Amelioration of Diabetes and Its Related Complications in Streptozotocin Induced Diabetic Rats by Herbal Formulations

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ABSTRACT

The study is aimed at development and evaluation of herbal suspensions (A-1, A-2, A-3, A-4, A-5, and A-6) for anti-diabetic potential in streptozotocin (STZ)-induced diabetic rats. The most stable formulations: A-3 at the doses of 125mg/kg BW/day and 250 mg/kg BW/day, and A-6 at the doses of 162.5 mg/kg BW/day and 325 mg/kg BW/day were evaluated for their anti-diabetic activity in STZ induced diabetic rats and also assessed for few diabetes-related complications by studying the biochemical parameters like total cholesterol, total protein, blood urea, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT), and the histopathology of the pancreas, kidney, liver and heart samples. Formulation A-6 (325 mg/kg) ameliorated diabetes by exhibiting a decrease in blood glucose level, total cholesterol, blood urea, SGOT, and SGPT; and an increase in body weight and total protein; by removing vacuolization and swelling of pancreatic islets in histopathological studies.

Key Words: *Murraya koenigii, Momordica charantia, Syzygium cumini, Pterocarpus marsupium*

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INTRODUCTION

As per WHO, diabetes is defined as "a metabolic multifactorial disorder, characterized by chronic hyperglycemia with disturbances of carbohydrate, protein, and fat metabolism due to the defects in insulin secretion, insulin action or both" [1] and it affects the physical, psychological and social health of human body [2]. All forms of diabetes are associated with various complications caused by chronic hyperglycemia like renal failure; cerebrovascular disease; coronary artery disorder; neurological complications; limb amputation; blindness; long-term damage, dysfunctions and failure of various organs and eventually premature death [3, 4]. Since a long term treatment is required for this disease, there is a demand for a safe therapy which would be effective for diabetes and its related complications. The best solution to this problem is herbal formulations in which various phytochemicals work mutually in a vibrant manner in order to produce maximum curative efficacy with the least side effects [5]. About more than 1200 plant species exhibit the antidiabetic potential and more than 200 natural products derived from plant sources are known to possess the blood glucose lowering action [6, 7]. Thus, the current investigation aims towards the development and pharmacological evaluation of a few oral herbal anti-diabetic suspensions prepared by utilizing some anti-diabetic herbs: *Momordica charantia* Linn. seeds (Cucurbitaceae), *Pterocarpus marsupium* Roxb. heartwood (Fabaceae), *Syzygium cumini* Skeels seeds (Myrtaceae) and *Murraya koenigii* Linn. leaves (Rutaceae) [8-11].

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MATERIALS AND METHODS

Collection and authentication of plant materials

The fresh fruits of *M. charantia* and *S. cumini* were procured from a local market of Ghaziabad. The heartwood of *P. marsupium* was purchased from Khari Baoli market, Delhi and the fresh leaves of *M. koenigii* were collected from the Herbal Garden, KIET School of Pharmacy, Ghaziabad. All the plant materials were authenticated by Dr. Sunita Garg, Chief Scientist, Raw Material Herbarium and Museum, Delhi (RHMD), CSIR-NISCAIR, New Delhi and assigned the voucher specimen/Accession numbers:

i. *Momordica charantia* seeds (Ref No. NISCAIR/RHMD/Consult/2015/2911/104-1)
ii. *Syzygium cumini* seeds (Ref No. NISCAIR/RHMD/Consult/2015/2911/104-2)
iii. *Pterocarpus marsupium* heartwood (Ref No. NISCAIR/RHMD/Consult/2015/2911/104-3)
iv. *Murraya koenigii* leaves (Ref No. NISCAIR/RHMD/Consult/2015/2911/104-4)

All their voucher specimens are preserved in the herbarium section of Department of Pharmacognosy, KIET School of Pharmacy, Ghaziabad, Uttar Pradesh.

Extraction of plant materials

All the crude drugs were separately crushed to smaller pieces, re-dried and coarsely powdered. Then, the seeds of *M. charantia* (4.0 kg) and *S. cumini* (2.5 kg) were defatted with petroleum ether. The defatted material of *M. charantia* and *S. cumini* and leaves of *M. koenigii* (3.5kg) were extracted with ethanol (95%) in a Soxhlet Apparatus for 72 hours. The heartwood of *P. marsupium* (3.0 kg) was extracted with ethanol by cold maceration for 21 days. All the extracts were individually concentrated under reduced pressure to yield a dark reddish orange mass - 270.9 g (6.77%) of *M. charantia*, a dark blackish brown mass - 110 g (4.4%) of *S. cumini*, a dark reddish brown mass - 210.58 g (7.02 %) of *P. marsupium* and a dark green mass - 199.7 g (5.71%) of *M. koenigii*.

Formulation of anti-diabetic herbal suspensions

The suspensions were formulated by utilizing the individual alcoholic extracts of the plant materials and appropriate suspending agent by applying trituration [12]. The calculation for the required amount of the individual extract was based on their Therapeutically Effective Dose (TED). The dose at which the individual alcoholic extracts of the plants exhibited the maximum anti-diabetic activity was found to be 150 mg/kg BW for *M. charantia* [8], 100 mg/kg BW for *P. marsupium* [9], 100 mg/kg BW for *S. cumini* [10] and 300 mg/kg BW for *M. koenigii* [11]. The quantity of individual extract required for preparing the suspensions was calculated using the ratio-proportion method. The composition of the formulations is mentioned in Table 1.

Pharmaceutical evaluation of the formulated suspensions

The formulated suspensions were evaluated pharmaceutically using the standard procedures for the following parameters: organoleptic characteristics [13], pH [14], sedimentation volume [15], viscosity [16], ease of redispersibility [17], crystal growth [18] and particle size [19].

Table 1: Formulation composition of Suspensions

Ingredients	Function	Formulations					
		A-1	A-2	A-3	A-4	A-5	A-6
M. charantia seeds	Anti-diabetic principle	1.5g	-	0.75g	0.5014g	0.49g	0.375g
P. marsupium heartwood	Anti-diabetic principle	-	1g	0.5g	0.3343g	0.33g	0.25g
S. cumini seeds	Anti-diabetic principle	-	-	-	0.3343g	-	0.25g
M. koenigi leaves	Anti-diabetic principle	-	-	-	-	0.90g	0.75g
CMC Sodium	Thickening & stabilizing agent	0.5% w/v	0.5% w/v	0.5% w/v	0.5% w/v	0.5% w/v	0.5% w/v
Tween-80	Suspending agent	0.1% w/v	0.1% w/v	0.1% w/v	0.1% w/v	0.1% w/v	0.1% w/v
Propylparaben	preservative	0.02% w/v	0.02% w/v	0.02% w/v	0.02% w/v	0.02%w/v	0.02%w/v
Methyl paraben	preservative	0.2% w/v	0.2% w/v	0.2% w/v	0.2% w/v	0.2% w/v	0.2% w/v
Distilled water q.s.	Solvent	100ml	100ml	100ml	100ml	100ml	100ml

Study of HPTLC (High-Performance Thin Layer Chromatography) profile

Sample preparation

Each formulation was separately extracted in chloroform. 10 ml of the formulation and 10 ml of chloroform was added into a 50 ml round bottom flask and then it was refluxed for about one hour. Then it was allowed to cool and the contents of the round bottom flask were poured into separating funnel. The chloroform layer was separated and used as the sample for HPTLC.

Stationary phase

HPTLC plates silica gel 60 F 254, size 10.0 × 10.0 cm manufactured by Merck KGaA were used as the stationary phase.

Sample application

CAMAG Automatic TLC Sampler 4 (ATS4) "ATS4_201152" S/N 201152 (1.02.18) with nitrogen as

spray gas was used for applying the samples. 10 µl of the sample was applied with the 25 µl syringe as a band of 0.0 mm width and 8.0 mm length, 8.0 mm from the bottom of the plate. The distance between the tracks was 10.0 mm.

Developing solvent system

After trying various solvent systems, the best resolution and the maximum number of spots were obtained in Benzene: Chloroform: Methanol - 2:4:0.5**.**

Development of chromatogram

The twin trough glass chamber was first saturated with the solvent Benzene: Chloroform: Methanol, 2: 4: 0.5 for 20 minutes and then the chromatogram was developed up to the distance of 75mm.

Detection

The air-dried plates were scanned by CAMAG TLC Scanner "Scanner_201368" S/N 201368 (2.01.02). The scanning of the plates was started at the position Y 5.0 mm and ended at Y 75.0 mm. The optimized optical system was used with a scanning speed of 20 mm/s and data resolution of 100 µm/step. All the chromatograms were scanned in absorbance mode at both 254 and 366 nm and also between 200 to 700 nm using the tungsten and deuterium lamp with slit dimension 6.0 × 0.45, micro. The colour of the resolved bands was noted and their R_f (Retention Factor) was calculated.

Accelerated stability studies for anti-diabetic herbal formulations

The formulated suspensions A-1, A-2, A-3, A-4, A-5, and A-6 were stored at 50 °C ± 2 °C and 75 ± 5 % RH for a period of three months [20]. The suspensions were withdrawn after a period of 30, 60 and 90 days and analyzed for above parameters physical characterization like colour, odour, pH, sedimentation volume, viscosity, redispersibility, crystal growth, particle size, and HPTLC.

Anti-diabetic activity

Animals used

The experiments were performed as per the ethical norms approved by the Institutional Animal Ethics Committee (IAEC). The animal study protocol was approved and assigned the Proposal No. IAEC/KSOP/B/15/004. Albino Wistar rats (age: 16-20 weeks), either sex, weighing 180-200 g were obtained from the Animal House, KIET School of Pharmacy, Ghaziabad. They were kept in polypropylene cages (not more than 3 animals per cage) under standard laboratory conditions (Room temperature: 25 ± 2 °C; Relative Humidity: 30-70% and Photoperiod: 12h of the light/dark cycle). They were provided with the standard rodent pellet diet (Pranav Agro Industries Ltd., Delhi) and tap water ad libitum. Cage card label and marking on tails was used for identifying them. They were acclimatized to animal house conditions for 7 days.

Acute oral toxicity studies

The acute oral toxicity study was carried out as per the OECD (Organization for Economic Cooperation and Development) guidelines, draft guidelines 423 adopted on 17th December 2001. Three animals per group were used. The most stable formulations were administered orally up to the dose of 2,000 mg/kg BW. The observations on behavior alteration of the animals were observed after 3, 24 and 48 h. Observations were made for the changes in their skin, fur, eyes and for any tremors.

Experimental induction of diabetes

Streptozotocin (STZ) induced hyperglycemia was used as the experimental model to study the effect of the most stable formulated polyherbal suspensions. The animals were fasted overnight (deprived of food for 16 hours and allowed free access to water). Then, diabetes was induced in rats by injecting STZ (Sigma-Aldrich - procured from Discovery Chemicals Pvt. Ltd., New Delhi) intraperitoneally after dissolving in freshly prepared cold 0.1 M citrate buffer, pH=4.5, at a dose of 55 mg/kg body weight [21]. 5% glucose solution was given to the animals, overnight, in order to overcome the drug-induced hypoglycemia. A week time was given for developing diabetes. The rats with hyperglycemia (blood glucose range of above 250 mg/dl) were considered as diabetic and used for further study. The treatment was started on the eighth day after STZ injection and this was considered as the first day of treatment. The treatment was continued for 28 days. The experimental design is mentioned in Table II.

Table 2: Experimental Design

Experimental Groups	Treatment Schedule
Group I (6 animals)	Control rats receiving 0.1M citrate buffer (pH=4.5)
Group II (6 animals)	Diabetic control (STZ -55 mg/kg, i.p., once)
Group III (6 animals)	STZ (55 mg/kg, i.p., once) + Glibenclamide (5mg/kg BW/day orally in aqueous solution)
Group IV (6 animals)	STZ (55 mg/kg, i.p., once) + Formulation A-3 : low dose (125 mg/kg BW/day orally)
Group V (6 animals)	STZ (55 mg/kg, i.p., once) + Formulation A-3 : high dose (250 mg/kg BW/day orally)
Group VI (6 animals)	STZ (55 mg/kg, i.p., once) + Formulation A-6 : low dose (162.5 mg/kg BW/day orally)
Group VII (6 animals)	STZ (55 mg/kg, i.p., once) + Formulation A-6 : high dose (325 mg/kg BW/day orally)

Assessment of parameters

The body weight and blood glucose levels were assessed on 0th, 7th, 14th, 21st and 28th days. The biochemical parameters: total cholesterol, total proteins, liver function tests-serum glutamate-oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and

kidney function test (blood urea) were determined at the end of the study by using the diagnostic kits (Erba Mannheim). Blood was collected from the animals by retro-orbital bleeding and these parameters were assessed on the serum of the rats obtained at the mentioned days. Trinder's method [22] was used for estimation of glucose. Total cholesterol was evaluated by the method of Allain *et al*. [23]. Total Protein was estimated by Biuret method [24]. SGOT and SGPT were determined by the Kinetic method given by the International Federation of Clinical Chemistry (IFCC) [25]. The method of Talke and Schubert [26] was used for estimation of urea.

Statistical analysis

The data in the tables are the mean ±SEM (n=6 Wistar rats per groups), *p < 0.05, **p < 0.01, ***p < 0.001 compared with multiple groups using Bonferroni's multiple comparison test followed by one-way ANOVA. a= significant difference as compared to control (Group-I), b= significant difference as compared to induced control (Group-II), c= significant difference as compared to standard (Group-III).

Histopathological studies

The rats were sacrificed after the completion of the treatment schedule and their pancreata, livers, kidneys, and hearts were collected. The tissues were then immediately fixed in 10% formalin solution to avoid decomposition. Embedding in paraffin wax was carried out by removal of water by alcohol dehydration and infiltration of xylene. Sections were then stained by hematoxylin-eosin (H&E) [27] and viewed under the light microscope and shot by photomicrography.

RESULTS

Pharmaceutical evaluation and stability studies (with HPTLC profile – Fig.1) of the anti-diabetic herbal suspensions are mentioned in Table 3 – 8.

Table 3: Pharmaceutical evaluation and stability study data of suspension A-1

Parameters	0th day	30th day	60th day	90th day
Colour	Faded yellow	Faded yellow	Faded yellow	Faded yellow
Odour	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.8	7.0	7.0	7.2
Sed. Vol.	1.16	1.21	1.23	1.26
Viscosity	90 cps	106 cps	108 cps	113 cps
Part. size	35-38 µ	35-38 µ	35-39 µ	36-41 µ
Redis (%)	100	95	95	90
Crys. gro.	None	None	None	None
No. of spots observed	9	8	7	7

Table 4: Pharmaceutical evaluation and stability study data of suspension A-2

Parameters	0th day	30th day	60th day	90th day
Colour	Reddish brown	Reddish brown	Reddish brown	Reddish Brown
Odour	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.78	6.66	6.5	6.45
Sed. Vol.	1.12	1.47	1.50	1.53
Viscosity	180 cps	91.7 cps	91.0 cps	91.0 cps
Part. size	56-59 µ	56-59 µ	60-63 µ	59-63 µ
Redis (%)	100	95	90	90
Crys. gro.	None	None	None	None
No. of spots observed	8	7	7	7

Table 5: Pharmaceutical evaluation and stability study data of suspension A-3

Parameters	0th day	30th day	60th day	90th day
Colour	Brownish orange	Brownish orange	Brownish orange	Brownish orange
Odour	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.66	6.66	6.65	6.65
Sed. Vol.	1.15	1.15	1.19	1.19
Viscosity	116 cps	122 cps	129 cps	129 cps
Part. size	39-45 µ	40-43 µ	41-43 µ	41-43 µ
Redis (%)	100	95	95	95
Crys. gro.	None	None	None	None
No. of spots observed	9	9	9	9

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Table 6: Pharmaceutical evaluation and stability study data of suspension A-4

Parameters	0th day	30th day	60th day	90th day
Colour	Light brown	Light brown	Light brown	Light brown
Odour	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.68	6.71	6.75	6.77
Sed. Vol.	1.11	1.44	1.53	1.57
Viscosity	138 cps	94.2 cps	89.9 cps	87.5 cps
Part. size	49-55 µ	50-53 µ	51-53 µ	51-53 µ
Redis (%)	100	90	85	85
Crys. gro.	None	None	None	None
No. of spots observed	7	6	6	5

Table 7: Pharmaceutical evaluation and stability study data of suspension A-5

Parameters	0th day	30th day	60th day	90th day
Colour	Dark brown	Dark brown	Dark brown	Dark brown
Odour	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.9	6.7	6.3	6.3
Sed. Vol.	1.13	1.26	1.31	1.34
Viscosity	101 cps	78.4 cps	67.0 cps	65.9 cps
Part. size	38-39 µ	41-45 µ	41-45 µ	41-45 µ
Redis (%)	100	90	85	85
Crys. gro.	None	None	None	None
No. of spots observed	7	8	7	6

Table 8: Pharmaceutical evaluation and stability study data of suspensions A-6

Parameters	0th day	30th day	60th day	90th day
Colour	Brownish green	Brownish green	Brownish green	Brownish green
Odour	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.7	6.7	6.75	6.75
Sed. Vol.	1.14	1.20	1.20	1.20
Viscosity	158 cps	125.6 cps	125.6 cps	125.6 cps
Part. size	54-57 µ	54-59 µ	55-59 µ	55-59 µ
Redis (%)	100	95	95	95
Crys. gro.	None	None	None	None
No. of spots observed	8	8	8	8

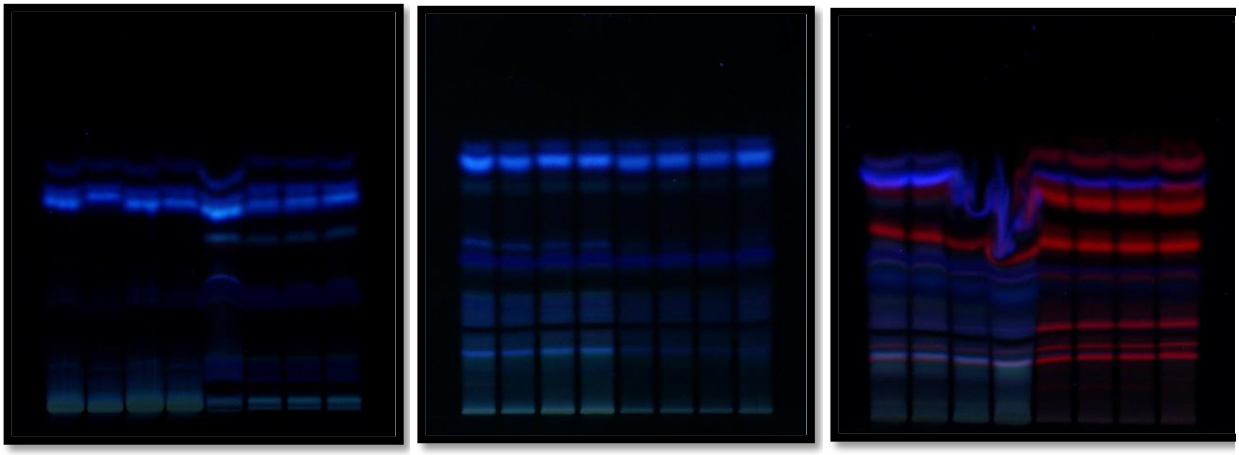

Fig.1.The HPTLC pattern of anti-diabetic herbal suspensions under 366 nm. From left to right: tracks 1-4 for A-1 (0th, 30th, 60th, 90th day) samples; tracks 5-8 for A-2 (0th, 30th, 60th, 90th day) samples; tracks 9-12 for A-3 (0th, 30th, 60th, 90th day) samples; tracks 13-16 for A-4 (0th, 30th, 60th, 90th day) samples; tracks 17-20 for A-5 (0th, 30th, 60th, 90th day) samples and tracks 21-24 for A-6 (0th, 30th, 60th, 90th day) samples.

Anti-diabetic activity

Acute toxicity studies

It was observed in the acute toxicity studies that there was no comparable loss in weight after 3, 24 and 48 h of oral administration of the formulations at the dose of 2,000 mg/kg BW. There were no changes in their skin, fur, and eyes. No tremors were observed. All the animals were in the same condition as before administering the dose and there was no mortality.

Effect on body weight

The basal body weight (mean) of all the groups ranged from 183 to 185 g and there was no intergroup variation. The normal control group showed a weight gain of 3.7% after 28 days. The STZ diabetic group exhibited a decrement of 12% in body weight after four weeks. The groups, treated with the standard drug-glibenclamide (standard anti-diabetic drug) and that treated with a high dose of formulation A-6 (325 mg/kg) showed the weight gain of 2.6% and 1.57%, respectively. These results were highly significant ($p<0.001$) as compared to the induced control group. The other groups treated with A-3 (125 mg/kg) and A-3 (250 mg/kg) did not produce any significant weight gain, while those treated with A-6 (162.5 mg/kg) showed a significant ($p<0.05$) weight gain as compared to the induced control group but were significantly less in comparison to normal control group (Fig. 2).

Effect on blood glucose level

The basal blood glucose level (mean) for the animals of the normal control group was 84.3 mg/dl and this remained almost the same throughout the experiment. The STZ diabetic rats exhibited a highly significant ($p<0.001$) increment of 35.82% in blood glucose level as compared to the normal control group. The percentage of decrement in blood glucose levels was 45.3%, 40.8%, 39.6%, 39.29% and 27.64% respectively for Glibenclamide, A-6 (325 mg/kg) dose, A-3 (250 mg/kg), A-6 (162.5 mg/kg) and A-3 (125 mg/kg) treated groups (Table-9 and Fig. 3).

Effect on total cholesterol

The STZ diabetic group showed an increment of 53.58 % in total cholesterol level as compared to the normal control group. In comparison to the STZ diabetic group, the treatment groups exhibited a decrease in total cholesterol level by 48.5 %, 46.42 %, 30.54 % and 13.49 %, for A-6 (325 mg/kg), Glibenclamide, A-6 (162.5 mg/kg) and A-3(250 mg/kg), treated groups, respectively (Table-10).

Effect on total proteins

The level of total proteins was found to decrease by 30.76 % in STZ diabetic rats as compared to the normal control group. In all the other groups, the total protein level increased after continuous treatment for 4 weeks. The formulation A-6 (162.5 mg/kg) was capable of increasing the total protein content by 24.05% which was similar to the increment shown by Glibenclamide group when compared to the induced control rats while there was an elevation of 19.42%, 17.56 %, and 6.84 %, respectively for A-3(250 mg/kg), A-6(325 mg/kg) and A-3 (125 mg/kg) (Table-10).

Effect on blood urea

The rats in the normal control group showed the basal urea level 29 mg/dl (mean). In the case of STZ induced diabetic animals, it was increased to 52.83 mg/dl. All the treatment groups tried to decrease this elevated level and attain the normal value. There was a decline of 31.86 %, 26.92 %, 19.04 %, 16.39 % and 12.31 %, respectively for A-6 (325 mg/kg), A-6 (162.5 mg/kg), A-3 (250 mg/kg) and A-3 (12 5 mg/kg) treatment groups (Table-10).

Effect on liver function tests (SGOT and SGPT)

The SGOT and SGPT levels were increased in the STZ diabetic rats by 52.13% and 65.63%, respectively as compared to the normal control group. These values were decreased in the treatment groups and tried to attain the level to near normalcy. The Glibenclamide treated group showed a decrement of 45.48 %, and 54.14 %, respectively in values of SGOT and SGPT as compared to STZ-induced groups. Of all the herbal suspensions, the treated group, A-6 (325 mg/kg) was capable of attaining the values similar to that of the standard group (Table-10).

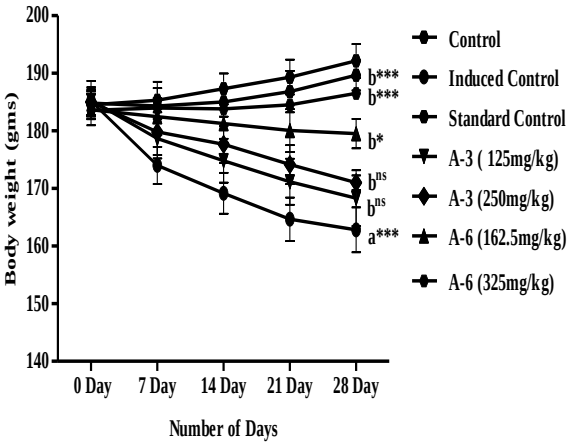

Fig. 2. Changes in body weight (g) on the experimental groups. The data in the figure are the mean ± SEM (n=6 Wistar rats per groups), *p < 0.05, **p < 0.01, ***p < 0.001 compared to multiple groups using Bonferroni's multiple comparison test followed by one-way ANOVA. a = significant difference as compared to control (Group-I), b = significant difference as compared to induced control (Group-II), c = significant difference as compared to standard (Group-III).

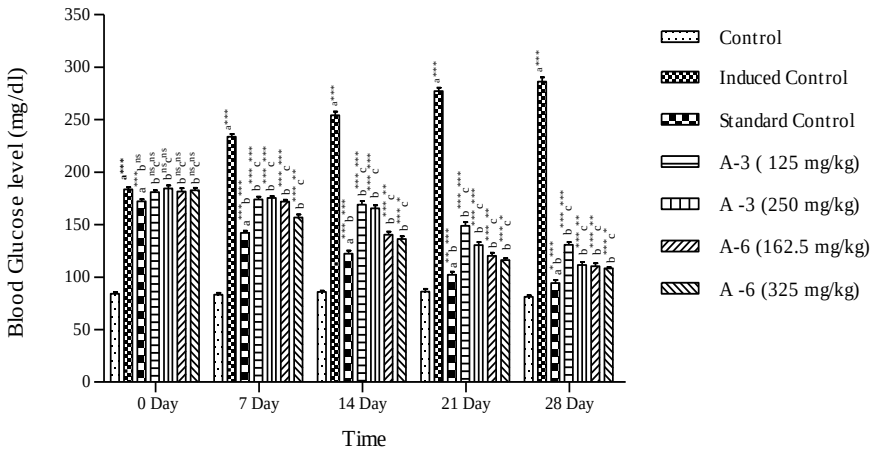

Fig. 3. Changes in blood glucose level (mg/dl) on experimental groups. The data in the figure are the mean ± SEM (n=6 Wistar rats per groups), *p < 0.05, **p < 0.01, ***p < 0.001 compared to multiple groups using Bonferroni's multiple comparison test followed by one-way ANOVA. a = significant difference as compared to control (Group-I), b = significant difference as compared to induced control (Group-II), c = significant difference as compared to standard (Group-III).

Table 9: Levels of blood glucose (mg/dl) in control and experimental groups. The data in the table are the mean ± SEM (n=6 Wistar rats per groups), *p < 0.05, **p < 0.01, ***p < 0.001 compared to multiple groups using Bonferroni's multiple comparison test followed by one-way ANOVA. a = significant difference as compared to control (Group-I), b = significant difference as compared to induced control (Group-II), c = significant difference as compared to standard (Group-III).

Experimental Groups	Blood Glucose Level (mg/dl)				
	0th day	7th day	14th day	21st day	28th day
Group I Control	84.26 ± 1.75	83.12 ± 1.97	85.09 ±1.16	86.12 ±2.66	81.12 ± 1.66
Group II Induced Control	183.65 ± 1.97 a^{***}	233.6 5 ± 2.72 a^{***}	264.10 ± 3.42 a^{***}	297.12 ± 3.23 a^{***}	326.12 ± 4.32 a^{***}
Group III Standard control	176.32 ± 1.86 $a^{***}b^{ns}$	142.32 ± 1.96 $a^{***}b^{***}$	122.26 ± 2.99 $a^{***}b^{***}$	102.26 ± 2.99 $a^{**}b^{***}$	94.26 ± 2.99 $a^{*}b^{***}$
Group IV A-3 (125 mg/kg)	180.90 ± 1.82 $b^{ns}c^{ns}$	173.90 ± 2.82 $b^{***}c^{***}$	168.90 ± 3.49 $b^{***}c^{***}$	148.90 ± 3.49 $b^{***}c^{***}$	130.90 ± 2.49 $b^{***}c^{***}$
Group V A-3 (250 mg/kg)	184.49 ± 2.77 $b^{ns}c^{ns}$	175.49 ± 1.77 $b^{***}c^{***}$	165.49 ± 2.97 $b^{***}c^{***}$	130.49 ± 2.97 $b^{***}c^{***}$	111.49 ± 2.97 $b^{***}c^{**}$
Group VI A-6 (162.5 mg/kg)	181.69 ± 2.93 $b^{ns}c^{ns}$	171.89 ± 1.93 $b^{***}c^{***}$	140.32 ± 2.93 $b^{***}c^{**}$	120.32 ± 2.96 $b^{***}c^{**}$	110.32 ± 2.96 $b^{***}c^{**}$
Group VII A-6 (325 mg/kg)	182.81 ± 2.11 $b^{ns}c^{ns}$	156.81 ± 2.81 $b^{***}c^{**}$	136.25 ± 2.81 $b^{***}c^{**}$	116.25 ± 1.81 $b^{***}c^{*}$	108.25 ± 1.21 $b^{***}c^{*}$

Table 10: Levels of total cholesterol, total proteins, blood urea, SGOT and SGPT in control and treatment groups. The data in the table are the mean ± SEM (n=6 Wistar rats per groups), *p < 0.05, **p < 0.01, ***p < 0.001 compared to multiple groups using Bonferroni's multiple comparison test followed by one-way ANOVA. a = significant difference as compared to control (Group-I), b = significant difference as compared to induced control (Group-II), c = significant difference as compared to standard (Group-III).

Experimental Groups	Total Cholesterol (mg/dl)	Total Protein (g/dl)	Urea (mg/dl)	SGOT (U/l)	SGPT (U/l)
Group I Control	85.58 ± 3.39	7.25 ± 0.34	29.00 ± 1.33	55.45 ± 3.32	21.94 ± 2.52
Group II Induced Control	184.31 ± 4.59 a^{***}	5.02 ± 0.10 a^{***}	52.83 ± 2.11 a^{***}	115.83 ± 5.31 a^{***}	63.83 ± 3.17 a^{***}
Group III Standard control	98.79 ± 3.22 b^{***}	7.01 ± 0.35 b^{***}	36.00 ± 0.64 b^{***}	63.15 ± 3.66 b^{***}	28.92 ± 2.86 b^{***}
Group IV A-3 (125 mg/kg)	186.95 ± 3.80 $b^{ns}c^{***}$	5.39 ± 0.22 $b^{ns}c^{**}$	47.04 ± 0.76 $b^{ns}c^{**}$	103.09 ± 4.41 $b^{ns}c^{***}$	57.92 ± 2.68 $b^{ns}c^{***}$
Group V	159.50 ± 4.73	6.22 ± 0.22	44.17 ± 2.80	95.57 ± 3.90	45.84 ± 2.44

A-3 (250 mg/kg)	$b^{**}c^{***}$	$b^{ns}c^{ns}$	$b^{*}c^{*}$	$b^{*}c^{***}$	$b^{**}c^{**}$
Group VI A-6 (162.5 mg/kg)	128.07 ± 4.30 $b^{***}c^{***}$	6.61±0.26 $b^{**}c^{ns}$	42.77 ± 1.65 $b^{**}c^{ns}$	88.96 ± 3.16 $b^{***}c^{***}$	38.58 ± 3.01 $b^{***}c^{ns}$
Group VII A-6 (325 mg/kg)	94.95 ± 3.58 $b^{***}c^{ns}$	6.93±0.25 $b^{***}c^{ns}$	38.61 ± 1.66 $b^{***}c^{ns}$	68.20 ± 2.95 $b^{***}c^{ns}$	33.92 ± 2.63 $b^{***}c^{ns}$

Histopathological study

The pancreata, livers, kidneys, and hearts of the normal control, STZ induced diabetic control, standard group and the formulations showed the best results, i.e., A-6: high dose and A-6: low dose were subjected to the histopathological studies.

Histology of pancreas

The histological investigations of the normal control group (Group I) pancreas exhibited their normal architecture. The islets of Langerhans consisted of the normal acini. There was no indication of any type of inflammation. The diabetic control group (Group II) showed the vacuolization, swelling up of the islet cells, presence of necrotic cells, loss in the shape and decrease in a number of islet cells. The pancreas sample from STZ + Gliben group (Group III) and STZ + A-6 high dose group (group VII) showed the normal islets of Langerhans. The histopathology of the pancreas from STZ+ A-6 low dose group (Group V) exhibited vacuolization and swelling up of the islet cells (Fig. 4 (A-E)).

Histology of kidneys

The histology of kidneys of the normal control group showed glomerulus surrounded by distal and convoluted tubules and normal Bowman's space. All other groups also exhibited similar histological features (Fig. 5 (A-E)).

Histology of livers

Histology of the liver samples from all the groups exhibited the normal portal triad (portal vein, bile duct, and hepatic artery) (Fig. 6 (A-E)).

Histology of hearts

The histology of heart from all the groups exhibited the normal myocardial blood vessel without any appreciable thickening (Fig. 7 (A-E)).

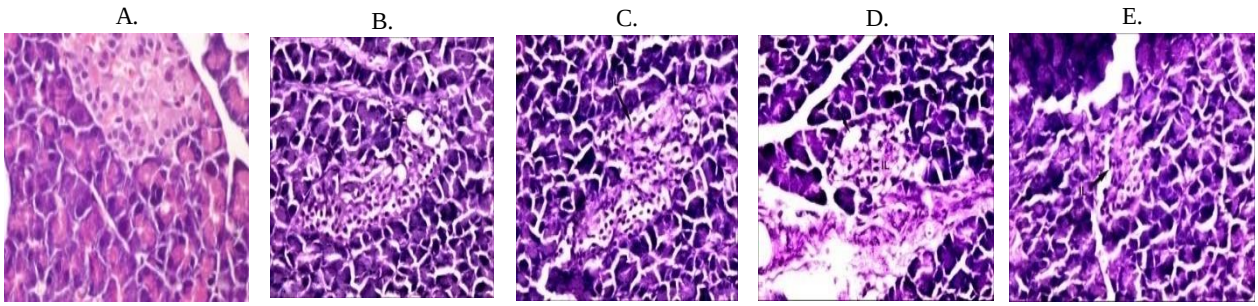

Fig. 4: High power photomicrograph (400x) of pancreas sample from normal control group showing islets with normal acini (A); Induced control group showing vacuolization and swelling up of the islet cells (arrow) (B); Standard control group showing the islets of Langerhans without vacuolization (C); A-6 (162.5 mg/kg) group showing vacuolization and swelling up of the islet cells (arrow) (D); A-6 (325 mg/kg) group showing the islet cells without vacuolization (E). IL=Islet of Langerhans

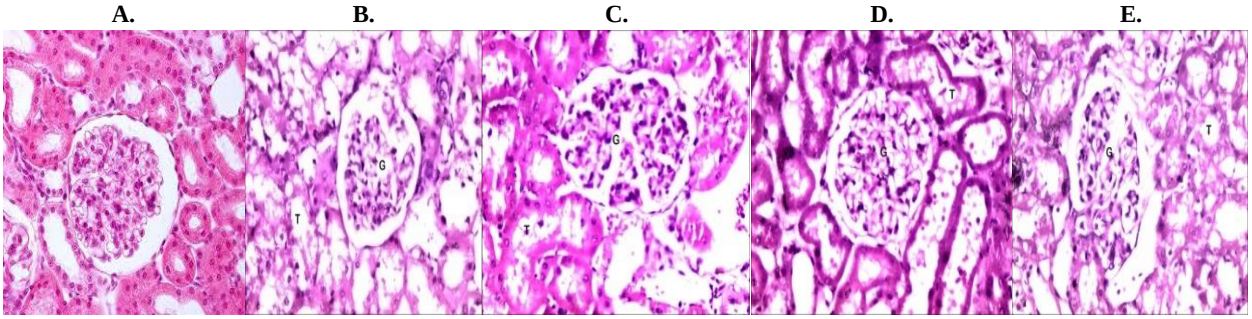

Fig. 5: High power photomicrograph (400x) of kidney sample from normal control group (A), Induced control group (B), Standard control group (C), A-6 (162.5 mg/kg) (D) group and A-6 (325 mg/kg) (E) group showing normal glomerulus surrounded by tubules and Bowman's capsule. G=Glomerulus, T=Tubules, B=Bowman's space

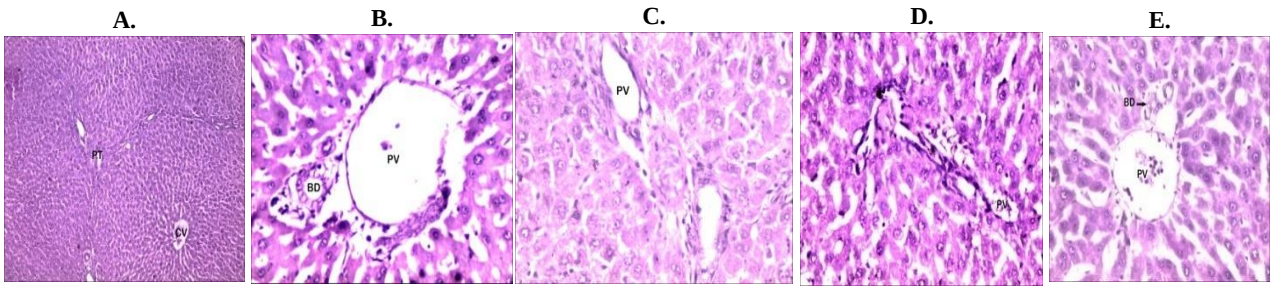

Fig. 6: High power photomicrograph (400x) of liver sample from normal control group (A), Induced control group (B), Standard control group (C), A-6 (162.5 mg/kg) (D) group and A-6 (325 mg/kg) (E) group showing a normal portal triad. PV= Portal Vein, BD= Bile Duct

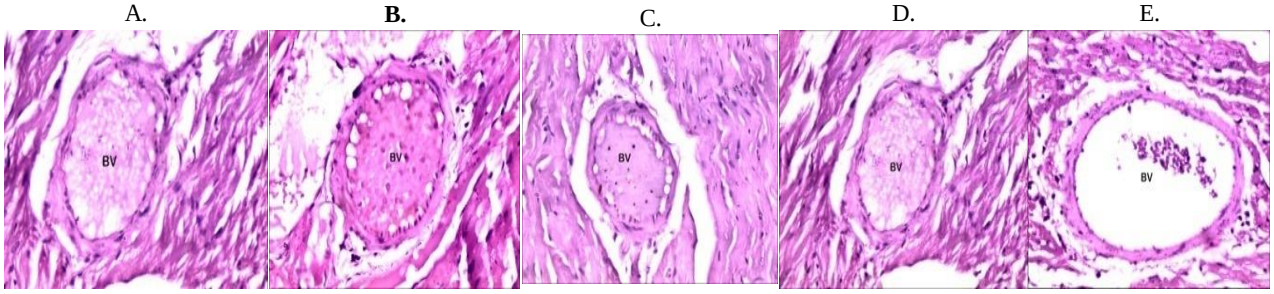

Fig. 7: High power photomicrograph (400x) of heart sample from normal control group (A), Induced control group (B), Standard control group (C), A-6 (162.5 mg/kg) (D) group and A-6 (325 mg/kg) (E) group showing a myocardial blood vessel. No appreciable thickening of the blood vessel wall is seen. BV=Blood Vessel

DISCUSSION

Suspensions provide high systemic effects through oral administration. Thus, for pharmacological evaluation of herbal drugs, suspensions: A-1, A-2, A-3, A-4, A-5, and A-6 were prepared to utilize the alcoholic extracts of *M. charantia* seeds, *P. marsupium* heartwood, *S. cumini* seeds, and *M. koenigii* leaves in different combinations along with the excipients. Water is the most popular solvent used for the preparation of suspensions because of its physiological compatibility, lack of toxicity, easy availability and good solubilizing property but at the same time, it acts as an excellent media for the growth of microorganisms. Thus, there was a requirement of a preservative which could prevent the risk of contamination by microbes. Parabens are the most commonly used preservatives as they are highly effective against microbes and possess very low toxicity to humans [28]. Investogations about carcinogenesis; reproduction toxicity; acute, subchronic & chronic toxicity; and absorption, metabolism & excretion studies are some of the *in-vivo* studies that have established the safety of parabens. Under FDA regulation, methylparaben and propylparaben are Generally Recognized As Safe (GRAS) [29]. Thus, 0.02% of propylparaben and 0.2% of methylparaben were used in combination as a preservative [30].

In order to enhance the wettability and dispersion of poorly soluble ingredients, a surfactant was required which could decrease the interfacial tension between solid particles and solvent during the preparation or reconstitution of a suspension. Thus, Tween-80 was used in a concentration of 0.1% as the suspending agent [30]. Formulation of suspensions requires certain stabilizers or thickening agents that prevent sedimentation or settling by modifying the viscosity by entrapping the solid particles in a viscous or 'gel-like' structure. As the water was used as a solvent, so a water-soluble wetting agent, i.e., CMC Sodium was used in a concentration of 0.5%. By taking the quantity of individual extract obtained by the ratio-proportion method and by using the simple trituration for preparing the suspensions, the stable formulations were obtained.

As the observations for pH, sedimentation volume, viscosity, and redispersibilty were almost constant on 0^{th}, 30^{th}, 60^{th} and 90^{th} days for suspensions A-3 and A-6, it led us to infer that A-3 and A-6 might be the most stable formulations.

HPTLC for each formulation: A-1, A-2, A-3, A-4, A-5, and A-6 were carried out in order to assess their stability. The 0^{th}-day samples and the 30^{th}, 60^{th} and 90^{th}-days samples obtained after storing the formulations at 50 °C ± 2 °C and 75 ± 5% RH were subjected to HPTLC analysis. The HPTLC profile for the 0^{th} day for each of the formulation was used as the standard. Then HPTLC profiles of 30^{th}, $60^{th,}$ and 90^{th}-day samples were compared with this standard to check whether the formulations are stable or not. After studying the HPTLC profile we concluded that the formulations A-3 and A-6 were the

most stable samples. These two chemically and physically stable formulations were selected for studying their potential in diabetes and their associated issues in STZ induced diabetic rats. The preliminary studies conducted by us indicated the non-toxic nature of the formulations on normal rats.

STZ induced experimental diabetes is a very popular model for type I diabetes. Glibenclamide is usually used as an insulin stimulant and standard anti-diabetic drug in STZ induced diabetes for comparing the anti-diabetic potential of various hypoglycemic agents. Severe weight loss is observed in STZ-induced diabetes [31] and our results also supported this statement. The diabetic rats treated with glibenclamide and A-6 (325 mg/kg) exhibited a marked increase in body weight of animals. It has been suggested that in diabetes, carbohydrate is unavailable for utilization as a source of energy, so it results in protein wasting leading to a loss in body weight [32]. Therefore, an increase in body weight of the treated groups is associated with enhancement of glucose metabolism.

Various research studies, performed separately on *M.charantia*, *P.marsupium*, *S.cumini*, and *M.koenigii* have suggested that they all decrease the blood glucose level by potentiating the effect of insulin in plasma by enhancing the insulin secretion from pancreatic β-cells or its responsiveness [33, 34]. The continuous treatment of diabetic rats with formulated suspensions and glibenclamide for 28 days showed a decrease in blood glucose level. The diabetic rats treated with a high dose of A-6 (325 mg/kg) exhibited the best hypoglycemic effects among all the groups treated with formulated suspensions. These formulations tried to control blood sugar level but not to the extent of glibenclamide.

The abnormality in the lipid profile is one of the most common complications associated with diabetes mellitus [35, 36]. The high levels of total cholesterol may lead to coronary complications, morbidity, and death in diabetics [37]. Insulin deficiency has been estimated to be the most probable reason for the elevated cholesterol level in STZ-induced diabetic rats [38]. In the current study, among all the groups, treated with herbal formulations, A-6 (325 mg/kg) was best capable of reducing the total cholesterol level and the results were better even than the glibenclamide treated group. This potential of this formulated suspension might be due to elevation in insulin secretion which finally led to decrement in cholesterol and fatty acid synthesis.

Earlier research investigations have reported that the level of total protein content decreases in STZ-induced diabetic rats because, during diabetes, the protein catabolism occurs which leads to the flow of amino acids into the liver where they act as the substrate for gluconeogenesis [39]. It has also been suggested that insulin deficiency is also responsible for the deranged glucagon-mediated regulation of cyclic AMP formation which ultimately leads to accelerated proteolysis [40]. All the treatment groups exhibited an increment in the total protein content which might be due to the inhibition of proteolysis caused by insulin deficiency. Formulation A-3 (125mg/kg) was the best in terms of elevating the total protein content.

The increased proteolysis in diabetic animals manifests the negative nitrogen balance. Thus, the concentration of urea in blood increases due to the disturbed nitrogen balance coupled with decreased protein synthesis [41]. The STZ diabetic rats treated with glibenclamide and A-6 (325mg/kg) significantly declined the level of blood urea and tried to reach the normal range.

Administration of herbal suspensions A-3 and A-6, both in low and high doses improved the liver function by decreasing the serum SGOT and SGPT levels. The elevated levels of SGOT and SGPT in diabetes are associated with the incidence of heart and liver diseases.

The histopathological studies of pancreas indicated that the formulation A-6 (325 mg/kg) showed similar observations as those with glibenclamide treated group. According to earlier investigations, irreversible variations in the pancreas have been observed after 10 days of streptozotocin treatment [42] and some research investigations have reported the decrement in a number of beta cells and their degeneration [43]. Our results also supported their observations. There was no vacuolization of cells in Glibenclamide and A-6 high dose treated groups indicating that these were beneficial in restoring the normal architecture of pancreas.

The histology of kidney, liver, and heart in all the groups showed normal architecture. In the kidney, neither there were any signs of inflammation in glomerulus nor any thickening of the basement membrane. Glomerulosclerosis, glomerular hypertrophy, tubular degeneration and narrowing in the Bowman's space are usually not observed in the early phase of diabetes [44]. In the liver, there was no degeneration of hepatocytes. Sinusoids also did not show any changes and there were no other signs of inflammation. This indicated that diabetic complications related to liver appear in case of chronic diabetes. These observations were contrary to the findings of earlier studies. Many changes in the liver histology have been observed only after 3 weeks of streptozotocin administration. These variations included portal vein congestion, periportal necrosis [45], hepatocytes contraction, sinusoid dialation [46], bile duct hyperplasia and infiltration of cells in portal area [47]. There were no changes in the heart histology in any of the groups. All showed normal myocardial blood vessels. These observations were in the support of the fact that there are minimal variations in the heart in the acute phase of diabetes [48].

CONCLUSIONS

Formulation A-6 (325 mg/kg) exhibited the best results showing a reduction in blood glucose level, total cholesterol, blood urea, SGOT, and SGPT while an elevation in body weight and total protein indicating its favorable effects in diabetes and its related complications. The histopathological report of the pancreas also supported the anti-diabetic potential of the formulation by removing vacuolization and swelling of pancreatic islets. The formulation A-6 might be acting as an anti-diabetic agent by potentiating the effect of insulin. *M.charantia* has been reported to show diarrhoeal effects while *P.marsupium* causes constipation due to its astringent property. None of these effects were observed in the animals treated by herbal suspension A-6. The formulation did not show any other toxic effects and it is estimated that the toxic effects caused by these individual herbs might have reduced due to the decreased quantity of each drug in the formulation. It might be evaluated for the abortifacient activity which is the main side effect of Momordica seeds. Further studies are essential for exploring the phytochemistry of the formulation.

Conflict Of Interest

None.

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