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In-Vivo Hypoglycemic, Antioxidant And Hepatoprotective Properties Of A Unani Combinatorial Therapy In Streptozotocin Induced Diabetic Rats.

Asema Mahveen[1], Mir Yousuf Ali[1], Zaibunnisa begum[2], Shahzadi Sultana[2], *NMA Rasheed*[2], *Asiya Farheen*[1], *Nazema Farheen*[1]

[1]*Ph.D Scholar, DrNTRUHS, Dept. of Ilmul-Advia (Pharmacology), GNTC, Hyderabad, Telangana*
[1]*Prof. & Dr, Ph.D Ph.D Guide, Dept. of Ilmul-Advia (Pharmacology), GNTC, Hyderabad, Telangana*
[2]*Prof. & H.o.D, Department of Ilmul-Advia (Pharmacology), GNTC, Hyderabad.*
[2]*Principal, GNTC, Hyderabad, Telangana, India.*
[2]*SRF, National Research Institute of Unani Medicine for Skin Disorders, Hyderabad.*
[1]*Ph.D Scholar, Dr.NTRUHS (Vijayawada), Dept. of OBG, GNTC, Hyderabad,*
[1]*M.D.,Dr.NTRUHS, Dept. of Kulliyat, GNTC, Hyderabad.*

***Corresponding author:**
Dr. Asema Mahveen, Dept. of Ilmul-Advia (Pharmacology)

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ABSTRACT: **Background:** The term Type 2 Diabetes mellitus designates not a single disease but a heterogenous collection of hyperglycemic syndromes phenotypically and genotypically different. According to W.H.O. Type 2 Diabetes Mellitus has already reached epidemic proportions currently affecting over 422 million people worldwide and one of the four leading non-communicable diseases highlighted in the targets of the WHO Global Monitoring Framework (2019-20). In Unani Literature such as Al-Qanoon Fil Tibb, Zakhira Khwazam shahi, Bayaz-e-Kabeer, Sharahe Asbab-wa-alamat mentioned clearly the effective use of various herbal medicine for Ziabetus shakri (Diabetes mellitus). Unani medicine procured from the plants has a special significance in the management of Diabetes mellitus.

Aims and Objective: The objective of the present study is to evaluate the hypoglycemic, antioxidant and hepatoprotective potential of unani combinatorial therapy in the better management of Diabetes mellitus.

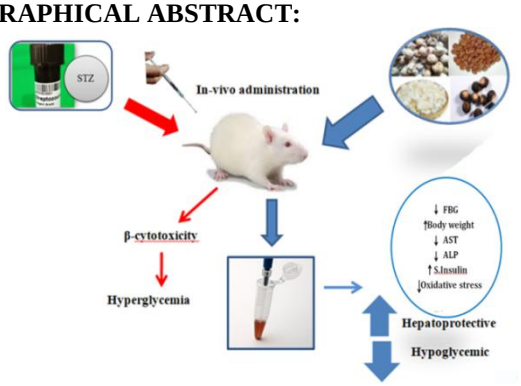
Materials and Methods: In the present study impact of combinatorial therapy in streptozotocin induced diabetic Wistar male albino rates was targeted. The groups were administered with different combinations of selected herbal drugs to the rats orally for 4 weeks. The efficiency of combination was evaluated in comparison with standard drug at its recommended dose by assessing FBG, Bodyweight, Serum insulin, AST and ALP and antioxidant properties.

Result: recorded result indicated the significant reduction in blood glucose concurrence with remarkable increase in Serum insulin levels which were compared to the control group ($P < 0.05$).

Conclusion: All the mentioned parameters were almost restored to the Normal Control group levels.

Keywords: Ziabetus shakri, Unani, Hypoglycemic, antioxidant, Hepatoprotective, Wistar rats, Diabetes mellitus, HbA1c, S.Insulin, combinatorial therapy, ALT, AST.

GRAPHICAL ABSTRACT:



I. INTRODUCTION

Diabetes Mellitus is recognized as syndrome of impaired carbohydrates, fats, and protein metabolism that have hyperglycemia and glucose intolerance as their hallmark caused by either lack of insulin secretion, defect in insulin action or both. [(1-3)]Diabetes Mellitus is one of the quickly growing and notable health challenges of

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21st century. According to W.H.O factsheet estimates that about 422 million people worldwide have diabetes, the majorly living in low and middle-income countries and about 1.6 million deaths attributed to diabetes each year.[4-6]Increasing evidence in both experimental and clinical studies suggest that oxidative stress place a major role in the pathogenesis of both types of Diabetes Mellitus as well as acts as a important part in diabetes related complications. [7] Clinical and experimental studies also suggest that augmented oxidative stress induces the complications of Diabetes Mellitus.[8] the excess in free fatty acids found in insulin-resistant state is known to be directly toxic to hepatocytes.[9]. The markers of liver dysfunction such as AST and ALP are principle indicators to measure liver health and involve in hepatic insulin resistance. Thus, there is a need to explore new treatment modules which play an important role in the correction of oxidative stress, as it improves survival and reduction of diabetic co-morbidities. This elucidates correcting the hyperglycemia is not the complete treatment of Diabetes Mellitus. In the present study, FBG, Body weight, Glycosylated hemoglobin (HbA1c) is the gold standard that reflects the glycemic control level, S.Insulin levels, Liver enzymes (AST and ALT),and antioxidant properties of herbal combinatorial drugs are evaluated.

Health Impact Of Diabetes Mellitus:

Adults with Diabetes Mellitus have two to three fold increased risk of heart attacks and stroke, neuropathy, can cause damage to blood vessels to cause foot ulcers and limb amputation, 2.6% of global blindness by diabetic retinopathy and also the leading cause of kidney failure. The presence of liver disease has received increased attention because of their long term health consequences and economic burden for national health services. [10-13]

LITERATURE REVIEW:

UNANI CONCEPT:

DEFINITION: According to Hakim Kabiruddin, Ziabetus shakri (Diabetes Mellitus) is a Fasaad-e-taghziya or defect occur in Kabid (Liver) aur urooq (vessels) at digestion level whose underlying etiology and detail description of occurrence is difficult. [14]

CAUSES AND TYPES: According to Shaikh-ur-rayees, in "Kitab-ul-Qanoon"(Canon of Medicine) says Ziabetus is a may be due to abnormal cold temperament or abnormal hot temperament.[15] Ziabetus shakri is a disease due to temperamental impairment in the organ as a whole locally or generalized due to "ghair-tabayee khilt-e balgham". According to Hkm Gulam Jilani, in "Maghzanul jawahar", Ziabetes is of two types according to causes and pathogenesis i.e, Ziabetus Haar and Ziabetus Barid. Ziabetus Haar is very common in both of them that's why the term Ziabetus implies to Ziabetes Haar. [16]

ETIOPATHOGENESIS:

The humoral theory was postulated by Father of Medicine Buqrat (Hipppcrates): He in his book Tabiyat-ul-Insaan (Human Nature) set forth his famous doctrine that "the body contains four major kinds of Akhlaat (Humors), a right proportion according to Quality and Quantity constitutes health and its disturbed proportion leads to disease". [17] Ibn-Sina (Avicenna) says, "All the body fluids including Akhlat Muharikah (Hormones) are called "Akhlat" which is the most important part of Human body, their imbalances in quality and quantity is the cause of illness. All the colourless and white fluids of the body irrespective of their location are Balgham (Phlegm) and it is mentioned in "Kulliyat-e-Asri" that Akhlat Muharikah are white and colourless fluids comes under Khilt-e-Balgham" [18-19] According to Jalinoos (Galen) and Ibn-Nafis, Balgham (Phlegm) is defined as white colourless fluids of the body performing diverse functions and second to dam (Blood) in superiority and whenever needed transformed, metabolized and mobilized to the blood and performs the nutritive and diverse functions. The temperament for all the kinds of Balgham performing diverse functions would also be different. Sue-Mizaj of certain Akhlaat Muharikah plays a dominant role, localized Sue-Mizaj will not always be localized phenomenon, and it may turn to generalized Sue-Mizaj and vice versa. [20] Any disturbance in the homeostatic conditions of the cells causes Sue-Mizaj (Abnormal Temperament) of cells/tissues locally and any aberration in both Kamiyat (Quantity) and Kayfiyat (Quality) causes Sue-Mizaj of entire body.[20]

Thus, correlation with the modern definition of Diabetes mellitus, Ziabetus Shakri is either due to lack of insulin i.e., local Sue'Mizaj (Accumulation of Ghayr–al-Tabayi Khilt Balgham) or due to insulin resistance i.e., generalized Sue'Mizaj (Abnormal temperament) of the body.

SELECTION OF DRUGS:

Ibn-Sina (Avicenna) says in "Kulliyat-e-Qanoon", the concept of temperament of drugs has got prime importance in Unani medicine. Choosing

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the drugs for treatment according to the distemperament (Sue-Mizaj) will have a great impact in ascertaining the health and management of the disease.[21] Selection of drugs for the study is in accordance to the ancient and authentic unani books which has given coded names with the following mentioned properties: Blood Purifier, (Mussaffi-e-Dam), Resolvent (Muhalil), Astringent (Qabiz), Carminative (Kasir-e-riyah), Stomachic (Muqawwi-e-Maida), Absorbent (Jazib-e-rutubat), Liver and Heart Tonic (Muqawwi-e-Kabid and Qalb), Detergent (Jaali), Dessicative (Mujaffif), Cooling (Mubarrid), Refrigerant (Mufarrih) and Detoxificant and Antiseptic (Dafe-e Taffun).[22-32]

II. MATERIALS AND METHODS

2.1. CHEMICALS

Streptozotocin was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Daonil ®; Glibenclamide, the Standard anti-diabetic drug obtained from Sanofi Aventis, Total antioxidant capacity (TAC) assay kit was purchased from caymann chemicals, trolox were obtained from Sigma (Sigma-Aldrich GmbH, St. Louis, MO), Carboxy methyl cellulose, phosphate buffer saline, Citric acid, distilled water, and other chemicals used were of analytical grade.

2.2. DRUG COLLECTION, IDENTIFICATION AND PREPARATION OF EXTRACTS:

The plant material was procured from the local market and identified by Botanist, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad before carrying out the study. The Drugs were cleaned, washed, shade-dried and coarsely ground. The extracts were prepared by using Aqueous and methanol as solvents. The Drugs were macerated in different combinations and Extracts obtained were filtered using Whattmann No.1 paper, concentrated, dried and kept in desiccators for further use. The extracts were stored at 4°C in airtight containers until used for further studies. [33]

2.3. ANIMALS

Albino Wistar rats weighing 180–200 g of either sex were selected for the study. They were fed a standard rat pellet and water from Reverse Osmosis Purifier (Kent). Research on animals was conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by the Institute's Animal Ethics Committee with CPCSEA No (Reg. number 1070/ac/07/CPCSEA).

2.4. DIABETES INDUCTION

Induction of diabetes mellitus: Diabetes was induced in overnight fasted rats by STZ (45mg/kg, i.p) after dissolving in freshly prepared cold citrate buffer. Induction was done by a single intraperitoneal injection to Albino Wistar rats of freshly prepared streptozotocin (STZ, sigma chemical company, St. Louis, MO, USA) solution in 10mM sodium citrate buffer (pH 4.5), at a dose of 45mg/Kg body weight. STZ induce fatal hypoglycemia as a result of massive pancreatic insulin release, the rats were provided with 5% dextrose solution after 6 hours of STZ administration for next 24 hours to prevent hypoglycemia. Diabetes was confirmed 72 h after induction by measurement of tail vein blood glucose levels with the glucose meter. [34, 35]

After 72 h rats with BGL greater than 200mg/dl and less than 400mg/dl were selected and observed for consistent hyperglycemia (Fasting blood glucose level greater than 200mg/dl and lesser then 400mg/dl) up to 7 days. Such animals were divided into 9 groups as follows:

2.5. EXPERIMENTAL DESIGN:

2.5.1. EXPERIMENTAL TREATMENTS

Animals were adapted for seven days before incipience of experiment and they were randomly classified into nine groups.

Group I: Normal Control group in which the six-rats not induced and received no treatment instead vehicle was daily administered P.O. i.e., 1% w/v CMC suspension for five-weeks and kept as positive control.

Group II: Diabetic control group with six-rats is injected with single dose of STZ at 45 mg/kg freshly dissolved in 0.1M citrate buffer chemically receiving no treatment. This group also receives vehicle P.O. (sodium CMC suspension) orally for five-weeks and kept as negative control.

Group III: Standard group with six-rats received glibenclamide at a recommended dose 5 mg/kg body weight/day orally for five-weeks after diabetes induction.

Group IV: Cr.WASB group with six-rats injected with single dose of STZ at 45 mg/kg freshly dissolved in 0.1M citrate buffer and then were treated with coded combination, Cr.WASB for five-weeks.

Group V: M.ALWC group with six-rats injected with single dose of STZ at 45 mg/kg and then were

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treated with Meth. Extract combination coded as M.ALWC for five-weeks.

Group VI: M.WAS group with six-rats injected with single dose of STZ at 45 mg/kg and then were treated with Meth. Extract combination coded as M.WAS for five-weeks.

Group VII: Aq.ALWC group with six-rats injected with single dose of STZ at 45 mg/kg and then were treated with Aqueous Extract combination coded as Aq.ALWC for five-weeks.

Group VIII: Aq.WAS group with six-rats injected with single dose of STZ at 45 mg/kg and then were treated with Aqueous Extract combination coded as Aq.WAS for five-weeks.

Group IX: Cr.B group with six-rats injected with single dose of STZ at 45 mg/kg and then were treated with coded combination, Cr.B for five-weeks.

2.6. Monitoring Body Weight & Biochemical Measurements:

The rats were weighted every week through the study period and at the end of the experiment. Fasting blood glucose was measured using Colorimetric Assay Kits (Cayman Chemical, Ann Arbor, Michigan). The insulin was assayed by Enzyme Linked Immunosorbent Assay (ELISA) method using Boehringer-Mannheim kit (Andersen et al., 1993), Estimation of HbA1c, Liver enzymes such as AST, ALT, and Trolox Equivalence Antioxidant Capacity (TEAC).[36,37]

2.6.1 Trolox Equivalence Antioxidant Capacity (Teac)

TEAC measures the antioxidant capacity of MO, as compared to the standard, Trolox. TEAC assay was carried out using the method described by Re et al., a stock solution was prepared from 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS (8 mM) and potassium persulfate $K_2S_2O_8$ (3 mM) formed $ABTS^+$, which was prepared 24 h before the analysis was performed. Trolox was used as the standard, 25 µL of extract and 275 µL of ABTS mix was added to the well. A Multiskan plate reader (Thermo Electron Corporation, Beverly, MA, USA) was used to read absorbance at 734 nm. Results were expressed as the extent of ABTS inhibition at 100 µg/µl concentration.[38,39]

2.7. Treatment, Blood Sample Collection & Homogenate Preparation:

Distilled water was used as the diluent for reconstructing the extract and administered via oral gavage for 4-5 weeks. At the end of the treatment, rats were fasted overnight and anaesthetized intraperitoneally with sodium pentobarbital injection (60 mg/kg). Sodium pentobarbital was used to ensure unconsciousness of rats while death occurred as well as guaranteeing rapid and painless death. This procedure was carried out in the animal house. Blood samples were obtained via the rat's abdominal aorta into a lithium heparin plasma separator tubes and serum clot activator tubes. Blood samples were centrifuged at 4000 g for 10 min at 4°C and then stored at -8°C to obtain plasma and serum.

STATISTICAL ANALYSIS:

All the data expressed as mean ± SEM were analysed by One-way Analysis of Variance (ANOVA) followed by Dennett's test and turkey's multiple post hoc test were utilized for determine the significant differences ($P < 0.05$) between groups for multiple comparison. The study results were analysed statistically by SPSS V. 21 software. A value of $p < 0.05$ was considered to indicate a significant difference between groups. Data are expressed as mean ± SD in antioxidant study.

III. RESULTS

Changes in body weight (Table: 1), Fasting blood glucose (Table: 2), Liver enzymes (Table: 3), Serum insulin & HbA1c (Table:4), and Trolox Equivalence Antioxidant Capacity (TEAC) in normal and experimental rats are presented (Table: 5).

Administration of STZ resulted in increase in Mean blood glucose level on day 0 when compared to normal control animals (group-I). Post treatment with various combinatorial therapies in STZ treated rats significantly reduced the increased blood glucose level as compared to group-II. Although the Coded groups Cr.WASB, M.ALWC and Aq. ALWC produced satisfying results with decrease in FBG, Liver enzymes (AST, ALT) and Glycosylated haemoglobin (HbA1c) and likewise Increase in bodyweights and Serum Insulin levels. The above mentioned three groups have also demonstrated with good antioxidant properties with highest ABTS inhibition at 100 µg/µl concentrations.

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Table 1: The effect of the various combinations on body weight of STZ-induced diabetic rats (Mean ± S.E.M), n = 9.

Treatment groups(n)	Bodyweight (gms) MEAN ± S.E.M.	
	Initial	Final
Normal Control group	194.21 ± 0.26	203.83 ± 2.02
Diabetic Ctrl group	193.08 ± 0.41	160.4 ± 3.16
Standard group	193.91 ± 1.39	199.58 ± 1.49
Cr. WASB	188.83 ± 0.56	198.25 ± 3.14
M.ALWC	191.9 ± 0.52	202.38 ± 3.13
M.WAS	189.28 ± 0.39	195.68 ± 2.59
Aq. ALWC	195.7 ± 0.33	201.75 ± 1.47
Aq. WAS	194.13 ± 0.41	198.31 ± 1.75
Cr. B	195.71 ± 0.53	200.18 ± 1.36

Each value expressed as means ±SEM, (rats = 6). *Values significant at P < 0.05 as against diabetic control.

Figure 1: the effect of various combinations on body weight of streptozotocin-induced diabetic rats (Mean ± S.E.M), n = 9

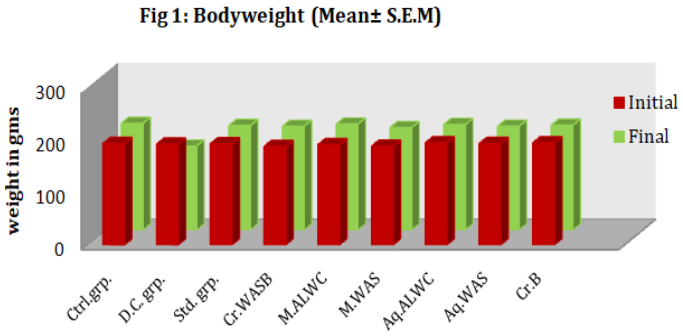

Table 2: The effect of the various combinations on Fasting Blood Glucose (FBG) on STZ-induced diabetic rats (Mean ± S.E.M), n = 9.

Treatment groups (n)	FBG (mg/dl)	MEAN ± S.E.M.
	Day 0 (Week 1)	Day 28 (Week 4)
Normal Controlgroup	70.6 ± 1.98	66.58 ± 2.24
Diabetic Ctrl group	380.6 ± 12.02	393.68 ± 6.37
Standard group	298.5 ± 7.34	142.11 ± 2.14
Cr. WASB	268.43 ± 7.56	73.61 ± 1.35

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M.ALWC	223.03 ± 12.5	70.9 ± 1.49
M.WAS	270.9 ± 4.33	146.03 ± 1.41
Aq. ALWC	237.95 ± 3.71	144.28 ± 1.56
Aq. WAS	270.53 ± 3.90	170.31 ± 3.35
Cr. B	270.68 ± 3.67	170.13 ± 2.62

Each value expressed as means ±SEM, (rats = 6). *Values significant at P < 0.001 as against diabetic control.

Table 3: The effect of the various combinations on Liver enzymes AST and ALT activities in STZ-induced diabetic rats. Data are the Mean ± S.E.M, n = 9.

Treatment groups(n)	**Liver Enzymes (U/L) MEAN ± S.E.M.**	
	AST (U/L)	**ALT (U/L)**
NormalControl group	26.3± 1.28	36.8 ±1.44
Diabetic Ctrl group	126.6 ± 1.64	70.6 ± 1.48
Standard group	46.9 ± 0.84	53.9 ± 1.19
Cr. WASB	35.2 ± 1.52	36.9 ± 0.88
M.ALWC	34.8 ± 1.54	38.7 ± 0.9
M.WAS	49.1 ± 1.23	54.1 ± 1.63
Aq. ALWC	49.2 ± 0.79	53.2 ± 1.95
Aq. WAS	68.7 ± 0.93	62.1 ± 0.94
Cr. B	69.3 ± 0.96	61.6 ± 1.17

Each value expressed as means ±SEM, (rats = 6). *Values significant at P < 0.05 as against diabetic control

Table 4: The effect of the various combinations on Serum insulin and HbA1C in STZ-induced diabetic rats. Data are the Mean ± S.E.M, n = 9.

Treatment groups	**Values (MEAN ± S.E.M.)**	
	Serum insulin (µU/ml)	**HbA1C**
N.Ctrl group	16.93± 0.79	5.33±0.21
D.Ctrl group	6.66 ± 0.65	14.83± 0.24
Standard group	12.78 ± 0.47	6.98± 0.14
Cr. WASB	15.31 ± 0.66	5.43± 0.24
M.ALWC	15.56 ± 0.61	5.91± 0.21
M.WAS	13.16 ± 0.36	7.05 ± 0.13
Aq. ALWC	13.3 ± 0.51	6.93± 0.17
Aq. WAS	9.9 ± 0.54	7.83± 0.28
Cr. B	10.51 ± 0.57	7.9 ± 0.16

Values are expressed as mean ± SEM of 6 rats in each group. *** p<0.001 when compared to control group).

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Table 5: Demonstrates the extent of ABTS + inhibition represented as percentage and TEAC values in treated groups.

Treatment groups	Values (MEAN ± S.E.M.)	
	Extent of Inhibition (%)	TEAC values
Cr. WASB	82.64	0.394±0.001
M.ALWC	78.42	0.352±0.002
M.WAS	70.61	0.312±0.001
Aq. ALWC	75.65	0.361±0.001
Aq. WAS	67.82	0.251±0.001
Cr. B	65.66	0.241±0.003

The extent of ABTS inhibition at 100 µg/µl concentration. Significant difference compared to control: *P < 0.05, Values are expressed in mean ± SD.

IV. DISCUSSION

Type 2 Diabetes mellitus includes the common major form of diabetes which results from defect(s) in insulin secretion and/or from insulin resistance, and often a combination of both**.**The body's response to insulin resistance is to enhance the β cell's secretion of insulin to maintain normal glucose tolerance.[40] The development of type 2 diabetes from the impaired glucose-tolerant state occurs as the result of an organized sequence of events. Initially, hepatic glycogenolysis and gluconeogenesis increase effecting Liver enzymes, resulting in enhanced basal hepatic glucose production. The final sequence of events is a progressive deterioration in β-cell function with subsequent decline in insulin-secreting ability [41] .Of critical importances is an understanding of how damaging the hyperglycemic state is at the tissue level. The hyperglycemic state induces the formation of harmful free radicals, increasing oxidative stress through non-enzymatic reactions and enzymatic processes. The hyperglycemic state is also responsible for the overproduction of superoxide anions by the electron transport system in the mitochondria. This may be the central mechanism that underlies all of the destructive pathways responsible for the diabetic paradigm.[42-45] Thus, in present study keeping in mind of all the pathological conditions, the parameters chosen are Fasting blood glucose (FBG), liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), antioxidant parameters (TEAC), S.Insulin, Glycosylated Haemoglobin (HbA1c) and bodyweight. The objectives of the study, for using various combinations of herbal drugs are indeed because of multiple complications in the Type 2 diabetes mellitus. Development of diabetes mellitus especially in rats by streptozotocin is easy, fast and reliable and sustained for a longer period is chosen as good diabetic model for study. [46]

The Body weight data represented in Table 1 clearly illustrated that rats injected with STZ showed significant decrease in body weight level comparison to the control group. No significant difference in body weight level in treated group and the control group. It was observed that the body weights of the Wister rats were increased after the test period, showed significant increase in body weight compared to diabetic group, One-way Analysis of Variance (ANOVA) followed by post hoc Tukey-Kramer Multiple Comparisons Test, P value is < 0.05, considered significant when compared to the Diabetic Control Group. The findings of Fasting Blood Glucose Levels (FBG) in Table 2 revealed that diabetic rat group showed a significantly higher glucose level compared with the control group. There were significant changes in fasting blood glucose level in Cr.WASB and M.ALWC groups, One-way Analysis of Variance (ANOVA) followed by post hoc Tukey-Kramer Multiple Comparisons Test, P < 0.001 when compared to Diabetic control group.

Data presented in Table 3 clearly illustrated that about the estimation of Liver Enzymes (ALT, AST) rats injected with STZ showed significant increase in serum ALT, AST levels comparison to the control group and treated groups, P ≤ 0.05.there is a marked decrease in Liver enzymes in Cr.WASB and M.ALWC when compared to other groups after treatment.

In Table 4, Estimation of Serum Insulin and HbA1c represented, Effect of treatment groups

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on increased Serum Insulin (µU/ml) and decreased HbA1c in Streptozotocin- induced diabetic rats before & after treatment observations were seen. Insulin level was found decreased in STZ-induced diabetic rats. Reversal of this effect was seen on treatment by the treated groups. This may be indicative of regeneration of the islet cells which is more prominent in Cr.WASB and M.ALWC groups.One-way Analysis of Variance (ANOVA) followed by post hoc Dunnett Multiple Comparisons Test, P value is < 0.001, considered significant when compared to the Control Group. In Table: 5, Data represented Total Equivalent Assay Capacity assay (TEAC) revealed satisfactory bioactivities of the extracts.. The extent of ABTS•+ inhibition for Cr.WASB, M. ALWC, M.WAS, Aq.ALWC, Aq.WAS, Cr.B extracts were 82.64%, 78.42%, 70.61%, 75.65%, 67.82%, and 65.66% respectively at 100 µg/µl concentration. The present study, demonstrated convincing TEAC value of 0.394±0.001, 0.352±0.002, 0.312±0.001, 0.361±0.001, 0.251±0.001 and 0.241±0.003, respectively.

V. CONCLUSION:

Combinatorial treatment approach is best suited for metabolic disorders such as diabetes mellitus that have multiple targets for drug action. In the present study, of all the various combinations, Cr.WASB, Methanol extract of ALWC and Aqueous extract of ALWC demonstrates anti-diabetic as well as antioxidant functions very effectively. Further the combinations act in synergistic way. This is a novel approach being evaluated for the first time in an effective manner and can lead to development of new therapy options in treatment of diabetes mellitus.

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DECLARATIONS

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Conflict of interest: None Declared.
Ethical approval: the study was approved by the Institutional Ethics Committee

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