SYNERGISTIC ANTI-DIABETIC EFFECTS OF COMBINED ETHANOLIC PLANT EXTRACTS, *Verbascum thapsus*, *Trigonella foenum graecum*, *Ficus semicordata* AND *Cocos nucifera* (VTFC), IN STREPTOZOTOCIN INDUCED DIABETIC RATS: A POLYHERBAL COMBINATION

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DOI: 10.47750/pnr.2023.14.S01.132

Abstract

Objective: To evaluate synergistic antidiabetic effects of combination of ethanolic plant extracts, *Vervascum thapsus* (Leaves), *Trigonella foenum graecum*(Seeds), *Ficus semicordata* (leaves) *and cocos nucifera* (Husk) in streptozotocin induced diabetic rats *Verbascum thapsus* (Mullein*), Trigonella foenum graecum* (Fenugreek or Methi), *Ficus semicordata* (Drooping fig) and *cocos nucifera* (Nariyal) are traditionally used in the treatment of various disease including diabetes mellitus. The anti-diabetic activity of each individual plant species is well documented, but their combined or synergistic effect is unknown, so current interest is to enhance therapeutic action and reduce the concentration of single herb, thereby decreasing adverse effects. Current study is evaluated the hypoglycaemic activity of the combined mixture of ethanol extract of Leaves of *Vebascum thapsus*, Seeds *of Trigonella foenum graecum*, leaves of *Ficus semicordata* and husk of *coconut* (VTFC) at different doses in streptozotocin induced diabetic rats. Diabetic rats were treated with combined ethanolic extracts of VTFC at doses 810 mg for 300 g rat (112.50 mg AEVT, 600 mg AETFG, 22.50 mg AECNH, 75 mg AEFS) calculated by design-expert software by using Box Behnken design per day or glibenclamide 5 mg/body weight for 7 days. VTFC extracts significantly lowered elevated fasting blood glucose level. The results were nearly equipotent with glibenclamide, so it is suggested that in addition to its hypoglycaemic activity VTFC protects liver, kidney blood, pancreas against diabetic injury. This is the first synergistic study of selected plant species to evaluate possible role for this mixture in treatment of diabetes.

Keywords:*Antidiabetic, polyherbal, Verbacum thapsus, Trigonella foenum graecum, Ficus semicordata, Cocos nucifera* husk

Introduction

Diabetes mellitus is a well-known chronic disease caused by low production of insulin by β cells of pancreas and a decrease in glucose absorption by the cells, causing elevation in the concentration of glucose in the blood. Diabetes is very speedily increasing in developing countries of the whole world due to which mortality and morbidity occurs in these countries.[1]hyperlipidemia, disorder in liver functions, renal dysfunctions are the complications of untreated diabetes mellitus.[2]May plants play an important role as hypoglycaemic agents due to less side effects and low cost however many traditional anti-diabetic plants awaiting poor scientific and medicinal evaluation for their ability to improve glucose metabolism.[3]

Verbascum thapsus (Family Scrophulariaceae) also known as common Mullein is a famous herbaceous plants that is distributed all over the Europe, North America, temperate regions of Asia. Numerous pharmacological activities has been reported in common mullein such as anti-infammatory, antiviral, antimicrobial, anticancer, a antihyperlipidemic and antiheppatotoxic.[4]Ethanolic extract of leaves of *Verbascum thapsus* showed anti-diabetic activity in alloxan induced diabetic rats.[5]As a herbal remedy for bronchitis, whooping cough, dry cough, tuberculosis, and asthma since ancient times, *Verbascum thapsus* is used, which is also known as woody mullein. The herb is also somewhat diuretic. Pneumonia, fever, allergies, and migraines may all be treated at home with this herbal cure.[6]

Trigonella foenum graecum (Fenugreek) is an annual herb belonging to the family Fabaceae. It is distributed to North America, South eastern Europe and central asia.[7]It is a traditional medicinal herb grown in India that has been used for thousands of years. This plant's numerous biological activities have been extensively studied.[8]Aqueous extract of *Trigonella foenum graecum* seeds showed anti-diabetic activity in alloxan induced type 2 diabetic rats.[9]hypocholeteromic and anti-diabetic activity.[10,11,12]In the United States and across the globe, it is a common medicine used as a nutraceutical.[13]

Ficus semicordata (Family-Moraceae), commonly known as bhue goolar, khaina, and khanayo, is a kind of fig. More than 750 species of trees, shrubs, and climbers are found in the tropical and subtropical regions of the world.[14]India it is distributed in sub-Himalayan forest from Manipur to Chenab, Udisha, West Bengal, Central India, Chota Nagpur. It is also distributed in Myanmar, Bangladesh.[15]Ethanol extract reported to have anidiabetic activity in streptozotocin induced diabetes mellitus.[16]Various specieses of Ficus showed anti-diabeitc, antioxidant ,antileprosy, anti ulcer activities etc.[17]

As a result of its wide range of medicinal uses, the coconut (*Cocos nucifera*) is sometimes referred to as the "tree of life." Many authors have reported it periodically to see whether it has biological effects or chemical constituents[18].*Cocos nucifera* belongs to family Arecaceae, due to considerable linking between polysaccharide's, lignins and phenolics. Mesocarp becomes fibrous and hard. In northeastern Brazil coconut husk have been traditionally used to cure arthritis and diarrhea. Antiviral, free radical scavenging activity, antibacterial activity, antileshmaniasis and antinociceptive activities has been reported in aqueous extract of coconut husk.[19]Aqueous extract of coconut husk showed antidiabetic activity in Alloxan induced diabetic rats.[20]

Due to effectiveness of the various parts of the above crude drugs for diabetes, it is proposed to formulate a combination of above four and evaluate the efficacy of combined drugs (Ethanolic extracts of VTFC) with reference to glibenclamide.

Combination of *Allium sativum*, *Gingiber officinale* and *Capsicum frutescens* evaluated for antidiabetic activity in alloxan induced diabetic rats and found to be equipotent with glibenclamide.[21] The polyherbal formulatins controlled diabetes instead of single drug due to synergistic effects and minimal side effects. It has been observed that synergistic effects can be produced by mixing medicinal plant material in complex polyherbal formulation enhance the bioavailability of active ingredients, increase the therapeutic effects and decrease toxicity.[22,23,24]

Materials and methods

Drugs and chemicals

Streptozotocin was purchased from Sisco research laboratories Pvt. Ltd Mumbai India, while glibenclamide was a product of advacare Pharma. Autoanalyzer used was a product of Aspen Diagnostics Pvt Ltd. All other chemicals and reagents were purchased from CDH Pvt, Ltd. Delhi.

Plant material

The plant materials were collected and purchased from various suppliers as well as various locations from India and identified and authenticated by Dr. Sunita Grarg (Former chief scientist, heard RHMD) and Mr. R.S.Jayasomu (Chief scientist, head RHMD) CSIR-NIScPR Delhi. Plant materials were separately screened for any foreign particles, dried and finely powdered with the help of grinder. The dried, powdered plant materials after defating with petroleum ether was subjected to successive solvent extraction with benzene followed by ethyl alcohol (90%) till solvent becomes colorless. All the extracts separately concentrated by rotatory evaporator and stored at -20 °C until use.

Box Behnken design for the optimization of polyherbal combination

Independent and dependent constraints for the optimization of polyherbal combination

Statistical-based mathematical models are commonly used for the optimization purpose. Therefore, it is necessary to explore the independent constraints which affect the desired properties of developed system (like dose or other characters)[45,46].Here, dose of the poyherbal combination was optimized by four factors-three levels of Box-Behnken design (BBD) using response surface methodology (Box-Behnken/BBD). The independent constraints were dose (mg) of four different extract like AEVT (A), AETFG (B), AECNH (C) and AEFS (D). The effect dose of these four extracts was observed on dependent constraints i.e. glucose concentration (mg dL-1) in blood (Y).The mathematical modeling was resultant of computer simulation programming with Design of Experiment software (version 8.0.6, State-Ease Inc., Minneapolis, USA). The models were accessible by 3D surface response graphs/ contour graphs indicating the relation between independent and dependent constraints. The best-fitted model was selected based on the value of R^2 with probability limits of the P value <0.05. The desirability function for concentration of blood glucose (mg dL-1) was possible minimum level, of optimize the polyhedral composition. The polynomial equation was also established to check the effect, interaction, and quadratic effect of independent variables on response.

Y (blood glucose concentration): $\beta 0+ \beta 1A+ \beta 2B+ \beta 3C+ \beta 4D + \beta 12AB+ \beta 13AC+ \beta 14AD + \beta 23BC + \beta 24BD+ \beta 34CD+ \beta 11A^2+ \beta 22B^2 +\beta 33C^2 +\beta 44D^2$ (1)

Here, Y is the response, β0 is constant, β1, β2, β3 and β4 are the linear coefficient, whereas β12, β13, β24 and β23 are the interaction coefficient two independent variables each time and β11, β22, β33and β44 are the quadratic coefficient observed values while A, B, C, D are the values of independent variables as shown in table 1.

Optimization by numerical optimization technique

All responses were optimized by numerical optimization i.e. desirability approach. In this approach a specific goal was assigned to each response as shown in table 1. The desirability function value of zero indicated the unacceptable response while a value >0-1 represented the closeness of the response to its target value. To confirm and validate the response surface methodology, an optimized formulation was designed and the prediction error was calculated by using the following equation.[47]

$$\text{Prediction error (\%)} = \frac{\text{Predicted value} - \text{Observed value}}{\text{Predicted value}} \text{x100} \quad (2)$$

In the desirability function approach, the desirability function was calculated. The best formulation with the best desirability function, fulfilling the maximum requirement of response variables was selected.

Preparation of selected drugs mixture

Mixer was developed by combining the dried ehanoilc extracts of the plant material based on Design of Experiment software (version 8.0.6, State-Ease Inc., Minneapolis, USA) by using three level and four factor Box Behnken design (BBD). Calculated doses of VTFC was 810 mg (112.50 mg AEVT, 600 mg AETFG, 22.50 mg AECNH, 75 mg AEFS).

Experimental animals

Male albino wistar rats (300-350 gm) were purchased from IVRI Bareilly, Uttar Pradesh India. The animals were housed in cages which were specially designed for rats. The temperature of animal house was maintained at 22±2 °C. All rats given free access to drinking water *ad libitum* and normal rat pellets. The study was approved by the institutional animal ethical committee of School of pharmacy, Bharat Institute of Technology, Meerut, U.P. India. All animals were acclimatized to experimental environment for one week before the study.

Diabetic Induction

Rats were fasted for sixteen hours, then a freshly prepared single intraperitoneal injection of Streptozotocin (55mg/kg body weight)[9] prepared in ice cold citrate buffer having 0.1 molarity and pH 4.5 was given to rats. The rats were allowed to drink 1% glucose solution immediately after the injection to prevent hypoglycemia condition. The normal saline alone was injected to control rats. After forty eight hours of the injection, fasting blood glucose level was estimated by using a glucometer (Accu-chek, manufactured by Roche Diabetes care Pvt. Ltd. Mumbai Suburban-400086, Maharashtra India). Rats with glucose levels greater than 139 mg/ dl were considered as diabetic and taken for the study.

Experimental design

The rats were divided in to 4 groups of six rats each

Group 1: Normal Control rats administered with 1 ml distilled water once daily for 7 days.

Group 2: Diabetic control rats administered with 1 ml distilled water once daily for 7 days

Group 3: Diabetic rats were treated with mixture of ethanolic extracts of VTFC (810 mg for 7 days).

Group 4: Diabetic rats treated with glibenclamide (5 mg/kg body weight) for 7 days.

The extracts and drug administered orally once daily for 7 days. After seven days rats were fasted for twelve hours and anaesthetized by intraperitonial injection of Ketamine hydrochloride (87 mg/kg body weight) as per guideline given in annexure 5 of CPCSEA . The blood was collected by cardiac puncture for biochemical assays. Then dissections of rats were done and kidney, pancreas, liver, heart and lungs were removed washed with cold saline and weight of each organ was noted.

Measurment of fasting blood glucose level in Rats

Fasting blood glucose levels was measured by using a glucometer throughout the experiment (0, 1, 3, 5 and 7 days). The blood samples were taken site was tail vain of Rats.

Measurement of hematological parameters

The Auto Analyzer (ASPEN Diagnostics PVT.LTD) was used to estimate RBC count MCV (mean corpuscular volume), hemoglobin, MCH (mean corpuscular hemoglobin, MCHC (mean corpuscular hemoglobin concentration). Other parameters were estimated platelets, WBC (white blood cells and white blood cells differential counts.

Biochemical analysis

The serum triglycerides (TG), total cholesterol, HDL-C (high density lipoprotein cholesterol), LDL-C (low density lipoprotein Cholesterol), total protein, albumin levels, as well as concentrations of urea, creatinine, calcium and globulin were measured by using Auto Analyzer.

The activities of Aspartic aminotransaminase (AST), alkaline phosphate (ALP), Alanine aminotransaminse (ALT),CK(Creatine kinase), GGT (Gmmma-glutamyle transferase) and lactate dehydrogenase were estimated by using randox assay kits.

Histopathological analysis

Liver, Pancreas, and kidney were fixed in 10 % formalin solution and then dehydrated by passing through a mixture of ethyl alcohol followed by water. Organs were processed and embedded in paraffin wax. Staining of cut sections were done with hematoxylin and eosin. Light microscopic examinations of slides were carried out.[25]

Statistical analysis

The mathematical modeling was resultant of computer simulation programming with Design of Experiment software (version 8.0.6, State-Ease Inc., Minneapolis, USA). A single factor parametric one way ANOVA was used to determine significant differences among treatment means. The best-fitted model was selected based on the value of R^2 with probability limits of the P value <0.05.

Acute oral toxicity test. The toxicity level of the combined extract was evaluated as per the guidelines of OECD 420 using a fixed dose procedure. The initial dose was chosen at 300 mg/kg (ethanolic extracts of VTFC) with the consideration that there were no in-vivo or in-vitro toxicity data from selected combined extracts. In the preliminary study of one rat with a dose of 300 mg/kg, no toxicity was observed so the dose was increase up to 2000 mg/kg. A single dose of 2 ml of 2000 mg/kg b. w. of the combined extracts (Ethanolic extracts of VTFC) was administered via agavage needle to each of the rats (n=5). After dosing of the combined extracts, rats were individually observed once every thirty minutes periodically during the first 24 hours, and daily thereafter for 14 days. The rats were observed for any change in fur lost, breathing pattern, tremors, convulsions, abnormal defecation (diarrhea), lethargy, severe pain, distress, and moribidity.[43,44]

Results and discussion

According to the literature, the BBD is an appropriate design for analyzing an independent, rotatable, or nearly rotatable quadratic response surface with no embedded factorial or fractional factorial design. This design requires only three levels and is a more efficient alternative to the full three-level factorial. In the present study, a total of 25 formulation compositions were obtained with one center and 24 factorial points (Table 2). BBD offers less number of runs as compared to its parallel central composite design as axial points are absent here. In glucose response, the quadratic model and lack of fit were found to be significant and non-significant, respectively. The higher value of the regression coefficient and P<0.05 explored a significant effect of independent constraints on dependent variable or glucose concentration in blood.

Table 1. Independent and dependent variables selected for BBD

Independent variable			**Dependent Variable**	**Goal**
Name and unit	**Level**			
	Lower (-1)	**Upper (+1)**		
AEVT (mg)	75	150	Concentration of Blood glucose (mg dL-1)	Minimum
AETFG (mg)	150	600		
AECNH (mg)	15	30		
AEFS (mg)	75	150		

Table 2. Composition of various batches of polyherbal formulation with their experimental & predicted values of responses

Batch Code	Independent variables				dependent variables		
	AEVT (mg)	AETFG (mg)	AECNH (mg)	AEFS (mg)	Concentration of Blood glucose (mg dL-1)		
					Exp. values	Pred. values	Residual
1	75.00	150.00	22.50	112.50	168.68	169.35	-0.67
2	150.00	150.00	22.50	112.50	149.35	148.74	0.61
3	75.00	600.00	22.50	112.50	126.46	127.50	-1.04
4	150.00	600.00	22.50	112.50	84.35	84.11	0.24
5	112.50	375.00	15.00	75.00	145.35	144.89	0.46
6	112.50	375.00	30.00	75.00	120.38	120.73	-0.35
7	112.50	375.00	15.00	150.00	124.92	126.00	-1.081
8	112.50	375.00	30.00	150.00	91.81	93.70	-1.89
9	75.00	375.00	22.50	75.00	153.75	152.04	1.71
10	150.00	375.00	22.50	75.00	125.42	126.18	-0.76
11	75.00	375.00	22.50	150.00	134.72	134.22	0.50
12	150.00	375.00	22.50	150.00	95.11	96.08	-0.97
13	112.50	150.00	15.00	112.50	163.52	164.68	-1.16
14	112.50	600.00	15.00	112.50	116.54	115.80	0.74
15	112.50	150.00	30.00	112.50	139.81	140.81	-1.00
16	112.50	600.00	30.00	112.50	84.12	83.21	0.91
17	75.00	375.00	15.00	112.50	164.62	165.69	-1.070
18	150.00	375.00	15.00	112.50	151.29	150.18	1.11
19	75.00	375.00	30.00	112.50	154.52	153.95	0.57
20	150.00	375.00	30.00	112.50	107.21	104.46	2.75
21	112.50	150.00	22.50	75.00	167.39	166.66	0.73
22	112.50	600.00	22.50	75.00	75.37	77.17	-1.80
23	112.50	150.00	22.50	150.00	107.94	106.45	1.49
24	112.50	600.00	22.50	150.00	89.41	89.46	-0.049
25	112.50	375.00	22.50	112.50	124.37	125.50	-1.13

*Value are expressed as mean±SD, n=3, Exp. = Experimental, Pred. = Predicted.

Table 3. Statistical model summary of selected dependent variables

FP (unit)	Regression parameters	Models			Best fitted model
		Linear	2FI	Quadratic	
Glucose conc in blood	SD	14.73	13.41	1.16	Quadratic model
	R^2	0.7833	0.8743	0.9993	
	Adjusted R^2	0.7400	0.7845	0.9984	
	Predicted R^2	0.6558	0.5747	0.9189	
	% CV	-	-	0.91	
	Adeq Precision	6894.16	8518.53	102.854	
	P-value	< 0.0001	0.0059	< 0.0001	

FP= Formulation parameters

Table 4. Analysis of variance table for responses with the best fitted quadratic model

Source	Glucose level (mg/dl)				
	Sum of the square	**DF**	**Mean square**	**F-value**	**P-value**
Model	20375.85	14	1429.74	1068.05	< 0.0001*
Residual	10.39	10	1.34	-	-
Lack of Fit	4.61	4	0.54	0.7	0.5990**
Pure Error	5.38	6	0.77	-	-
Total	20386.24	24	-	-	-

*Significant $\alpha<0.05$, **Non significant $P>0.05$

Effect of independent constraints on blood glucose concentration

The polynomial equation as given below and the 3D surface response plot and contour plots (Fig 1 and Fig. 2) show the effect of independent constraints on glucose level. Based on the outcomes achieved from experiments and interpretation made by BBD, it was perceived that model terms *viz* A, B, C,D, AB, AC, AD, BC, CD, B^2, and D^2 showing a significant ($P<0.05$) impact on the glucose concentration in blood while BD, A^2,& C^2 exhibiting non-significant impact on the blood glucose level. The F-value was 1068.05 remarking that the quadratic model was significantly fitted with a non-significant lack of fit (F= 0.7, P=0.5990). The ANOVA of the quadratic model for response studied and it revealed that it is considerably fitted with the highest value of R^2 (0.9993), acceptable precision (7.02), and coefficient of variance (0.91). A reasonable agreement was observed between predicted R^2 (0.9189) and adjusted R^2 (0.9984).The statistical model summary and ANOVA parameters are depicted in Tables 3 and 4, respectively.

Glucose concentration = +124.37-16.00 * A-26.62* B-14.12* C-11.98* D -5.70*A* B-8.75* A*C-3.07*A*D-2.18 * B * C+1.12* B * D-2.04 * C * D +2.62 * A2 -4.57 * B2 +1.32 * C2-9.87 * D2

As per the above equation, the all extract viz AEVT (A), AETFG (B), AECNH (C) and AEFS (D) explores a negative impact on the glucose concentration in blood. Among all four types of extract, B extract exhibiting more prominent negative effect followed by A, then C and lowest effect was due to D. The interaction effect of two extract together i.e. AB, AC, AD, BC, CD, exhibited significant negative impact on blood glucose level while the integration effect of BD was positive. Similarly, quadratic effect was also studied. The quadratic effect of B and D was displayed a negative on glucose level but the quadratic effect of A and C was positive which was not significant.

Fig. 1: Effects of combined ethanol extracts of VTFC on body weight and organ weights

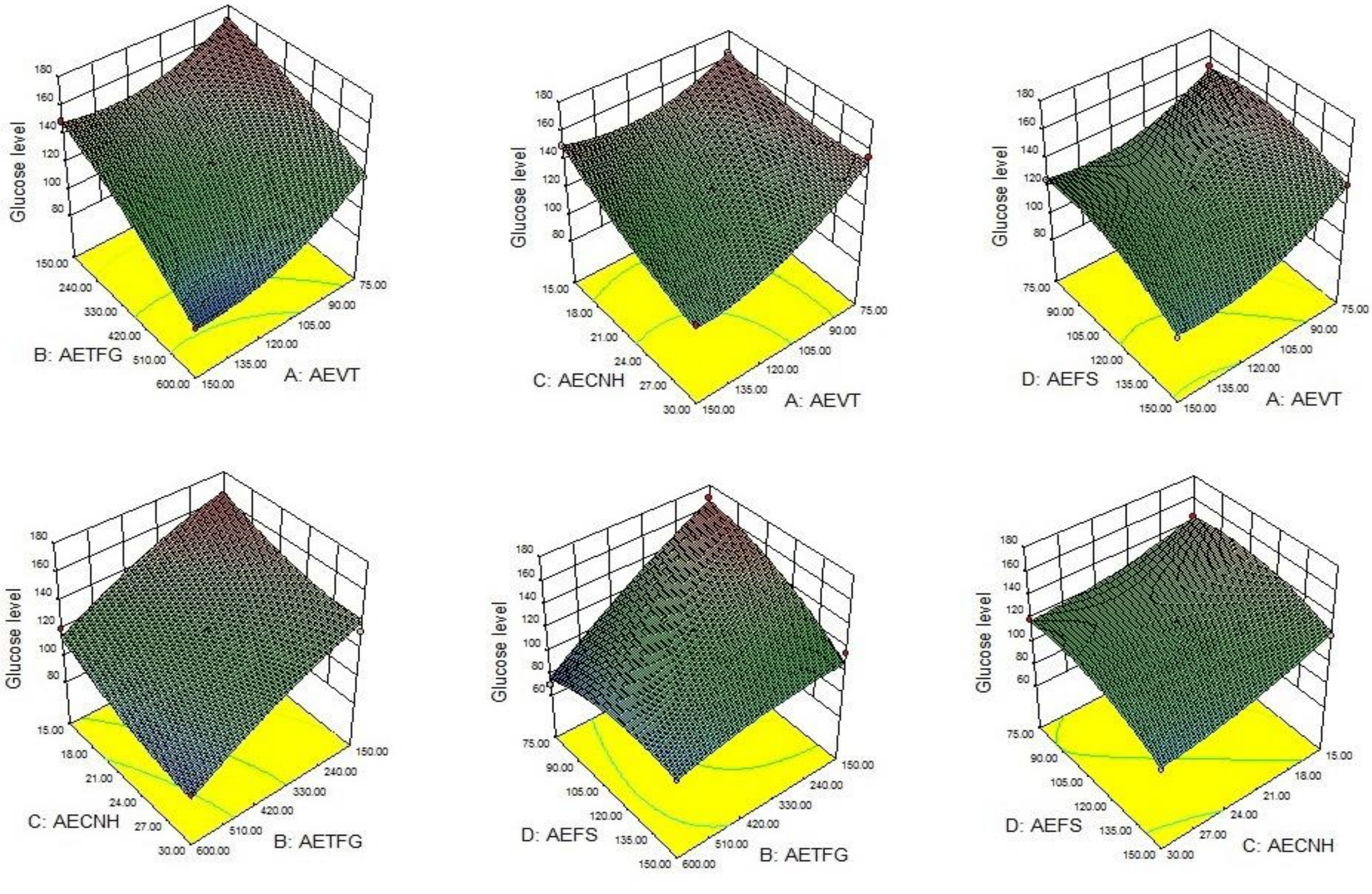
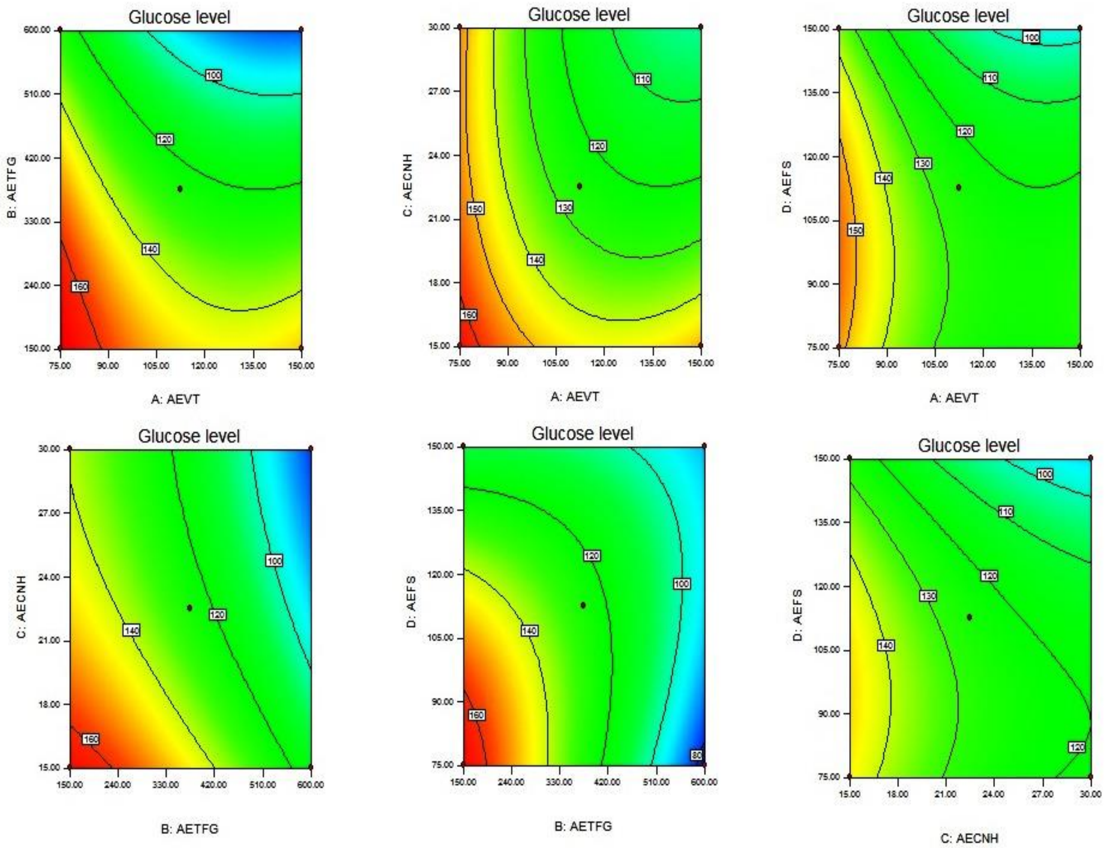


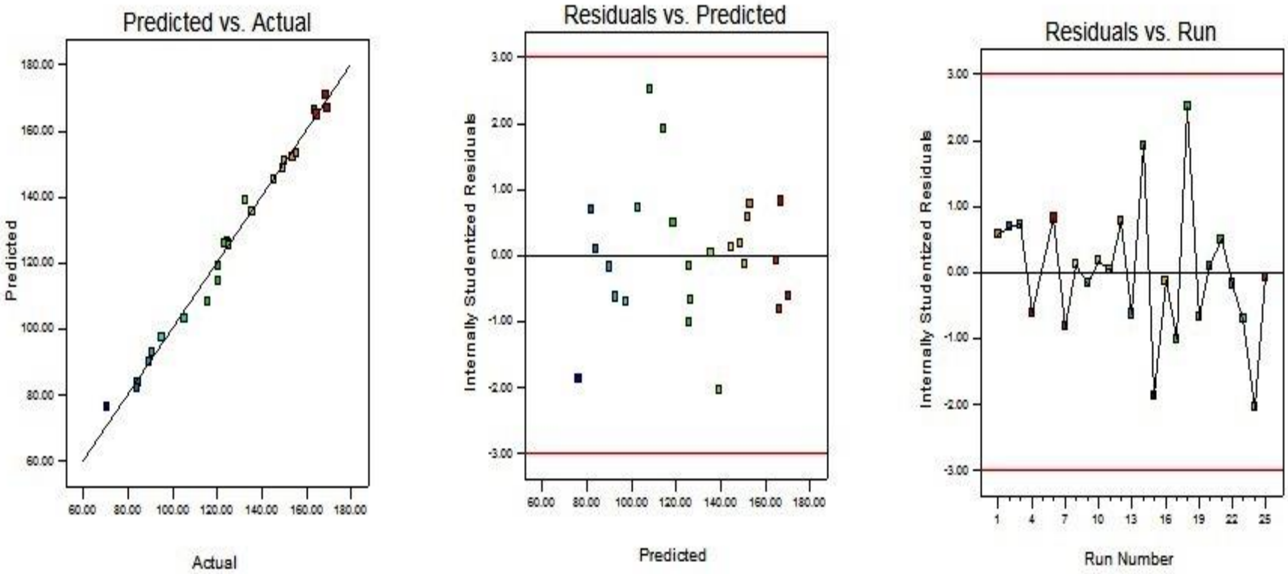
Fig.2: Contour plots generated by BBD showing the effect of independent constraints on Blood glucose concentration



Process variable control

To determine process control, various diagnostic tools like plots between the predicted versus actual response values, residual versus predicted values, and residual versus run values, were analyzed. Fig. 3 indicates that all the process and formulation variables were well controlled and obtained values were found to be in the accepted range.

Fig.3: Process diagnostic plots between predicted versus actual (A); residual versus predicted (B); and residual versus run responses (C) showing that responses for glucose level in blood.



Optimization by numerical optimization technique

Numerical optimization was made to select the optimized formulation as well as to validate the response surface methodology. A total of 55 point predictions were obtained by the software which were individually studied. Out of 55-point predictions made by software, formulation with a composition of 112.50 mg AEVT,600 mg AETFG,22.50 mg AECNH,75 mg AEFS with the predicted value of glucose level 77.17 was selected . To confirm and validate the optimization process, the selected optimized combination was prepared in the laboratory and glucose level was obtain the experimental (observed) values. The experimental values for glucose level were found to be 75.37 mg/dl. The prediction error was calculated as per the equation given in the procedure and it was found to be 2.33 %.The desirability function is based on the conversion of all responses into a dimensionless value (desirability function). The value of the desirability function ranges from 0 to 1. The value 0 is observed when the factors give undesirable results, whereas the value 1 is attributed to optimal response for the factors under study.[48] For the optimized composition, the desirability function was 0.9213 indicating the robustness of the optimized combination (Table 5). It was observed and elucidated from the results that all the obtained values were aligned with the predicted values, indicating BBD combined with the desirability function is a promising approach for the optimization of glucose level.

Table 5. Composition, experimental (observed), predicted, and desirability values of polyherbal formulation.

Independent constraints	**Optimal value**	**Response**	**Observed value**	**Predicted value**	**Prediction error (%)**	**Desirability function**
AEVT (mg)	112.50	Glucose conc in blood	75.37	77.17	2.33	0.9213
AETFG (mg)	600.00					
AECNH (mg)	22.50					
AEFS (mg)	75.00					

Effects of combined ethanol extracts of VTFC on body weight and organ weights

The initial and final body weights of rats in various experimental groups are presented in figure 4. The normal rats (group1) showed steady body weight gain through the seven days of treatment, diabetic control rats (group 2) showed significant ($p<0.01$) weight loss. Treatment with ethanoilic extract of VTFC 810 mg (group 3) and glibenclamide (group 4) for 7 days leads to a significant increase in body weight. The combined ethanolic extracts of VTFC prevented loss of body weight in comparison to the standard drug glibenclamide.

Relative liver weight was significantly ($p<0.001$) higher in diabetic rats than in control rats (Table 6). The ethanolic extract of VTFC at 810 mg/kg body weight also showed an effect equal to glibenclamide in decreasing the liver weight. Same observations were reported by using plant extracts for treatment of diabetes.[19,26,27]

Figure 4: Effect of VTFC on body weight change in control and streptozotocin induced diabetic rats. Values are significantly different at $p<0.05$. D810=Diabetic+810 mg VTFC, DG=Diabetic+5 mg/kg glibenclamide

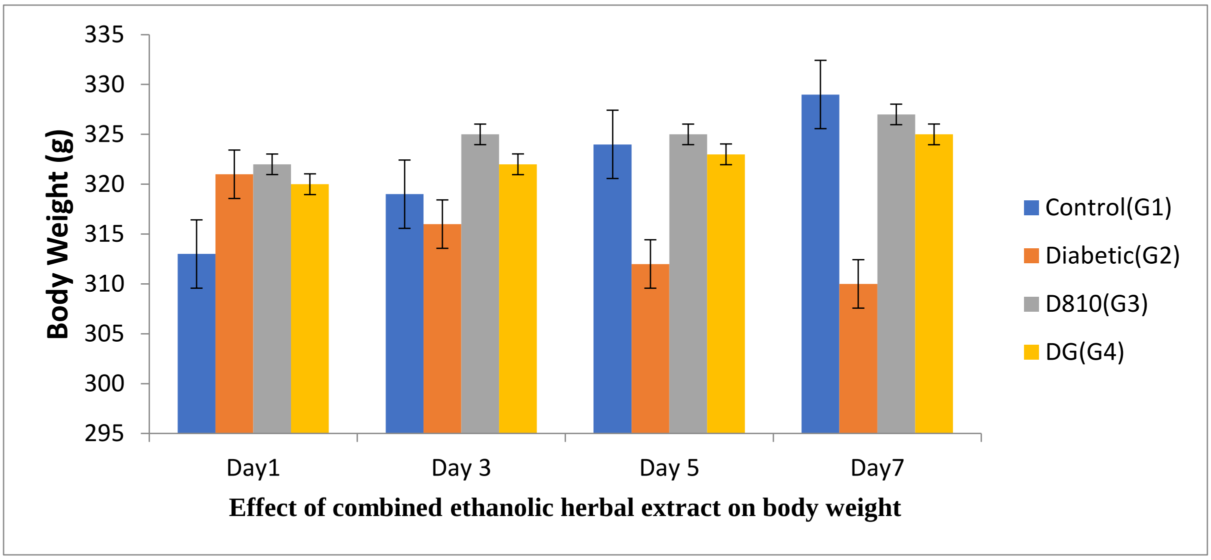


Table: 6 Effects of ethanolic extracts of VTFC on organ weight(g/100 g)in control and STZ diabetic rats(n=6,mean±SD)

Treatment(mg/kg)	**Liver**	**Kidney**	**Pancreas**	**Lung**	**Heart**
Control	0.9±0.13^{a}	0.19±0.63^{a}	0.23±0.76^{a}	0.09±0.02^{a}	0.06±0.01^{a}
Diabetic	1.39±0.18^{b}	0.27±0.96^{b}	0.37±1.17^{b}	0.14±0.03^{b}	0.08±0.07^{b}
D 810 mg	0.7±0.12^{a}	0.18±0.07^{a}	0.32±0.04^{b}	0.1±0.03^{b}	0.06±0.05^{a}
DG	1.09±0.12^{b}	0.20±0.16$^{a\ b}$	0.26±0.03^{a}	0.12±0.04^{b}	0.07±0.06^{a}

D810=Diabetic+810 mg VTFC,

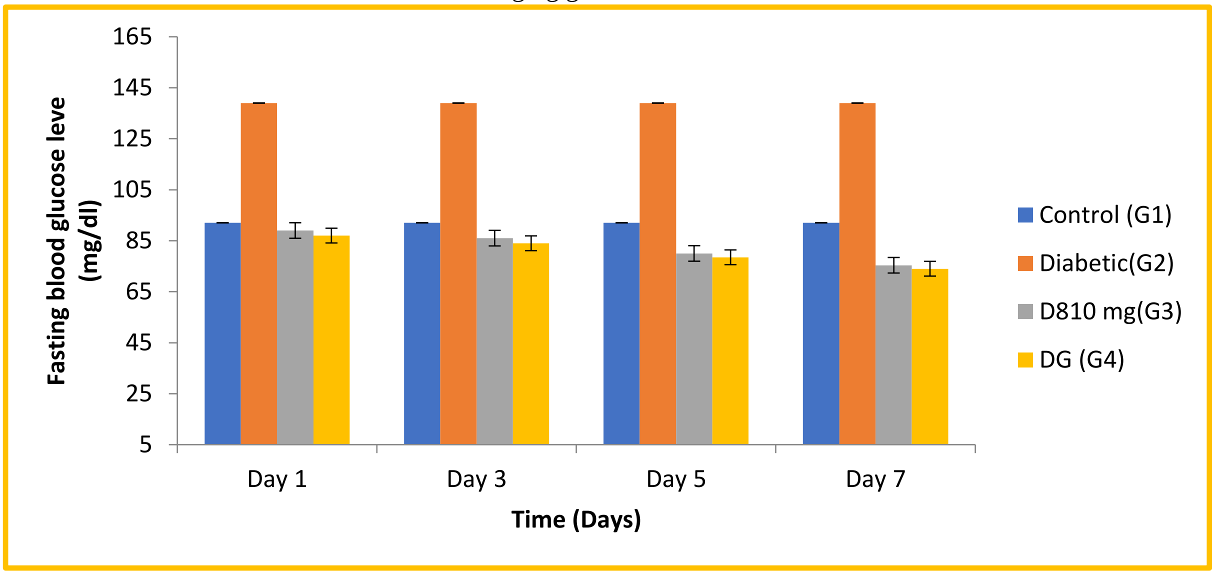
DG=Diabetic+5 mg/kg glibenclamide

Values followed by different superscript letters in same column are significantly different at $p<0.05$

Effects of VTFC on serum glucose levels

The effect of combined ethanol extract of VTFC and glibenclamide on hyperglycaemia was measured by using fasting blood glucose level. Diabetic control rats (group 2) was showed higher ($p<0.01$) hyperglycaemia when compared to normal control (group 1) rats (Figure 5). But when ethanolic extracts of VTFC at dose 810 mg (group 3) were given to rats (wt 300 gm) it completely prevent the hyperglycemic condition. The ethannolic extracts of VTFC significantly ($p<0.01$) decrease blood glucose level in STZ induced diabetic rats. The antihyperglycemic action of ethanolic extract of VTFC may be due to improved insulin sensitivity or inhibition of endogenous glucose production.[28]

Figure.5: Effects of VTFC on fasting blood glucose in control and streptozotocin induced diabetic rats. Values are significantly different at $p<0.05$. D 810=Diabetic+810 mg Combined VTFC mixture, DG= Diabetic+ 5 mg/kg glibenclamide.

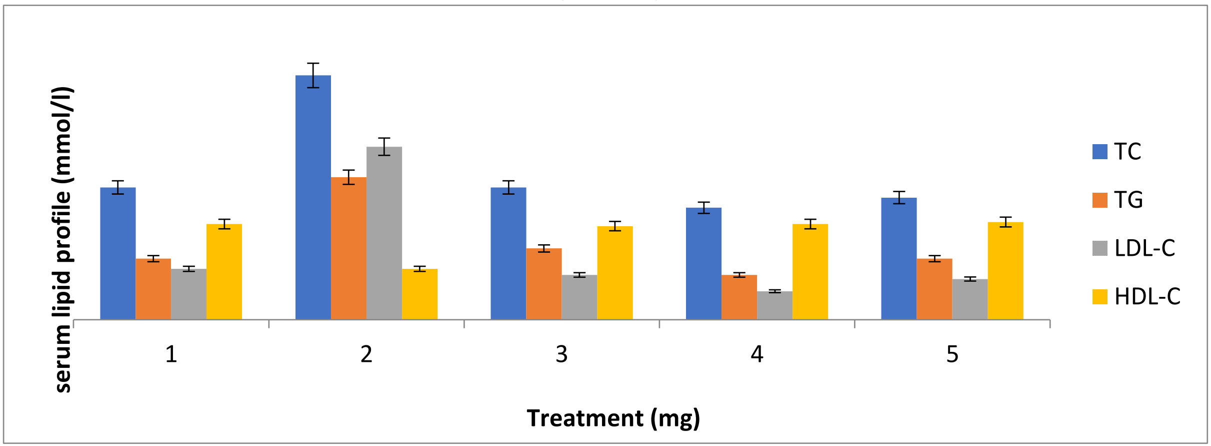


Effects of ethanol extracts of VTFC on serum lipid profile

Total cholesterol (TC),Triglycerides (TG), low density lipoprotein cholesterol(LDL-C) was significantly elevated in diabetic control while HDL-C (high density lipoprotein cholesterol) was decrease as compared to control rats (Figure 6).Treatment with ethanol extract of VTFC at dose 810 mg /kg body weight for seven days showed marked decreased in level of low density lipoprotein level cholesterol (LDL-C), triglycerides (TG),total cholesterol(TC) but concentration of high density lipoprotein cholesterol(HDL-C) increase to control levels in all treated rats.

Generally coexistence of hyperglycaemia and dyslipidemia is occurs in diabetic rats, also lipid profiles abnormalities are one of the most common problem of diabetes.Variation in lipid metabolism results from uncontrolled hyperglycemia which is a risk for myocardial infarction and coronary heart diseasease.[29] Present study showed that efficiency of insulin level in blood causes elevated level of glucose and hypercholesterolaemia in rats. However increased blood lipid level were restored to near normal in VTFC groups (group 3) with a concomitant elevation in high density lipoprotein cholesterol (HDL-C).Observations of present study matches the observation reported by researcher treated diabetic rats with extracts of some other combined species of medicinal plants.[21,30]

Figure 6: Effect of VTFC on lipid profile in control and streptozotocin induced diabetic rats. Values are significantly different at p<0.05.TC= Total Choleterol, TG=Triglycerides, LDL-C+ Low density lipoprotein Cholesterol, HDL-C= High density lipoprotein Cholesterol



Effects of combined ethanolic extracts of VTFC on haemotological parameters

To observe the harmful effects of foreign constituent's and VTFC extracts on blood constituents of rats, hematological parameters were assessed. In the present study significant reductions in hemoglobin, RBCs, hematocrit, MCH (mean corpuscular hematocrit), MCV (mean corpuscular volume), MCHC (mean corpuscular hematocrit concentration) in diabetic control rats were reported (Table7). Anaemia is the common pathophysiology associated with diabetes mellitus.[31,32] However STZ induced diabetic rats significantly elevated white blood cells counts (WBC), basophils, eosinophils, monocytes, neutrophils and lymphocytes compared to normal and VTFC treated rats. This showed disablement of hematological functions in diabetic rats and may be attributed to a defense reaction against STZ induced diabetic rats. Increase in the WBC count in the human being is related with cardiovascular disease, and cerebrovascular, carotid atherosclerosis diseases.[33] Platelets aggregation found in diabetic patients with long term poor glycemic control because of lack or deficiency of insulin.[34]Decrease level of platelets in diabetic rats induced with STZ was confirmed in the present study in compared to normal control rats. If this condition is persist for longer duration then external and internal hemorrhage will occur and finally death. However ethanolic extracts of VTFC at dose of 810 mg (for 300 g rats) and glibenclamide markedly elevated the platelets counts. This is the indication of ability of ethanolic extract of VTFC to stimulate the biosynthesis of clotting factors in blood coagulation or clotting during hemorrhage or severe bleeding.[35]

Table 7: Effects of ethanolic extracts of VTFC on haematological indices in control and STZ induced diabetc rats (n=6, maen±SD)

Index	**Control(Group 1)**	**Diabetic(Group 2)**	**D 810 mg (Group 3)**	**DG(Group 4)**
RBCs(109/l)	7.52±0.68	6.82±0.17	7.79±0.35	7±0.27
Hb(g/dl)	14.6±0.45	11.07±0.22	14.14±0.39	14.47±0.62
MCV(fl)	62.67±3.14	60.57±2.47	62±1.49	64±2.49
MCH (pg)	21.33±1.37	19.56±0.78	21±0.53	21±0.51
MCHC(g/dl)	32.67±0.52	30.69±0.79	32±0.44	31.96±0.59
Platelets(x10^9/l)	769.7±12.8	674.6±15.30	749.6±12.34	727.4±10.49

WBCs($x10^9$/l)	9.97±1.18	17.03±2.78	7.92±1.34	8.99±1.61
Neutrophils(%)	3.26±0.26	11.33±2.17	4.62±1.30	2.26±1.17
Lymphocytes(%)	6.04±0.12	15.24±2.84	4.49±0.12	4.14±0.28
Eosiniphils(%)	0.67±0.36	1.38±0.49	0.49±0.01	0.6±0.02

RBCs= Red blood cells;Hb=Haemoglobin; MCV=Mean corpuscular volume; MCH=mean corpuscular haematocrit;MCHC=mean corpuscular haematocrit concentration; D810 mg=Diabetic +810 mg ethanolic extract of VTFC;DG=Diabetic+5 mg/kg glibenclamide, values followed by superscript letters different from control for the same parameter are significantly different at p<0.05

Effect of ethanol extracts of VTFC on cellular toxicity markers

Globulin, bilirubin, total protein, albumin concentrations were decreased in diabetic control. Urea content, creatinine, total protein, urea contents were increased, while significant elevation (p<0.01) in the activities of serum AST (Aspartate aminotransaminase), ALP(Alkaline phosphatse),GGT (gama glutamyl transferase), ALT (alanine amino transaminase),CK (creatine kinase) and cholinesterase were observed (Table 8) The decrease in toxicity markers reflect the significant impact on kidney and liver functions caused by STZ induced diabetes. The ethanolic extract of VTFC at the dose of 810 mg (for 300 gm rat) was showed equipotent to glibenclamide in restoring the levels of these markers to normal. Levels of urate, urea and creatinine increased in serum in untreated diabetic rats seen in this study could be due to deficiency of insulin and the consequent inability of glucose to reach extra hepatic tissues which is responsible for gluconeogenesis as an alternative source of glucose.[36]The increased levels of cretinine, urate and urea in the serum could therefore signify kidney impairment. Markers are significantly showed reduction which is the indication of potential of ethanolic extracts of VTFC to protect the cells against diabetic injury. The elevation in alkaline phosphatase (ALP) activity seen in the present study may be a result of hapatic cells damage, which leads to leakage of the enzymes from the damaged tissues the serum.[36,37] Gama glytamyl trasferase (GGT) and aspartate transaminase (AST) are the important indicators of damage of hepatic cells. Their increase in diabetic rats as seen in the present study is the indication of severe damage to the liver cells. When ethanolic extracts of VTFC treated diabetic rats observed, the activities of these enzymes decreases and reached to control level which indicates the activity of liver was preserved by this extract.

The increases in cellular toxicity markers, which are responsible for alteration of liver functions in diabetic control rats are due to STZ induction. Ethanol extracts of VTFC at dose of 810 mg was found to be equipotent to glibenclamide in restoring the increased markers to control levels. So it is possibly suggested that the ethanoic extract of VTFC play a important role in reducing the liver and kidney problems as well as cardiovascular diseases via decreasing the serum uric acid, urea and creatinine caused by diabetes.

Table 8: The effect of ethanolic extracts of VTFC on some markers of biochemical parameters in control and STZ induced diabetic rats(n=6, mean±SD)

Index	**Control(G1)**	**Diabetic(G2)**	**D810 mg (G 3)**	**DG (G 4)**
Total Protein(g/dl)	63.5±1.41	60.44±2.07	63.22±1.03	62.59±1.11
Albumin(g/dl)	28.83±1.09	26.4±1.00	29.01±0.84	29.5±0.01
Globulin (g/dl)	34.67±1.78	32.70±1.77	34.44±1.18	33.9±1.11
Total bilirubin (g/dl)	6.67±1.09	21±9.86	13±2.84	8.70±1.77

Creatinine(mmol/l)	64.58±3.13	98.55±1.09	8.44±3.11	79.22±3.10
Urea(g/l)	5.7±0.24	20.10±1.77	6.33±0.40	5.17±1.21
Uric acid (g/l)	0.24±0.02	0.33±0.18	0.15±0.03	0.22±0.02
ALP (U/I)	326.3±43.40	771±20.60	286.3±48.61	333±33.22
AST (U/I)	167.67±22.25	428.5±67.57	166.4±19.33	217.3±20.52
ALT(U/I)	36.00±4.35	98±16.73	59.77±5.29	219.1±20.51
GGT(U/I)	2.00±0.45	15.6±1.90	4±0.44	5.60±0.49
ChE(U/I)	0.8±0.00	9.1±3.03	0.7±0.02	1.04±0.01

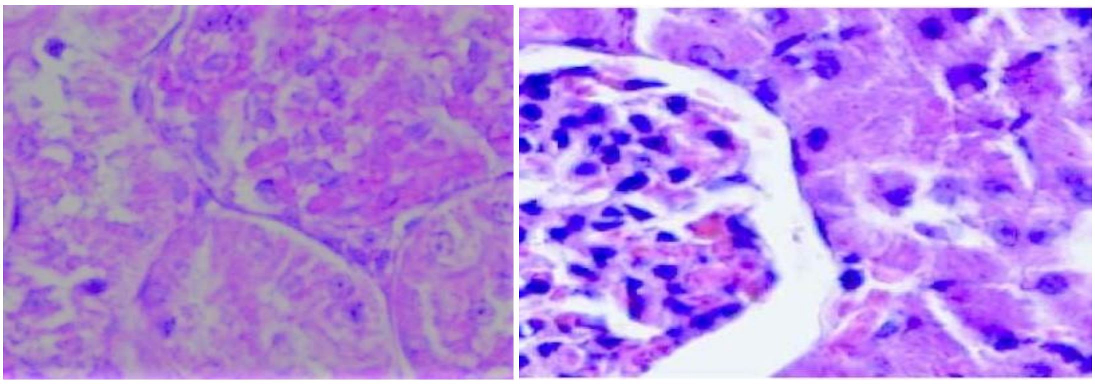
ALP=Alkaline Phasphate, AST=Aspartate aminotransaminase, ALT=Alanine aminotransaminase, GGT=Gamma glutamyl transferase, ChE=Cholenesterase, D810 mg=Diabetic+ethanolic extracts of VTFC 810 mg (300 gm rat), DG=Diabetic+5 mg/kg glibenclamide. Values followed by superscript letters different from the control for the same parameter are significantly different at $p<0.05$

Histopathological Examination

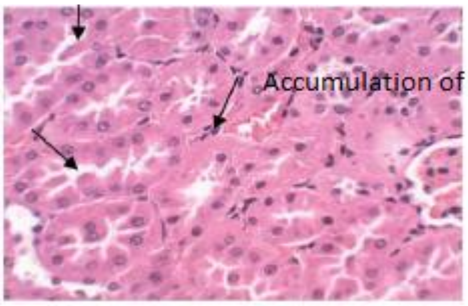
Hitopathology of Kidney

As seen in figure 7a**,** control rats shows normal, glomeruli, renal tubules and corpuscles, Figure 8b diabetic rats shows tubular degeneration, tubular necrosis and acute cellular swelling in their kidneys. Failure of kidney and its dysfunctions are main complications diabetes mellitus. Elevated level of glucose in blood may lead to oxidative stress, which is one of the causative factor for diabetic related kidney disorders like tubular atrophy, apoptosis and necrosis.[38]Administration of ethanol extracts of VTFC or glibenclamide (group 3 & 4) improve and restore the kidney functions. The group 3 (administered ethanol extracts of VTFC 810 mg) showed better regenerative capacity over glibenclamide.

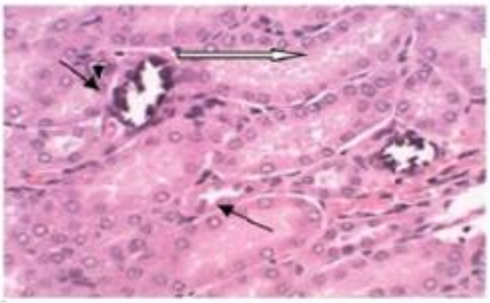
Figure 7: Histopathology of Kidney in normal and streptozotocin induced diabetic rats (a) Normal Control rats- Normal renal corpuscles and tubules (G1), (b) Diabetic rats- Cellular degeneration and swelling, widening of Bowmans capsules, Glomerular atrophy(G2), (c) Accumulation of protein in glomerular saces and mild atropy (G3), (d) Fatty changes in glomerular epithelia and (white coloured arrow and mild cellular degeneration (black coloured arrow) (G4).



(a) Control (G1) (b) Diabetic (G2)



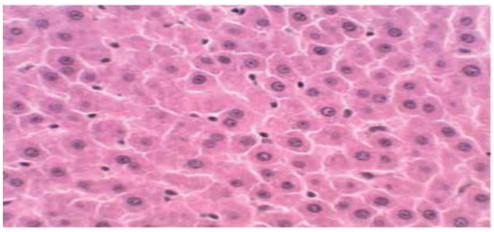
(c) D810 mg (G3)

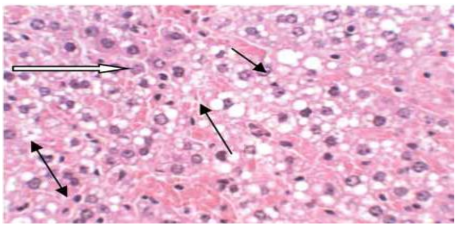


(d) Glibenclamide (G4)

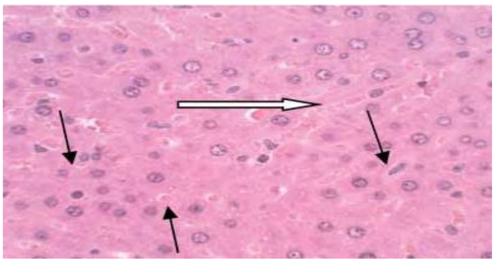
Histopathology of Liver

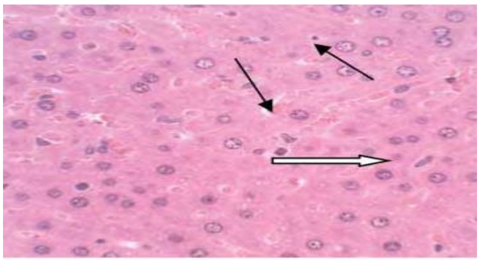
As seen in Figure 8a normal control rats (G1) showed normal hepatic cells with normal nucleus and well preserved sinusoids. But diabetic control rats (G2) fatty deposits, sinusoidal dilation with congestion, vacuolation of hepatocytes, mild portal inflammation severe degeneration and necrosis. Degeneration of hepatocytes in untreated diabetic rats could be due to insulin deficiencyand oxidation of fatty acids causing to deposition of triglycerides in hepatocytes.[39]However when ethanol extract of VTFC or glibenclamide (G3 & G4) administered to diabetic rats, they showed normal hepatocytes and minimum necrosis. So hepatoprotective activity seen in ethanol extract of VTFC may be due to free radical scavenging activities of the constituents (flavonoids, polyphenols) present in the combined extract of VTFC.[40]





(a) Control (G1) (b) Diabetic rats(G2)



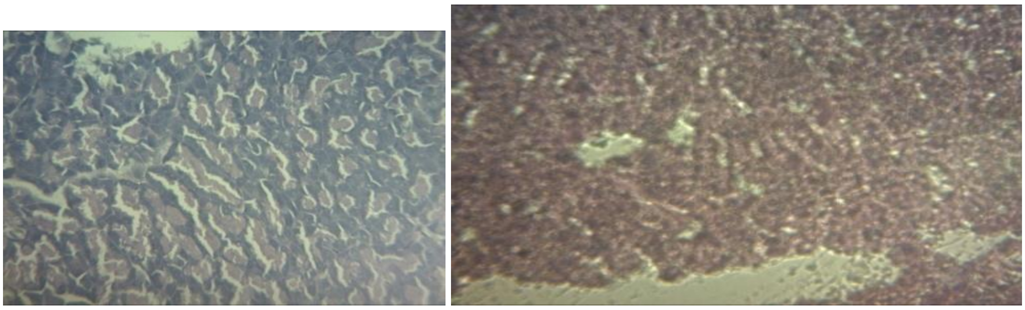


(c) D810 (G3) (d) DG (G4)

Figure 8: Histopathological changes in Liver of normal rats and streptozotocin induced diabetic rats (a) Control rats showing normal histology (G1), (b) Diabetic rats showing degeneration in white colored arrow & fat deposition in white colored arrow, necrotic cells dark brown & swelling in cells (G2), (c) D 810 black colored arrow showing reduced fatty change, White arrow showing mild congestion in sinusoidal cells, cells also showing mild granular degeneration (G3), (d) Black arrow showing reduced degeneration, white arrow showing moderate hyperplasia (G4)

Histopathology of Pancreas

(a) Normal Control group (G1) (b) Diabetic control group(G2)



(c) D810 mg (G3) (d) DG (Standard drug G4)

Figure 9: Histopatological changes in pancreas of control and streptozotocin induced diabetic wistar rats. Normal control (a): Normal pancreaticislets of Langerhans and acini, (b) Diabetic control: Degeneration and shrinkage, vacuolar changes and necrotic change, haemosiderosis. (c) D810: reduced necrosis and degeneration. (d) DG: Reduced cellular degeneration and vacuolar swelling, Mild haemosiderosis.

Figures 9a, islets of Langerhans in pancreas andacini are seen normal in control rats (**G1)**. In contrast Figure 9b histopathological changes in diabetic control rat's shows, haemosiderosis, degeneration, necrosis, pancreatic hyperplasia (G2). Mild to moderate hyperplasia, degeneration, Minimum necrosis, are seen in diabetic rats treated with glibenclamide and ethanol extract of VTFC (G4 & G3). This noticeable depletion in extent of injuries in diabetic rats treated with combined ethanol extract of VTFC could be due to elimination of again damage to pancreas, prevention of β cells death and recovery of partially injured β cells. Treatment of diabetic rats with different combined plant extracts has been reported to possess recovery of degeneration of pancreatic cells.[41,42]

Conclusion

The result of present study showed that the combined ethanoic extracts of leaves of *Vebascum thapsus*, seeds *of Trigonella foenum graecum*, leaves of *Ficus semicordata* and *Cocos nucifera*husk (VTFC) has strong hypoglycemic, hypolipidaemic, hepatopretective potential in Streptozotocin induced diabetic rats. The oral administration of combined ethanol extracts of VTFC modulated organ and body weight, attenuated blood and cellular toxicity markers, reduction in blood glucose level and repaired damage to liver cells, pancreatic cells and kidney in diabetic rats. This combined mixture at the dose of 810 mg is effective in significantly reducing the blood glucose level. This combined mixture is synergistically viable but because of the hypoglycaemia as side effect the dose and combination is adjusted for additive effect**.** Further studies on the combined VTFC ethanol extracts, clinical trials and mechanism of action are required to make more potent anti-diabetic formulations.

References

1. RS Renjith, AM Chikku, T Rajamohan. "Cytoprotective, antihyperglycemic and phytochemical properties of Cocos nucifera (L.) inflorescence''; *Asian Pacific Journal of Tropical Medicine,* 2013; 804-810

2. Mona Abd-Elzaher El-Shemy. "Antidiabetic and anti-hyperlipidemic effects of virgin coconut oil in rats''; *Egypt. J. Vet. Sci,* 2018; 49(2), pp. 111-117.

3. S. Saranya, S. Pradeepa., S. Subramanian. "Biochemical evaluation of antidiabetic activity of *Cocos nucifera* flowers in STZ Induced diabetic Rats'';*Int. J. Pharm. Sci. Rev. Res., Jun,* 2014; 26 (1) : 67-75.

4. Muhammad Riaz, Muhammad Zia-Ul-Haq, Hawa Z.E. Jaafar. "Common mullein, pharmacological and chemical aspects''; *Brazilian journal of Pharmacognosy*,(2013); 23: 948-959.

5. Anusha Pothamsetty, M. Janarthan1, Md. Faheemuddin, Mohsina Hussain, Md. Mazher Ahmed. "Evaluation of anti-Diabetic activity of the plant leaves of *Verbascum thapsus* in alloxan induced diabetic rats'';*International Journal of Pharmaceutics & Pharmacology,* 2017; 1(4): 118.

6. Gorkem Dulger, Tulay Tutenocakli, Basaran Dulger. Anti-staphylococcal activity of *Verbascum thapsus* L. against methicillin-resistant *Staphylococcus aureus*. Konuralp Tıp Dergisi,2017; 9(1) : 53-57.

7. Rosengarten F Jr. 1969. The Book of Spices. Available from: cabdirect.org

8. Sara Oufquir, Mehdi Ait Laaradia, Zineb El Gabbas, Kenza Bezza, Jawad Laadraoui, Rachida Aboufatima, Zahra Sokar, Abderrahman Chait. *Trigonella foenum-graecum* L. sprouted seed extract: Its chemical HPLC analysis, abortive effect, and neurodevelopmental toxicity on mice. HindawiEvidence-Based Complementary and Alternative Medicine, 2020; Article ID 1615794, 1-10.

9. Sajad Arshadi, Mohammad Ali Azarbayjani, Fatemeh Hajaghaalipor, Ashril Yusof,Maghsoud Peeri, Salar Bakhtiyari, Robert S. Stannard, Noor Azuan Abu Osman and Firouzeh Dehghan. "Evaluation of *Trigonella foenum-graecum* extract in combination with swimming exercise compared to glibenclamide consumption on type 2 Diabetic rodents''; *Food & Nutrition Research,* 2015; 59: 29717.

10. Basch E, Ulbricht C, Kuo G, Szapary P, Smith M. Therapeutic applications of fenugreek. Altern Med Rev 2003; 8(1): 20_7.

11. Kaviarasan S, Naik GH, Gangabhagirathi R, Anuradha CV, Priyadarsini KI. In vitro studies on antiradical and antioxidant activities of fenugreek (Trigonella foenum graecum) seeds. Food Chem 2007; 103(1): 31_7.

12. Miraldi E, Ferri S, Mostaghimi V. Botanical drugs and preparations in the traditional medicine of West Azerbaijan(Iran). J Ethnopharmacol 2001; 75(2_3): 77_87.

13. Kalyan C. Nagulapalli Venkata, Anand Swaroop, Debasis Bagchi, Anupam Bishayee. ''A small plant with big benefits: Fenugreek (*Trigonella foenum-graecum Linn*.) for disease prevention andhealth promotion"; Mol. Nutr. Food Res, 2017; 61(6) : 1-26.

14. Abhay Jayprakash Gandhi, VJ Shukla, RN Acharya. Qualitative, quantitative screening and antifungal study of *Ficus semicordata*Buch. -HAM. Ex Sm. International Journal of BotanyStudies, 2019; 4 (6) : 82-85.

15. Bakshi DN, Sensarma P, Pal DC. A Lexicon of Medicinal Plants in India. Calcutta: Naya Prokas; 2001. p. 194-5..

16. Virender Kaur, Kumud Upadhyaya, Milind pande. "Bioassay-guided evaluation of *Ficus semicordata* for antidiabetic activity''; *Int J Pharm Pharm Sci,* 2017; 9 (3), 71-77.

17. Ponnuvel Deepa, Kandhasamy Sowndhararajan, Songmun Kim, Se Jin Park. ''A role of Ficus species in the management of diabetes mellitus: A review''; *Journal of Ethnopharmacology*,2018; 215: 210–232

18. Isabella F.D. Pinto, Railmara P. Silva, Adriano de B. Chaves Filho, Lucas S. Dantas,Vanderson S. Bispo, Isaac A. Matos, Felipe A.M. Otsuka, Aline C. Santos, and Humberto Reis Matos. Study of antiglycation, hypoglycemic, and nephroprotective activities of the green dwarf variety Coconut water (*Cocos nucifera L.*) in alloxan-induced diabetic rats. J Med Food, 2015; 00 (0), 1–8.

19. Heenataj. B, Kushmitha. V, Dr. N.G. Ramesh Babu, Dr. I. Seethalakshmi. Antioxidants and cytotoxicity analysis of Coconut husk extract. International Journal of Engineering Research and Management (IJERM), 2017; 04 (08), pp.5-8.

20. Emojevwe Victor and Jeroh E. Antidiabetic effects of the *Cocos nucifera* (Coconut) Husk Extract. Journal of Medical and Applied Biosciences,2012; 4: 16-18.

21. G .A. Otunola and A.J.Afolavan. Antidiabetic effect of combined species of *Allium sativum, Gingiber officinale* and *capsicum frutescens* in alloxan induced diabetic rats. Frontiers in life science,2015;8(4),314-323.

22. Zhou X, Seto SW, Chang D, Kiat H, Razmovski- Naumovski VR, Chan K, Bensoussan A. Synergistic effects of Chinese herbal medicine: A comprehensive review of methodology and current research. Front Pharmacol. 2016;7:201. doi: 10.3389/fphar.2016.00201, PMID 27462269.

23. Van Vuuren S, Viljoen A. Plant-based antimicrobial studies–methods and approaches to study the interaction between natural products. Planta Med. 2011;77(11):1168-82. doi: 10.1055/s-0030-1250736, PMID 21283954.

24. Williamson EM. Synergy and other interactions in phytomedicines. Phytomedicine. 2001;8(5):401-9. doi: 10.1078/0944-7113-00060, PMID 11695885.

25. Ramesh Babu K, Yogesh, Raghavendra H, Kantikar SM and Prakash KB. "Antidiabetic and histopathological analysis of fenugreek extract on alloxan induced diabetic rats'';*Int.J.Drug Dev. & Res.*, April-June 2010; 2(2) : 356-364.

26. Shetti AA, Sanakal RD, Kaliwal BB. 2012. Antidiabetic effect of ethanolic leaf extract of *Phyllantus amarus* in alloxan induced diabetic mice. Asian J Plant Sci Res. 2:11–15.

27. Sunmonu TO, Afolayan AJ. 2013. Evaluation of antidiabetic activity and associated toxicity of Artemisia afra aqueous extract inWistar Rats. Evid based Complement Altern Med. doi:10.1155/2013/929074.

28. Kuroda M, Mimaki Y, Sashida Y, Mae T, Kishida H, Nishiyama T. 2003. Phenolics with PPAR-ligand-binding activity obtained from liquorice *(Glycyrhizza uralens* roots) and ameliorative effects of glycerine on genetically diabetic KK-Ay mice. Bioorg Med Chem Letters. 13:4267–4272.

29. Khan HA, Sobki SH, Khan SA. 2007. Association between glycaemic control and serum lipid profile in type 2 diabetic patients: HbA1c predicts dyslipidemia. Clin Exp Med. 7:24–29.

30. Kumar S, Kumar V, Prakash O. 2011. Antidiabetic, hypolipidemic and antioxidant activities of *Callistemon lanceolatus* leaves extract. J Herbs Spice Med Plant. 17:144–153.

31. Akindele OA, Babatunde AI, Chinedu FM, Samuel OA, Oluwasola CA, Oluseyi AA. 2012. Rat model of food induced nonobese- type 2 diabetes mellitus; comparative pathophysiology and histopathology. Intern J Physiol, Pathophysiol Pharmacol.4:51–58.

32. Colak S, Geyi Koghu F, Aslan A, Deniz GY. 2014. Effects of lichen extracts on haematological parameters of rats with experimental insulin-dependent diabetes mellitus. Toxicol Ind Health. 30:878–887.

33. Elkind MS, Cheng J, Boden-Albala B, Paik NC, Sacco RL. 2001. Elevated white blood cell count and carotid plaque thickness: the Northern Manhattan stroke study. Stroke. 32:842–849.

34. Jarald E, Joshi SB, Jain DC. 2008. Diabetes and herbalmedicines. Iran J Pharmacol Ther. 7:97–106.

35. Adebayo OJ, Adesokan AA, Olatunji LA, Buoro DO, Soladoye AO. 2005. Effect of ethanolic extract of *Bougainvillea spectabilis* leaves on haematological and serum lipid variables in rats. Biokemistri. 17:45–50.

36. Gastaldelli A, Simona B, Pettiti M, Toschi E, Camastra S, Natali A, Landau BR, Ferrannini E. 2000. Influence of obesity and type 2 diabetes on gluconeogenesis and glucose output in humans: a quantitative study. Diabetes. 49:1367–1373.

37. Wills DE (1985). Biochemical Basis of medicine. 3rd Edn., John Wright and sons Ltd., Bristol, England, pp. 267-268.

38. Kumar D, Zimpellmann J, Robertson S, Burns KD. 2004.Tubular and interstitial cell apoptosis in the streptozotocindiabetic rat kidney. Nephron. 96:e77–e88.

39. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, Scott DK, O'Doherty RM. 2010. Depletion of liver kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes. 59:347–357.

40. Atta AH, Elkoly TA, Mouneir SM, Kamel G, Alwabel NA, Zaher S. 2010. Hepatoprotective effect of methanol extracts of *Zingiber officinale* and *Circhonium intybus*. Indian J Pharmaceut Sci. 72:564–570.

41. Jelodar GA, Maleki M, Motadayen MH, Sirus S. 2005. Effect of fenugreek, onion and garlic on blood glucose and histopathology of pancreas of alloxan-induced diabetic rats. Indian J Med Sci. 59:64–69.

42. Abdollahi M, Zuki ABZ, Goh YM, Rajion MA, Rezaeizadeh A, Noordin MM. 2011. Effects of *Momordica charantia* on pancreatic histopathological changes associated streptocotozin-induced diabetes in neonatal rats. Histol- Histopathol. 26:13–21.

43. 43.(E.N. Sholikhah, M. Mustofa, D.A.A. Nugrahaningsih, F.S. Yuliani, S. Purwono, S. Sugiyono, S. Widyarini, N. Ngatidjan, J. Jumina, D. Santosa, M. Koketsu, Acute and subchronic oral toxicity study of polyherbal formulation containing Allium sativum L., Terminalia bellirica (Gaertn.) Roxb., Curcuma aeruginosa Roxb., and Amomum compactum Sol. ex. maton in rats, Biomed. Res. Int. (2020) 2020, https://doi.org/10.1155/2020/8609364.)

44. 44.OECD, Test no. 420: acute oral toxicity-fixed dose procedure, OECD Guidelines for the Testing of Chemicals, OECD, Paris, 2001.

45. Mohd Yasir, Ameeduzzafar Zafar, Kaveripakkam M. Noorulla, Abdurazak J. Tura, Udai Vir Singh Sara, Dharamveer Panjwani, Mohammad Khalid, Misbahu J. Haji, Wondesen Gadisa Gobena, Teshome Gebissa, Debesa D. Dalecha, Nose to brain delivery of donepezil through surface modified NLCs: Formulation development, optimization, and brain targeting study, Journal of Drug Delivery Science and Technology, Volume 75, 2022, 103631.

46. Ameeduzzafar, Alruwaili NK, Imam SS, et al. Formulation of Chitosan Polymeric Vesicles of Ciprofloxacin for Ocular Delivery: Box-Behnken Optimization, In Vitro Characterization, HET-CAM Irritation, and Antimicrobial Assessment. AAPS PharmSciTech. 2020;21(5):167. Published 2020 Jun 5. doi:10.1208/s12249-020-01699-9

47. Yadav, P., Rastogi, V. & Verma, A. Application of Box–Behnken design and desirability function in the development and optimization of self-nanoemulsifying drug delivery system for enhanced dissolution of ezetimibe. Futur J Pharm Sci 6, 7 (2020). https://doi.org/10.1186/s43094-020-00023-3

48. Marasini N, Yan YD, Poudel BK, Choi HG, Yong CS, Kim JO. Development and optimization of self-nanoemulsifying drug delivery system with enhanced bioavailability by Box-Behnken design and desirability function. J Pharm Sci. 2012 Dec;101(12):4584-96. doi: 10.1002/jps.23333. Epub 2012 Sep 28. PMID: 23023800.