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Mechanistic Explorations of Antidiabetic Potentials of *Sansevieria Trifasciata*

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ABSTRACT: There has been a great resurgence of interest in phyto-therapy in treating chronic diseases like Type 2 diabetes. The current research aims to explore the mechanistic anti-diabetic potentials of the leaves and rhizomes of *Sansevieria trifasciata* (ST). Chemo-profiling by phytochemical tests and GC-MS analysis have shown the presence of phenolics, alkaloids, terpenoids, flavonoids, saponins, steroids and glycosides. The median lethal dose was found to be 500 mg/kg on acute toxicity studies. The extract showed statistically significant ($p<0.001$ and $p<0.05$) hypoglycemia on fasting and oral glucose challenge; body weight ($p<0.001$) determination and quenching of TBARS ($p<0.001$); the facts being further supported by histopathological assessment. The flow cytometric data revealed the beneficial role of plant extract in preventing apoptotic cell death under hyperglycemic conditions and results of Western blot analysis showed reduced expressions of the vascular inflammation markers on administration of plant extract.

KEYWORDS: Phytotherapy; Chemoprofiling; Hypoglycemia ; Western Blot; Flow Cytometry.

INTRODUCTION

Constant escalations in the number of diabetics world-wide and the failure of conventional therapy in-spite of tremendous strides in modern medicines, to restore normoglycemia without adverse effects again calls for naturopathy and alternative medicine. Diabetes being multi-factorial and with secondary complications, prevention of hyperglycemia is the central dogma for its management. Till yet no such oral hypoglycemic exists which can achieve tight glycemic control without side effects. Dietary adjuncts, lifestyle interventions and resurgence of interest in phyto-therapy automatically made its way[1-3]. Natural hypoglycemics attracted attention due to ease of incorporation in everyday diet, easy affordability, less adverse incidences, and safety aspects on a long term basis. Ethno botanical literature reports about more than 800 anti-diabetic plants species[4].

Sansevieria trifasciata (ST), also known as snake plant or mother-in-law's tongue from family Agavaceae is an herbaceous, succulent, perennial plant, growing to a height of 90 centimeters. Leaves form a basal rosette, are flat, thick, leathery, sword-shaped, and variegated with grayish white transverse markings. Flowers are whitish green, up to 5 centimeters long. The plant is native to India and widely distributed in Philippines, Malaysia etc. Though mostly used for ornamental cultivation and as air purifying plant, literature surveys have shown that the plant has significant analgesic and antipyretic effect, used in diabetes, ear ache, pharyngitis, skin itches and urinary diseases. Related species have shown significant

antioxidant and anti-microbial potentials[5-8]. However the plant is readily available from cheap sources and very limited studies have been done on its anti-diabetic actions. The current research aims to explore the anti-diabetic potentials of the leaves and rhizomes of the plant mechanistically using assay procedures, immunoblotting and flow cytometric analysis.

MATERIALS & METHODS

Chemicals

Acetic anhydride, conc sulphuric acid, methanol, deoxycholic acid, EDTA etc. All chemicals used were of analytical grade and other diagnostic reagents were purchased from Merck (India) and Sigma Aldrich (India).

Instruments

UV spectrophotometer (Perkin Elmer Lambda 25 UV-vis); GC-MS (Agilent technologies 6890N Network GC system for gas chromatography); Western Blot; Flow cytometer FACS scan (Becton Dickinson).

Methodology

Collection of plant material

Leaves and rhizomes of the plant were collected from in and around of the areas of IIT Kharaghpur and authenticated by Biotechnologist Prof (Dr.) Shanta K Adiki, Nirmala College of Pharmacy, Guntur, South India.

Preparation of plant extract

Collected leaves and rhizomes of the plant were washed thoroughly in tap water and then dried at 35°C. Size reduction was done in electrical grinder (Bajaj GX 11) and passed through sieve # 40 to get uniform size. About 250 gm powder was macerated with methanol for about 5 days. After collecting the filtrate it was vacuum evaporated to get the methanol extract (extract coded as STE).

Phytochemical Investigations of the STE

Phytochemical analysis of the major bioactive compounds of interest of STE was performed using the methods of Harbone (1984), Trease and Evans (1989) and other literature methods. The extract was analyzed for glycosides (Keller Killiani and Borntrager's tests), alkaloids (Mayer's and Dragendorff's reagents), saponins (Foam test), carbohydrates (Molisch test), anthocyanins (sodium hydroxide test), phenols, flavonoids etc.

The total polyphenol content of the STE was determined by UV spectrophotometry (Perkin Elmer Lambda 25 UV-vis) at 760nm using Folin-Ciocalteu reagent by the method of Othman *et al.*, 2007 and Modnicki *et al*., 2009[9-10]. The concentrations of the total polyphenols were determined in Gallic equivalents (GAE) per gram of the extract. The polyphenol content was calculated by the formula:

$$X = \frac{5.6450 \times A}{m}$$

Where X is total phenolic compounds (%), A is absorbance of investigated extract and m is mass (g) of the investigated sample.

The total flavonoid content of the extracts was determined by the method of Djeridane *et al*., 2006 which is based on the formation of a complex of flavonoid-aluminium, and the concentration of the flavonoids was expressed in terms of quercetin equivalent (QE) per gram extract[11].

GC-MS analysis of the volatile constituents

Volatiles were identified and quantified using GC-MS (Agilent technologies 6890N Network GC system for gas chromatography) as per Yang et al., 2009 under the following chromatographic conditions: column length of 30 mm with internal diameter 0.25 mm with 0.25 mm film thickness of 5% phenyl methyl siloxane, with fused silica capillary column. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injection port temperature was 225°C with a split ratio of 5:1. Column temperature was held at 40°C for 1 min and then programmed 5°C/min to 280°C and held for 5 min. As per the MS conditions ion source was maintained at 230°C, electron energy 70eV, multiplier voltage 1247V, GC-MS interface zone temperature maintained at 280°C with a scan range of 35-350 mass units. The volatiles were identified by comparing their mass spectra and relative abundances with NIST 02 and WILEY 7 spectral libraries of compounds[12,13].

Acute toxicity studies of the extract

Acute toxicity studies were carried out to evaluate any possible toxic effect or changes in normal behavior of Wistar rats as per the methodology given by Achi et al., 2012. Thirty wistar rats were dived into five groups with six rats in each group. The first group served as the control which received only normal saline; second, third, fourth and fifth group received STE at doses of 100, 200, 400, 500 mg/kg body weight, intraperitonially. All rats received their respective doses daily with free access to food and water at all times. Behavioral changes, signs of toxicity, mortality were observed amongst animals at

intermittent hours for a period of 24 hrs. Observations also included changes in skin, eyes, mucous membrane, respiratory etc. Further animals were observed for a period of 14 days for symptoms of toxicity or death or latency of death. The LD50 values were determined according to Lorke's method by administering extracts in normal saline via oral or intraperitoneal route. The numbers of dead animals are to be expressed in percentage[14].

Anti-diabetic potentials of the extract in animal models

Swiss albino male rats, weighing approximately 120-130 g were acclimatized under laboratory conditions for two weeks prior to the experiments. All the experiments with animals were carried out according to the guidelines of the institutional animal ethical committee and full details of the study were approved by the CPCSEA. Diabetes was induced in the experimental animals with an intraperitoneal injection of STZ at a dose of 60 mg/kg body weight dissolved in citrate buffer (0.1 M, pH 4.5). STZ-injected animals exhibited massive glycosuria and hyperglycemia within a few days and the diabetic nature of the animals was confirmed by measuring blood glucose concentration 72 h after STZ injection in the overnight-fasted rats. The rats with blood glucose above 350 mg/dL were considered to be diabetic. The animals were divided into four groups (consisted of six rats in each) and they were treated as follows:

Group 1 (Control) - Animals received only water as vehicle.
Group 2 – Animals received STZ injection at a dose of 65 mg/kg body weight.
Group 3 – Animals received extracts orally at a dose of 50 mg/kg body weight once a day for 15 days after diabetic induction.
Group 4 - Animals received extracts orally at a dose of 100 mg/kg body weight once a day for 15 days after diabetic induction.
Group 5 - Received standard drug Glibenclamide (0.5mg/kg, i.p) once a day for 15 days. Blood was withdrawn from the tail vein and the fasting blood glucose levels were determined on day 0, 5, 10 and 15 days using single touch glucometer (Accu-check).

Oral glucose tolerance test carried out as per Soltani et al., 2007 where wistar rats were fasted overnight and were dived into three groups with six rats in each group. Group 1 rat served as normal control who were administered 2 g/kg glucose per oral (p.o.); Group 2 and 3 received the STE at doses of 50 and 100 mg/kg and glucose was administered to them 30 mins prior to the administration of extracts. Next blood was withdrawn from tail vein before administration of glucose and at 30, 60 and 120 mins after oral glucose administration and glucose level measured by single touch glucometer (AccuCheck, Roche Diagnostics, USA)[15-18].

At the end of the experiments, all the animals were sacrificed with an overdose of barbiturate and blood samples were drawn from the caudal vena cava, collected in heparinized tubes, and centrifuged at 1500 rpm for 10 min to obtain serum. For the biochemical analysis and immunoblotting, cardiac tissues were lysed in radioimmunoprecipitation assay (RIPA) buffers (50 mMTris pH 8, 150 mMNaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 mg/mL leupeptin, 2 mg/mL aprotinin, 1 mM EDTA, 10 mMNaF, and 1mM$NaVO_4$). After centrifugation and collecting the clear supernatant total protein concentrations were determined by BCA assay (Pierce/Thermo Scientific, Rockford, IL, USA). For histopathological examinations, tissues from the normal and experimental animals were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 mm thickness were stained with hematoxylin and eosin to evaluate under light microscope.

TBARS determination in tissues of normal and STE treated diabetic rats

TBARS (Thiobarbituric acid reactive substances) in tissues was estimated by the method of Fraga et al., 1981. 0.5 mL tissue homogenate, 0.5 mL saline and 1.0 mL 10% TCA were mixed well and centrifuged at 3000 rpm for 20 min. 1.0 mL of the protein free supernatant and 0.25 mL of thiobarbituric acid (TBA) reagent were mixed well and boiled for 1 hr at 95°C, tubes cooled at room temperature and absorbance measured at 532 nm[19-24].

Measurement of intracellular ROS production

Intracellular ROS production was estimated by using 20, 70- dichlorofluoresceindiacetate (DCFDA) as a probe following the method as described elsewhere. Briefly, 100 ml of tissue homogenates were incubated with the assay media (20 mMtrisHCl, 130 mMKCl, 5 mM MgCl2, 20 mM NaH2PO4, 30 mM glucose and 5 mM DCFDA) at 37˚C for 15 min. The formation of DCF was measured at the excitation wavelength of 488 nm and emission wavelength of 610 nm for 10 min by using fluorescence spectrometer equipped with a FITC filter[25,26].

Assay of intracellular NAD and ATP level

NAD and ATP play an important role in STZ induced DNA damage in diabetes mellitus. Levels of these parameters were, therefore determined in the experimental animals. NAD was assayed in the homogenates by the colorimetric method. Intracellular ATP concentration was estimated using the commercial

assay kit available from Abcam (Cambridge, MA, USA)[27-30].

Immunoblotting

Samples containing 30 mg proteins were subjected to 10% SDS-PAGES and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% non-fat dry milk to prevent nonspecific binding and then incubated with primary antibodies overnight at 12˚C. The primary antibodies used in the present study were anti p38 (1:1000), anti phosphorylated p38 (1:1000 dilution), anti ERK1/2 (1:1000), anti phosphorylated ERK1/2 (1:1000), anti-NF-kB (p65 subunit) (1:250 dilution), anti phosphorylated NF-kB (p65 subunit) (1:500), anti-cytochrome C (1:1000), anti cleaved caspase 3 (1:1000) and anti PARP (1:1000) antibodies. The membranes were washed in TBST (50 mmol/L TrisHCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate HRP conjugated secondary antibody (1:4000) for 2 h at room temperature and developed using the ultrasensitive ECL substrate (Millipore, MA). The intensity of each immunoblotting band was measured using the using NIH-imageJ software[27-30].

Determination of mitochondrial membrane potential (Δψm)

Mitochondria were isolated from the heart tissue of experimental rats. Analytic flow cytometric measurements for the membrane potential of isolated mitochondria were performed using a FACScan flow cytometer with an argon laser excitation at 488 nm. Mitochondrial membrane potential was estimated on the basis of cell retention of the fluorescent cationic probe rhodamine 123[26].

Determination cytochrome C expression in mitochondrial and cytosolic fractions

Cytosolic and mitochondrial protein fractions of cardiomyocytes were extracted as per literature methodologies. The purity of mitochondrial fractions was verified by immunoblotting for a reliable mitochondrial marker, prohibitin. These fractions were subjected to immunoblot analysis for the investigation of cytochrome C expression as described above.

Flow cytometric analysis of cardiomyocyte apoptosis:

To investigate the mode of cell death involved in the studied disease pathophysiology, the cardiomyocytes were isolated from the heart tissue of experimental animals and analyzed by flow cytometry. Cardiomyocytes were washed with PBS, centrifuged at 800 g for 6 min, resuspended in ice-cold 70% ethanol/PBS, centrifuged at 800 g for a further 6 min, and resuspended in PBS. Cells were then incubated with propidium iodide (PI) and FITC-labeled Annexin V for 30 min at 370C. Excess PI and Annexin V were then washed off; cells were fixed and then stained cells were analyzed by flow cytometry (Becton Dickinson, Mountain View, CA) equipped with 488 nm argon laser light source; 515 nm band pass filter for FITC-fluorescence and 623 nm band pass filter for PI-fluorescence using Annexin V/PI binding assay. The Annexin V binding assay measures the fluorescence generated by Annexin binding to externalized phosphatidylserine of apoptotic cells. On the other hand florescence generated by PI binding reflects the necrotic cells. A dot plot of PI fluorescence (y-axis) versus FITC-fluorescence (x-axis) has been prepared. The data was analyzed by Cell Quest software[25].

RESULTS & DISCUSSION

The methanol extract of the plant on preliminary phytochemical screening showed the presence of alkaloids, terpenoids, flavonoids, tannins, tri-terpenes, saponins, steroids and glycosides. The polyphenolic and flavonoid content on triplicate determination were found to be 301.25 ± 3.23 and 7.02 ± 1.02 respectively. GC-MS analysis of the extract showed the presence of compounds like ketones (25.9%), alcohols (22.6%), terpenoids (12.9%), about 18.8% of phenolic compound, methyl salicylate was determined in the species; relative concentration of isopropyl myristate was determined to be 20.7. Results of toxicity studies and anti-diabetic potentials of the extract (STE) are presented in **Table 1-4**. Results of TBARS level in tissues of normal and STE treated diabetic rats are presented in **Table 5**. STZ induced diabetic animals showed increased ROS production in cardiac tissue which was found to be quenched with the treatment with STE (plant extract). Results of histopathological assessments (**Fig. 1-3)** have shown that treatment with STE extract reduced the changes induced by STZ-injection and kept the organ almost similar to that of normal. The flow cytometric data revealed that hyperglycemia increased the number of Annexin V staining but very little PI binding, indicating apoptosis as the major cause of cell death in this disease pathophysiology. Treatment with the plant extract (STE) shows reduction in the number of apoptotic cells which indicates the beneficial role of STE in preventing apoptotic cell death under hyperglycemic condition (**Fig.4**). Levels of vascular inflammatory markers (VEGF, ICAM-1, IL-6 and MCP-1) were significantly high in STZ-induced diabetic rats but results of western blot analysis have shown that the administration of STE showed reduced expressions of the vascular inflammation markers and thus it's another suggestive beneficial role in the disease pathophysiology.

Table 1: **Determination of the median lethal dose of the STE**

Treatment	Dose (mg/kg)	No. of animals used	No. of survival	No. of death	MLD
Control	10	10	10	0	
Extract	100	10	10	0	
	200	10	10	0	500mg/kg
	400	10	7	3	
	500	10	5	5	

Table 2: **Impact of STE extract and its effect on Fasting Blood Sugar levels**

Treatment regimen of different Groups	Blood glucose level (0 hr)	Blood glucose level (10^{th} day)	Blood glucose level (15^{th} day)
Saline (5 ml/kg)	72.46±4.0	71.14±2.9	70.22±2.9
Streptozotocin (60mg/kg)	258.20±14.0*	278.80±8.2*	289.49±9.8*
STZ with MEST (100 mg/kg)	278.22±25.6	82.12±1.6**	80.22±4.9**
STZ with glibenclamide (0.5 mg/kg)	293.02±18.50	72.15±1.4**	72.29±1.6**

Measurement of values as mean ±SEM from n=6, * $p < 0.001$ compared with control group which is basically treated with saline and **$p<0.001$ compared with STZ control group.

Table 3: **Body weight determination of normal and STE treated diabetic rats**

Treatment regimen of different Groups	Initial body weight	Final body weight
Saline (5 ml/kg)	178.25±7.2	190.20±4.53
STZ (60 mg/kg)	179.36±7.9	140.97±5.6*
STZ with MEST (50 mg/kg)	169.37±4.4	160.91±3.7
STZ with MEST (100 mg/kg)	187.9±7.6	168.7±3.6
STZ with glibenclamide (0.5 mg/kg)	188.7±8.6	174.00±9.2**

Determination of values as mean ± SEM from n = 6,* $p< 0.001$ compared with saline Control group, ** $p < 0.05$ compared with STZ-control group.

Table 4: Effect of STE on oral glucose tolerance test

Treatment regimen of different groups	Initial plasma glucose concentration	Plasma glucose concentration after 1 hr	Plasma glucose concentration after 2 hrs
Control (2g/kg glucose)	75±2.0	95.21±3.4	122.2±3.90
Group 1-(2g/kg glucose+50mg/kg STE)	78.2±3.5	98±1.9	96±2.21
Group 2-(2g/kg glucose+100mg/kg STE)	81.20±3.8	92±2.4	78.5±3.05

Table 5: TBARS determination in tissues of normal and STE treated diabetic rats

Treatment regimen of different groups	TBARS level in kidney tissue	TBARS level in hepatic tissue
Saline (5 ml/kg)	0.05±0.007	1.42±0.5
STZ (60 mg/kg)	1.79±0.05*	2.32±0.1**
STZ with MEST (50 mg/kg)	1.13±0.08#	1.56±0.1***
STZ with MEST (100 mg/kg)	1.27±0.02	1.55±0.2***
STZ with glibenclamide (0.5 mg/kg)	1.02±0.08##	1.49±0.06***

Units- m moles/100g of wet tissue. Values are expressed as mean ± SEM from n = 6, *p < 0.01, ** p < 0.001 compared with saline control group. # P < 0.05, ***p< 0.001 compared with STZ-control group. ## P < 0.01 compared with STZ-control group.

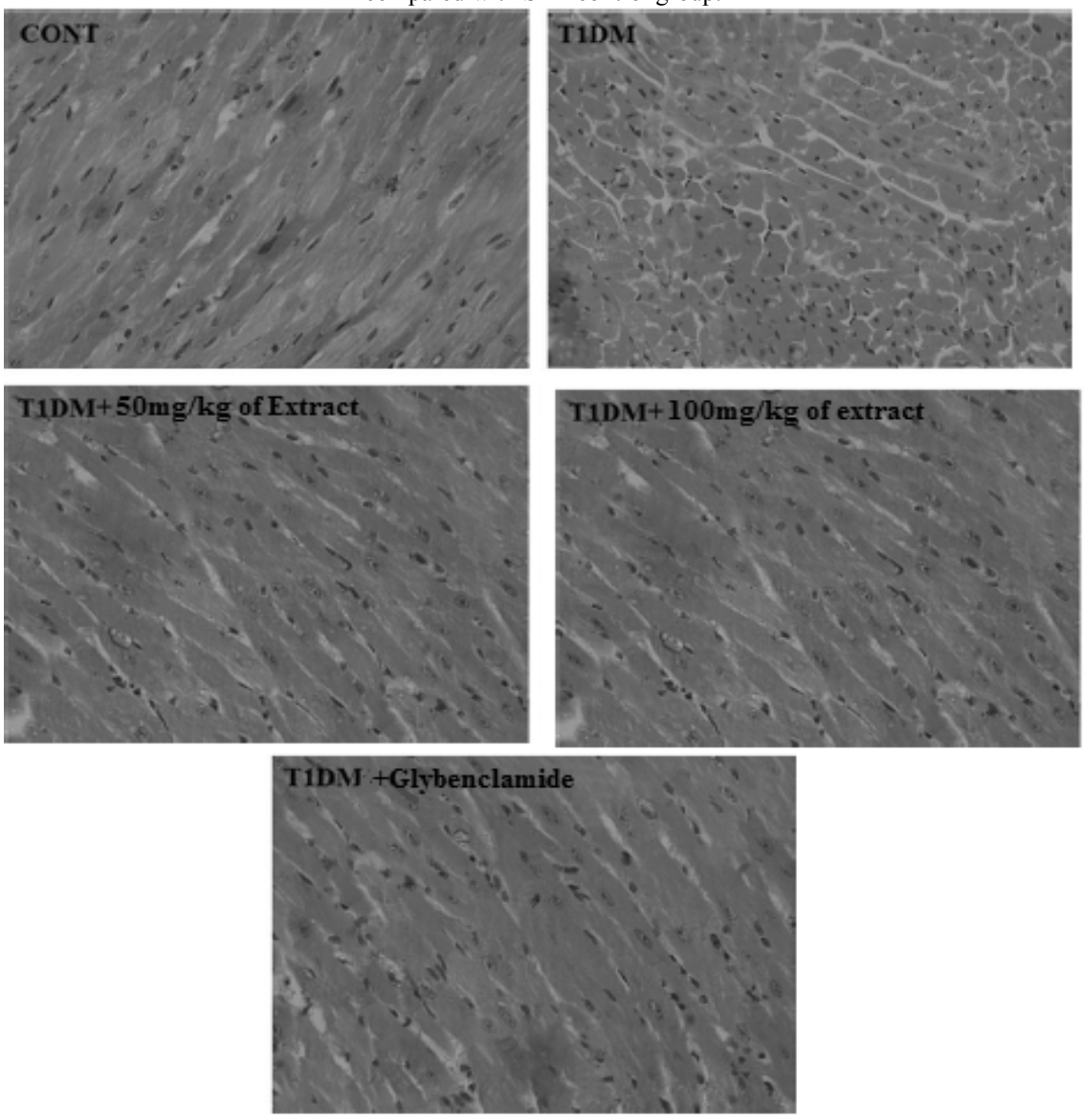

Fig.1: **Histological changes in cardiac tissue (stained with hematoxylin and eosin dye)**

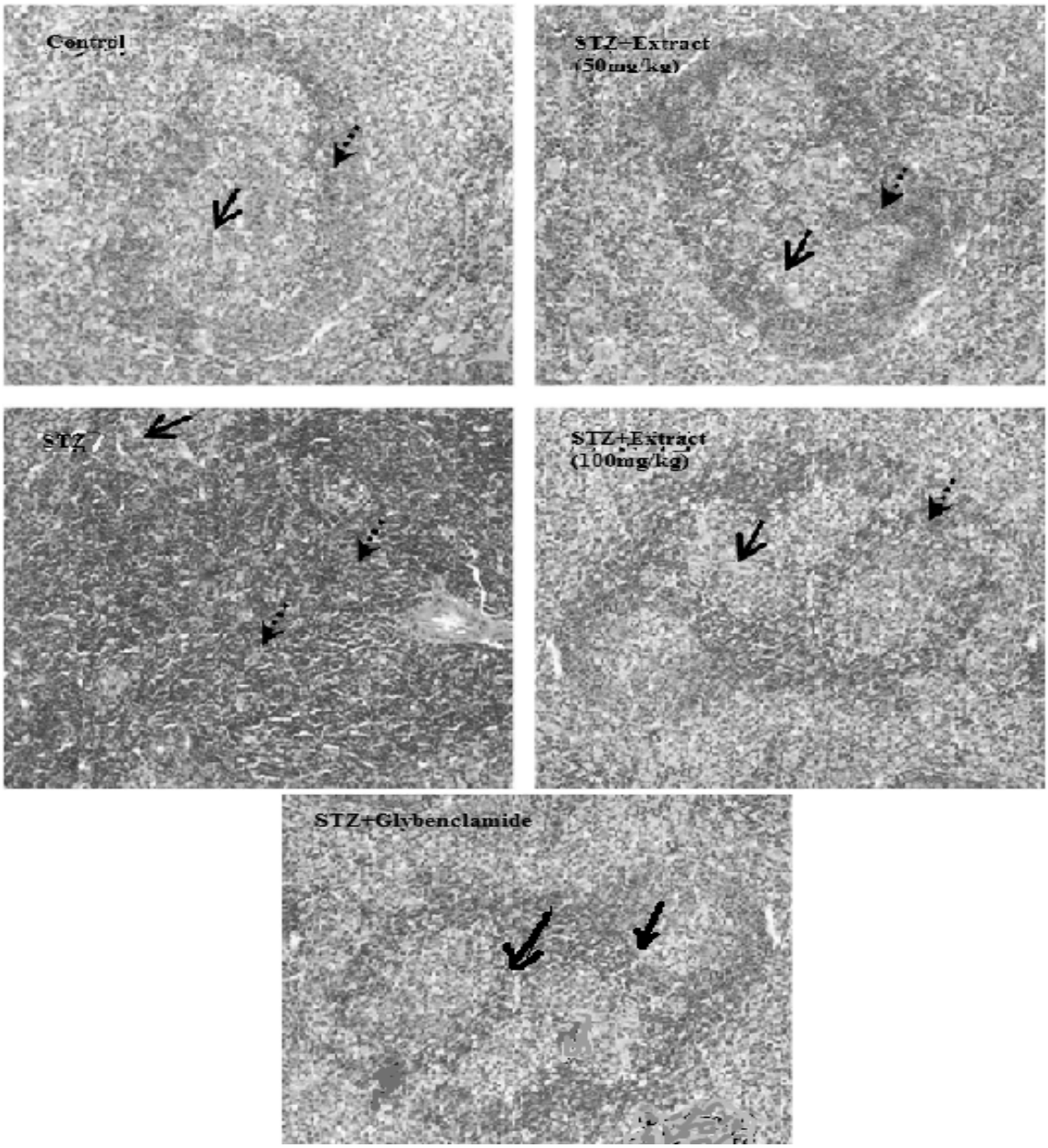

Fig.2: **Histological changes in spleen tissue (stained with hematoxylin and eosin dye)**

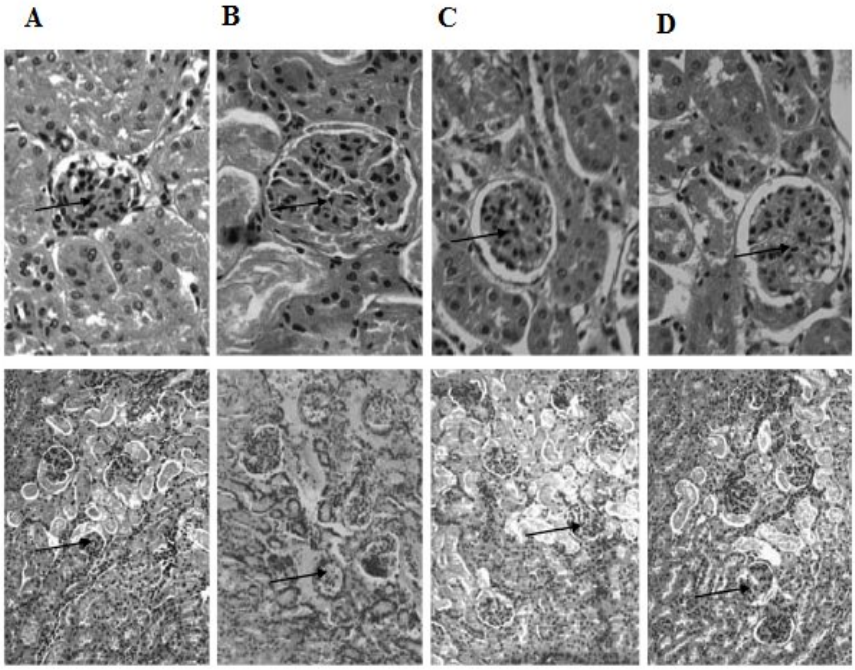

Fig.3: **Haematoxylin and eosin stained kidney section. (A) Kidney section from normal animals showing normal appearance of glomeruli (marked with arrows); (B) STZ treated animals, showing multiple foci of hemorrhage, necrosis and cloudy swelling of tubules (C) kidney section from extract + STZ showing almost normal appearance of glomeruli (marked with arrows) and tubules in kidney and (D) kidney section from Extract+ glibenclamide represents microscopic magnification (100×) and panel B represents microscopic magnification (1000×).**

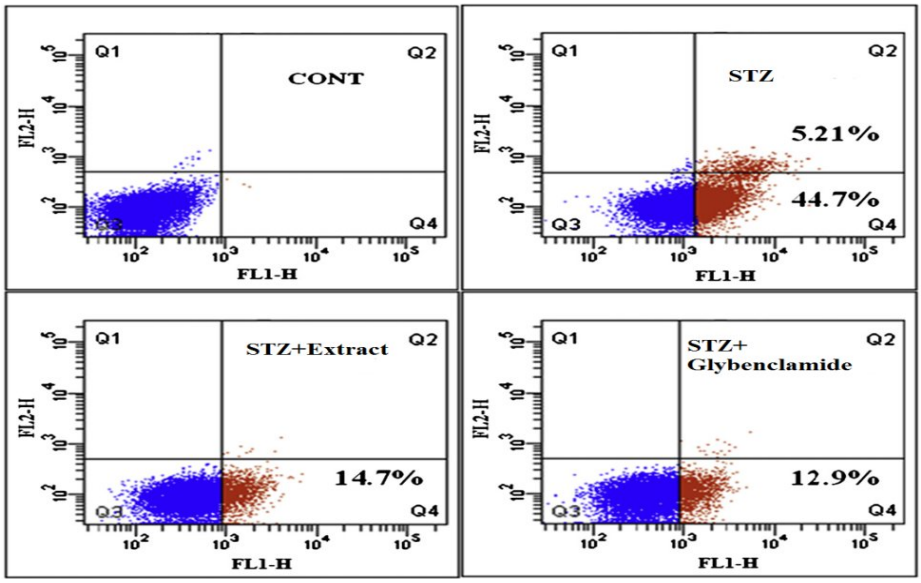

Fig.4: **FACS analysis of cardiomyocytes isolated from the experimental animals. Cell distribution analyzed using Annexin V binding and PI uptake. The FITC and PI fluorescence measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results expressed as dot plot representing as one of the six independent experiments. Cont: cells isolated from the heart tissue of normal animals; T1DM: cells isolated from the heart tissue of the diabetic animals; T1DM+Extract: cells isolated from the heart tissue of the diabetic animals treated with AA and T1DM+Glibenclamide: cells isolated from the heart tissue of the diabetic animals treated with Glibenclamide.**

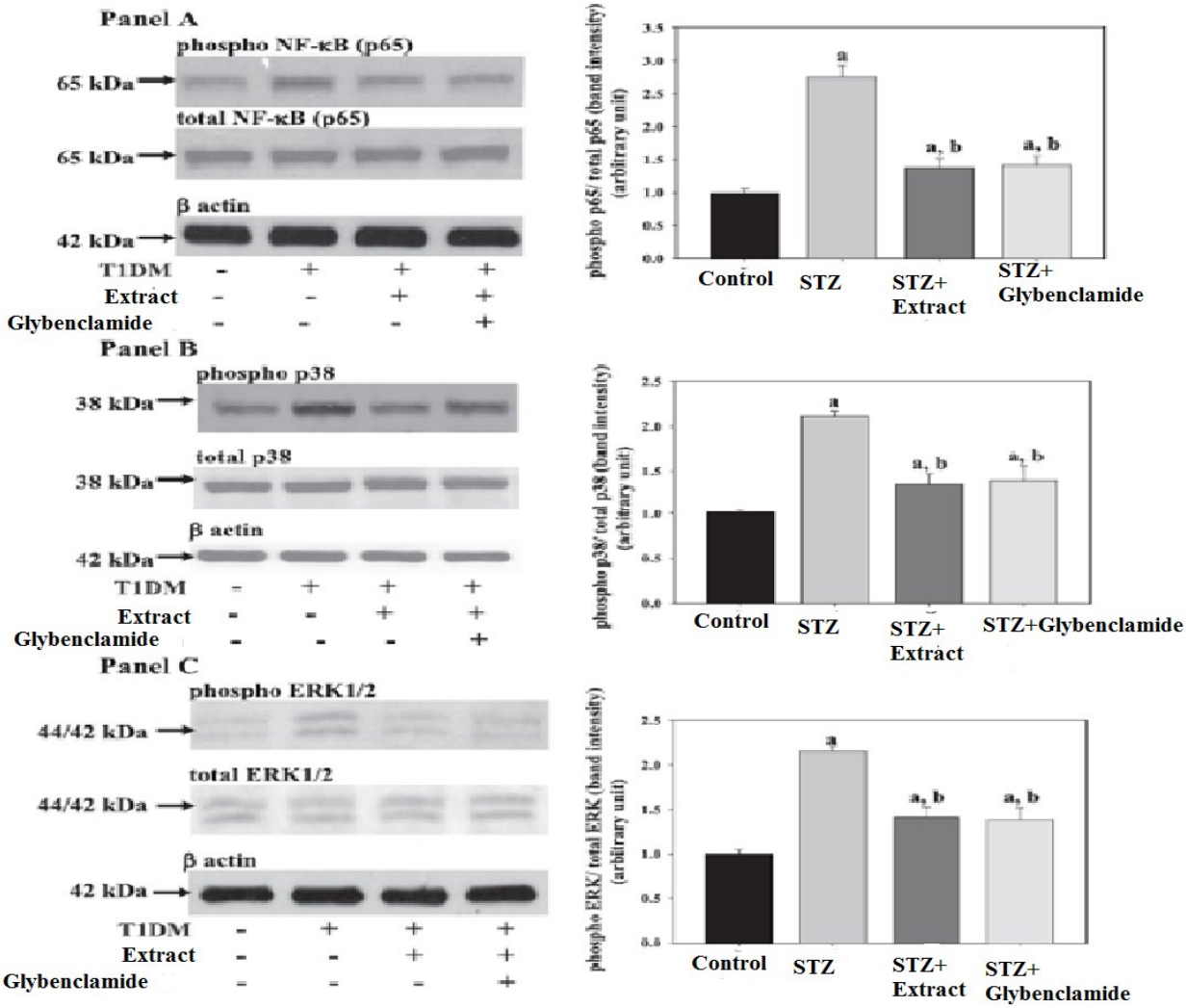

Fig.5: Western blot analysis of NF-kB (Panel A), MAPKs (p38: Panel B; ERK1/2: Panel C) in the cardiac tissue of diabetic and experimental animals. Cont: normal animals; TIDM: STZ treated diabetic animals; T1DM+Extract: animals administered with Extract after the induction of diabetes and T1DM+Glybenclamide: animals treated with glibenclamide after the induction of diabetes. Data are mean ± SD, for 6 animals per group and were analyzed by one-way ANOVA, with Student Newmane Keuls post hoc test. Significant differences were attributed at $p < 0.05$. The letter "a" indicates significant difference from "Cont" and "b" indicates significant difference from "TIDM" (untreated diabetic).

CONCLUSION

The current research made an in-depth exploration on the mechanistic role of *Sansevieria trifasciata* in combating Type 2 diabetes and the experimental results are in accordance with the fact that *S. trifasciata* has immense potentiality to serve as a good source of bioactive components that can be used as important lead compounds or therapeutic bullets in treating Type 2 diabetes. With the aid of computational biology further studies can be carried out in this regard.

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