestinal glucose absorption, improving peripheral glucose uptake, lowering fasting plasma insulin levels and increasing insulin sensitivity. In case of type 2 DM, metformin is often prescribed in combination with other medications. Numerous studies have shown that metformin can be repurposed for anticancer treatment. As a supportive therapy metformin can be used as anti-aging, cardiovascular protective and neuroprotective agents[7]. The reduction in cancer incidence and mortality in patients with diabetes using metformin was proved by meta-analysis studies. Cancer incidence reduced by 31% and cancer mortality reduced by 34%[8]. Metformin, with the chemical name of 1,1-dimethylbiguanide hydrochloride, is freely soluble in water, sparingly soluble in ethanol and insoluble in acetone, ether, or chloroform[9]. Metformin is a solid base with a pKa of 12.4[10]. Supreme oral bioavailability of Metformin is 40 to 60%, with mean plasma half-life after oral administration is 4.0 and 8.7 hours[11]. The rapid advancement in nanotechnology evolves the numerous novel drug delivery strategies including nanoparticles. Nanoparticles can be designed to conquer the known disadvantages of the drug utilized for the nano based drug delivery formulations, the low solubility/bioavailability and high cytotoxic side effects[12]. The unique structural, chemical, mechanical, magnetic, electrical, and biological properties were exhibited by nanoparticles. The emerging properties of nanoparticles lead to the increased usage of inorganic nanoparticles with a greater impact on drug delivery, imaging, and therapeutic functions. In case of nanoparticles the optical and electronic properties are inter-dependen[13]. Nanoparticles overcome the main critical issues encountered with conventional pharmaceutical treatments such as the nonspecific distribution, rapid clearance, uncontrollable release of drugs and low bioavailability[10]. Nanoparticles show characteristic properties of variations in colours and variation in size and shape, which can be utilized for therapeutic/Bioimaging applications. Nanoparticles consist of several kinds of material being classified as non-degradable and biodegradable. Biodegradable systems have an advantage over non-degradable systems in that they are nontoxic bio-tolerable, bio-compatible, biodegradable and water soluble. Depending upon the method of preparation of nanoparticles, Nano spheres or Nano capsules can be obtained[14]. Here in the present study aimed towards the development of metformin nanoparticles using natural biodegradable polymer starch for the effective control of diabetes mellitus.

2. MATERIALS AND METHODS

Metformin hydrochloride was gifted by Apex labs Chennai, India. Potassium dihydrogen phosphate, Sodium hydroxide, Dimethyl sulphoxide, Tween 80, Citric acid, Starch, Methanol and all other chemicals were analytical grade purchased from Sigma-Aldrich (Bangalore, India). Milli Q water was filtered through a 0.22 µm filter and used throughout the analysis. All other chemicals were of analytical grade and used without further purification.

2.1 Compatibility analysis

The compatibility of starch, Metformin, Metformin nanoparticles was checked by analysing FTIR spectra using FTR spectrometer (JASCO FT/IR 6300) at wavelength ranging from 4000- 400 cm^{-1}. Potassium bromide pellet press technique was adopted for sample preparation.

2.2 Preparation of Metformin Nanoparticles by Solvent Evaporation

Weighed amount of sodium hydroxide (750 mg) in 35 ml

water was treated with starch 2.5g (7.2mM) under magnetic stirrer at 250 C for 30 minutes. To the above whole content add 200mg of Tween 80 dissolved in 10ml of distilled water along with different amount of Metformin (7.7mM, 15.4mM and 23.1mM), followed by this 5 ml distilled water containing 500 mg citric acid was added left under magnetic stirrer for 30 minutes at 25°C. The resulting drug encapsulated cross-linked starch nanoparticles were subsequently precipitated by 50 ml of absolute ethanol and purified by means of centrifugation (4500 rpm for 10 minutes) and washed twice with 80/20 absolute ethanol/water to remove unreacted compounds.Final wash is done with absolute ethanol and dried at 25° C.

2.3 Particle size and zeta potential analysis

Particle size distribution and zeta potential of Metformin nanoparticles was measured using Zeta sizer (Nano ZS90 series, Malvern Instruments, UK) by diluting Metformin nanoparticle at 1:10 ratio using Milli Q water.

2.4 Morphology

The developed Metformin nanoparticles were visualized for its morphology using scanning electron microscopes.

2.5 XRD analysis

The amorphous nature of Metformin nanoparticles in comparison to Standard Metformin drug was checked using a powder X-ray beam diffractometer at a voltage 40 KV and current of 20 mA.

2.6 Thermal analysis

Thermal behaviour for Metformin nanoparticles in comparison to Standard Metformin drug was recorded using differential scanning calorimeter (DSC 8000 Perkin Elmer Pvt Ltd) by heating the samples in aluminium pan under nitrogen gas with controlled temperature(30 - 300 °C) at a ramp rate of 10 °C/ min.

2.7 Quantification of Metformin nanoparticles - UV Spectrophotometric method

UV Spectrophotometric method for the estimation of Metformin was performed at the wavelengths of 234 nm**.** The linearity of Metformin was performed at the selected wavelength conditions.

2.8 Encapsulation efficiency

Metformin nanoparticles were treated with phosphate buffer saline (PBS).Centrifuged at 10000rpm for 15 minutes. Absorbance of the supernatant was measured using UV spectrophotometer at the wavelength of 234 nm (Metformin) and the amount of drug is-entrapped was calculated as drug entrapment efficiency using the formula

$$\text{Entrapment efficiency (\%)} = (Ta\text{-}Tf)/Ta \times 100$$

Where, Ta: Total amount of drug used in formulation and Tf: Amount of free drug in the supernatant

2.9 In vitro release studies

The *in vitro* release of Metformin from the Metformin nanoparticles was checked using the dialysis bag method. The release studies were performed by placing 1 ml of Metformin nanoparticles formulation with 1 ml of phosphate buffer and the sink condition was maintained by placing the dialysis bag (Mol. Wt. cut off 12,000 - 14,000 Da; pore size, 0.2 µm) in a glass beaker containing 100 ml phosphate buffer pH 7.4 kept under magnetic stirring condition at 37 ˚C. Periodically, 2 ml of sample was withdrawn and replaced with 2 ml of phosphate buffer (pH 7.4) at different time intervals. The amount of Metformin released from the Metformin nanoparticles was measured by UV-Visible spectrophotometer at the wavelengths of 234 nm (Metformin).

2.10 In vivo release studies

2.10.1 *Selection & acclimatization of animals and diabetes induction*

Wistar strains of 18 male albino rats weighing between 180-220 gm are used for this study. The animals were housed in large spacious cages and they were fed with commercial pellets and access to water *ad libitum*. The animals were well acclimatized to the standard environmental condition of temperature (22^0c ± 5^{0C}) and humidity (55 ±5%) and 12 hr light dark cycles throughout the experimental period. Animal experimentation was performed in accordance with the Institutional Animal Ethics Committee (IAEC) guidelines (IAEC approval No- 245/PO/RCB/B1/S/18/CPCSEA). Diabetes mellitus is induced in wistar rats by single intraperitoneal injection of freshly prepared solution of Streptozotocin (25 mg/kg BW) in physiological saline after overnight fasting for 12 hrs**.** The development of hyperglycemia in rats is confirmed by plasma glucose estimation 72 hrs posts Streptozotocin injection. The rats with fasting plasma glucose levels of >160-200 mg/dl were used for this experiment.

2.10.2 Experimental procedure

In the experiment a total of 18 rats (12 diabetic surviving rats & 6 normal rats) were used. Diabetes was induced in rats 3 days before starting the experiment. The rats were divided into 3 groups after the induction of Streptozotocin diabetes. In the experiment 6 rats were used in each group. Group-I acts as (Normal control) consist of normal rats given with 10 ml/kg of normal saline, orally. Group-II as (Toxic control) Diabetic control received 25 mg/Kg of Streptozotocin through I.P. Group-III acts as Diabetic control received metformin nano preparation dissolved in sterile distilled water at a dose of (40mg/Kg I.P) for 28 days.

2.10.3 Sample collection

After 28 days of treatment, body weight, blood glucose, haemoglobin, glycosylated haemoglobin, plasma insulin, total cholesterol, triglycerides, HDL-cholesterol and phospholipids and glycogen content levels were determined. Blood(5ml) was collected from the eyes (venous pool) by sino-ocular

puncture in EDTA coating plasma tubes for the estimation of blood parameters.

2.10.4 Estimation of blood glucose

Blood glucose was estimated by a commercially available glucose kit (One Touch Ultra) Johnson Johnson based on glucose oxidase method.

2.10.5 Plasma insulin

Plasma insulin was determined by the ELISA method using a Boehringer–Mannheim kitwith an ES300 Boehringer analyzer (Mannheim, Germany).

2.10.6 Estimation of blood parameters

The hematological parameters such as Total haemoglobin, Plasma lipids (HDL-cholesterol), phospholipids and glycosylated haemoglobin were determined by standard protocol.

2.10.7 Hepatic glucokinase and hexokinase activity

The part of liver for each test was perfused with ice cold 0.15M KCl and 1mM EDTA solution and homogenized twice its weight of ice cold buffer (0.01 cysteine and 1mM EDTA in 0.1 ml Tris-HCL, pH 7.4) and centrifuged for 20 minutes 4^0C. Glucose phosphorylation was assayed by means of glucose 6 phosphate dependent spectrophotometric method.

2.10.8 Glucose-6-phosphatase activity

The part of the liver for each test was homogenized with 40 times its weight of ice cold buffer (0.1 citrate-KOH, pH 6.5) and filtered through cheesecloth. Glucose-6-phosphatase activity was measured by phosphate release by the method Marjorie. The determination of phosphoric acid concentration in assay mixture was done calorimetrically.

2.10.9 Glycogen Content

The tissue sample was digested by hot concentrated 30 % KOH and treated with anthrone reagent. Glycogen content was determined colorimetrically.

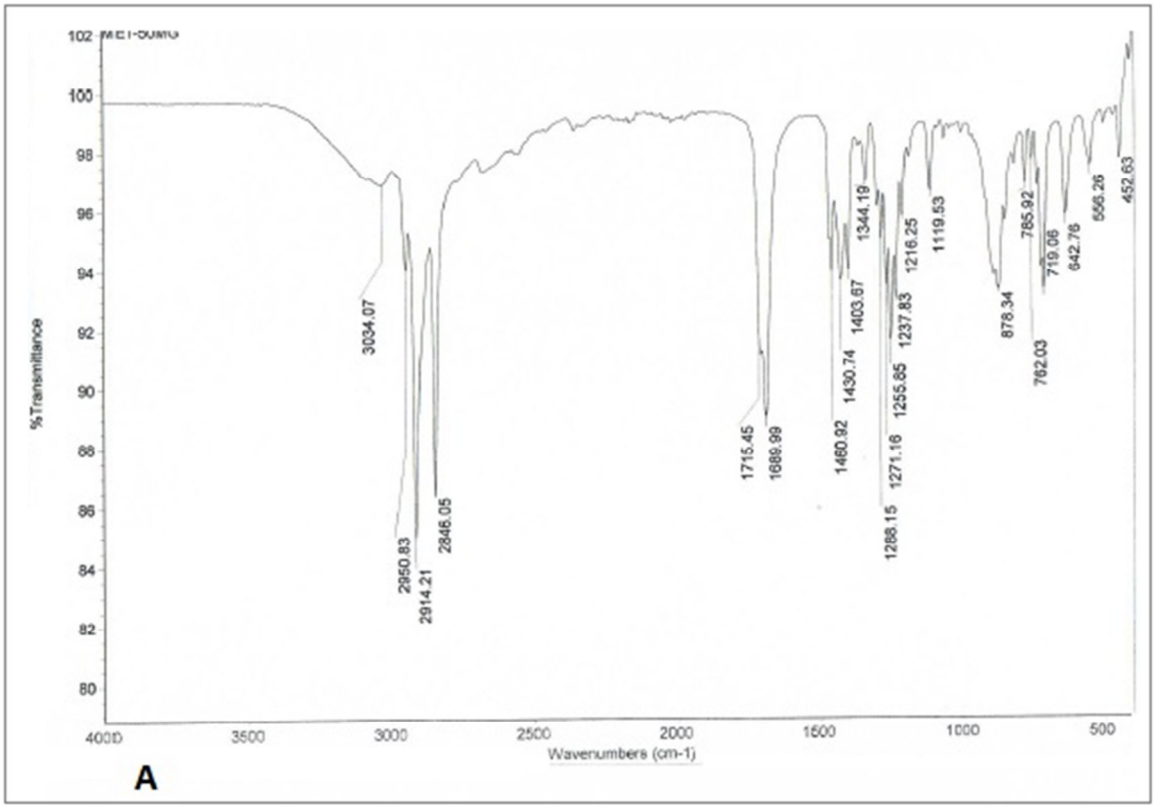
3. STATISTICAL ANALYSIS

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA), and the group means were compared by Newman-Keuls multiple range test (NKMRT). Values were considered statistically significant at $p<0.01$.

4. RESULTS AND DISCUSSION

4.1 FTIR spectroscopic analysis

The compatibility study for drug and the excipients were carried out using FT-IR spectrometer. Here, FT-IR analysis was performed to check the structural and functional groups of developed nanoparticles. Metformin nanoparticles showed characteristic absorption band at 3287 cm^{-1}, 3034 cm^{-1}, 3261 cm^{-1} which may be attributed due to hydrogen bonded O-H group stretching of starch molecules. The absorption bands at 2950 cm^{-1}, 2914 cm^{-1}, 2846 cm^{-1}, 2923 cm^{-1} may be attributed due to unsymmetrical stretching of C–H (CH_2 group) of starch molecules (Fig. 1). The absorption band at 1638 cm^{-1} and 1639 cm^{-1} was due to the presence of bonded water in starch. The peak at 1363 cm^{-1} represented the angular deformation of C–H (CH 3 group) of starch molecules. The peak at 931 cm^{-1} and 993 cm^{-1} was related to the C–O–C of a-1,4 and a-1,6 glycosidic linkages . Other peaks, at 1148 cm^{-1} and 1015 cm^{-1} were associated with C–O bond and C–C bond of starch nanoparticles[15–17].



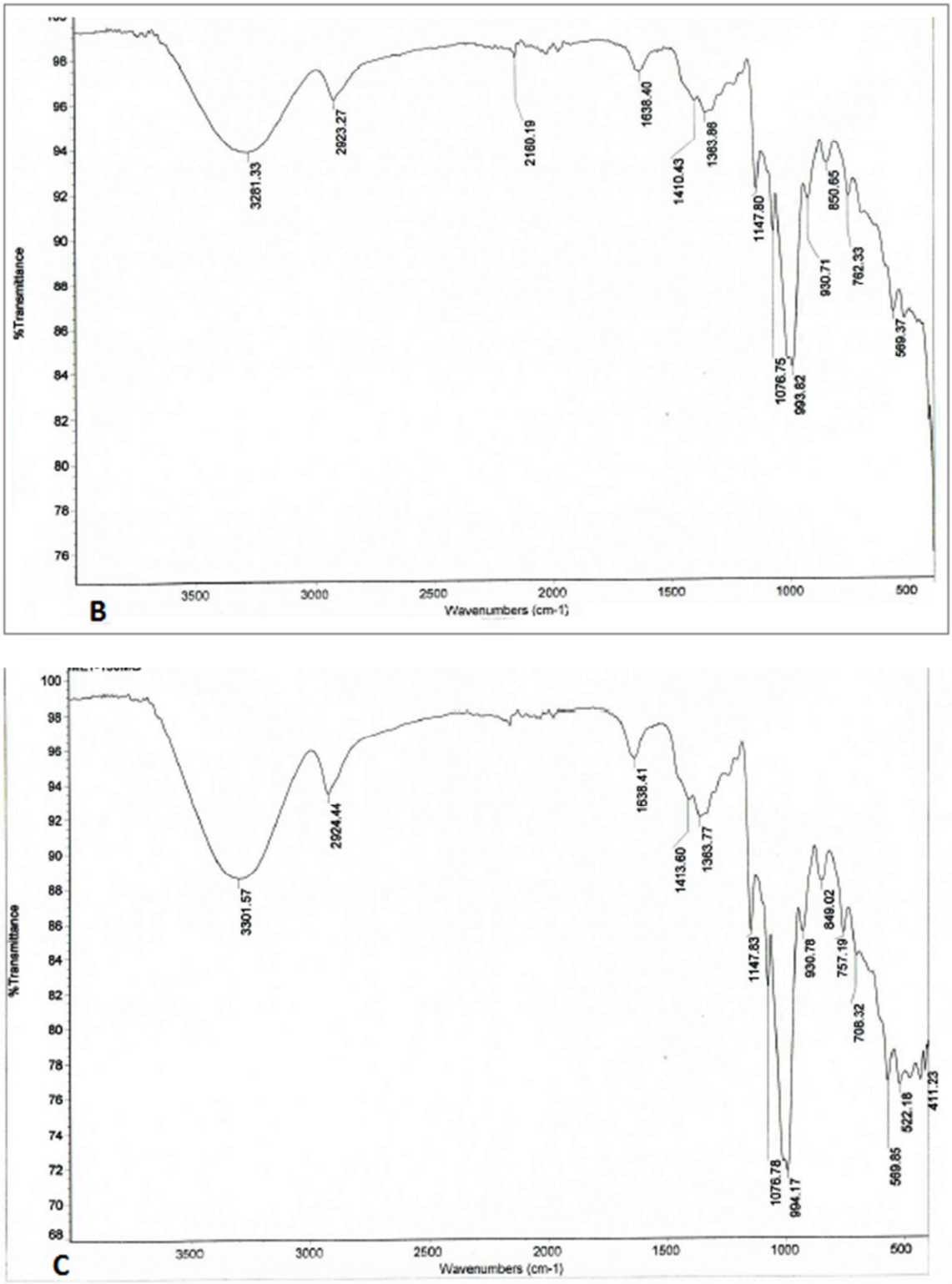


Fig 1. FT-IR spectra of (A) Metformin nanoparticles (1:1) (B) Metformin nanoparticles (1:2) and (C) Metformin nanoparticle (1:3)

Starch showed characteristic absorption band at 3285 cm^{-1}, 2923 cm^{-1} which may be attributed due to hydrogen bonded O-H group stretching of starch molecule. The absorption bands at 2923 cm^{-1} may be attributed due to unsymmetrical stretching of C–H (CH_2 group) of starch molecules. The absorption band at 1638 cm^{-1} was due to the presence of bonded water in starch. The peak at 1410 cm^{-1} and 1364 cm^{-1} represented the angular deformation of C–H (CH 3 group) of starch molecules. The peak at 931 cm^{-1} and 993 cm^{-1} was related to the C–O–C of a-1,4 and a-1,6 glycosidic linkages.

4.2 Development of Metformin nanoparticles

The Metformin nanoparticles were prepared using solvent evaporation technique. Herein, starch, tween 80 and citric acid were used for the preparation of Metformin nanoparticles. Here aqueous solution of tween 80 acts as a steric barrier and this supports the production of smaller particles with uniform size distribution. In addition, citric acid used in the formulation may support the stability of the developed Metformin nanoparticles. By varying the starch concentration and Metformin concentrations different trials

were prepared and these trials were checked for its size, zeta potential and drug content. Previously, Doxorubicin-Loaded Glycyrrhetinic Acid-Biotin-Starch Nanoparticles was reported using by an N, N′-dicyclohexylcarbodiimide (DCC)/4-dimethylaminopyridine (DMAP)-mediated one-step esterification reaction. By varying the starch to metformin drug concentration in molar ratio 1:53.47, 1: 10.05, 1:5.34, 1:1.06 and 1:0.21 different trials were performed. Previously metformin loaded alginate nanoparticles were developed by Sandeep Kumar et al; 2016 they observed an encapsulation efficiency of 78 %. Their developed metformin nanoparticles showed enhanced efficiency compared to pure metformin[18]. Smaller stable Metformin nanoparticles with good encapsulation efficiency (79.97%) are obtained as per previous studies. Recently, newer technology in formulation development of solvent-free solid dispersions (SDs) for poorly soluble anti-inflammatory drugs Mefenamic acid and flufenamic acid was achieved using microwave technology in order to offer an enhanced in vitro dissolution rate and vivo anti-inflammatory effects[19].

4.3 Particle size and zeta potential analysis of Metformin nanoparticles

The mean particle size and zeta potential of Metformin nanoparticles are shown in Table. The particle size for different Metformin nanoparticles of 1:1, 1:2 and 1:3 were in the range of 118.9 ± 15.96– 160.7± 22.54 nm. Whereas, the zeta potential ranges from 18.9 ± 0.81– 160.7 ± 6.81mV (Table 1). The zeta potential value indicates that the developed metformin nanoparticles are positively charged. Results conclude that the particle size of metformin nanoparticles gets increased on increasing the starch concentration. Among the different trials the nanoparticle prepared at 1:3 ratio showed the lowest particle size of 118.9 ± 15.96 nm (Fig. 2). Previous reports on particle size reveals that different size-fractions of drug-loaded microspheres showed quite distinct drug loading and release kinetics which may be achieved by control on microparticle size by fractionation technique[20].

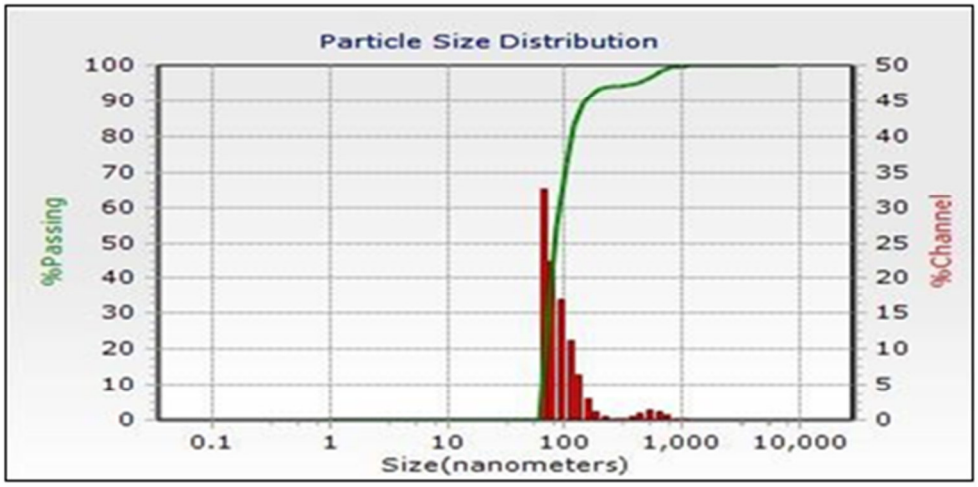


Fig 2: A typical particle size data of metformin nanoparticles

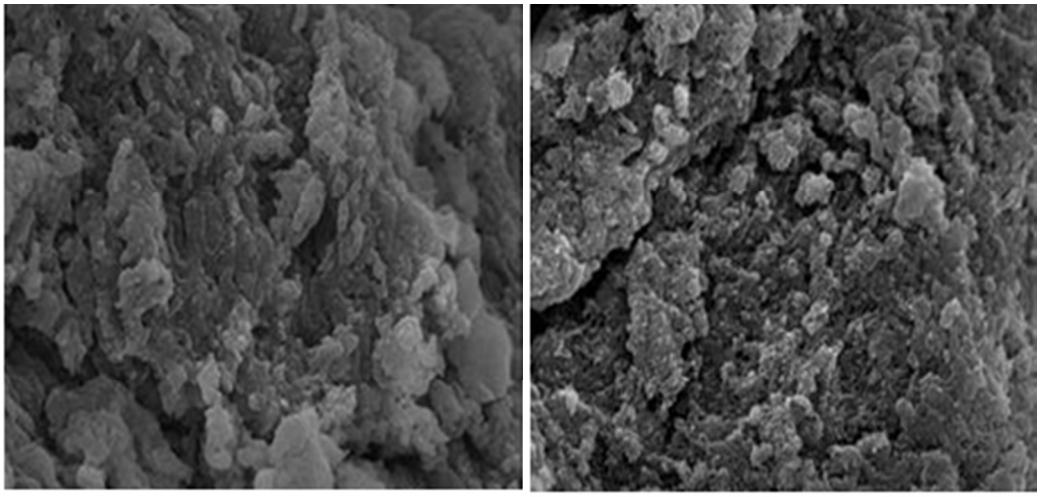
Table.1 Formulation layout for the development of Metformin Nanoparticles

Formulation Code	Concentration (Starch: Metformin)	Average particle size (nm) ± SD	Zeta potential (mV) ± SD	Metformin Encapsulation efficiency (%) ± SD
MF-NPs - 1	1:1	160.7± 22.54	18.9 ± 0.81	73.20 ± 0.81
MF-NPs - 2	1:2	140.6 ± 24.15	31.7 ± 5.07	79.97 ± 0.9
MF-NPs - 3	1:3	118.9 ± 15.96	160.7 ± 6.81	67.84 ± 1.50

All values are expressed mean ±SEM (n=3)

4.4 Morphology of Metformin nanoparticles

The morphology of Metformin nanoparticles was checked using scanning electron microscopy and found the particles are spherically shaped with aggregate nature.



A B

Fig 3: Scanning electron microscopy image of Metformin Nanoparticles - X1000 magnification (A) and X5000 magnification (B)

4.5 XRD analysis of Metformin nanoparticles

XRD diffraction spectra of starch nanoparticles exhibit sharp diffraction peaks between the 2θ values (22.74, 17.76, 16.64, 14.86) with an average peak intensity of nearer to 500 to 600 %, especially the peak at 2θ (22.74) shows higher peak intensity of nearer to 600 % which indicates the crystalline nature of starch nanoparticles. No sharp diffraction peaks, especially the peaks of drugs were masked which indicates the non-crystalline nature of formulation (Fig. 3).

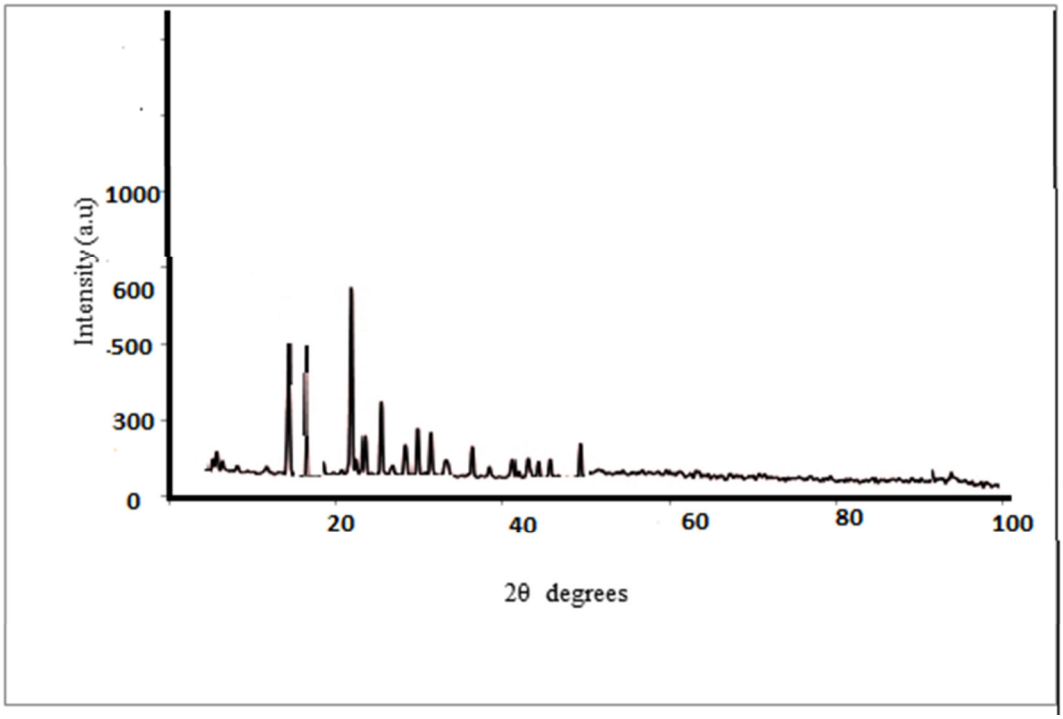


Fig.4 XRD Spectra of Starch nanoparticles

XRD diffraction spectra of metformin nanoparticles exhibit Sharp diffraction peaks between the 2θ values (12) with an average peak intensity of nearer to 400 %, especially the peak at 2θ (12) shows higher peak intensity of nearer to 400 % which indicates the crystalline nature of starch nanoparticles. The absence of major peaks for metformin nanoparticles may be due to the non- crystalline nature of metformin nanoparticles which may be encapsulated within the nanoparticles. Whereas the XRD diffraction spectra of Standard metformin exhibit diffraction peaks between the 2θ values (12) with an average peak intensity of nearer to 160 % (Fig. 5 and 6).

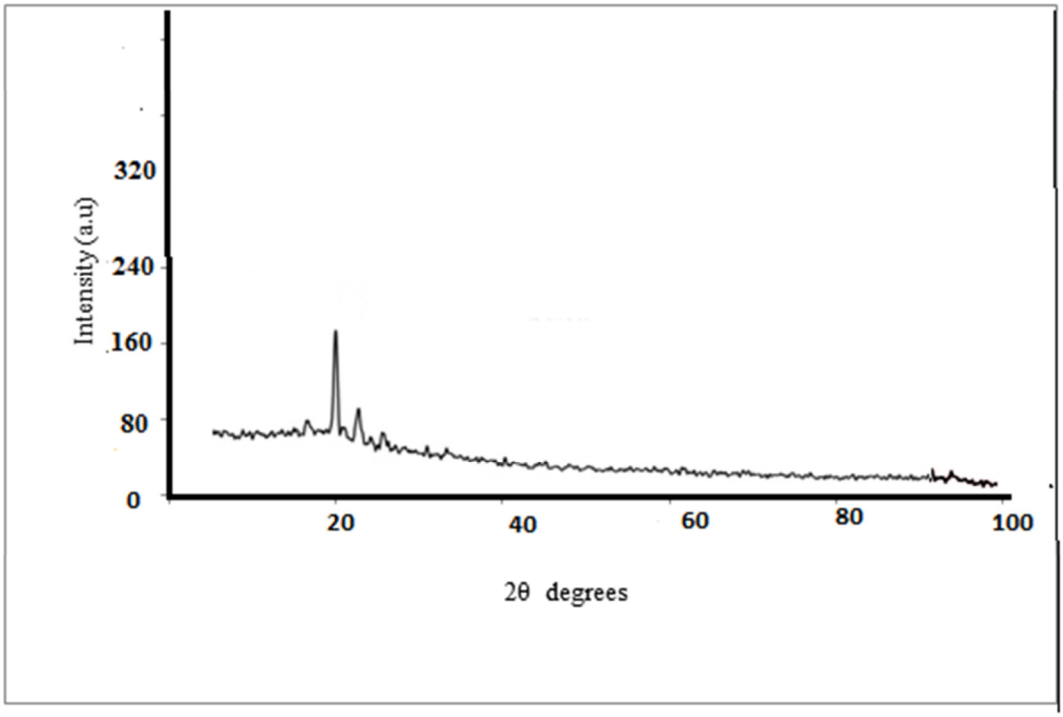


Fig.5 XRD Spectra of Standard Metformin

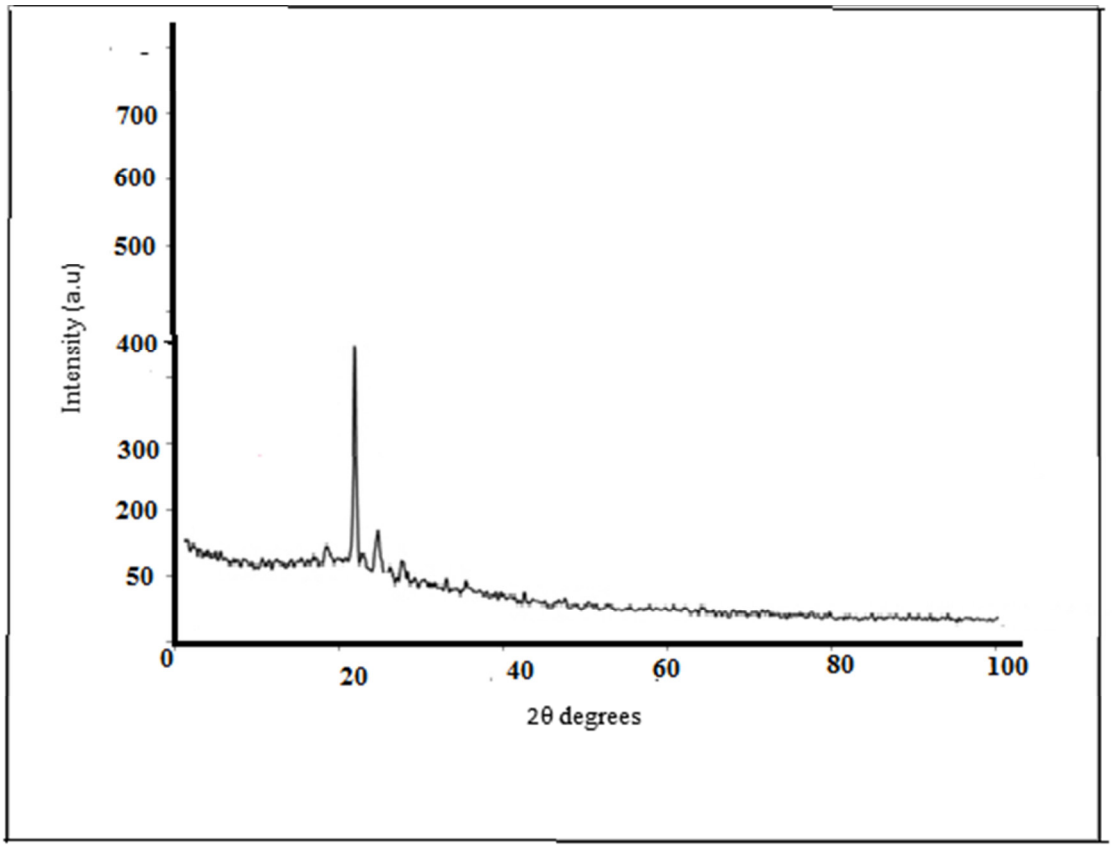


Fig.6 .XRD Spectra of Metformin nanoparticles

4.6 *Encapsulation efficiency of Metformin nanoparticles*

Higher drug encapsulation efficiency is desired to achieve better therapeutic effect on target site. The amount of metformin incorporated into the metformin nanoparticles was determined for three different formulations shown in the range of 67.84 ± 1.50 to 79.97 ± 0.9 % as shown in Table .1Among the different trials the metformin nanoparticles developed at the ratio of 1:2 showed the higher encapsulation efficiency of 79.97 ± 0.9 %.

4.7 *Thermal analysis*

The melting endothermic peak of starch nanoparticles appears at 100 °C. However, Starch-Metformin Nanoparticles possess a mild melting indicated by a broad

peak at 100.0 °C, 300.0 °C and350.0 °C (Fig. 7). In the DSC thermograms of Starch-Metformin Nanoparticles, the peak due to starch is found to be slightly shifted and no melting endothermic peak of metformin was observed which indicates that metformin that is encapsulated inside the nanoparticles may be in amorphous. This interaction occurred via hydrogen bond formation between starch nanoparticles and metformin resulted in entrapping metformin molecules within the hydrophilic polymer and inhibiting its crystallization. These results comply with the previously reported formulation of diacerein solid dispersion system[21].

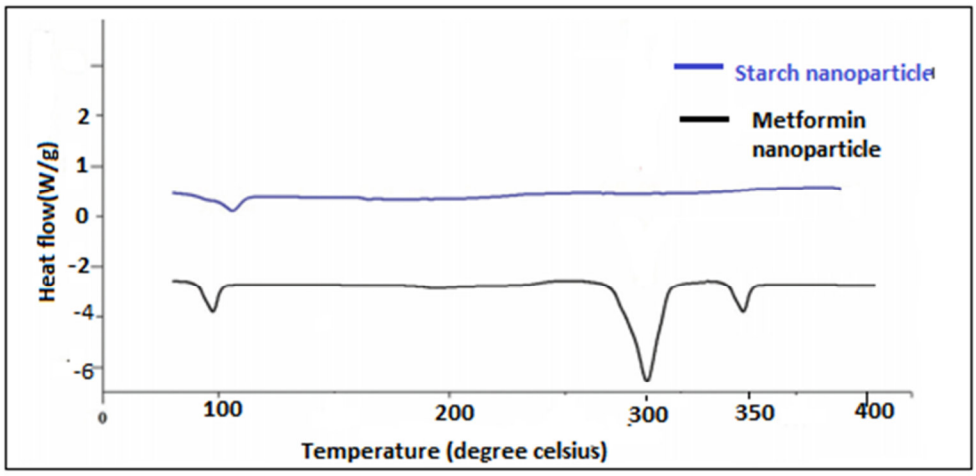


Fig. 7 DSC of Starch Nanoparticles

4.8 *In vitro* release studies

The *in vitro* release profile of metformin nanoparticles (1:1) analyzed by UV Spectrophotometer showed an release of 4.41 % at 72 h, which indicates a sustained release profile. Whereas metformin nanoparticles (1:2) also showed a sustained release profile of 10.12 % at 72 h, the metformin nanoparticles (1:3) showed a sustained release profile of 22.74 % at 72 h as shown in Fig. 8. The results indicate that metformin nanoparticles (1:3) showed superior release profile compared to metformin nanoparticles (1:1) and metformin nanoparticles (1:2).

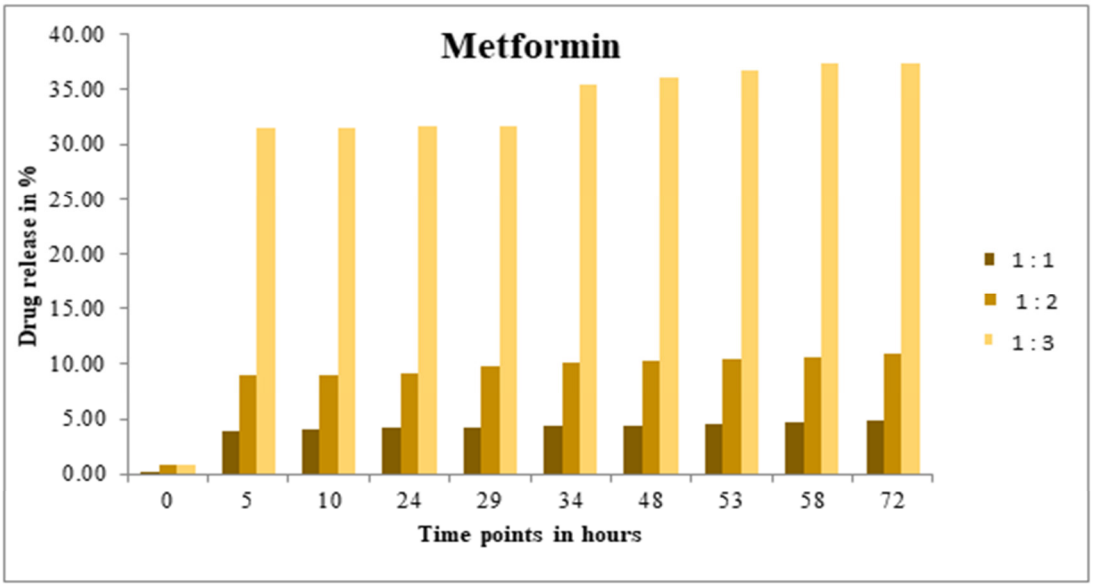


Fig 8. *In vitro* release profile of Metformin Nanoparticles

The in vitro drug release studies of MN3 showed controlled drug release profile as compared to pure metformin. The 100% drug from pure metformin was released within 1 h whereas, only 20.52% drug was released in the first 1 h, and 100% metformin was released further for 30 h from MN3. The drug release profile of MN3 shows a sustained release pattern with the passage of time. The encapsulated drug released slowly with the eruption of polymeric layers and completed up to 30 h. The cumulative drug release profile of MN3 and pure metformin is shown in Fig. 5. Previously it has been reported for alginate based metformin nanoparticles shown an encapsulation efficacy of 78%, with enhanced efficiency and response for alginate based metformin nanoparticles relative to pure metformin in the treatment of diabetes mellitus[18]. Metformin loaded pectin (PCM) nanoparticles showed drug entrapment efficiency of 68 ± 4.2 % with prolonged release characteristics in the treatment of Type 2 Diabetes Mellitus[22].

4.9 *In vivo* studies [23–25]

The levels of initial and final blood glucose, and change in

body weight , in normal rats, and treatment control animals in each group was shown in Table. The mean body weight of diabetic rats (G2) was significantly decreased as compared to normal control rats. The body weight of diabetic control rats treated with nanoparticles of metformin at a dose of 40mg/kg was increased the body weight non-significantly as compared to normal control animals. Fasting blood glucose level was significantly increased 185.30 ± 4.60 in diabetic animals as compared to normal animals. However the level of fasting blood glucose, returned to near normal range in diabetic rats treated with nanoparticles of metformin at a dose of 40mg/kg respectively. Table 2 illustrates the levels of total hemoglobin, glycosylated hemoglobin and plasma insulin in normal rat and treatment control animals in each group. The levels of total hemoglobin, and plasma insulin levels were decreased significantly whereas glycosylated hemoglobin levels were increased significantly as compared to normal control rats. However the level of total hemoglobin, glycosylated hemoglobin and plasma insulin, returned to near normal range in diabetic rats treated with nanoparticles of metformin at a dose of 40 mg/kg respectively. Table 3 shows the level of serum total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), Low density lipoprotein (LDL) and phospholipids of normal and experimental animals in each group. Total cholesterol, triglycerides, high density lipoprotein, Low density lipoprotein (LDL) and phospholipids levels were significantly increased, whereas HDL-C level was decreased in streptozotocin induced diabetic rats as compared to normal rats. Treatment of normal and streptozotocin induced diabetic rats with nanoparticles of metformin at a dose of 40mg/kg for 28 days resulted in marked decrease in total cholesterol, triglycerides, Low density lipoprotein(LDL) and phospholipids levels and increase in HDL-C levels as compared to streptozotocin induced diabetic rats.

4.9.1 Effect of nanoparticles on Glycogen Content

Glycogen content of liver tissue was estimated on the 28th day in non-diabetic control, diabetic control drug, treated group and positive control group as shown in Table No. 4. In diabetic control rat liver glycogen content decreased significantly as compared to non-diabetic control. Treatment with nanoparticles of metformin at a dose of 40 mg/kg, increase in liver glycogen content in comparison to diabetic control.

4.9.2 Effect of nanoparticles on Hepatic Enzymes

To establish diabetic, plasma glucose was determined 72hr after streptozotocin administration. Only those rats with over 180 mg were included in the study. On the 28th day, hepatic enzymes Hexokinase, Glucokinase and substrate Glucose-6-phosphate were estimated in saline control (group I), diabetic control (group II) and treatment controls (groups III).The result has been compiled in Table 5. As compared to non-diabetic control values, the mean level of enzymes Hexokinase, Glucokinase and substrate Glucose-6-phosphate values decreased in diabetic control. Treatment with nanoparticles of metformin at a dose of 40 mg/kg respectively for 28 days led to a rise in percentage of these parameters (P<0.001) as compared to diabetic control.

Table 2. Effect of Metformin nano particles on initial and final body weight and blood glucose in normal and treated animals.

GROUP	Body weight (g)		Blood glucose (mg / 100ml)	Blood glucose (mg / 100ml)
	Initial	Final	Initial	Final
G1	205 ± 5.20	240 ± 5.55	85.70 ± 2.30	93.60 ± 2.45
G2	225 ± 5.30	180 ± 4.80**(a)	92.20 ± 2.35	185.30 ± 4.60**(a)
G3	210 ± 5.15	245 ± 5.45	94.80 ± 2.60	105.40 ± 2.90*(b)

*Values are expressed as mean ± SEM. Values were compared by using analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests. ** (a) Values are significantly different from normal control G1 at P<0.001. ** (b) Values are significantly different from Diabetic control G2 at P<0.01.*

Table 3. Effect of Metformin *nano particle preparation* on plasma insulin, Hemoglobin & Glycosylated hemoglobin in normal and treated animals.

Groups	Haemoglobin (gm/100ml)	Glycosylated haemoglobin HbA_1 (%)	Plasma Insulin (µU/ml)
G1	13.60± 1.25	0.40 ±0.07	30.10 ± 2.20
G2	8.80 ± 0.80**(a)	0.96 ±0.16**(a)	13.50 ± 1.40**(a)
G3	13.20 ± 1.08**(b)	0.44 ±0.09**(b)	33.60 ± 2.60**(b)

*Values are expressed as mean ± SEM. Values were compared by using analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests.** (a) Values are significantly different from normal control G1 at P<0.001. ** (b) Values are significantly different from Diabetic control G2 at P<0.01.*

Table 4. Serum lipids of Normal and experimental groups

Groups	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	Phospholipids (mg/dl)	LDL (mg/dl)
G1	87.85 ± 2.60	93.40 ±2.40	52.40 ±1.80	128.30 ±2.25	17.45 ± 1.20
G2	185.30 ±5.20**(a)	150.30 ±3.12**(a)	35.65 ±1.20**(a)	202.20 ±5.24**(a)	40.20 ± 2.40**(a)
G3	115.60 ± 3.10**(b)	99.60 ±2.85**(b)	38.30 ±1.33	142.30 ±3.20	23.80 ± 1.55**(b)

*Values are expressed as mean ± SEM. Values were compared by using analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests. ** (a) Values are significantly different from normal control G1 at P<0.001. ** (b) Values are significantly different from Diabetic control G2 at P<0.01.*

Table 5. Effect of Metformin *nano particle preparation* on glycogen content (mg/gm tissue)	
Groups	**Liver Tissue Glycogen Content (mg/gm tissue)**
Group I	40.25 ± 1.88
Group II	12.40 ± 0.76*[a]
Group III	36.55 ± 1.70*[b]

Values are expressed as mean ± SEM. Values were compared by using analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests. ** (a) Values are significantly different from normal control G1 at P<0.001. ** (b) Values are significantly different from Diabetic control G2 at P<0.01.

Table 6. Effect of Metformin nanoparticles on enzymes involved in carbohydrate metabolism in rats			
Groups	**Hexokinase (µg/mg)**	**Glucose-6-Phosphate (µg/mg)**	**Glucokinase (µg/mg)**
Group I	0.218 ± 0.015	0.390 ± 0.016	27.50 ± 1.38
Group II	0.094 ± 0.004*[a]	0.134 ± 0.008*[a]	6.85 ± 0.26*[a]
Group III	0.168 ± 0.009*[b]	0.325 ± 0.010*[b]	22.80 ± 0.95*[b]

Values are expressed as mean ± SEM. Values were compared by using analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests. ** (a) Values are significantly different from normal control G1 at P<0.001. ** (b) Values are significantly different from Diabetic control G2 at P<0.01.

Streptozocin causes massive reduction in insulin release, through the destruction of β-cells of the islets of Langerhans. In our study, we have observed a significant increase in the plasma insulin level when Streptozocin induced diabetic rats were treated with nanoparticles of metformin at a dose of 40mg/kg, and this could be due to potentiation of the insulin effect of plasma by increasing the pancreatic secretion of insulin from existing β- cells of islets of Langerhans or its release from bound insulin. In uncontrolled or poorly controlled diabetes there is an increased glycosylation of a number of proteins including haemoglobin and α-crystalline of lens[24,26].Glycosylated haemoglobin (HbA_1C) was found to increase in patients with diabetes mellitus to approximately 16 %[25] and the amount of increase is directly proportional to the fasting blood glucose level[27]. During diabetes the excess glucose present in blood reacts with haemoglobin. Therefore, the total haemoglobin level is decreased in Streptozotocin induced diabetic rats[28]. Administration of nanoparticles of metformin at a dose of 40mg/kg respectively for 28 days prevents a significant elevation in glycosylated haemoglobin thereby increasing the level of total haemoglobin in diabetic rats. This could be due to the result of improved glycaemic control produced by nanoparticles of metformin at a dose of 40 mg/kg respectively. The body weight was decreased in Streptozotocin diabetic rats. Nanoparticles of metformin at a dose of 40 mg/kg respectively, increases the body weight in Streptozotocin induced diabetic rats. The ability of nanoparticles of metformin at a dose of 40mg/kg respectively to protect massive body weight loss seems to be due to its ability to reduce hyperglycemia.The level of serum lipids are usually elevated in diabetes mellitus, and such an elevation represents the risk of coronary heart disease (CHD)[29]. Lowering of serum lipids concentration through diet or drug therapy seems to be associated with a decrease in the risk of vascular disease[30]. The abnormal high concentration of serum lipids in diabetic subjects is mainly due to increased mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone sensitive lipase. However, glucagon, catecholamines and other hormones enhance lipolysis. The marked hyperlipidaemia that characterized the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depots. In the Streptozotocin -induced diabetes mellitus, the rise in blood glucose is accompanied by an increase in serum cholesterol and triglycerides. The levels of cholesterol and triglycerides and Low density lipoprotein (LDL) levels were brought to near normal by the treatment with nanoparticles of metformin at a dose of 40 mg/kg in Streptozotocin induced diabetic rats.The effect of nanoparticles of metformin at a dose of 40 mg/kg on diabetic hypertriglyceridemia could be through its control of hyperglycemia. The level of HDL-cholesterol was decreased in diabetic rats when compared with normal rats[31–35].Our results clearly show that the level of HDL-cholesterol was increased in Streptozotocin induced diabetic rats when treated with nanoparticles of metformin at a dose of 40 mg/kg respectively. These results suggest that nanoparticles of metformin at a dose of 40 mg/kg respectively have protective effect against Streptozotocin induced diabetes and its complications. As reported earlier in the current study also the liver glycogen content was reduced significantly in diabetic control as compared to non-diabetic control[36–39]. Treatment with nanoparticles of metformin at a dose of 40mg/kg respectively, prevented this alteration in glycogen content of liver tissue, but could not normalize the content of glycogen of the non-diabetic control. This prevention or depletion of glycogen in the liver is possibly due to either stimulation of insulin release from β-cells[40] or due to the insulin mimetic activity of some components of the plants resulting in direct peripheral glucose uptake. Decreased enzymatic activity of Hexokinase, Glucokinase and substrate glucose-6- phosphate has been reported in diabetic animals resulting in depletion of liver and muscle glycogen[41–43].The present study also had similar results. Treatment with nanoparticles of metformin at a dose of 40 mg/kg, significantly increased the hexokinase, Glucokinase activity and glucose-6-phosphate level in the liver, indicating an overall increase in glucose influx by nanoparticles of metformin at a dose of 40 mg/kg, seems to have an overall effect of increase in glucose utilization.

5. CONCLUSION

Metformin loaded Starch nanoparticles were effectively prepared by solvent evaporation technique. The prepared nanoparticles were analysed using particle size distribution,

zeta potential and SEM for its size and shape. The analysis confirmed the incorporation of the drug within the nano carrier. In vitro release of metformin from nanoparticles was significantly prolonged to 72 hours. The formulated nanoformulations showed sustained release of the drug hence may rise the drug retention time in blood circulation. Animal studies endorse formed metformin nanoparticles further lowering the blood glucose levels, possible beneficial effects by diminishing the levels of total cholesterol, triglycerides, LDL- cholesterol, VLDL-cholesterol and aggregate HDL-cholesterol. This study will be useful for the future studies aiming at the development of metformin HCl-loaded nanoparticles with the high encapsulation efficiency for efficient treatment of type 2 diabetes mellitus.

6. AUTHORS CONTRIBUTION STATEMENT

Dr.Revathi A Gupta and Mr.P.Odaya kumar contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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8. CONFLICT OF INTEREST

Conflict of interest declared none.

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