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Phyto-chemical Standardization of Herbal Formulation (PMM3) for Blood Sugar Attenuating Actions in Streptozotocin induced Rats

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ABSTRACT

The present study was intended to prepare herbal formulation, PMM3 using purified and modified parts of five common Indian herbs like, *Trigonella foenum-graccum*, *Tinospora cordifolia*, *Scoparia dulcis*, *Adhatoda vasica* and *Cassia occidental*. PMM3 was standardized using physico-chemical, phyto-chemical, UV-VIS spectral, HPTLC, AAS and GC methods. The phenolics and flavonoids contents were assessed. Anti-hyperglycaemic activities of PMM3 was evaluated on Streptozotocin induced (50mg/kg, i.v) diabetic rats. PMM3 (50-150 mg/kg, p.o) exhibited best potentiality in reducing blood glucose within 14 days treatment in comparison with Diabecon® (Himalaya, *India*) at the same dose. The preset observation identified formulation PMM3 for anti-hyperglycaemic effect.

Key words: *Diabetes, Herbs, Streptozotocin, HPTLC, Quercetin.*

INTRODUCTION

Diabetes is the silent cause of death. In 2015, WHO estimated diabetes killed 1.6 million people globally and expected to double within 10 years.[1] At present, more than 62 million diabetic individuals diagnosed with the disease.[2] Besides lifestyle modification, insulin injection and oral hypoglycaemic are commonly recommended. The use of natural products, particularly herbal nutraceuticals has a long folkloric history for the treatment of blood sugar abnormalities. Indian traditional remedies for diabetes are usually mixed formulations containing blood sugar lowering herbs in combination with immune modulators, hypocholesteremic, antioxidants and detoxicants.[3-4] There are several herbal formulations present in the markets, but still require more research to develop effective drugs to treat diabetes and its complications. Moreover, most of the marketed herbal drugs did not have scientific reports. Earlier, it has been reported that *Tinospora cordifolia* stem, *Trigonella foenum-graccum* seed, *Scoparies dulcis* areal part, *Adhatoda vasica* leaves, *Cassia occidentalis* leaf showed promising blood sugar and blood lipid lowering actions.[5-7] Several pharmaceutical researches are undergoing to modify their physicochemical properties of these herbs. In this regards, the present study was attempt to purify and characterized the bioactive anti-hyperglycemic principles derived from five non-toxic herbs that are commonly used as species in Indian cousins. Based on our earlier reports, one formulation (PMM3) were prepared and examined following the recommended guidelines of WHO (2007) and Indian Pharmacopeia

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(2007) for new drug development.[8-9] The physico-chemical properties, chemical standardization, heavy metals contents, pesticide residues were examined. Further, pharmacological anti-hyperglycemic properties were carried out in Streptozotocin induced diabetes rats and compared with marketed anti-diabetic formulation.

Materials and Methods

Test Drug Preparation

Tinospora cordifolia stem growing on *Azadirachta indica* tree, *Trigonella foenum-graccum* seed, *Scoparies duleis* areal part, *Adhatoda vasica* leaves, *Cassia occidentalis* leaf were collected from Salipur, Odisha and identified by Botanical Survey of India, West Bengal. *Trigonella foenum-graccum* seeds were powdered and soaked with raw milk (1:2 w/v) and kept overnight (12 h) at 37ºC. Thereafter, washed with warm distilled water (37ºC), dried and again soaked with raw milk. This procedure was applied repeatedly 15 times. Finally, purified dried fenugreek powder (FGM) was kept in air dried container for further use.[7] The white powdered starch was extracted by cold extraction and drying process from *Tinospora cordifolia* stem growing on *Azadirachta indica* tree. The dried *Scoparies dulcis* areal part, *Adhatoda vasica* leaves and *Cassia occidentalis* leaves were powdered.[6] The new herbal formulation PMM3 was prepared by mixing with powdered of individual five plant's proportionately as milk modified *Trigonella foenum-graccum* seed (50%), starch from *Tinospora cordifolia* stem growing on *Azadirachta indica* tree (25%), powdered *Scoparies dulcis* areal part (15%), powdered *Adhatoda vasica* leaves (5%) and powdered *Cassia occidentalis* leaves (5%). Finally, PMM3 was kept in air dried container for further use.

Standardization of PMM3

Physico-chemical Properties

The physico-chemical properties like, loss on drying, total ash, acid insoluble ash, alcohol soluble extractive, water soluble extractive and pH of test formulation PMM3 were determined according to the guidelines of Indian Pharmacopeia.[9]

Phytochemical Group Analysis

The group analysis of PMM3 was examined for alkaloids by Dragendroff's test, sterols and triterpenoids by Liberman Buchard test, saponins by Froth test, tannins by lead acetate test, carbohydrates by Fehling's test, reducing sugars by Benedict test, proteins by Millon's test, phenolics by ferric chloride test, flavonoids by aluminum chloride test and glycosides by Borntrager's test.[6-7]

Estimation of Total Phenolics

The amount of total phenolics in PMM3 was determined as described earlier.[10] To 0.2ml of PMM3 solution in methanol (1 mg/ml), 1.0 ml of Folin-Ciocalteu reagent was added, mixed and incubated in the dark for 5 min. Thereafter, 0.8 ml of sodium carbonate solution (7.5%) was added and further incubated in the dark for 30 min. Finally, 3 ml of distilled water was added and the absorbance at 765 nm was measured. The total phenolics content was expressed as gallic acid equivalent (GAE) in mg per g. of extract.

Estimation of Total Flavonoids

For the estimation of flavonoids present in PMM3, 0.2 ml of PMM3 (1 mg/ml) was mixed with 0.8 ml methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After 30 min the absorbance of the reaction mixture was measured at 415 nm. The total flavonoid was expressed as quercetin equivalent (QE) in mg per g of extract.[10]

UV Spectral Analysis

The methanolic and aqueous solutions of PMM3 were examined under visible and UV light for spectral analysis. The sample was scanned in the wave length ranging from 200-400 nm using UV-VIS spectrophotometer and characteristic peaks were detected.[11]

Standardization of test formulation by HPTLC

The powdered PMM3 was mixed in methanol (1 mg/ml), filtered and spotted on a pre-coated silica gel plates (Merck, $60F_{254}$, 10x10 cm) using Camag Linomat

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5 applicator and processed in a solvent system (toluene : ethyl acetate : formic acid = 5:4:1) for 30 min. The densitometric scanning was performed on Camag TLC Scanner 3 at absorbance 280 nm (D2 lamp) operated by multi level winCATS planar chromatography manager.[12] The gallic acid and quercetin was used as a standard. The obtained unknown peaks were individually marked as Rf and area percent were measured. The known peak for gallic acid and quercetin was quantified and compared.

Heavy Metal Analysis

Four heavy metal contaminants - mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As) - were analyzed in test formulation, PMM3 and measured using atomic absorption spectrophotometer (Perkin Elmer, USA) following Indian Pharmacopeia (2007).[9]

Pesticide Residual Analysis

PMM3 were extracted by standard procedure, impurities were removed by partition and individual pesticides, viz. DDT, Pyremethrin and BHC were measured by GC (Agilent Technologies, 7890B) as recommended by AOAC (2005).[13]

Pharmacological Study

Animals

Swiss albino mice and Wistar rats were maintained in animal house for at least 10 days prior to experimentation. Recognized guidelines for the care and use of the animals were followed (CPCSEA guidelines for laboratory animal facility, 2003).[14] Permission for the experiments was obtained from the institute animal ethics committee (02/IEAC/IPT/09). The room temperature was maintained at 23±2°C and humidity at 40-60%. A 12 hour light-dark cycle was also maintained. The animals were fed supplementary feed for rats and water. The food was withdrawn as per experimental protocol.

Acute toxicity study

The homogenous suspension of PMM3 was prepared freshly, using 0.5% (w/v) carboxyl methyl cellulose (CMC) using a mortar and pestle. The different groups of mice were administered various doses (0.5-2 g/kg p.o.) of extracts. The mice were then critically observed for clinical symptoms, behavioural changes and mortality up to 72h period following OECD guidelines No.423.[15]

Selection of doses

The effective doses of blood sugar lowering action of PMM3 was selected at 50, 100 and 150 mg per kg orally.[5-7] Diabecon® (Himalaya, India), a proprietary Ayurvedic formulation was used as prototype at the dose of 150 mg/kg orally.[16]

Streptozotocin induced Diabetes in Rats

Streptozotocin (STZ) was dissolved in ice-cold citrate buffer (0.1 M, pH 4.9) and injected intravenously through the tail vein in rats (except normal control) at the dose of 50 mg/kg.[10,17] The diabetic state was confirmed 3 day after STZ injection by measuring fasting blood glucose (one-touch Accu-chek sensor glucometer). The animals having marked hyperglycaemia (fasting plasma glucose > 200 mg/dl) were selected for the study. All the animals were treated with the following dose of PMM3 and Dibecon® (Himalaya, India) once daily for 14 days as follows:

Group	Treatment	Dose/duration
1	Normal Control	0.5% CMC, 2 ml/kg b.w. orally
2	STZ diabetes + Control	0.5% CMC, 2 ml/kg b.w. orally
3	STZ diabetes + Diabecon Tablet	150 mg/kg b.w. orally
4	STZ diabetes + PMM3	50 mg/kg b.w. orally
5	STZ diabetes + PMM3	100 mg/kg b.w. orally
6	STZ diabetes + PMM3	150 mg/kg b.w. orally

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After the experimental regimen, blood glucose was monitored 1h after last dose given as described earlier.

STATISTICAL ANALYSIS

The data generated during the study were expressed as means ± standard error of mean. The data were analyzed statistically using software based statistical package (spss version 20, IBM, USA). The percent changes were also calculated.

RESULTS

Standardization of PMM3

The physicochemical properties of test formulation PMM3 is given in Table 1. The results depicted that loss of drying was 6.69%, total ash was 8%, water soluble extractive was 12% and pH was 6.3.

Table 1: Physicochemical properties of PMM3

Parameters	Result (mean ± S.E.M)
Loss on drying (%w/w)	6.69±0.26 (6)
Total ash (%w/w)	8±0.42 (6)
Acid insoluble ash (%w/w)	1.4±0.03 (6)
Alcohol soluble extractive (%w/v)	0.6±0.008 (6)
Water soluble extractive (%w/v)	12±0.14 (6)
pH	6.3±0.001 (6)
The number in the parentheses indicate the number of samples carries out.	

The result of phyto-chemical group analysis of PMM3 are showed in Table 2. The formulated test compound, PMM3 has several bioactive groups like alkaloids, tannins, glycosides, flavonoids and phenolics.

Table 2: Phyto-chemical group Analysis of PMM3

Parameters	PMM3
Reducing sugars	-
Non-reducing sugars	+
Proteins	-
Tannins	+
Alkaloids	+
Triterpenoids	-
Glycosides	+
Flavonoids	+
Phenolics	+
Saponins	-
Sterols	-
+ present, - absent	

Table 3 represented that PMM3 has moderately high amounts of phenolics (23.18 mg GAE/g) and flavonoids (2.94 mg Q/g).

Table 3: Determination of Total Phenolics and Flavonoids in PMM3

Parameter	Results (mean ± S.E.M)
Total phenolics (mg GAE/g)	23.18±5.93 (6)
Total flavonoids (mg QE/g)	2.94±3.05 (6)
N=6 in each test; GAE = Gallic Acid Equivalent; QE = Quercetin Equivalent	

UV-V is spectral analysis of methanolic extract of PMM3 exhibited five characteristic bands at 268.2, 328.4, 333.4, 340.8 and 346.6 nm (Fig. 1), whereas water extract showed only one peak at 271.2 nm (Fig. 2). The maximum absorbance of methanolic extract of

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PMM3 was reported at 268.2 nm, more or less similar to water extract (271.2 nm).

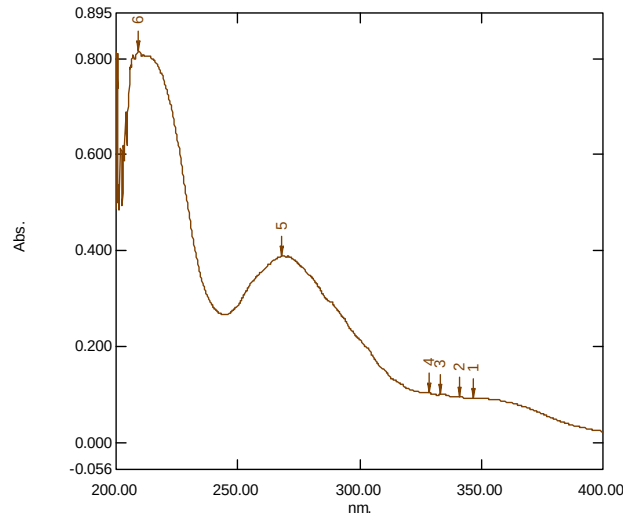

Fig. 1: UV-VIS spectrum of PMM3 (methanolic extract)

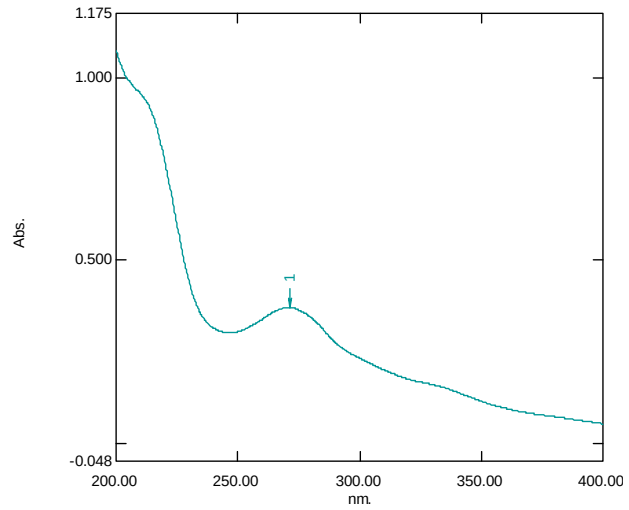

Fig. 2:. UV-VIS spectrum of PMM3 (aqueous extract)

HPTLC chromatogram of PMM3 illustrated distinct features of bioactive compounds and peaks. Fig. 3 represented HPTLC chromatogram of standard gallic acid (Rf. 0.49) and Fig. 4 for standard quercetin (Rf 0.63). PMM3 has 11 distinct peaks including gallic acid and quercetin (Fig. 5). The standard curve of gallic acid denoted the values of Y=953.6+11.77X and r^2=0.9963, whereas, for quercetin Y=-924.4+9.87X and r^2=0.99989.The concentrations of gallic acid present in PMM3 was 60.46 µg/g and quercetin was 39.34 µg/g (Table 4).

Environmental contaminants particularly heavy metals like arsenic, lead, cadmium and mercury and pesticides like DDT, BHC and Cypermethrin were examined in PMM3. The test formulation PMM3 did not cross the limit values of tested contaminants (Table 5).

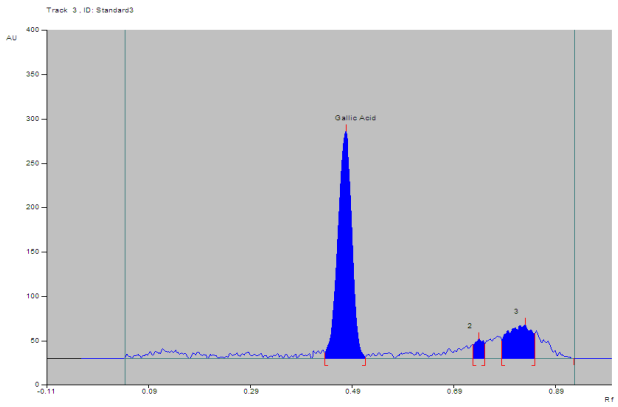

Fig. 3: HPTLC of gallic acid

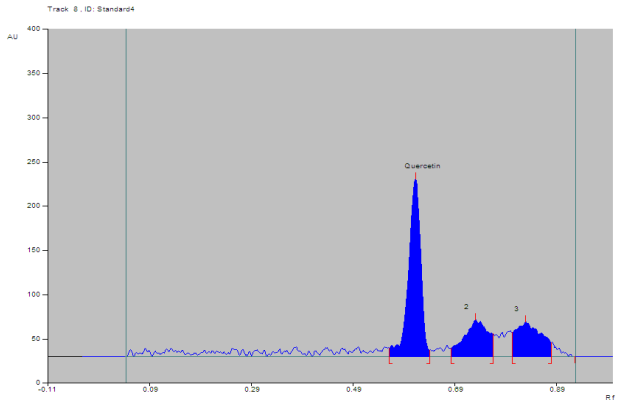

Fig. 4: HPTLC of quercetin

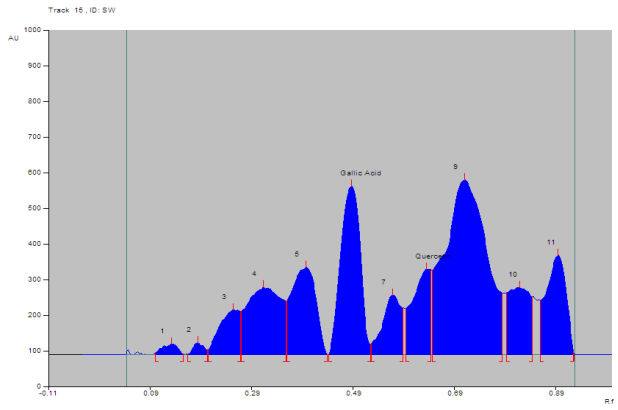

Fig. 5: HPTLC of PMM3

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Table 4: Compounds of PMM3 in HPTLC

SN	Rf (Area %)
1.	0.13 (0.75)
2.	0.18 (0.57)
3.	0.25 (4.03)
4.	0.31 (10.39)
5.	0.4 (9.57)
6.	0.49[a] (14.8)
7.	0.57 (4.89)
8.	0.63[b] (6.67)
9.	0.71 (33.05)
10.	0.82 (6.49)
11.	0.89 (8.81)
gallic acid Y=953.6+11.77X and r^2=0.9963; quercetin Y=-924.4+9.87X and r^2=0.99989. a=gallic acid 60.46±0.07 µg/g PMM3 and b=quercetin 39.34±0.09 µg/g PMM3	

Table 5: Environmental Contaminants Analysis of PMM3

Heavy Metals in PMM3	Limit	Value
Arsenic	5 ppm	<0.01 ppm
Lead	10 ppm	0.15 ppm
Cadmium	0.3 ppm	<0.01 ppm
Mercury	0.2 ppm	<0.01 ppm
Pesticides in PMM3	**Limit**	**Value**
DDT	0.01 ppm	37.057 bpm
Cypermethrin	0.05 ppm	79.720 bpm
BHC	0.03 ppm	60.264 bpm

Table 6: Effect of PMM3 on blood glucose in streptozotocin induced diabetic rats

Test Drug	Dose	Fasting blood glucose (mg/dl)		
		Day 0	Day 7	Day 14
Normal Control (without diabetes)	2 ml/kg, 5% CMC	72.1±6.96	75.9±10.75	73.1±8.05
Diabetes Control (with STZ)	2 ml/kg, 5% CMC	70.7±7.95 (a)	388.6±12.89 (a)*[411.9%]	446.1±10.09 (a)* [510.2%]
Diabetes + Diabecon Tablet	150 mg/kg	71.4±9.22 (b)	214.1±11.61 (b)* [-44.9%]	199.6±10.42 (b)* [-55.2%]
Diabetes + PMM1	50 mg/kg	70.8±8.60 (b)	276.8±10.28 (b)* [-28.7%]	258.7±8.4 (b)* [-42%]
Diabetes + PMM1	100 mg/kg	70.5±6.73 (b)	236.4±11.20 (b)* [-39.1%]	201.5±10.14 (b)* [-54.8]
Diabetes + PMM1	150 mg/kg	72.2±5.04 (b)	208.5±9.04(b)* [-46.3%]	184.8±12.82 (b)* [-58.5%]

Pharmacological Studies

Acute toxicity studies revealed that the test formulation PMM3 has safe up to 2 g/kg body weight dose in mice. Streptozotocin elevated blood glucose up to 411.9% within 7 days and 510.2% within 14 days compared to normal control rats. The standard oral herbal agent, Diabecon® reduced blood glucose 44.9% within 7 days and 55.2% within 14 days. Moreover, test formulation PMM3 exhibited dose dependent significant ($p<0.05$) anti-hyperglycaemic action, similar or even better than standard marketed herbal agent (Table 6).

Discussion

Fenugreek or *Trigonella foenum-graecum* is one of the oldest medicinal plants, originating in India, China and Northern Africa to treat diabetes. Mitra &

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Bhattacharya (2006)[18] reported that fenugreek powder showed significant anti-diabetic and dislipidaemic potentiality in Indian diabetic patients. Our previous studies reported that modified fenugreek exhibited more beneficial action on alloxan diabetes rats than fenugreek powder.[7] The bioactive water soluble anti-hyperglycaemic principle of *Tinospora cordifolia* has been identified and known as amylopectin. This principle has been shown to enhance insulin secretion and improve glucose metabolism, thereby lowering blood sugar.[19] Extracts of *Scoparies dulcis* have been also reported for anti-hyperglycaemic and antioxidant action.[20] *Adhatoda vasica* is most well known for its effectiveness in treating respiratory conditions.[21] *Cassia occidentalis* leaf has been used as a folk medicine for laxative and purgative and liver disorders.[22] In combination with purified or modified parts of these five herbs, PMM3 exhibited rich sources bioactive components, namely alkaloids, tannins, glycosides, flavonoids and phenolics. The gallic acid and quercetin are known antioxidants and uses as therapeutic agents.[10] Moreover, heavy metals and pesticide residues on PMM3 are within permissible limits.

The method of Streptozotocin (STZ) action in pancreatic β cell depletion has been studied extensively over the years. STZ radically damages pancreatic β-cells, consequential in hypoinsulinemia and hyperglycemia.[23] It is generally assumed that STZ is taken up via the cell membrane GLUT2 glucose transporter and causes DNA alkylation and eventual β cell death.[24] In this study, STZ rats showed prominent hyperglycaemia. Test formulation, PMM3 has distinct anti-hyperglycaemic properties, even better than herbal marketed formulation Diabecon®. Diabecon® is a proprietary formulation (Himalaya, India), which contain the extracts of *Balsamodendron mukul, Gymnema sylvestre, Pterocarpus marsupium, Glycyrrhiza glabra, Casearia esculenta, Eugenia jambolana, Asparagus racemosus, Boerhaavia diffusa, Sphaeranthus indicus, Tinospora cordifolia, Swertia chirata, Tribulus terrestris, Phyllanthus amarus, Gmelina arborea, Gossypium herbaceum, Berberis aristata, Aloe vera, Shilajeet* and powders of *Momordica charantia, Piper nigrum, Ocimum sanctum, Abutilon indicum, Curcuma longa, Rumex maritimus* and *Trikatu*.[18] On the basis of results obtained, it can be concluded that PMM3 has effective blood glucose reducing action and may be helpful in the therapeutic management of diabetes in near future.

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