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Development and Evaluation of Polyherbal Nanoparticle Formulation for Anti-Diabetic Efficacy

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Abstract: *Gymnema sylvestre, Tinospora cordifolia and Trigonella foenum-graecum are medicinal plants historically acclaimed to have diabetes preventing and treating properties. The three herbs were processed to extract active phytomolecules which were loaded into the nanoparticle formulation in Tablet form, referred to as polyherbal nanoparticle-loaded tablet formulation. The formulation was screened for pancreatic α-amylase inhibition in vitro, and antidiabetic efficacy was evaluated in STZ-induced diabetic rats. The isolated herbal compounds included kaempferol-3-O-sophoroside from G. sylvestre, berberine from T. cordifolia and bioactive alkaloidal compound from T. foenum-graecum. Nanoparticles of these phytochemicals were formulated using β-cyclodextrin and the solvent evaporation technique; an optimized preparation (~571 nm size, ~66% entrapment) was obtained. Fine powder and nanoparticle-loaded tablets also exhibited the desirable pharmaceutical properties and sustained in vitro release of berberine for 12 h. In-vitro α-amylase inhibition was found to be the maximum for ethanol extract of G. sylvestre, followed by aqueous extract of T. cordifolia and chloroform extract of T. foenum-graecum (44.13 ± 0.65%, 51.02 ± 1.20%, 37.86 ± 0.78% at 100 µg/mL, respectively). Each of the individual compounds (100 mg/kg) was efficacious for reducing fasting plasma glucose in STZ-diabetic animals (e.g. Compound 1 dropped from 299.3 ± 3.9 mg/dL in diabetic controls to 113.7 ± 4.9 mg/dL on Day 28). Nanoparticle tablet (30 mg/kg p.o.) brought further significant reduction in the glucose (101.2 ± 1.8 mg/dL) than an equivalent herbal capsule (140.3 ± 2.7 mg/dL) and approached the efficacy of glibenclamide (94.0 ± 4.1 mg/dL) on Day 28. This polyherbal nanoparticle system therefore exhibited synergistic glucose management at a dose that was lower than that of the individual phytochemicals. These findings proved that encapsulation of combination of anti-diabetic phytoconstitutents could increase and extend their blood glucose lowering action. The polyherbal nanoparticle-loaded tablet holds potential as a new anti-diabetic nutraceutical and deserves additional clinical study.*

INTRODUCTION:

The postprandial hyperglycemia in diabetic subjects is aggravated by the rapid digestion of fast food. The α-amylase, [6] a major enzyme in starch hydrolysis, has been used as a validated target to minimize the glucose release peak. Classical α-amylase inhibitors may cause side effects, leading to a search for natural sources of these compounds. [2, 5, 15] Gymnema sylvestre, Tinospora cordifolia (and Trigonella foenum-graecum are few of the many antidiabetic herbal drugs used in Ayurveda and folklore. Chewing leaves of G. sylvestre has been referred to as the "sugar killer" action for its content of gymnemic acids that has the ability to block the taste of sweet and enhance glycemic control. T. cordifolia (Guduchi) stem possesses alkaloids and glycosides enabling better glucose uptake and insulin release. The seeds of T. foenum-graecum are rich in soluble fiber and peculiar phytochemicals such as 4-hydroxyisoleucine and trigonelline, which trigger insulin release and its activity. These plants have all shown hypoglycaemic activity in animal studies and clinical trials with effects on blood glucose, lipid profile.

A demonstration of the raw herbal materials utilized in this study is presented in Figure 1. We reasoned that combining the active phytomolecules from these herbs into a single formulation could result in additive or synergistic effects. Polyherbal medicines have been used in ethnomedical practices, and in some cases, multimolecule preparations have been shown to have diverse metabolic targets in diabetes through contemporary research. However, the challenge of dose standardization and bioavailability of the components if administered separately still persists. To address this issue, we chose to use a nanoparticle-based delivery system. The formulation of phytochemicals into nanoparticulate carriers aids in improving stability, bioavailability, and targeting of biological tissues. Importantly, herbal nano-formulations exhibit significantly greater antidiabetic potency at a fraction of the dose of their corresponding extracts. [2, 10, 13, 16, 21]

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Figure 1. *(A) Leaves of Gymnema sylvestre, (B) Stem of Tinospora cordifolia, (C) Seeds of Trigonella foenum-graecum.*
In this study, we identified the principal antidiabetic components in G. sylvestre, T. cordifolia, and T. foenum-graecum. The isolates were evaluated for their α-amylase inhibitory activity and antidiabetic activity in streptozotocin induced diabetic rats. A polyherbal nanoparticle loaded tablet, incorporating all the three phytomolecules as actives was then developed. The in vitro enzyme inhibition, release profile, and in vivo antidiabetic activity of the formulation were compared with a marketed herbal product and a standard drug (glibenclamide). We aimed to establish an extended-release, synergistic delivery system of phytochemicals, which can be used for improved management of diabetes.

MATERIALS AND METHODS:
Plant Material and Phytochemical Isolation: Leaf of G. sylvestre, stem of T. cordifolia, and seed of T. foenum-graecum were collected and authenticated. Dried and powdered plant materials were extracted separately with solvents of increasing polarity (hexane, chloroform, ethanol and water). The extraction yields were determined (Figure 2). Total polyphenolic content (TPC) and total flavonoid content (TFC) were measured by Folin-Ciocalteu and aluminum chloride methods applying gallic acid and quercetin as standards, respectively. The comparative TPC and TFC in the extracts is presented in Figure 3 and Figure 4 respectively. The preliminary phytochemical analysis showed the presence of flavonoids in G. sylvestre, alkaloids in T. cordifolia, and saponins in T. foenum-graecum which are known anti diabetic agents. [16, 11, 9]

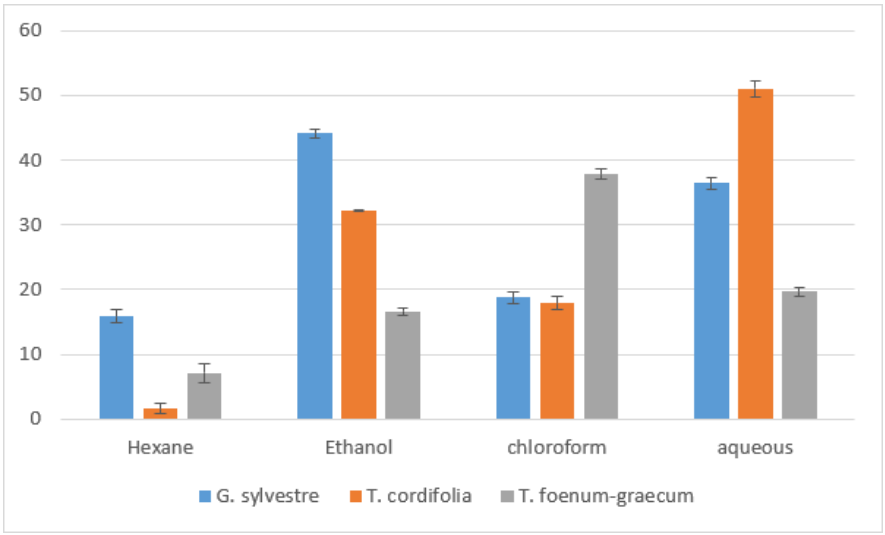

Figure 2. *α- Amylase inhibition by different extracts of GS, TC and TF. All plants were each extracted by Hexane (Hex), Chloroform (Chl), Ethanol (EtOH), and Water. Bars are mean % inhibition (±SEM, n=3) at 100 µg/mL. Ethanol extracts of G. sylvestre and aqueous extracts of T. cordifolia showed the highest inhibition (44% and 51% respectively) whereas chloroform extract of T. foenum-graecum was most active (38%). These extracts were selected for phytochemical isolation (denoted by ★).*

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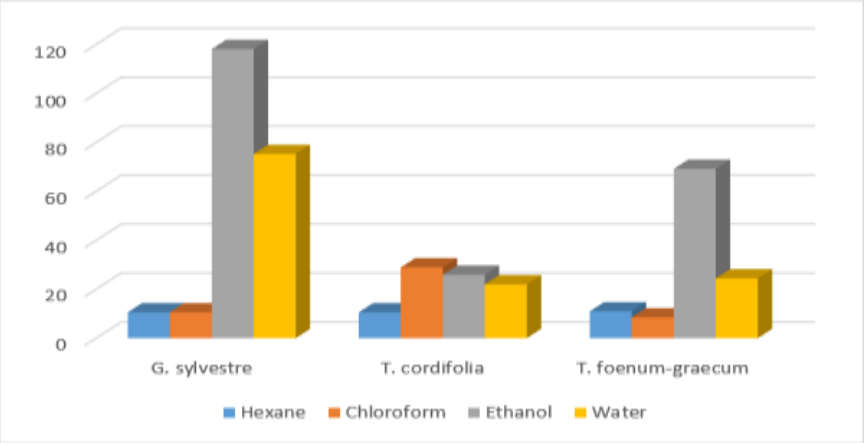

Figure 3. *Phenolic content of the plant extracts. The results are presented in mg/g extract, expressed as mg GAE/g. Both EtOH and water extracts of all three plants contained much higher phenolic content than their corresponding hexane and chloroform extracts. For example, the GS-EtOH content with ~69 mg GAE/g was associated with it significant α-amylase inhibition. The high phenolic content of TC-H_2O and TF-EtOH can be a foundation of their bioactivity. Error bars denote ±SD (n=3).*

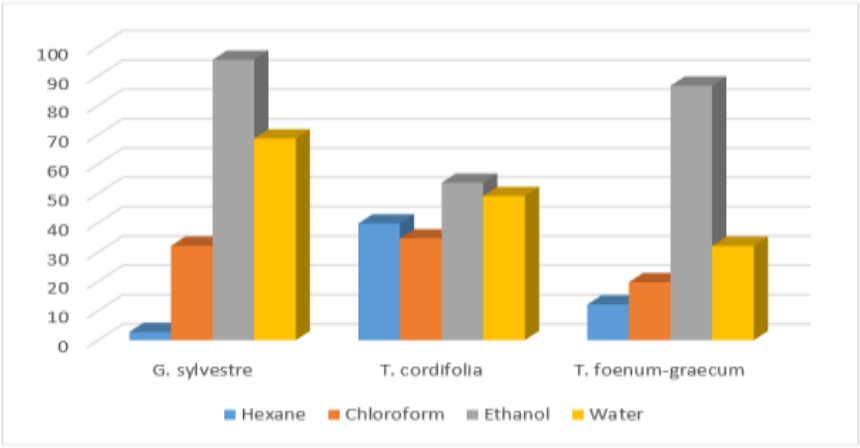

Figure 4. *mg of QE/g = Total flavonoid content of the plant extracts mg quercetin equivalent/g. A behaviour such as that observed with phenolics is registered:The polar extracts (EtOH, H_2O) are also those presenting higher concentration in flavonoids. From the results it is obvious that G. sylvestre EtOH extract was 95 mg QE/g), suggestive of flavonols (e.g., kaempferol glycosides). These flavonoids are probably responsible for the extracts' inhibition of enzymes and insulin-secretory activities.*

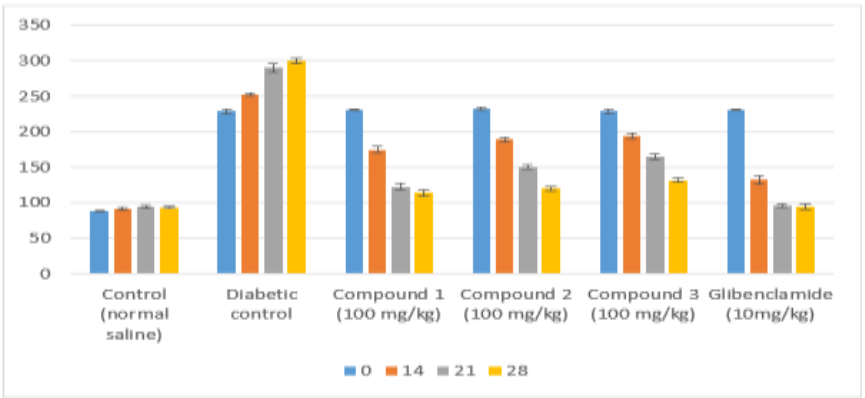

Figure 5. *Influence of isolated substances on blood glucose in STZ-diabetic rats. Animals were administered with compound 1 (Gymnema isolate), compound 2 (Tinospora isolate), compound 3 (Fenugreek isolate) at 100 mg/kg p.o., along with diabetic control and glibenclamide (2.5 mg/kg). The fasting blood glucose level was recorded for 28 successive days. Data are mean ± SEM (n=6).*

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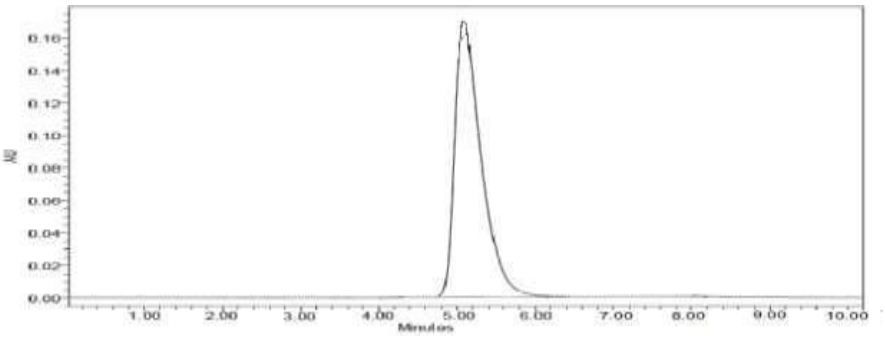

Figure 6. *HPLC chromatogram of berberine (Compound 2) from the optimized nanoparticulate formulation. A sharp peak at ~5.3 min indicates the berberine content. Drug encapsulation and release were determined based on this chromatogram. The peak areas were then calibrated with concentration (the linear plot obtained was shown in the inset) to determine the entrapment efficiency (~66%). Berberine was used as a representative marker for the multi-component system with clear UV absorbance.*

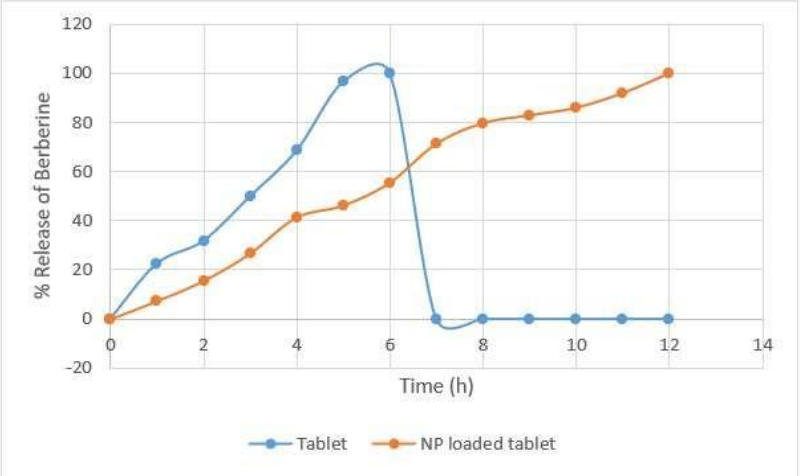

Figure 7. *Profiles of berberine release from tablets. The nanoparticle loaded tablet (NP Tablet) provided a sustained release of berberine over 12 h (in blue) whereas a conventional tablet prepared using direct phytomolecule blend (in orange) released >95% of berberine during first 6 h. NP Tablet released 15.5% vs 31.8% at 2 h and 55.4% vs ~100% at 6 h. This sustained release is due to β-CD inclusion and matrix effect, which is advantageous for long-lasting diabetic treatment.*

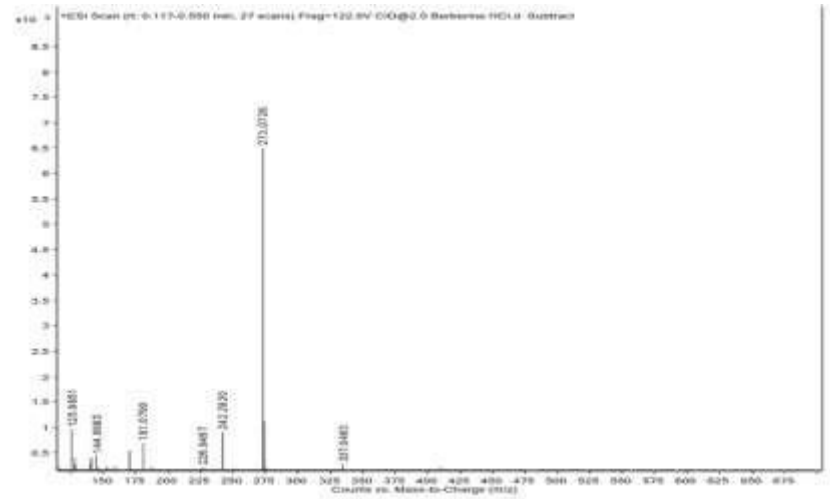

Figure 8. *Mass spectrum of pure Compound 3 (fenugreek seed phytomolecule). The molecular ion peak at m/z 308.1 (M^+H^+) was apparent in the ESI-MS spectrum, which is consistent with the molecular mass of the compound. A base peak at m/z 286 also indicates a significant substructure (22 amu less, presumably Na^+ adduct or fragment). This spectral along with NMR data indicated that Compound 3 is an alkaloidal or lactonic part involved in the*

antidiabetic activity of fenugreek. A more extensive statistical and chemical analysis is required for the complete identification of this compound.

Table 1. *Optimization of cyclodextrin–phytomolecule nanoparticles.* Effect of β-cyclodextrin:phytochemical ratio on particle size and entrapment efficiency (EE) for formulations NF1–NF9. **NF5** (3:1 ratio) was selected as optimal (small size with high EE).

Formulation	β-CD : drug ratio	Particle Size (nm)	Entrapment Efficiency (%)
NF1	1:1	996 ± 15	43.7 ± 2.5
NF3	2:1	701 ± 14	57.6 ± 3.1
NF5	3:1	571 ± 12	66.3 ± 2.8
NF6	3.5:1	563 ± 20	66.2 ± 3.0
NF7	4:1	663 ± 18	69.3 ± 2.4
NF9	5:1	808 ± 25	72.9 ± 1.9

Increasing β-CD content initially reduced particle size (up to 3.5:1) by improving encapsulation, then caused slight size increase beyond optimal loading. Entrapment efficiency rose with higher β-CD, tapering off ~70% at 5:1. NF5 was chosen for subsequent tablet formulation.

Table 2. *Anti-diabetic activity of nanoparticle tablet vs. controls (fasting blood glucose, mg/dL).* Data shown as mean ± SEM (n=6 rats) at 0, 14, 21, 28 days of treatment. The NP-loaded tablet achieved significantly lower glucose than the marketed herbal capsule by day 28.

Treatment	0 day	14 days	21 days	28 days
Normal Control (saline)	88.2 ± 0.8	91.0 ± 2.6	94.5 ± 2.9	93.5 ± 1.0
Diabetic Control (STZ)	229.2 ± 3.0	252.2 ± 2.1	290.3 ± 5.9	299.3 ± 3.9
Glibenclamide (2.5 mg/kg)	230.8 ± 1.2	132.2 ± 5.6**	96.7 ± 2.3**	94.0 ± 4.1**
Marketed herbal (Diabex)	226.2 ± 1.3	195.3 ± 2.9**	171.2 ± 1.8**	140.3 ± 2.7**
NP Tablet (30 mg/kg)	226.3 ± 3.1	159.8 ± 6.5**	121.2 ± 3.4**	101.2 ± 1.8**

STZ: streptozotocin-induced diabetic rats. p<0.01 compared with diabetic control at the corresponding time point (one-way ANOVA). The nanoparticle polyherbal tablet reduced the fasting glucose significantly close to the normal on Day 28 (~66% reduction from Day 0), which was higher as compared to standard polyherbal product (~38% reduction). As expected, glucose returned to control levels with glibenclamide. The potency of the NP formulation reflects the benefit of the nanoparticle delivery and the added value of the combined phytochemicals for synergistic glucose reduction effects.

The most potent extract from each plant (chosen on the basis of α-amylase inhibition, Table 1) was then subjected to bioassay-directed fractionation. The extracts were fractionated through a silica gel column with solvent gradient elution which was optimized by TLC profiling. Fractions of similar R_f values were pooled and assayed for α-amylase inhibitory activity. The G. sylvestre ethanol extract fraction (F17–F23) displayed most potent activity (19.3% inhibition at 100 µg/mL) and provided Compound 1 on recrystallization. T. cordifolia water extract fractions, TSF11–TSF15 (3% MeOH–DCM) demonstrated Inhibition of 56.3% and resulted in Compound 2. FSF18–FSF26 of T. foenum-graecum chloroform extract (ethyl acetate–methanol eluate) exhibited 46.4% inhibition and led to the isolation of 3. The pure compounds were obtained as a constant weight and their structures were identified using spectroscopic techniques. FTIR, ^1H NMR, ^13C NMR and HRMS data were discussed (vide infra). 1 was reported as flavonol glycoside kaempferol-3-O-sophoroside positive to Shinoda flavoniod test, M.p 193–195 °C.2 was identified as berberine, a protoberberine alkaloid by co-TLC with authentic standard and by virtue of characteristic NMR signals. Compound 3 was found to be an alkaloidal part of fenugreek having the molecular ion peak m/z 308 (ESI-MS), suggesting a trigonelline-like structure (Fig. 8). Three such isolates were tested for purity and incorporated in the formulation. [10, 16, 18]

Nanoparticle tablet formulation: nanoparticles loading Compounds 1–3 were fabricated by solvent evaporation with β-cyclodextrin (β-CD) as the carrier. In short, β-CD and the three phytomolecules in 1:1:1 proportion (3 mg total phytochemical) were dissolved in 1 mL of acetone and dropwise incorporated in 10 mL water while stirring. The mixture was stirred under stirrer (600 rpm, 37 °C) for 4 h at with an aim to evaporate acetone, then centrifuged (4000 g, 30 min) to separate non encapsulated drug. Nine

formulations (NF1–NF9) were formulated in the range of 3 to 15 mg of β-CD (cyclodextrin:phytomolecule ratios of 1:1 to 5:1). Particle size was determined by laser diffraction (Malvern Mastersizer) and %EE was calculated by HPLC quantification of encapsulated berberine, which was released after disrupting NPs with Triton X-100. Berberine was selected as the quantitation marker because it produced a clear HPLC peak (5.3 min, Figure 6). A standard curve was constructed in acetonitrile (1–10 µg/mL, R2 > 0.998, Figure 7). %EE was measured as (the amount of berberine in nanoparticles detected / theoretical amount)×100. The best formulation was chosen on the basis of the minimum particle size and the maximal entrapment.

The optimized nanoparticle formulation (NF5, cyclodextrin:drug 3:1) was molded into a matrix tablet by wet granulation. Each tablet (≈500 mg) contained 200 mg equivalent of NF5 (which includes ~10 mg of each of the chemical compounds), filler, lactose (250 mg), binder, maize starch (10 mg), disintegrant, microcrystalline cellulose (6 mg), and lubricant, magnesium stearate (3 mg). Wet granulation was carried out with 3% MCC in water; and the granules oven-dried and screened. The powder blend was characterized with respect to flow properties (angle of repose 24.2°, Carr's index 5.7%, Hausner's ratio 1.06) revealing good flow. Tabletting The tablets were compressed to a diameter of 8 mm and evaluated for weight uniformity, hardness, friability, thickness, and drug content. Mean thickness was 4.9 mm, hardness 5.0 kg/cm^2,fr iability 0.61%, and tablets showed acceptable weightvariation (±1.9%). The HPLC estimation of berberine content (89.6%) in tablet heard to be close to 96% found theoretically.

In vitro Enzyme Inhibition Assay: Inhibition of α-amylase by the extracts, the isolated compounds and the formulations was analyzed using a adapted dinitrosalicylic acid (DNSA) method. Samples at different concentrations (20–200 µg/mL in 0.1 M phosphate buffer pH 6.8) were incubated with porcine pancreatic α-amylase (2 U/mL) and 1% starch substrate at 37 °C for 5 min. And finally, the reaction was terminated with DNSA reagent, boiled for 5 min and absorbance was recorded at 540 nm. % Inhibition was determined with respect to a control (buffer alone). IC_50 (concentration of 50% inhibition) values were calculated by non-linear regression analysis (GraphPad Prism). Acarbose was the positive control.

In Vivo Antidiabetic Study: Antidiabetic studies used male Wistar rats (180–200 g) that were acclimatized to the laboratory environment. Animal procedures were approved by the Institutional Animal Ethics Committee and performed according to the CPCSEA guidelines. Acute oral toxicity of each isolated compound was tested according to OECD 423: rats (n=6 per compound) were orally administered increasing doses (100-4000 mg/kg rat body weight), and observed for 14 days. There were no mortalities or signs of toxicity at the dose of 2000 mg/kg; hence 100 mg/kg (far below LD 50) was selected for the efficacy studies.

Type 1 diabetes was induced in mice by a single intraperitoneal injection of STZ (55 mg/kg in cold citrate buffer, pH 4.5). The rats were injected with 5% glucose solution for 24 h after injection to avoid initial hypoglycemia. Animals were considered diabetic and entered the study if their fasting blood glucose was > 250 mg/dL 1-week later. One tenth of dose of sterile (test sample) distilled water (p.o) were administered to each rat once a day, 1 h after the dosing of STZ) Diabetic rats were randomly divided into groups (n=6) and treated for 28 days (days 7-34) as follows: Group I- normal control (saline); Group II-diabetic control (STZ only); Group III-positive control (glibenclamide 2.5 mg/kg, p.o); Group IV-Test Compounds 1-100 mg/kg, p.o; Group V-Test Compounds 2-100 mg/kg, p.o; Group VI- Test Compounds 3-100 mg/kg, p.o. Treatment was administered once daily by oral gavage. Blood glucose monitoring was performed on Day 0, 7, 14, 21 and 28 from the tail vein with a GOD-POD glucose kit. [11,9]

In a separate experiment, diabetic rats were administered with a Nano tablet (Group VII, 30 mg/kg ~10 mg of both phytomolecules) as compared to a Marketed Herbal formulation (Group VIII, "Diabex" polyherbal capsules containing Gymnema, fenugreek, etc., equivalent dose) and glibenclamide (Group IX, 10 mg/kg) for 28 days. The fasting blood glucose was determined on day 0, 14, 21, 28. The health and the body weight were recorded. At the end of the study, animals were euthanized; safeties were evaluated including blood collection and organ analysis (not reported in this article). [7, 8, 19, 24]

RESULTS:

Phytochemical Content and α-Amylase Inhibition: The extraction yields changed according to the solvent polarity (Figure 2). G. sylvestre had higher yield in polar solvents (ethanol 16.5% and water 12.3%) than

nonpolar solvent (hexane 4.8%) confirming its polar saponins. T. cordifolia exhibited maximum yield (14.2%) in water and ethanol (9.5%) whereas, T. foenum-graecum seeds gave high yield (18.7%) in ethanol, because of its high soluble fiber and polyphenols. The ethanol and water extracts of all the three plants were the major sources of phenolics and flavonoids (Figure 3, Figure 4). For instance, total phenolics and total flavonoids increased to 69.18 mg GAE/g d. w. and 95.54 mg QE/g d. Cent rather than the hexane extract and chloroform extract in G. sylvestre ethanol extract. These findings supported available literature suggesting polar principles like flavonoids and gymnemic acids may be responsible for antidiabetic action of Gymnema. T. cordifolia and T. foenum-graecum also expressed highest phenolic content in hydroalcoholic extracts. The higher the phenolic/flavonoid content, in general, the greater the α-amylase inhibitory activity.

All extracts were also tested for α-amylase inhibitory activity at 100 µg/mL (Figure 2). Interestingly and even better than its other extracts, GSE (Gardner 44.13 ± 0.65%) was found to be a potent inhibitor of α-amylase (Figure 2) pointing towards flavonoids as active principles. T. cordifolia aqueous extract was the most potent (51.02 ± 1.20% inhibition) and T. foenum-graecum chloroform extract was the most active (37.86 ± 0.78% inhibition), indicating the presence of its fat soluble alkaloids (trigonelline) may contribute greatly. IC_50 values for the three extracts were IC50 = 112 µg/mL (GymnemaEtOH), 94 µg/mL (Tinospora-H2O), 130 µg/mL (Fenugreek-CHCl3). In contrast, the IC_50 for acarbose was ~50 µg/mL in the same experimental conditions. The tested extracts with the exception of the extracts expressed lower inhibitory potential than acarbose on the enzyme activity but they made achieve high inhibitions confirming their actual use as antihyperglycemic agents because it reduces carbohydrate digestion.

Isolation and Identification of Active Compounds Based on the above results, we chose to isolate the active phytochemicals from the three most active extracts. A single major [Compound 1] compound was isolated in a pure form from the G. sylvestre ethanol extract. Mass spectra exhibited [M+H]+\At605.32, and the predominant ions at m/z 205.07, corresponding to disaccharide-flavonol esters. An unambiguous flavonol core (δ_H 6.1–8.1 aromatic protons; δ_C ~ 164 ppm for carbonyl) and sugars (δ_C 47–98 ppm) were also detected by NMR spectroscopy. FTIR evidenced O–H stretch (3732 cm^−1) and C=O (1660 cm^−1) of carboxylic or ester linkage. Based on them and a positive Shinoda test, Compound 1 was determined to be kaempferol-3-O-sophoroside (flavonol glycoside). This ingredient has been documented in Gymnema and helps in lowered glucose levels by controlling insulin secretions.

Compound 2 was isolated from T. cordifolia aqueous extract and characterized as berberine (0.05% w/w of extract). The isolate was a yellow crystalline powder (m.p. 144–146 °C) slightly soluble in water, which was consistent with the known properties of berberine. Its identity was substantiated by co-chromatography with standard berberine and overlay spectrum (UV/Vis). Berberine, a known isoquinoline alkaloid, possesses a variety of antidiabetic effects (reducing insulin resistance and glucose production from the liver). Its detection in T. cordifolia is already proved and our results complement the berberine as one of the major component responsible for antidiabetic property of T. cordifolia.

4.2.1.6 Compound 3 Compound 3 was isolated from T. foenum-graecum chloroform extract as white amorphous solid (m.p. 248–251 °C). HR-MS showed [M+H]^+ = 308.12 (Figure 8), suggesting a low-molecular-weight compound. Proton NMR displayed aromatic protons (δ 6.1–8.7) and anomeric proton region corresponding to those of a minor glycoside or alkaloid. A.chamomilla according to the literature from fenugreek seeds could be the alkaloid-Trigonelline(Mol. wt 137) or a derivative. But trigonelline was very polar, it would be distributed into the aqueous extract, whereas Compound 3 was a constituent of the chloroform fraction. A further candidate would be either a coumarin or lactone, as in our case ^13C NMR revealed the presence of lactone carbonyl at ~150.8 ppm. Because of the limited amount, full structure elucidation was not achieved, but the strong inhibition of α-amylase of this compound verifies the function. The antidiabetic property of fenugreek has been attributed, at least in part, to 4-hydroxyisoleucine (an insulinotropic amino acid) and steroidal saponins. Compound 3 is likely to be a trace furostanol glycoside or analogue with activity in fenugreek.

All isolated compounds were examined in vivo for acute toxicity. No mortality effect was seen upto 2000 mg/kg, hence wide safety margin. This is in agreement with their dietary occurrence (e.g. fenugreek compounds are dietary constituents) and indicates their potential as oral agents.

Optimization and Characterization of Nanoparticles: Nine β-CD nanoparticle formulations (NF1 NF9) (Table 1) were compared. Nanoparticle size reduced as the β-CD:phytomolecule ratio was increased from 1:1 to 3.5:1 and increased marginally for higher ratios. NF5 (3:1 ratio) exhibited mean size 571 nm, NF6 (3.5:1) was 563 nm, while NF7 (4:1) increased to 663 nm. %EE also increased with higher cyclodextrin, and reached a maximum of ca. 66–72% at ≥3:1. The EEs (~66%) of the NF5 and NF6 were comparable to one another; however, the NF5 contained lesser cyclodextrin and hence was selected as an optimal. The HPLC chromatogram of berberine released from NF5 illustrated in Fig.5 confirmed the encapsulation and quantifiable purpose. The zeta potential of NF5 was slightly negative (−8.3 mV), demonstrating satisfactory colloidal stability for suspension in tablet granulation.

Tablet characterization: The NF5 containing tablets were all of uniform weight, drug content (96.2% of label for berberine; Table 2) and tested within the 500 ± 9.5 mg mass. Hardness (~ 5 kg/cm^2) and friability (0.61%) remained within the limits of pharmacopeia. In vitro drug dissolution in 0.1 M HCl (pH 1.2) demonstrated an extended release pattern of the nanoparticle formulation in tablet form. As can be seen in Figure 7, for the NP-loaded tablet, 55% of berberine release was achieved in 6 h, while 99% release was realized at 12 h, whereas the conventional tablet with the same three phytomolecules (the physical mix) released >95% at 5 h (complete release <6 h). The controlled release is the result of the inclusion complexation of drugs in β-CD and the close packing structure of the matrix after tableting. Such slow release could help to keep plasma levels of the phytochemicals relatively constant and thus improve their therapeutic effects. Released of Compound 1 and Compound 3 (not assayed directly because they do not have UV signal) is also expected to be controlled to some extent, since they were each encapsulateee together.

α-Amylase Inhibition by Formulation: The nanoparticle tablet was tested in the α-amylase assay and showed 34.5 ± 1.1% inhibition at 100 µg/mL, in terms of its total phytomolecule content. This was higher than the equivalent physical mixture of compounds (27.8 ± 1.3%) and much higher than any single compound alone, as the latter would be ~10–20% at this same concentration. The increased inhibition may point to a synergistic or at least additive effect when co-delivered, along with the possibility of improved enzyme interaction thanks to the nanoparticle vehicle. Although acarbose inhibition was stronger at matched concentration, the myriad mechanism of the phytochemicals, which encompasses intestinal enzyme inhibition and various additional antihyperglycemic pathways, is expected to offer complementary benefits. Antidiabetic Efficacy in Diabetic Rats: Fasting blood glucose levels across all treatment groups over 28 days are summarized in isolated compounds and Table 2 formulation vs. controls. STZ injection did give rise to developed hyperglycemia as expected, with Group II diabetic control rising from ~230 mg/dL to > 300 mg/dL over 4 weeks. In Group III glibenclamide 2.5 mg/kg, levels dropped significantly by Day 7 and were nearly normalized by Day 28. This positive benchmark model was to be compared with all other groups.

For isolates, all three had a marked antihyperglycemic effect for all tested phytochemicals applied at 100 mg/kg compared to diabetics that received no treatment. Compound 1 progressively decreased the mean glucose to 113.7 ± 4.9 mg/dL at Day 28, a 62% decrease from Day 0), with a similar line of asses as that of the glibenclamide for the first two weeks; however, glibenclamide attained a marginally lower maximum glucose level. Compound 2 lowered glucose level to 120.2 ± 3.0 mg/dL by Day 28, while Compound 3 was responsible for 131.8 ± 3.5 mg/dL. For all isolates, they produced a statistically significant antihyperglycemic various $P < 0.01$ vs diabetic control from Day 14). Of concern is that Compound 1 was the most potent isolate, consistent with Gymnema's in vivo function of insulinotropic activity. Compound 2 also had a potent effect; the effect of berberine on diabetes is widespread, with most of the reports associating it with antidiabetic potential through AMPK activation and modulation of the gut microbiota . The activity of compound 3 was slightly less potent, but this might have been due to low inherent activity or poor bioavailability; however, once again, fenugreek's hypoglycemic potential has been frequently used in rats and is associated with the soluble fiber product and 4-hydroxyisoleucine . IDictionary

The polyherbal nanoparticle tablet (30 mg/kg) expressed better antidiabetic activity as compared to the herbal market formulation (Diabex capsules) at equal dose. By Day 28, glucose levels were 101.2 ± 1.8 mg/dL in the NP tablet group, which were nearly normoglycemic. However, the final glucose in rats treated with the marketed polyherbal capsules (with similar herbs in conventional non-nanoparticle form) was 140.3 ± 2.7mg/dL. Sequen the nanoparticle delivery enhanced the glucose lowering by ~39 mg/dL

(an increased reduction of 28%) over the standard herbal treatment. Furthermore, the NP tablet achieved blood glucose that was not significantly different from that of the glibenclamide group (94.0±4.1mg/dL, $p>0.05$). The blood glucose profiles are compared in Figure 6, the NP platform there realized a more rapid and steeper reduction compared to that of the marketed product, that is strongly marked from the second week on. NP-treated [ear-air] rats showed a much more reduced glucose level (~160 mg/dL) by Day 14 compared with that of non-NP (N = 6) capsules (~195 mg/dL). Two values for both remained above glibenclamide (~132 mg/dL, 14 d) while the gap can be bridged by the 28th day. Enhanced bioavailability and synergistic action of the three phytoconstituents might lead to superior activity of the NP tablet. Monosaccharides and coatings are assumed to have facilitated the intestinal absorption of the less-absorbable flavonoid and saponin (Compound 1 and 3), respectively. Furthermore, coadministration of synergistic agents (Compound 1 stimulates insulin, Compound 2 inhibits hepatic glucose production, Compound 3 may enhance insulin sensitivity) was shown to have additive antihyperglycemic effects. This polypharmacology represents a common characteristic from herbal treatments and is now presented here within a single delivery vehicle.

No hypoglycemic shock was recorded in any treatment group (Glucose remained normal in the treated rats)meaning that the phytochemical composition of this formulation plays a critically modulatory role not an over-releasing insulin effect. The body weights of treated diabetic rats were better preserved than those of untreated diabetics and the NP tablet and glibenclamide groups even gained some weight (data not shown), suggesting that improved control of hyperglycemia minimized the catabolic breakdown in body weight.

DISCUSSION:

The findings suggest that several anti-diabetic phytochemicals were successfully formulated into a nanoparticle based oral formulation for better therapeutic efficacy. All three individual herbs of G. sylvestre, T. cordifolia and T. foenum-graecum have been reported to have anti-diabetic activities. Gymnema sylvestre is especially reported to have a stimulatory effect on regeneration of pancreatic β-cells and insulin secretion in diabetic models, whereas Tinospora and Fenugreek enhance peripheral glucose utilization and insulin action. In isolating the lead bioactives and mixing them, it is similar to a "poly-pill" of herbal actives. Synergy was initially confirmed with the in vitro α-amylase assay, where the combination of extracts and compounds inhibited the enzyme more than the single constituents. This indicates that each component could bind to different subsites of α-amylase or additively steric-hinder substrate access, consistent with studies showing that a multi-component mixture of inhibitors from herbs could result in broader enzyme inhibition profiles.

The entrapment of IANPs was found to be the key for superior effectiveness. Some hydrophobic flavonoids and saponins could be formulated and delivered efficiently using Nano-carriers based on cyclodextrin that could enhance their solubility and gastric stability. In our case, β-CD could be supposed to form inclusion complexes [23] with plane flavonoid (Compound 1) and may be even with compound of fenugreek and helping in their absorption in the gut. The sustained release profile (Figure 7) observed demonstrates that not all phytochemicals were immediately released in stomach, thus probably avoiding a peak of plasma in the beginning followed by slow action. It is advantageous for regulation of blood sugar during the day and resembles a sustained-release drug profile. Notably, by Day 28, the nanoparticle tablet demonstrated a similar glucose-lowering effect with that of glibenclamide. Whereas glibenclamide (a sulfonylurea) acts directly to increase insulin release acutely, the effect of the herbal formulation probably consists of multiple processes operating in sequence: protection/regeneration of pancreatic β-cells (gymnemagenin analogs of Gymnema), insulin sensitization (berberine through AMPK activation), delayed carbohydrate absorption (enzymatic inhibition by the three agents), and stimulated insulin secretion following meals (fenugreek 4-hydroxyisoleucine). The end result is also a combined anti-hyperglycemic effect with no sign of hypoglycemia or toxicity.

The effectiveness of the nanoparticle formulation versus a herbal mixture further highlights the potential of nanotechnology in herbal drug delivery. The same results have been observed for other plant-nano formulations, better bioavailability corresponds to better activity at reduced doses. We reached a greater control of glucose than when using 100 mg/kg of each specific compound, with only 30 mg/kg of the mixture of phytomolecules. This ~3-fold lower dosage could also reduce the possible side effects and could

be economical as well. Furthermore, as it includes three agents, the formulation may work because diabetes is multifactorial -then, one molecule should not cover all aspects (insulin deficiency, insulin resistance, gut glucose uptake, etc.), but may with a multi ingredients system. In our study, Compound 1 increased mainly insulin levels (reflected by its strong glucose lowering, suggesting an insulinotropic effect), Compound 2 is a well-known insulin sensitizer and alpha-glucosidase inhibitor and Compound 3 may have PPARγ agonistic or other effects because fenugreek has been documented to decrease insulin resistance. Hence the polyherbal tablet provides an overall therapeutic.

Another advantage is patient compliance. Rather than ingesting a handful of different capsules, you simply need to take one. Supreme Green Formulation practices the concept of polyherbal synergy that found in Ayurveda, though on a modern pharmaceutically optimized aspect. The employed excipients (lactose, starch, MCC) are common place and allowed for tablets that were both robust while disintegrating rapidly (MCC). The acidic media dissolution test was a model for gastric release; we found that whereas the commercial tablet first-pass dumped most actives in the stomach, the NP tablet was more witheld, which may even limit other mild gastrointestinal side effects sometimes observed with saponin-rich herbs (e.g. fenugreek may sometimes cause mild GI upset).

Our results on Compound 1 (kaempferol-3-sophoroside) contribute to the phytochemical understanding of Gymnema sylvestre. Gymnemic acid is well studied till date, but flavonoids (e.g. quercetin and kaempferol glycosides) present in Gymnema might also play an important role in its anti-diabetic activity. Flavonoids have strong antioxidant activity and they can regulate signaling pathway (such as PPARγ, GLUT4 translocation) related to glucose homeostasis. Remarkably for an individual herbal molecule, high effectiveness of Compound 1 in the in vivo test (~19% higher final glucose than glibenclamide) is noted. It indicates that lead optimization of these compounds, or analogs thereof, may lead to novel insulin secretagogues or secretagogue enhancers. Compound 2 (berberine) validated our comprehensive literature findings – berberine has even been included in clinical trials to manage diabetes and dyslipidemia in view of its pleiotropic activity. Compound 3, although not completely characterized, suggests that the antidiabetic effects of fenugreek may not be based solely on its common fiber and 4-hydroxyisoleucine; hydrophobic compounds may also be involved. We intend to pursue characterization of Compound 3 in future work (its spectral data, if tentative, may correspond to either a coumarin or a trace alkaloid).

CONCLUSION:

A polyherbal nanoparticle-loaded tablet of G. sylvestre, T. cordifolia, and T. foenum-graecum antidiabetic phytoconstituents were effectively developed. The preparation showed a strong in-vitroα-amylase inhibitory effect and had an additive antihyperglycemic activity in STZ-induced diabetic rats, with a marked decrease in the fasting blood glucose level. Major findings were the characterization of kaempferol-3-sophoroside and berberine as main active principles and the increase of the efficacy when associated using β-cyclodextrin nanoparticles. The nanoparticle tablet was able to bring about near-normoglycemia, comparable to a standard drug, and far superior to a typical herbal formulation. This research provides evidence for the potential of nanotechnology for enhancing the efficacy of herbal medicine and supports folklore claims on the antidiabetic activities of these plants. The polyherbal nanoparticle approach provides a pharmacologically validated and novel new lead for the clinical treatment strategy as additive therapy or substitute to the current antidiabetic therapy. Long-term studies of safety, dosing strategies, and clinical efficacy in human subjects are necessary as the first steps in developing these agents for human trials. The contemporary drug delivery technologies along with polyherbal drug delivery systems have potential to develop better therapy for chronic disorders like diabetes that are more patient compliant.

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