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Development, evaluation and quality control of new antidiabetic ayurvedic polyherbal combination

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The current study is designed to developed the new polyherbal combinations of emerging medicinal plants *Morus alba* (Moraceae), *Annona squamosa* (Annonaceae), *Nelumbo nucifera* (Nelumbonoceae) and *Psidium guajava* (Myrtacea) having effective antidiabetic activity with traditionally used medicinal plants. The formulation were also subjected for the determination of quality control parameters like the heavy metal content, thin layered chromatography (TLC) and high performance thin layered chromatography (HPTLC) profile with an aim to establish their protocols. All 3 formulations, (quoted as FA, FB and FC), are prepared from 10 medicinal plants on the basis of reported mode of action of these medicinal plants used in diabetes. Two dose level 200 and 400 mg/kg of formulations were chosen to evaluate the oral glucose tolerance test (OGTT) in normal rats and antidiabetic activity in streptozotocin (STZ) (60 mg/kg) induced diabetic 'albino wistar' rats. Glibenclamide (5 mg/kg) was used as standard. Blood glucose level was measured at 0 h, after 3 h on 5, 10 and 15th day. All 3 combinations were compared on the basis of biochemical analysis (total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), urea, creatinine) of blood plasma. Blood glucose level was determined by glucometer (Escencia entrust, Bayer Health Care). Study showed that FB was found extremely significant in reducing the blood glucose level as compared to all others. FB (200 and 400 mg/kg) showed maximum reduction in average blood glucose level (BGL) to 61.2 and 64.02%, with maximum decrease in average blood glucose level to 67 and 61.4 mg/dl near to standard glibenclamide (66.2%). The results obtained herein indicate that all 3 formulations showed effective antidiabetic activity in order FB > FC > FA and FB was proved to be most beneficial combination.

Key words: Polyherbal formulation, antidiabetic therapeutics, medicinal plants, *Annona squamosa, Morus alba, Nelumbo nucifera*

INTRODUCTION

Diabetes is often considered as one of the major causes of death in developing countries. India has been considered as diabetic capital with highest number of diabetic patients in the world. "International Journal of Diabetes in

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Developing Countries" says that there is alarming rise in prevalence of diabetes which has gone beyond the epidemic form to pandemic one. The International Diabetes Federation estimated that the number of diabetes patients in India doubled from 19 million in 1995 to 40.9 million in 2007. According to their projections, the figure will rise to 69.9 million by 2025. The World Health Organization estimates that mortality from diabetes and heart disease cost India about $210 billion every year and is expected to increase to $335 billion in the next 10 years (www.HindustanTimes.htm).

Ayurveda, the Indian system of medicine recognizes diabetes since 2500 BC. Several drugs and formulations have been prescribed in Ayurveda for its treatment. Some of the plant drugs like Gurmar (*Gynmea sylvestre*) Asclepiadaceae, Vijaysar (*Pterocarpus marsupium*) Fabaceae, Jamun (*Syzium cumini*) Myrtaceae, Karela (*Momordica charantia*) Cucurbitaceae etc are still very popular as home remedies for control of diabetes. In recent years, much attention has been focused on identifying new herbal drug through the concept of polyherbalism which is also very peculiar in Ayurveda although it is difficult to explain in term of modern parameters. 'Sanghar Samhita' highlights the concept of synergism behind polyherbal formulations.

Recent study was also based on concept of polyherbalism in Ayurveda and here the fundamental aspect of designing new polyherbal combination was mode of action of chemical constituents in plants responsible for its antidiabetic activity. Some of the emerging antidiabetic plant drugs are Sharifa (*Annona squamosa)*, Amrud (*Psidium guajava*), Bimbi (*Morus alba*), Kamal (*Nelumbo nucifera*) etc. For the present study, 3 polyherbal formulations have been developed comprising of both traditionally and emerging plant drugs on the basis of mode of action of their active ingredients and their comparative antidiabetic activity was evaluated in animal models.

MATERIALS AND METHODS

Plants

Fresh leaves of *A. squamosa* (Moraceae)*, M. alba* (Moraceae)*, N. nucifera* (Nelumbonoceae)*, G. sylvestre* (Asclepiadaceae)*, P. guajava* (Myrtaceae)*, Coccinia indica* (Cucurbitaceae), fruits of *Momordica charantia* (Cucurbitaceae), fruits of *Piper longum* (Piperacea) and seeds of *Syzium cumini* (Myrtaceae), were collected from the nearby village of Barabanki (Uttar Pradesh) India and heart wood of *Pterocarpus marsupium* (Fabaceae) were procured from market at lucknow India. They were dried properly and were then authentified from National Botanical Research Institute Lucknow India. Voucher specimens no. NBRI-SOP-202 has been submitted in the institute. The plant materials were washed thoroughly with distilled water, dried, powdered and was extracted with ethanol: water (40:60) in Soxhlet. The resulted hydroalcoholic extract was filtered and concentrated under reduced pressure to give a semisolid residue.

Experimental design of polyherbal formulations

From hydro alcoholic extracts of 10 plant drugs, 3 formulations were developed (Table 1) by incorporating different extracts in such a way that it covers most of the targeted sites for better antidiabetic action and the quantity of content of formulation was based on literatures.

Experimental animals

Albino Wistar rats of same age group and body weight 150 to 200 g were selected for all the experiments. Animals obtained from Indian Toxicological Research Centre Lucknow, India, were housed in polypropylene cages at an ambient temperature of 25 to 30°C and 45 to 55% relative humidity with a 12 h each of dark and light cycle in an animal house of King George Medical College, Lucknow. Animals were fed pellet diet and water *ad libitum.* The Institutional animal ethical committee of King George Medical College, Lucknow approved the experimental protocol with reference to letter no.66/IAH/Pharma.

Acute toxicity study

Acute toxicity studies were carried out in adult female albino rats by Acute Oral Toxicity method of the Organisation for Economic Co-operation and Development (OECD) Guide line No. 423. Animals were divided into 5 groups of three animals each and were acclimatized for 5 days. Each formulation was administered orally at a dose level of 250, 500, 1000 and 2000 mg/kg b.wt to overnight fasted rats. The rats were observed for clinical signs of toxicity and death prior to dosing. The animals were observed continuously for 2 h and occasionally for further 4 h for general behavioral and finally for any mortality after 24 h till 14 days.

Selection of dose

For the assessment of antidiabetic activity two dose levels were chosen in such a way that one dose was approximately 1/10th of the maximum dose during acute toxicity studies and the other high dose was twice that of one-tenth dose (200 and 400 mg/kg).

Oral glucose tolerance test

Overnight fasted rats were weighed and divided in to four groups with 5 rats in each group for each formulation. Group I served as control and received only vehicle that is, distilled water and group II received standard drug glibenclamide (5 mg/kg b.wt), group III and IV received test formulations (200 and 400 mg/kg b.wt for each formulation). After 30 min, rats of all groups were loaded orally with glucose 2 g/kg b.wt. Blood glucose level were determined by glucometer (Escencia entrust, Bayer Health Care) before and at 30, 60, 120, 150 and 180 min after loading with glucose.

Induction of diabetes

Diabetes was induced by a single intraperitonial injection of freshly prepared streptozotocin at a dose of 60 mg/kg in normal saline to a group of overnight fasted rats. After 3 days of STZ administration, blood glucose level (BGLs) were estimated and rats having BGL > 250 mg/dl were used for study. During experiment the animals were divided into five groups with 5 animals each for each formulation (Bangar et al., 2009; Jain et al., 2013). Animals were divided in following manner for each formulation:

Group I: Normal control given distilled water.
Group II: Negative control (treated with STZ 60 mg/kg b.wt i.p).

Table 1. Design of polyherbal formulations on the basis of targeted sites of action.

Formulation	Plant added	Part used	Reported mode of action for antidiabetic activity	Quantity (mg)
A	*Annona squamosa*	Leaves	Antioxidant, increase in insulin secretion (Gupta et al., 2005)	50
	Morus alba	Leaves	a-Glucosidase and alpha amylase inhibitor (Habeeb et al., 2012; Sudha et al., 2011)	170
	Coccinia indica	Leaves	Inhibit glucose-6-phosphate (Shibib et al.,1993; Hossain et al., 1992)	50
	Nelumbo nucifera	Leaves	Increase glucose utilization (Huralikuppi et al.,1991)	50
	Gymnea sylvestre	Leaves	Act by regeneration of beta cells, retards the absorption of sugar from GIT, it increases utilization of glucose (Kanetkar et al., 2007; Baskaran et al., 1990)	130
	Piper longum	Fruits	Bioavailability enhancer (Patil et al., 2011; Myung Joo Kang et al., 2009)	50
B	*Annona squamosa*	Leaves	Antioxidant, increase in insulin secretion (Gupta et al., 2005)	60
	Morus alba	Leaves	α-glucosidase and alpha amylase inhibitor (Habeeb et al., 2012; Sudha et al., 2011)	180
	Nelumbo nucifera	Leaves	Increase glucose utilization (Huralikuppi et al., 1991)	60
	Syzium cumini	Seeds	Alpha amylase inhibitor (Sudha et al., 2011)	110
	Pterocarpus marsupium	Heartwood	Regeneration of beta cells (Chakravarthy et al., 1980)	90
C	*Nelumbo nucifera*	Leaves	Increase glucose utilization (Huralikuppi et al., 1991)	60
	Gymnea sylvestre	Leaves	Act by regeneration of beta cells, retards the absorption of sugar from GIT, it increases utilization of glucose (Kanetkar et al., 2007; Baskaran et al., 1990)	210
	Psidium guajava	Leaves	Alpha amylase inhibitor (Sudha et al., 2011)	120
	Momordica charantia	Fruit	Enhance glucose uptake in muscle tissue, Gluconeogenic enzyme inhibitor (Shibib et al., 1993).	60
	Piper longum	Fruit	Bioavailability enhancer (Patil et al., 2011; Myung Joo Kang et al., 2009)	50

Group III: Standard (Treated with Glibenclamide 5mg/kg b.wt after 3rd day of STZ injection).
Group IV: (Treated with Formulation orally dose of 200 mg/kg b.wt after 3rd day of STZ injection).
Group V: Treated with Formulation orally dose of 400 mg/kg b.wt after 3rd day of STZ injection).

Assessment of biochemical parameters

After 15th day of treatment, 2 to 4 ml blood was collected from orbital plexus of rats and allowed to clot for 30 min at room temperature. The blood samples were centrifuged at 5000 rpm for 20 min and serum was separated and analyzed for various biochemical parameters (HDL, LDL, SGOT, SGPT, TG, urea, creatinine) of blood.

Statistical analysis

The data were expressed as mean ± SEM. The data of OGTT and antidiabetic activity were analyzed by two way ANOVAs.

Quality control of developed formulations

Thin layer chromatography and HPTLC fingerprinting

For HPTLC analysis, 5 µl of the test solution and 5 µl of standard solution were loaded in the 6 mm band length in the aluminum plate coated with silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The mobile phase used for the analysis of non-polar compounds was Toluene: ethyl acetate (93: 7). The plate was dried and visualized under UV 254 and 366 nm. The plate was dipped in aniseldehyde and heated at 105°C till the spots appeared.

Safety assessment

Determination of heavy metal content by atomic absorption spectrometry (AAS): As quality control parameter, all 3 formulations (A to C) were tested for presence of heavy metal content by acid digestion method (Bushra et al., 2011; Suganya et al., 2012). Estimation of heavy metals lead, arsenic, cadmium and mercury was done by atomic absorption spectrometry in Biotech Park Lucknow, results were found within the permissible limits of WHO guidelines

RESULTS

Acute toxicity studies

All the 3 formulations treated rats showed no discernible behavior change up to 2 g/kg b.wt. No mortality was observed at any of the dose level used during 72 h observation period.

Glucose tolerance test in normal test

The plasma glucose levels of normal rats reached change peak at 30 min after the oral administration of glucose 2 g/kg b.wt and gradually decreased to average blood glucose level that is, 63 mg/dl in 180 min. It was found that FA at a dose

Table 2. Oral glucose tolerance test (blood glucose level expressed in mean ± SE).

Group	Fasting BGL	30 min	60 min	120 min	150 min	180 min
Control (2 ml/kg)	66.4±3.20	99.8±3.54	116.4±1.86	123.8±1.94	133.8±2.55	127.8±2.39
Standard (5 mg/kg)	60.4±2.94	104.4±3.61	86±3.67	74.6±2.69	64±1.97	58.8±1.95
F-A						
200 mg/kg	68.6±1.32	105.6±4.34	95.6±4.77	88.6±3.66	78.6±5.12	74.6±5.13
400 mg/kg	63.2±2.74	98.8±4.85	89.8±3.69	82.4±2.73	77±3.63	72.4±3.70
F-B						
200 mg/kg	65.6±2.44	107.2±4.55	95±5.11	87.2±4.01	81.8±3.41	75.6±2.69
400 mg/kg	64±2.42	97±4.30	86.8±3.30	80.2±3.05	73.6±3.54	68.4±3.01
F-C						
200 mg/kg	69±1.30	105.8±2.87	95.6±1.72	86.2±1.01	78.8±1.01	73.6±1.50
400 mg/kg	64.6±2.37	101.4±1.24	87.2±1.46	78±2.07	70.8±1.98	63.2±1.93

of 200 mg/kg b.wt showed maximum decrease in average blood glucose level to 10.9% at 150 min. FB with dose 200 mg/kg b.wt and 400 mg/kg showed maximum decrease to 10.2 and 10.5% at 60 min and maximum reduction to 14% was observed at 60 min with dose of 400 mg/kg of FC formulation (Table 2).

Screening of antidiabetic activity in experimentally induced diabetes rats

Glucose levels measured in blood of normal and experimental rats are given in Table 3. Streptozotocin treated diabetes rats showed significantly increased levels of blood glucose. All the 3 formulations showed a significant decrease in blood glucose in diabetic rats. All the 3 formulation showed significant reduction in blood glucose level on daily oral administration with a slight decrease in body weight. On repeated administration of vehicle, formulations or glibenclamide for 15 days, a sustained and significant ($P < 0.0001$) decrease in the average blood glucose of diabetic rats was observed in all 3 formulations.

Maximum reduction in Blood glucose level to 64.02 % was seen at 15 day after administration of 400 mg/kg of FB in a dose dependent manner as compared to the vehicle treated group. FA (200 mg/kg b.wt and 400 mg/kg b.wt) showed effective decrease in average Blood glucose level to 49.7% and 51.8 % between 0 day to 15th days with a maximum of 43.6 mg/dl and 54 mg/dl between 10 to 15 day. FB (200 mg/kg and 400 mg/kg b.wt) showed maximum reduction in average blood glucose level to 61.2 % from 0 day to 15 day and 64.02% with maximum decrease in average blood glucose level to 67 mg/dl between 10 to 15 day and 61.4 mg/dl near to standard Glibenclamide (66.2%).

FC showed greater decrease in average blood glucose level i.e. 59.8% at a dose of 200 mg/kg b.wt as compared to dose of 400 mg/kg b.wt which showed 54.8% decrease in average blood glucose level from 0 to 15 day in diabetic rats.

Biochemical parameters

Serum TG, total cholesterol and LDL-cholesterol were found to be increased significantly ($P < 0.0001$) in STZ induced diabetic rats as compared to non-diabetic control. HDL cholesterol was found to be significantly decreased in diabetic rats. Treatment with all three formulations produced a significant reduction in elevated serum TG, TC, LDL-cholesterol level in diabetic rats. Maximum decrease in average cholesterol, SGOT and creatinine level from 0 day to 15th day were found with the treatment of diabetic rats from FB at a dose of 200 mg/kg b.wt whereas maximum decrease in average TG was found with FA at a dose of 400 mg/kg b.wt. FA with dose of 400 and 200 mg/kg b.wt showed maximum decrease in average SGPT and urea and LDL cholesterol level that is, 69.8% near to glibenclamide, 43.36 and 39.6%. Interestingly, it was found that FB with dose of 200 mg/kg showed maximum increase in HDL level from 0 to 15th day that is, 45.04% same as that of glibenclamide (Table 4).

Assessment of TLC and HPTLC fingerprinting

All the 3 formulations were subjected to HPTLC analysis by the solvent system Toluene: EAA (93:7) at biotech park Lucknow and detected under UV 254 and 366 nm (Figure 1.1, Plate A). It is evident from Table 5 that in formulation A, there are 8 spots at the following 0.03, 0.12, 0.16, 0.22, 0.25, 0.29, 0.84, 0.91 as shown in (Figure 1.1) indicating the occurrence of at least 8 components in

Table 3. Comparison of antidiabetic effects of formulation A, B and C on blood glucose level in STZ induced diabetic rats (long term study of 15 days daily once).

Group	Fasting blood glucose level (mg/dl)	After 3rd day of STZ injection (0 h) (mg/dl)	After 3 h (mg/dl)	Day 5th (mg/dl)	Day 10th (mg/dl)	Day 15th (mg/dl)
Normal control	69.6±3.94	77.6±2.99	77.4±2.15	76.4±1.07	78.0±1.14	80.2±1.35
Diabetic control	68.2±2.15	307.4±7.31	308±7.31	327.2±7.84	343.6±6.20	359.4±5.99
Standard glibenclamide	66.8±2.22	294.6±16.87	266.8±15.33	212.4±13.51	153.8±11.59	99.4±5.37
Test F-A 200 mg	78.8±3.21	286.2±9.12	272.4±8.26	230.4±5.20	187.4±2.67	143.8±5.18
Test F- A 400 mg	64.0±2.16	290.4±9.21	276.4±9.19	236.8±7.84	193.8±6.88	139.8±4.43
Test F- B 200 mg	63.8±3.74	321.8±19.64	302.0±18.58	247.8±16.35	191.6±13.22	124.6±6.72
Test F-B 400 mg	71.6±2.37	300.2±12.61	278.4±12.54	225.6±10.40	169.4±8.33	108.0±2.74
Test F-C 200 mg	71.2±3.96	274.2±8.89	257.8±8.51	213.8±6.87	163.8±5.45	110.0±3.70
Test F-C 400 mg	74.2±3.15	295.6±5.93	283.8±7.83	238.4±5.79	188.8±3.97	133.4±2.69

P values < 0.0001 Values are Mean ± SE from 5 animals in each group and are significantly different from control, diabetic control.

Table 4. Effect of formulations on biochemical parameters of blood.

Parameter	Normal Control		Diabetic control		Standard glibenclamide		Test A 200 mg		Test A 400 mg		Test B 200 mg	
	0 Day	After 15 day	0 Day	After 15 days	0 Day	After 15 days	0 Day	After 15 days	0 Day	After 15 days	0 day	After 15 days
Cholesterol	52.7±2.65	52.66±2.25	95.24±4.79	112.78±6.20	107.08±6.49	48.3±3.14	101.4±5.70	75.96±5.24	103.8±6.10	65.64±5.47	114.54±6.67	70.36±6.79
Triglycerides	66.68±3.03	68.1±3.51	112.06±14.27	146.62±17.53	133.9±10.81	66.42±4.32	121.48±5.13	85.25±4.57	118.94±6.42	67.62±6.47	132.48±11.00	86.98±11.53
SGOT	20.36±3.77	19.54±3.88	59.88±4.15	70.28±4.13	54.54±3.96	19.26±2.34	52.34±2.29	26.28±3.77	51.52±2.98	22.88±3.22	62.00±2.31	26.48±2.45
SGPT	23.5±2.91	26.26±2.64	63.6±2.35	77.34±3.12	70.42±1.00	20.76±1.32	74.44±5.24	34.6±5.28	69.7±3.50	21.04±1.38	67.02±4.46	31.76±4.85
Creatinine (mg/dl)	0.63±0.12	0.71±0.11	1.55±0.03	1.78±0.04	1.71±0.07	0.93±0.10	1.67±0.03	1.36±0.08	1.70±0.06	1.23±0.16	1.69±0.06	0.802±0.08
Urea (mg/dl)	26.9±1.48	27.48±1.40	74.54±9.17	82.08±7.57	80.0±4.15	27.1±2.83	75.08±2.26	42.52±2.22	74.72±2.89	37.9±3.81	87.66±7.73	61.02±8.89
HDL (mg/dl)	37.32±1.34	38.34±1.24	22.08±2.06	13.5±1.41	20.82±2.46	38.18±1.12	20.54±1.17	26.8±1.75	26.16±3.69	33.24±3.37	18.08±2.26	32.9±1.90
LDL (mg/dl)	25.72±1.57	27.32±2.17	67.2±3.95	72.6±3.55	62.16±2.45	25.00±1.42	69.12±2.20	51.18±3.38	63.06±2.90	38.04±3.20	69.54±4.19	47.56±3.64

Parameter	Normal Control		Diabetic control		Standard glibenclamide		Test B 400 mg		Test C 200 mg		Test C 400 mg	
	0 Day	After 15Day	0 Day	0 Day	After 15 Day	0 Day	After 15 Day	0 Day	After 15 days	After 15 days	0 Day	After 15 days
Cholesterol	52.7±2.65	52.66±2.25	95.24±4.79	105.28±6.76	72.46±6.48	114.14±5.15	92.38±4.95	105.08±7.31	84.02±5.25	112.78±6.20	107.08±6.49	48.3±3.14
Triglycerides	66.68±3.03	68.1±3.51	112.06±14.27	127.46±9.11	94.64±8.74	140.72±8.13	119.64±5.98	131.96±12.38	111.98±10.84	146.62±17.53	133.9±10.81	66.42±4.32
SGOT	20.36±3.77	19.54±3.88	59.88±4.15	65.38±3.34	40.72±3.48	61.86±8.38	41.68±7.99	63.96±7.04	49.6±9.18	70.28±4.13	54.54±3.96	19.26±2.34
SGPT	23.5±2.91	26.26±2.64	63.6±2.35	70.12±3.69	45.66±4.11	91.36±6.90	77.58±7.85	88.06±4.09	70.82±3.62	77.34±3.12	70.42±1.00	20.76±1.32
Creatinine (mg/dl)	0.63±0.12	0.71±0.11	1.55±0.03	1.658±0.04	0.984±0.06	1.58±0.02	0.926±0.06	1.622±0.03	1.25±0.15	1.78±0.04	1.71±0.07	0.93±0.10
Urea (mg/dl)	26.9±1.48	27.48±1.40	74.54±9.17	94.76±5.61	77.9±5.37	140.12±13.0	84.04±9.82	65.8±5.22	52.76±5.20	82.08±7.57	80.0±4.15	27.1±2.83
HDL (mg/dl)	37.32±1.34	38.34±1.24	22.08±2.06	20.14±1.46	28.98±1.24	21.8±2.31	26.48±2.13	16.42±1.66	28.1±1.76	13.5±1.41	20.82±2.46	38.18±1.12
LDL (mg/dl)	25.72±1.57	27.32±2.17	67.2±3.95	75.44±3.85	65.56±3.97	78.46±2.75	61.18±7.55	94.88±2.92	79.36±4.00	72.6±3.55	62.16±2.45	25.00±1.42

P values: <0.0001 significantly different from control, diabetic control. Values are mean ± SE from 5 animals in each group.

Table 5. Chromatogram analysis of FA.

Peak	Start R_f	Start Height	Max R_f	Max height	Max (%)	End R_f	End height	Area	Area (%)	Assigned substance
1	0.01	0.1	0.03	291.4	42.21	0.07	0.5	5879.6	44.00	Unknown*
2	0.10	0.3	0.12	98.8	14.30	0.14	1.3	1419.2	10.62	Unknown*
3	0.14	1.4	0.16	30.6	4.44	0.18	0.1	502.7	3.76	Unknown*
4	0.19	0.3	0.22	21.9	3.17	0.23	9.5	454.3	3.40	Unknown*
5	0.23	9.6	0.25	34.5	4.99	0.27	18.5	664.2	4.97	Unknown*
6	0.27	18.9	0.29	185.9	26.92	0.32	9.1	3659.0	27.38	Unknown*
7	0.81	5.3	0.84	13.4	1.93	0.85	12.9	358.3	2.68	Unknown*
8	0.90	12.6	0.91	14.1	2.04	0.95	5.0	425.6	3.18	unknown*

Table 6. Chromatogram analysis of FB.

Peak	Start R_f	Start Height	Max R_f	Max height	Max (%)	End R_f	End height	Area	Area (%)	Assigned substance
1	0.01	0.1	0.03	291.4	42.21	0.07	0.5	5879.6	44.00	Unknown*
2	0.10	0.3	0.12	98.8	14.30	0.14	1.3	1419.2	10.62	Unknown*
3	0.14	1.4	0.16	30.6	4.44	0.18	0.1	502.7	3.76	Unknown*
4	0.19	0.3	0.22	21.9	3.17	0.23	9.5	454.3	3.40	Unknown*
5	0.23	9.6	0.25	34.5	4.99	0.27	18.5	664.2	4.97	Unknown*
6	0.27	18.9	0.29	185.9	26.92	0.32	9.1	3659.0	27.38	Unknown*
7	0.81	5.3	0.84	13.4	1.93	0.85	12.9	358.3	2.68	Unknown*
8	0.90	12.6	0.91	14.1	2.04	0.95	5.0	425.6	3.18	Unknown*

Table 7. Chromatogram analysis of FC.

Peak	Start R_f	Start Height	Max R_f	Max Height	Max (%)	End R_f	End Height	Area	Area (%)	Assigned substance
1	0.01	5.8	0.03	196.7	62.04	0.07	0.2	3802.6	59.39	Unknown*
2	0.07	0.3	0.10	59.2	18.67	0.13	2.0	976.9	15.26	Unknown*
3	0.13	2.1	0.15	11.7	3.69	0.17	0.5	210.5	3.29	Unknown*
4	0.26	2.2	0.29	24.5	7.72	0.31	1.3	453.3	7.08	Unknown*
5	0.90	21.1	0.91	25.0	7.87	0.96	5.1	959.2	14.98	Unknown*

Table 8. Concentration of heavy metal content in all polyherbal formulation.

Formulation code	Result (µg/l)			
	Pb	Cd	Hg	As
FA	1.7	Not detected	Not detected	Not detected
FB	3.6	Not detected	Not detected	0.2
FC	4.0	Not detected	Not detected	Not detected

formulation A. It is also clear from the Table 5 and chromatogram as shown in Figure 1.1 that out of 8 components, the components with Rf (0.03, 0.12 and 0.29) at 366 and 254 nm were found to be more predominant as percentage area is more with 44, 10.62 and 27.38%, respectively and remaining component were found to be in less quantity as the percentage area for all spots were less. From Table 6, it was found that in Formulation B there are 10 spots at following Rf (0.01, 0.05, 0.08, 0.11, 0.16, 0.21, 0.29, 0.33, 0.71 and 0.99), as shown in track 2, Figure 1.2, indicating the occurrence of at least 10 components in the formulation. It is clear from Table 8 and chromatogram (as shown in Figure 1.2, Plate A) that out of 10 components, the component with

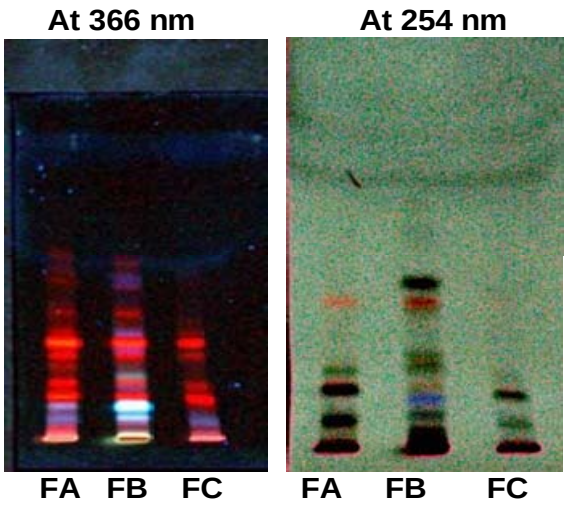


Figure 1. TLC chromatogram of polyherbal formulations (FA, FB and FC).

Rf value 0.08 and 0.29 at 366 and 254 nm were found to be more predominant as the percentage area is more with 24.95 and 42.23% and remaining component were found to be of very less quantity. Table 7 indicate that in formulation C there are 5 spots with R_f (0.03, 0.10, 0.15, 0.29, 0.91) as shown in track 3, Figure 1.3, indicating the occurrence of at least 5 components predominantly. Out of 5 components, components with R_f 0.03 and 0.10 and 0.91 at 366 and 254 nm were found to be more predominant as shown in the chromatogram (Figure 1.3, Plate A), the percentage area is more with 59.39, 15.26 and 14.98% and remaining components were found to be very less in quantity as the percentage area for all the spots less than 7%. One more solvent (Ethyl acetate: Glacial acetic acid: formic acid: Water) (100:11:11:25) was tried for the analysis of Flavonoids in the formulation.TLC of all formulations were performed in (EAA: Glacial acetic acid: Formic acid: Water) it was observed in UV chamber under 366 nm and it was that TLC of formulation A showed 3 spots at 0.53, 0.61, 0.77. Out of the 3 spots, the spot at R_f 0.53 corresponding to standard R_f s shown in Figure 2.

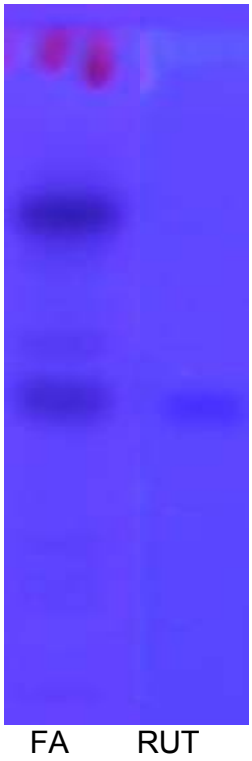


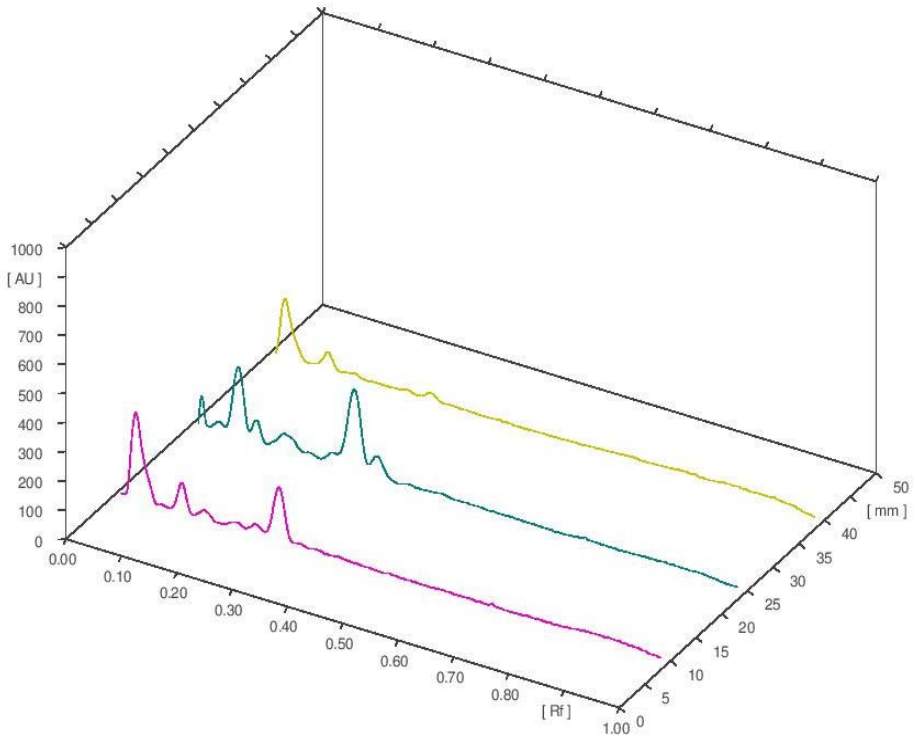
Figure 2. TLC of Formulation A with standard rutin under UV 366 nm.

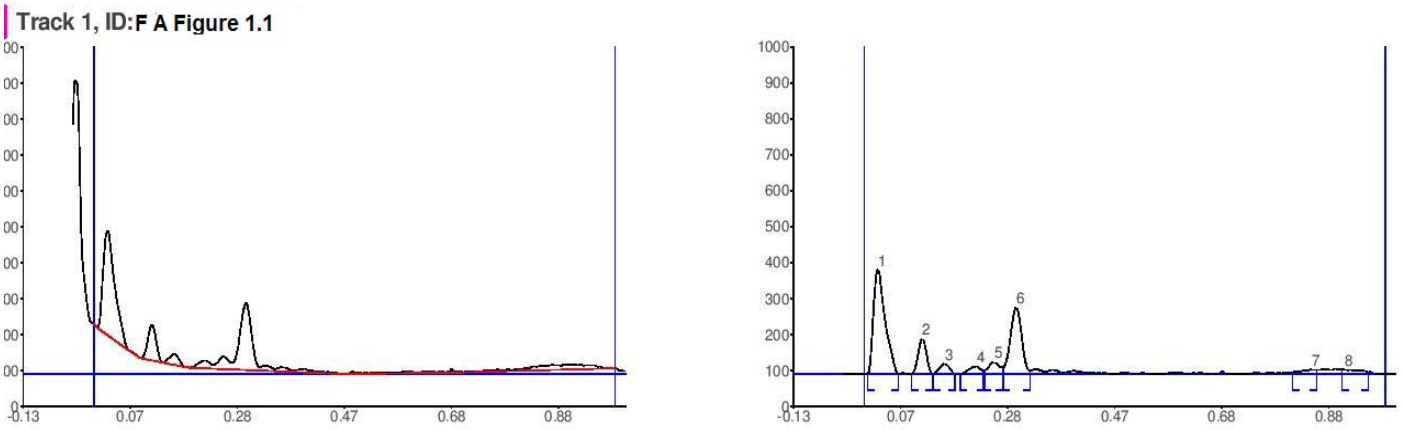
Assessment of heavy metal content

The content of heavy metal lead (Pb), arsenic (As), cadmium (Cd) and mercury (Hg) in ayurvedic formulation (A to C) was found to be within permissible limits as laid down by Guidelines for Quality standardized herbal formulations, WHO (2004) as mentioned in Table 10.

DISCUSSION

A large number of plants drugs and their preparations are used globally for the treatment of diabetes from centuries. In Ayurveda, the Indian system of medicine, which dated back to 2500 BC, a number of plant drugs like Vijaysar (*Pterocarpus marsupium*), Jamun (*Syzium cumini*), Karela (*Momordica charantia*), Gurmar (*Gynmea sylvestre*),





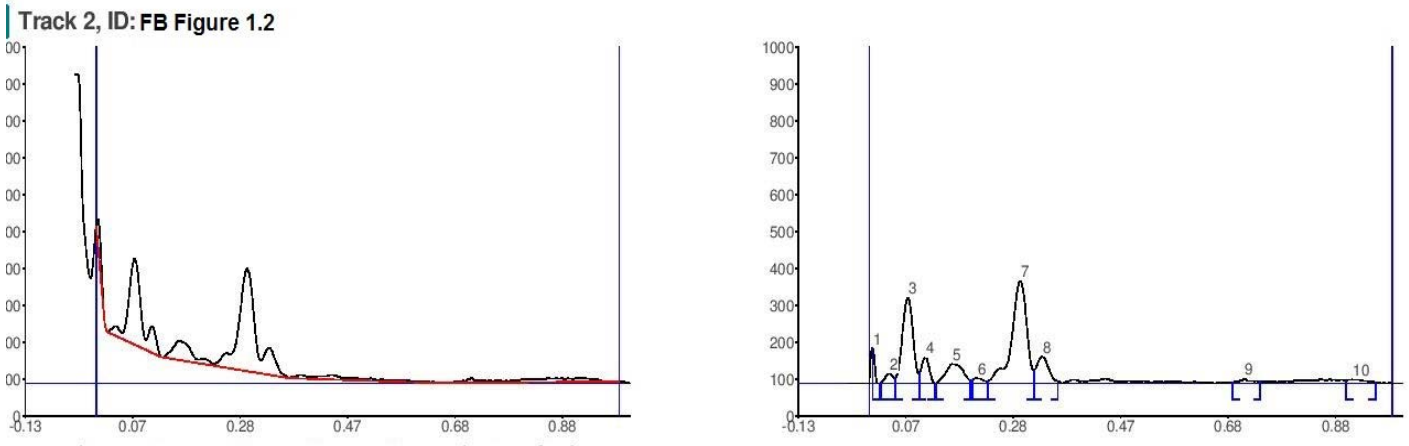


Plate A. TLC and HPTLC fingerprinting.

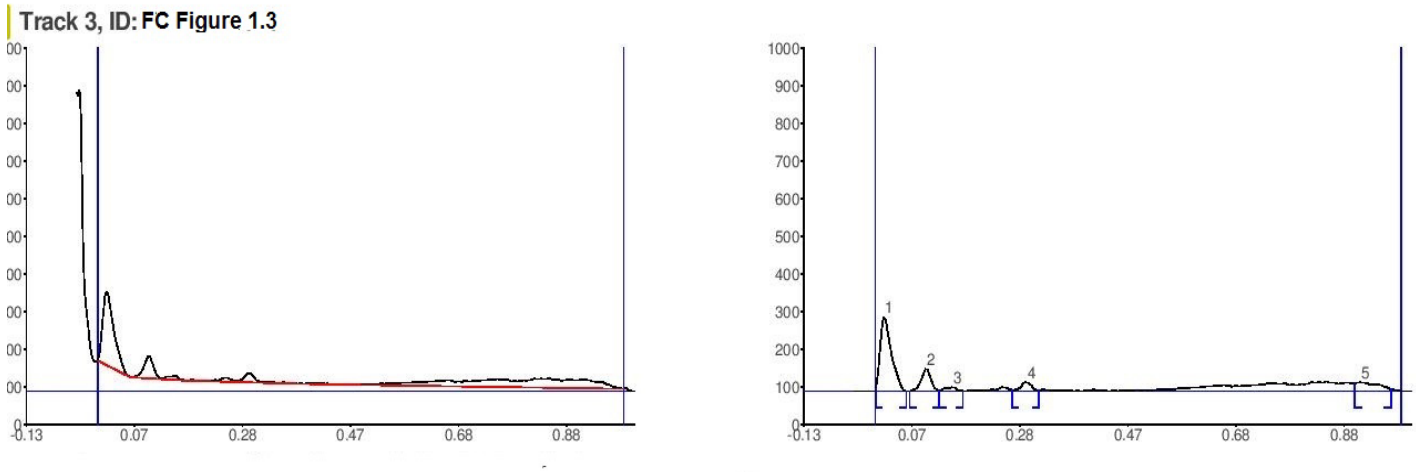


Plate A. Contd.

Kunduru (*Coccinia indica*) have been prescribed for the purpose and most of them are still being used as common home remedies for the treatment of diabetes. With the effort of several scientists a number of plant drugs which may be categorized as emerging anti-diabetic plants have been identified and mode of action evaluated. For the present study, plant drugs which are traditionally used as a home remedies and emerging plants have been selected on the basis of different mode of actions for developing new combinations with enhanced antidiabetic activity.

All 3 formulations (FA, FB and FC) have been developed with the combination of traditional and emerging antidiabetic medicinal plants on the basis of their mode of action to achieve the best synergistic antidiabetic combination, out of which FA and FB contain mixture of emerging antidiabetic plants with traditional plants (both the formulation contains different combination of same emerging plants with different traditional plants) and FC contains the mixture of traditional plants. Their antidiabetic activity was evaluated in albino rats using glibenclamide as standard. STZ induced diabetic model proved to be useful to study the comparison of their ability to reduce blood glucose level with improvement in loss of body weight. The animals were divided into 5 groups with 5 animals each. The treated group received formulations FA, FB and FC in 2 dose level for each formulation (200 and 400 mg). Although all 3 showed the good antidiabetic activity but FB was found to be best in these combinations. FB (200 and 400 mg/kg b.wt) showed maximum reduction in average BGL to 61.2% from 0 to 15th day and 64.02% with maximum decrease in average BGL to 67 mg/dl between 10 to 15th day and 61.4 mg/dl near to standard glibenclamide (66.2%). Interestingly it has been noted that with increase in dose of FC from 200 to 400 mg/kg b.wt, the average decrease in blood glucose level is decreased, so it is concluded that FC with 200 mg/kg b.wt is more potent in reducing the blood glucose level than 400 mg/kg b.wt. FB contain emerging medicinal plants *Annona squamosa, Morus alba, Nelumbo nucifera* combined with *Syzium cumini* and *Pterocarpus marsupium* which are traditional antidiabetic medicinal plants. So this combination proved to be successful antidiabetic combination with synergistic effect as compared to others. FA also contain emerging antidiabetic medicinal plants *A. squamosa, M. alba* and *N. nucifera* with *Coccinia indica, Gymnea sylvestre* and *Piper longum* but the combination was not proved to be as effective as FB and FC.

Many plants have been reported to contain substances like glycosides, alkaloids, terpenoids, flavonoids and tannoids etc. which have been proved to be of antidiabetic action by their different mode of action. *A. squamosa* is one of emerging antidiabetic medicinal plant which act on more than one site namely pancreas, muscle tissue, intestine (uptake of glucose through specific receptor) through number of pathways. This plant drug proved to be having oxygen radical scavenging activity, inhibit glucose-6-phosphate, gluconeogenic enzymes and increase the insulin secretion from β cell of pancreas, *M. alba* is known to possess α-amylase inhibitory activity, so these increased glucose disappearance rate may be due to the increased insulin release by regeneration of β cells of pancreases; apart from regulation of carbohydrate metabolism these combination also showed to maintain the metabolism of lipids.

It has been found that diabetic rats showed increase in the production of TG, TC, LDL-cholesterol which correlates with earlier findings where there is an increase in lipid levels and is observed not only in diabetic animals but also in diabetic patients (Kalita and Chakravarty, 2011). All formulation produced significant decrease in hypercholesterolemia and hypertriglyceridemia in diabetic rats. The possible mechanism for decreased lipid levels could be either insulin releasing effect or insulin sensitizing activity because insulin has been proved to inhibit in

adipose tissue and suppress the release of lipids (Kavishankar et al., 2011). The main function of HDL-cholesterol is to transport the cholesterol from peripheral tissues to liver and thereby it acts as a protective factor.

All 3 formulations proved to be effective in increasing the lower HDL level in diabetic rats. This shows that combination may help to increase the transport of peripheral tissue cholesterol to liver and thereby decrease the blood cholesterol. In the present study, it was noted that FB with dose of 200 mg/kg b.wt increase the HDL cholesterol level same as that of glibenclamide. Diabetic rats showed an increase in serum urea and creatinine levels which may be due to hyperglycemia that causes osmotic dieresis and depletion of extracellular fluid volume. Study indicated that FB showed maximum decrease in average creatinine level and FA showed decrease in blood glucose level and hence decreased the osmotic dieresis and depletion of extracellular fluid volume. Earlier studies reported that oxidative stress is one of the complications associated with diabetes and may be due to hyperglycemia which increases the oxygen free radicals in the body due to reduction in anti-oxidative enzymes like SGOT, SGPT observed in diabetic rats (Kalita et al., 2011; Kavishankar et al., 2011; Guerci et al., 1999; Loci et al., 1994; Karthic et al., 2008). Treatment with all 3 formulations significantly increases the SGOT and SGPT levels to maximum with FB and FA.

Although all 3 formulations showed antidiabetic, antihyperlipedemic and antioxidant activity but best synergistic effect was observed with FB > FC > FA which may be due to the presence of active principles having different mode of action through different pathway with different site of action like pancrease, liver and intestine. Therefore the idea for the developing the formulation on the basis of mode of action of medicinal plants were proved to be beneficial. In standardization of modern formulations, however, the concentration of heavy metals may be excessive because poor quality control allows for contamination, adulteration or improper purification. The Government of India, Department of AYUSH, Ministry of Health and Family Welfare has issued new safety standards. There is the mandatory testing for heavy metals in every batch of ayurvedic herbs manufactured by all licensees for all exporting purposes. The permissible limit of heavy metals in Ayurveda with herbal ingredients is lead: 10, cadmium: 0.30, arsenic: 10 and mercury: 1 ppm. In all formulations, the levels of heavy metals were found to be within the prescribed limit. The preliminary HPTLC analysis of all formulation shows the presence of many phytochemicals, the separation, identification and characterization of the bioactive compound from the plants is to be evaluated and reported in near future.

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