Green bioactive loaded oral nanomedicines for efficient control of Diabetes.

Herbal Antidiabetic Nanomedicine

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Abstract— **World Health Organization predicts daibetes to rank 7th among global mortality causes by 2030. As per *NEWSTRAITSTIMES* reports one out of every five above the age of 30 suffer from diabetes in Malaysia and an estimated prediction unfolds 4.5 million diabetics in Malaysia above the age of 18 by 2020. Current antidiabetic drugs suffer from serious side effects and plant-based green bioactives provide a useful alternative. Plant bioactive silymarin is reported to be beneficial both type 1 and type 2 diabetes. The compound however is constrained due to solubility and bioavailabilty limitations and nanomedicine design was construed as the probabale answer. Oral treatment with Sm nanomedicines had a marked restorative effect on streptozotocin induced diabetic rats as evident from biochemical and histological observations. Nanomedicines evaluated show sufficient promise to emerge as patient friendly alternative therapeutics in management of diabetes.**

   Keywords—Silymarin; Nanomedicine; streptozotocin; oral treatment; patient friendly.

I.   INTRODUCTION

Diabetes mellitus is a pathological state experessed by elevated blood glucose level, low blood insulin level or reduced sensitivity of target organs to insulin. The disease is expected to rank 7th among global mortality causes by 2030 as per WHO projection[1]. As per *NEWSTRAITSTIMES* Malaysia one out of every five Malaysian people above the age of 30 suffer from diabetes and with a estimated prediction of 4.5 million diabetics in Malaysia above the age of 18 by 2020. Current antidaibetic drugs like biguanides, sulphonyl ureas and thiazolidenes suffer from unwanted side effects which renewed thrust in favour of plant products as antidiabetics. Although plant bioactives are mostly safer than synthetic drugs, they suffer from solulibility and permeability which limits their therapeutic usefulness.
          Silymarin (Sm) is one of the oldest traditional herbal medicines used for treatment of diverse pathological conditions. Sm is composed of four flavonolignan isomers; silybin, isosilybin, silychristine and silydianin amongst which, silybin (Sb), is the principal bioactive and constitutes 34% by mass of Sm[2]. Sm is reported to be effective in type 1 and type 2 diabetes patients [3] along with successful reports of reducing both fasting and mean daily glucose, triglycerides and total cholesterol levels. Sm is biopharmaceutic class IV type compound with low water solubility and permeablity and warrants urgent delivery developments for further explorations in diabetics.

Spotlight in the drug delivery arena is still on oral formulations with an optimum pharmacokinetic profile. Nano-scale oral drug delivery was expected to provide answer to biopharmaceutical problems of Sm and induce patient compliance. FDA approved polymer Poly DL-lactide-co-glycolic acid (PLGA) was used for the nanomedicine design by polymer dispersion methods. Water soluble Vit- E derivative TPGS (D-α-tocopheryl polyethylene glycol succinate) was used as stabilizer and oral bioavailability enhancer [4] for PLGA Sm nanomedicines. Sm nanomedicines were challenged against streptozotocin induced diabetics as therapeutic alternative in systemic hyperglycemia.

II.   EXPERIMENTAL METHODOLOGY

A. Synthesis of Silymarin Nanomedicines

  Green Silymarin Nanomedicines (Smnp) were synthesized following a easy to scale-up solvent diffusion technique. Briefly, 10 mg of Sm and 50 mg of PLGA were codissolved together in 3 mL of acetone. The organic phase was then added into a 30 mL of aqueous solution containing 0.05% (w/v) of Vit-E TPGS. The addition was controlled by a syringe pump at a rate of 15µL/sec under magnetic stirring. Stirring was continued for an additional period of 12 h to evaporate off the organic phase. Nanomedicines formed were then recovered by ultracentrifugation (Hitachi Koki, Japan) at 30,000 rpm for 30 min at 4^0C. Smnp's were further washed two times with HPLC grade water to remove unbounded drug, polymer and stabilizer.

B. Particle size, zeta potential and morphology.

The particle size and size distribution of nanoparticles prepared were measured in Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) against a 4 mw He–Ne laser beam with a back scattering angle of 173^0c. Zeta potential of Sm nanomedicines was analyzed in the same instrument following the particle electrophoretic light scattering under an applied field. Morphology of Smnp was analyzed in atomic force microscopy (Nanoscope 3A, Veeco, USA) and micrographs were traced in tapping mode using RTESP tip with 267–328 KHz resonance frequency at a scan speed of 1.2 Hz. Surface topography of Smnp were also sketched by negative staining method in FEI Tecnai TM Transmission Electron Microscope (Netherland) at 80 KV.

C. Drug loading and Release Studies

Amount of Sm payload in nanomedicines was determined from the amount of Sm originally taken and the amount remaining in the supernatant after harvesting the nanomedicines following a validated HPLC method. A mixture of 85% phosphoric acid–methanol–water (0.5:46:64, v/ v) served as mobile phase with C-18 column. The elution has been made in an isocratic mode at a flow-rate 1 ml / min and the detection at 288 nm. The quantitative analysis is based on silybin standard required 25 min for one run.

For drug release studies nanomedicines were dispersed in phosphate buffer (100 mM, pH 7.4) was transferred into dialysis bags (MW cut off 12.4 KD). Bags were placed in glass vials containing 100 mL of buffer in a shaker bath maintained at 70 rpm, 37^0C. At predetermined time intervals, 10 mL of phosphate buffer solution was removed for analysis and the release medium replaced with fresh buffer in order to maintain the sink conditions. The Sm release at definite time intervals was estimated by previously described HPLC method.

D. In-vivo Antidiabetic evaluationsof Sm nanomedicines

Male Wistar rats weighing 170–200 g were procured from Central Research Institute, (Kolkata, India). Animals were acclimatized under standard laboratory conditions of relative humidity 50 ± 10%, temperature 22 ± 2^0C and 12/12 light dark cycle for 2 weeks prior to the start of experiments. Access to water was ad libitum and standard pellet food (Hindustan Uniliver, India) supply was provided twice a day till the start of the experiments. This study was carried out in strict accordance with the recommendations in the Guide for the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Glucose tolerance test (GTT) was carried out at the start of the experiment to assess the glucose homeostasis in normal conditions and to detect pre-diabetic condition if present. Animals were fasted for a period of eight hours prior to analysis with water ad libitum. Each test animal was then challenged intraperitoneally with a freshly prepared aqueous D-glucose solution (2.0 g/kg). Blood glucose level was measured in the blood samples taken from the tail vein

after 30, 60, 90 and 120 min of glucose injection and also initially at the 0th time. Individual data was recorded and animals with normal glucose homeostasis were considered for further experiments.

Diabetes was induced in overnight fasted groups by single intraperitoneal injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight[5] in freshly prepared citrate buffer (0.1 M pH 4.0).These animals were fed with standard pellet food and a 5% glucose solution ad libitum for 72 h. Afterwards the glucose solution was replaced with water. The control animals received the vehicle alone. The diabetic state was assessed by measuring fasting glucose level of blood taken from the tail vein. Rats with a blood glucose level above 250 mg/dL were considered as diabetic and were used in further experiments.

Study design involved four groups of 6 animals each with the following treatment schedule

1. Group C – Normal control received normal saline only

2. Group D – STZ only induced rats served as diabetic control

3. Group SmT – STZ induced diabetic rats treated orally with Sm 50 mg/kg b.w for 28 days (diabetic and Sb treated)

4. Group SmnpT – STZ induced diabetic rats treated ip with Smnp equivalent to 50 mg/kg Sm payload for 28 days (diabetic and Smnp treated).

Blood Glucose Estimation

Fasting blood glucose (FBG) concentration was monitored in blood samples every week during the entire experimental period and at the end of the treatment in the morning, using a glucometer (Dr. Morepen Gluco One Blood glucose monitoring system BG 03, India) with maximum measuring capacity of 600 mg/dL.

Intraperitoneal glucose tolerance test (IPGTT)

IPGTT test was performed twenty four hours after the last dose of treatment following the procedure as discussed earlier. Briefly a sterile 20% glucose was injected (ip) at a dose of 2.0 g/kg body weight. Blood was collected from the tail vein to estimate glucose level before (0th time) and 30, 60, 90 and 120 min after glucose injection by glucometer.

Biochemical and Histopathological Studies

After completion of IPGTT studies, animals were fasted overnight and FBG levels were checked. Body weight of each animal was recorded before and every week till the end of the 30 days experimental period. Blood samples (1.5–2.0 ml) were collected by cardiac puncture under light anesthesia. Samples were collected in marked vials added with or without anticoagulant for plasma and serum analysis and were stored at -20^0C until further studies. The animals were finally euthanized by using CO_2 gas and the tissue samples were collected for analysis. Pancreas specimens stored in 10% formalin for histopathological examinations.The tissue

samples were processed by using paraffin block techniques in wax. The samples were then sectioned (>5 m) stained with hematoxylin- eosin and mounted with neutral DPX medium. Photograph of stained sections were captured with a camera attached to a light microscope (B1 series, Motic, Xiamen, China).

Serum insulin levels were measured using rat insulin ELISA kit[6]. Cholesterol and triglyceride levels by using estimation kits (Span Diagnostics Limited, India) following the manufacturer's protocol. Serum fructosamine was determined by nitroblue tetrazolium (NBT) reduction assay[7]. Glycohemoglobin (HbA_{1c}) content in blood was estimated by the ion-exchange resin method [8].

The glycogen content was estimated according to the method of Murat and Serfaty. Briefly, liver tissues were homogenized in ice-cold citrate buffer (0.1 M, pH 4.2) at a ratio 1:9 (w/v), followed by centrifugation at 10,600 g for 30 min at 4^0C. The free glucose content in the supernatant was then measured by GOD/PO method using the assay kit (Span Diagnostics Limited, India). Amyloglucosidase (2 mg, Sigma, USA) was added to the homogenate (500 mL) and was further incubated for 4 h at 37^0C. The total glucose content after incubation was then measured similarly. The glycogen content in the liver was calculated as the difference between total and free glucose.

Serum malondialdehyde (MDA) level as a measure of lipid peroxidation was assayed by the help of thiobarbituric acid (TBA) method [9]. Liver tissues were homogenized (tissue homogenizer, TH 02, Omni International, Kennesaw, GA) in 10 mM potassium phosphate buffer containing 0.1 mM EDTA, pH 7.4, at a proportion of 1:9 (w/v). The homogenate was centrifuged at 6000 g for 10 min at 4uC. The resultant supernatant was used for the determination of catalase and SOD activities and estimation of glutathione (GSH) content[10].

III. RESULTS AND DISCUSSION

Nanomedicine Characterization

Nanoparticulation of biopharmaceutic class IV type compounds is a useful strategy that leads to dissolution and biopharmaceutic improvements leading to enhancement of bioactivity. Average hydrodynamic diameter of the synthesized Smnp recorded by dynamic light scattering method was 219.7 nm and the PDI was 0.121. Zeta potential of Smnp -30.56 mV which indicated high degree of stability of the nanomedicine formulation. AFM analysis of Smnp revealed smooth surface topography of mostly spherical geometry with a certain degree of coalescence (Figure I A). Transmission electron microscopy images were similar and showed discrete nanoparticles nearly spherical shape (Figure I B).
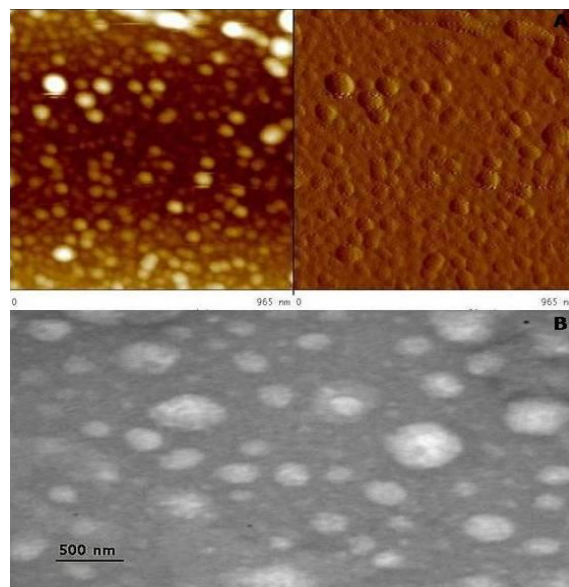

Figure I. Smnp Surface Morphology in (A)AFM and (B) TEM.

Drug Loading and Release studies

High Sm loading in PLGA nanomedicines (89.84%) was observed after routine reverse phase HPLC analysis.Time-dependent cumulative Sm percentage release from Smnp was studied and almost 81% of the initial Sm mass load (Figure II) was accountable during the study period. The release response for Smnp was biphasic in nature. A rapid release of AG occurred up to 96 hours due to some nanoparticle surface adsorbed Sm molecules. followed by a sustained release over a period of 288 hours. The release however, was steady in later hours and continued for a prolonged period of 288 hours.
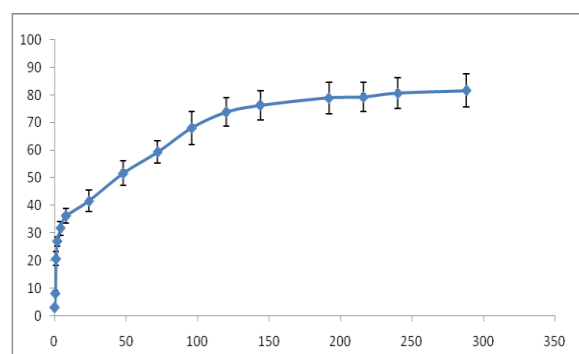

Figure II. Drug release profile of Smnp.

Antidiabetic study of Sm nanomedicines

Fasting blood glucose was monitored in different groups of rats at different time intervals. STZ treatment caused a steep rise in blood glucose in comparison to that in the control group (Figure III). But Smnp treatment significantly reduced the blood glucose level compared to free Sm treatment. Similar results were also observed incase of glucose tolerance test (Figure IV).
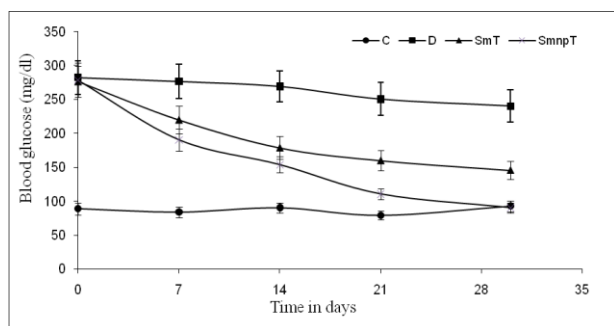
Figure III. Blood glucose level in different groups during the 4 weeks experimental period. Results were expressed as mean ± SD (n = 6).
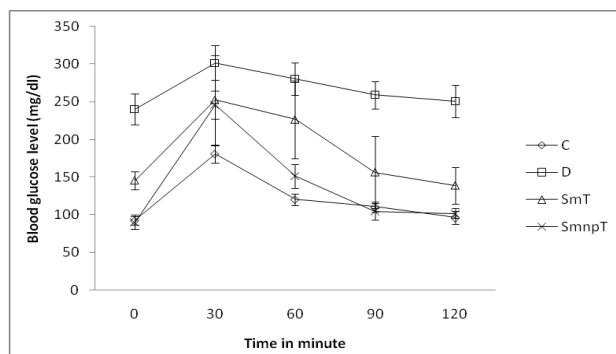

Figure IV. Intraperitoneal glucose tolerance test in different groups of rats. Results were expressed as mean ± SD (n = 6).

Gain in body weight in the diabetic rats after treatment with Smnp was observed indicative of steady recovery of animals from the prior diabetic conditions (Figure V). However there was no significant gain in body weight of diabetic rats treated with Sm.
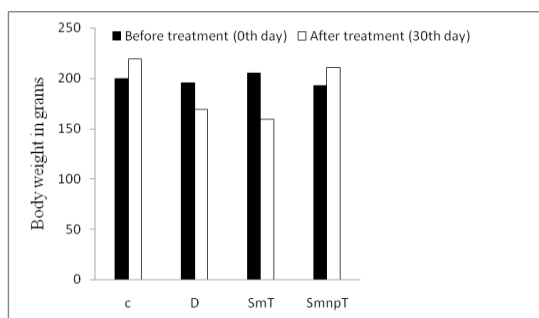

Figure V. Effect of Sm and Smnp treatment on body weight in rats.

Smnp treatment also produced a marked regulation of serum insulin, cholesterol and triglyceride values compared to Sm treatment in diabetic rats.

Table I. Effect of Sm and Smnp on serum insulin, cholesterol and triglyceride levels in diabetic rats.

Animal group	Insulin (µg/lit)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Control (C)	0.62±0.06	72.3±6.20	53.33±5.16
Diabetic (D)	0.14±0.01	173.3±9.88	171±14.51
D + Sm (SmT)	0.27±0.06	120.5±12.18	131.5±13.80
D + Smnp (SmnpT)	0.57±0.14	78±6.33	83.83±9.02

Results expressed as mean ± SD (n = 6).

Fructosamine is the first stable product of protein modification by glucose and its serum level rises in diabetes. Persistent hyperglycemia effects in glycation of haemoglobin which leads to the formation of HbA_{1c}, which is relative to blood glucose levels. Smnp treatment showed normalizing effect on both fructosamine and HbA_{1c} levels compared to Sm treatment.

Table II. Effect of Sb and CSbnp on glycohemoglobin (HbA_{1c}) and fructosamine levels in diabetic rats.

Animal group	Serum fructosamine (µmol/µg of protein)	% of HbA_{1c}
Control (C)	0.45±0.02	1.89±0.65
Diabetic (D)	3.02±0.04	6.45±0.23
D + Sm (SmT)	1.92±0.05	3.93±0.10
D + Smnp (SmnpT)	0.86±0.03	2.98±0.14

Results expressed as mean± SD (n = 6)

Excess glucose is converted into glycogen under the influence of insulin. Glycogen contents measured in the liver tissues showed reduced glycogen level in diabetic group. Maximum increase in liver glycogen content was however observed in nanomedicine treated group that could be reasoned due to nano size assisted transport of Sm to the liver. Besides Smnp treatment also produced a restorative effect on the oxidative stress markers like MDA and also the overall antioxidant status of diabetic animals expresses through SOD, CAT and GSH levels.
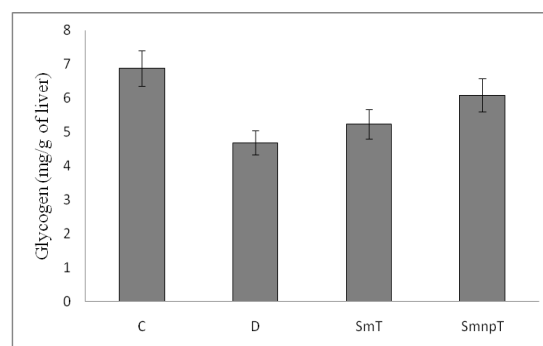

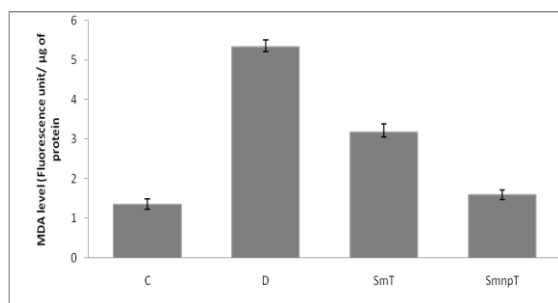Figure VI. Effect of Sm and Smnp on liver glycogen content. Results were expressed as mean ± SD (n = 6).

Figure VI. Effect of Sm and Smnp on serum MDA levels. Results were expressed as mean ± SD (n = 6).

Table III. Effect of Sb and CSbnp over antioxidant status of liver in diabetic rats.

Animal groups	SOD (U/gm of tissue)	Catalase (mU/mg of tissue)	GSH (μM/mg of tissue)
Control (C)	6.94±0.78	156.5±7.22	4.93±0.18
Diabetic (D)	3.22±0.52	73.4±6.26	2.99±0.11
D + Sm (SmT)	4.22±0.06	103.33±9.54	2.03±0.12
D + Smnp (SmnpT)	5.88±0.02	125.44±6.88	3.66±0.16

Results expressed as mean± SD (n = 6)

Histopathological analysis of diabetic pancreatic tissue slices of animals exhibited shrinkage of islet cells, reduced number of islet cells and signs of central necrosis.
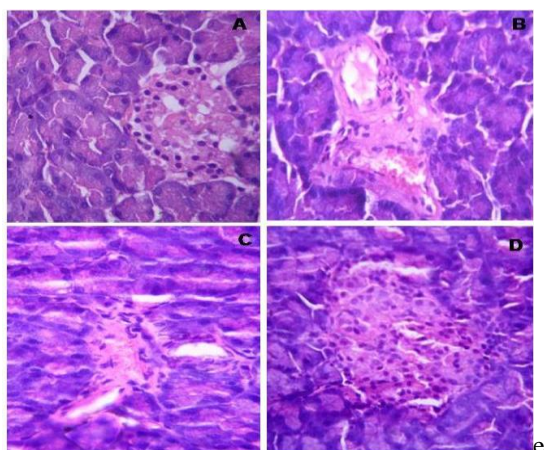

Figure VII.Histopathology examination of pancreas sections. (A) Normal Pancreas, (B) Diabetic Pancreas, (C) Sm treated pancreas, (C) Smnp treated pancreas (Magnification 100X).

Pancreatic tissue histoarchitecture showed a marked improvement in the structural integrity after Smnp treatment compared to Sm treatment.

IV. CONCLUSIONS

Although Sm has been explored as an alternative therapeutic in diabetes, oral Sm nanomedicines was experimented for the first time in diabetes with a view to increase patient compliance. Smnp contribution in diabetes control was profound and remarkable recovery response was observed in the test animals in insulin levels and reduced glycated hemoglobin parameters. Liver concentric effects of nanomedicines could be one reason that facilitated antioxidant defence mechanism and aided in beta cell regeneration. The study demands further pharmacokinetic evaluations of Sm nanomedicines to help their entry to clinical stages. Nanomedicines evaluated show sufficient promise to emerge as patient friendly alternative therapeutics in management of diabetes.

References

[1] World Health Organization (2013) Diabetes. Geneva: WHO. Available: www.who.int/mediacentre/factsheets/fs312/en/?. Accessed 2014 March 24.

[2] Kvasnicka F, Biba B, Sevcik R, Voldrich M, Kratka J. Analysis of the active components of silymarin. J Chromatogr A. 990, 239–245, 2003.

[3] Huseini HF, Larijani B, Heshmat R, Fakhrzadeh H, Radjabipour B, et al. The efficacy of silybum marianum (L.) Gaertn. (Silymarin) in the treatment of type II diabetes: a randomized, double-blind, placebo-controlled, clinical trial. Phytother Res. 20, 1036–1039, 2006.

[4] Zhang Z, Songwei Tan S, Feng SS. Vitamin E TPGS as a molecular biomaterial for drug delivery. Biomaterials. 33, 4889–4906, 2012.

[5] Malekinejad H, Rezabakhsh A, Rahmani F, Hobbenaghi R. Silymarin regulates the cytochrome P450 3A2 and glutathione peroxides in the liver of streptozotocin-induced diabetic rats. Phytomedicine. 19, 583–590, 2012.

[6] Shivanna N, Naika M, Khanum F, Kaul VK. Antioxidant, anti-diabetic and renal protective properties of Stevia rebaudiana. J Diabetes Complications. 27, 103–113, 2013.

[7] Johnson RN, Metcalf PA, Baker JR. Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. Clin Chim Acta. 127, 87–95, 1983.

[8] Goldstein DE, Little RR, Wiedmeyer HM, England JD, McKenzie EM. Glycated hemoglobin: methodologies and clinical applications. Clin Chem 32, B64–B70, 1986.

[9] Yagi K. Lipid peroxides and human diseases. Chem Phys Lipids. 45, 337–351, 1987.

[10] Roy P, Das S, Auddy RG, Saha A, Mukherjee A. Engineered andrographolide nanoparticles mitigate paracetamol hepatotoxicity in mice. Pharm Res 30: 1252–1262, 2013.