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IN-VITRO ANTI-DIABETIC, ANTIOXIDANT AND ANTI-APOPTOTIC EFFECTS OF SELECTED INDIAN MEDICINAL PLANTS USING RINM5F CELLS

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Keywords:

Diabetes, Indian medicinal plants, *In-vitro*, Antioxidant, Apoptosis, Insulin

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ABSTRACT: The major pathogenic mechanisms of diabetes are chronic hyperglycemia and glucotoxicity. Excess production of reactive oxygen species due to hyperglycemia results in oxidative stress and changes in mitochondrial energy metabolism promote β cell dysfunction, eventually leading to apoptosis and the development of diabetic complications. Although several oral antihyperglycemic agents are available, their adverse effect profile affects their prolonged use. Medicinal plants, having a low toxicity profile, are being looked at as alternatives for diabetes treatment. The present study was thus planned to elucidate the mechanisms of action of 5 medicinal plants*viz. Azadirachta indica*, *Momordica charantia*, *Gymnema sylvestre*, *Syzygium cumini*, *and Trigonella foenum graecum* selected on the basis of Ayurveda in diabetes using RINm5F cells. The effects of these plants on Streptozotocin (STZ) induced oxidative stress was assessed using variables like lipid peroxidation in terms of malondialdehyde (MDA) release, modulation of apoptosis, cell cycle analysis using flowcytometry, and insulin release using commercially available rat insulin Elisa kits. All the plant extracts in the presence of STZ inhibited the release of MDA as compared to STZ-treated cells. Maximum reduction was observed with *Azadirachta indica* and *Gymnema sylvestre* extracts compared to STZ treated cells, suggesting the ability to protect from cell death. Maximum and significant insulin release was observed at 200 µg/ml of *Syzygium cumini* followed by *Gymnema sylvestre* and *Momordica charantia* as compared to STZ treated cells. Our findings demonstrate that these plants have the potential to modulate diabetic conditions through their anti-oxidant property, anti-apoptotic and cytoprotective actions.

INTRODUCTION: Diabetes mellitus is a serious metabolic disorder characterized by hyperglycemia resulting from either a defect in insulin secretion or insufficiency of insulin. Type 1 Diabetes mellitus (insulin-dependent) is caused due to the functional loss of insulin-producing beta cells in the pancreatic islets of Langerhans.

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Type 2 Diabetes mellitus (insulin-independent) is the more common form of diabetes, accounting for 90-95% of cases resulting from insufficient insulin production or insulin resistance [1]. According to the World Health Organization, diabetes was the 9th leading cause of death, with an estimated 1.5 million deaths directly caused by diabetes [2].

In 2021, 537 million cases of diabetes were reported worldwide and are likely to increase up to 783 million by 2045 [1]. Oxidative stress plays a pivotal role in Diabetes mellitus. Increased oxidative stress leads to the enhancement of lipid peroxidation in the diabetic state.

Lipid peroxidation is enhanced due to increased oxidative stress in the diabetic condition. Overproduction of reactive oxygen species (ROS) generated due to hyperglycaemia causes oxidative stress and changes in mitochondrial energy metabolism, which promotes β cell dysfunction, eventually leading to apoptosis and diabetic complications. Diabetes causes significant morbidity and mortality due to microvascular and macrovascular complications and demands a multi-therapeutic approach [3]. Several therapies are currently available for diabetes including insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and glinides [4-6].

Metformin, thiazolidinediones, acarbose, miglitol, glibenclamide *etc* are commonly used antidiabetic drugs to achieve better glycaemic regulation. Many of these oral antidiabetic agents show several adverse effects such as nausea, abdominal pain, abnormal weight gain, allergic reaction, dark urine and complications in pregnancy; therefore, considering these ide effects of the synthetic drugs, research on exploring more effective and safer hypoglycaemic agents with 'fewer' lesser side effects is still a challenge [7]. Medicinal plants have a low toxicity profile with minimal or no side effects; therefore, they are being looked at for the treatment of diabetes. Several medicinal plants have been described as having antidiabetic, anti-hyperglycaemic, and antioxidant properties. The anti-hyperglycaemic effects of plants are often due to their ability to improve the performance of the pancreatic tissue by causing an increase in insulin secretion or inhibiting the intestinal absorption of glucose.

According to ethnobotanical information, about 800 plants may possess antidiabetic potential, whereas ethnopharmacological surveys suggest that more than 1200 plants used in traditional medicine systems exhibit hypoglycaemic properties [8, 9]. *Azadirachta indica, Momordica charantia, Gymnema sylvestre, Syzygium cumini, and Trigonella foenum graecum* are 5 such plants that are known to possess anti-hyperglycaemic and antioxidant properties [10-14]. Aqueous and ethanol extracts of *Momordica charantia* and *Azadirachta indica* have previously been shown to have an antihyperglycemic effect in STZ or alloxan-induced diabetic rats [15-19]. Seeds of *Trigonella foenum graecum* have demonstrated hypoglycemic and hypocholesterolemic effects in animal models [20, 21]. The seeds of *Syzygium cumini* have been reported to have potent antimicrobial, antioxidant activity due to the presence of gallic and ellagic acids [22, 23]. However, the mechanism by which these plants exert their protective effects on the progression of 'Diabetes mellitus (DM)' remains unknown (especially pancreatic β-cell mass). Therefore, the present study was undertaken with the objective to evaluate the potential of these 5 medicinal plant extracts on STZ-induced oxidative damage using RINm5F cells. The effects of these plants on streptozotocin (STZ) induced oxidative stress was assessed using variables like lipid peroxidation in terms of malondialdehyde (MDA) release, modulation of apoptosis, cell cycle analysis, and insulin release.

MATERIALS AND METHODS:

Chemicals: All the chemicals and reagents were purchased from Sigma (St Louis, MO, USA). Antibiotic mixture & serum supplement were obtained from HiMedia Laboratories Pvt. Ltd. Cell culture media was purchased from Gibco Life technologies.

Study Drugs: Aqueous (Aq), hydroalcoholic (HA), and alcoholic (Al) extracts of the selected 5 plants *viz., Azadirachta indica, Momordica charantia, Syzygium cumini, Trigonella foenum graecum,* and *Gymnema sylvestre* were obtained directly from Nashik. A certificate of analysis was obtained along with the plant material. Quality control of plant material was carried out as per WHO guidelines. A concentration range from 2.5µg/ml to 200µg/ml was selected for the various assays. Aqueous extracts of all plants and hydroalcoholic extract of *Azadirachta indica* were dissolved in distilled water and further diluted in RPMI, a cell culture medium. Alcoholic and hydroalcoholic extracts of the remaining 4 plants were dissolved in Dimethyl sulfoxide (DMSO) and further diluted in a cell culture medium to achieve the required concentrations. Glibenclamide was used as the standard drug and dissolved in DMSO.

Cell Culture: The rat insulinoma cell line (RINm5f) was purchased from the National Centre of Cell Sciences (NCCS), Pune. Cells were grown in poly-L-lysine coated 75cm^2 flasks in RPMI1640

medium supplemented with 100 units/ml of penicillin, 100µg/ml streptomycin, and 10% fetal bovine serum maintained in humidified incubator in the presence of 5% CO_2 at 37°C. Cells were cultured to 80 % confluence and used for further analysis.

***An in-vitro* assay using RINm5F Cell Line:**

Viability Assay: The viability assay of the various plant extracts and standard drug-using RIN cells was carried out using MTT assay. RIN m5F cells of 1×10^5 were seeded in 96 well microplates and incubated at 37°C overnight. After 24 h, the cells were treated with the various plant extracts at concentrations ranging from 2.5µg/ml to 200µg/ml and the plate was incubated for 24 h at 37°C. Following incubation, the cells were treated with 5mg/ml of MTT dye and the plate was further incubated for 4 h at 37 °C. The plate was removed after 4 h and 1N HCL: Isopropanol (1:24) was added in each well to dissolve the purple formazan crystals. The absorbance was measured by using ELISA reader at 550nm.

Lipid Peroxidation Assay: Lipid peroxidation was measured to assess the oxidative damage in terms of Malondialdehyde (MDA) levels and expressed as nmoles/mg of protein. The MDA release from RIN cells treated with STZ was determined by Thiobarbituric Acid Reactive Substances (TBARS) method. Briefly, 2 x 10^6 cells/ml of RIN-5F cell line were seeded with a complete medium of RPMI 1640 and 10% FBS in a 24-well plate. The cells were then treated with different concentrations of the plant extracts and standard anti-diabetic drug in the presence/absence of STZ (5mM), and the plate was then further incubated for 24hours. After the treatment, the cells were trypsinized and re-suspended in 1ml of ice-cold Phosphate-Buffered-Saline (PBS). 0.5ml of the cell suspension was used for MDA estimation and the remaining 0.5ml for protein estimation.

The assay mixture contained 0.5ml of cell suspension, 0.5ml of 0.5 M KCl in 10mM tris HCl, 0.5ml of 30% Trichloroacetic acid (TCA) and 0.5ml of 52mM Thiobarbituric acid (TBA). The assay mixture was heated at 80°C for 50 minutes, and after cooling, the mixture was centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. These concentrations of MDA were extrapolated from the standard curve constructed with 1,1,3,3-tetraethoxypropane. The cell protein concentration in the remaining cell suspension was determined by Folin Lowry method, and the protein concentrations were extrapolated from the standard curve constructed with Bovine Serum Albumin (BSA).

Apoptosis and Cell Cycle Analysis: To investigate the anti-apoptotic potential of 5 selected medicinal plant extracts on RINm5f cell line, Annexin V and Propidium Iodide (PI) double staining was performed to assess possible induction of cell death with STZ. RINm5f cells were treated with different plant extracts with/without STZ(2mM) and apoptosis was measured by flow cytometry. Annexin V+/PI+ - was determined as apoptosis.

Measurement of Annexin V-fluorescein Isothiocyanate (FITC) Apoptosis: Cells (1×10^5) were seeded in 24 well plates. After 48 h, cells were treated with different concentrations of drugs with and without STZ. After 72 h, control, i.e., untreated cells and STZ treated cells, were trypsinized, washed in PBS, and re-suspended in Annexin binding buffer. 5µl of Annexin V FITC and 5µl of Propidium Iodide (PI) was added to the cell suspension and incubated in the dark for 15 minutes at room temperature. The cells were then washed with Annexin V Binding buffer and centrifuged at 311 × g for 10 min at 4°C, and the supernatant was then decanted. The cells were re-suspended in Annexin V binding buffer, and the cells were fixed on ice with 2% Formaldehyde for 10 min. The cells were washed twice with PBS and were centrifuged at 393 × g for 10 min at 4 °C. The pellet was re-suspended in RNAase A and incubated for 15min at 37 ^{0}C. Apoptosis was measured immediately on the Image Stream platform (Amnis Corporation, EMD Millipore).

Cell Cycle Analysis: Cells (1×10^5) were seeded in 24 well plates. After 48 hours, cells were treated with different concentrations of drugs with and without STZ. After treatment, the cells were trypsinized and centrifuged at 311 × g for 10 min at 4°C and decant the supernatant. Lysing buffer (100mM Tris HCL, 154mM NaCl, 1mM $CaCl_2$, 0.5mM $MgCl_2$, 0.1% NP 40, 50µg/ml Propidium

Iodide, 40µg/ml RNAase) was added to the cell pellet. Propidium Iodide (50µg/ml) and RNAase (40µg/ml) were added to the lysing solution. The lysing solution containing the cells was incubated in the dark at room temperature for 15 min. The stained cells are then acquired through the Flow cell of the Flow cytometer. These stained cells were acquired using the Cell Quest Pro software of the Flow Cytometer.

Insulin Secretion Assay: Insulin secretion was studied to assess the effect on functional damage, the cell supernatant was collected and insulin secretion was measured using a rat insulin ELISA kit.

Statistical Analysis: The results were analyzed for statistical significance by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test using the Graphpad Instat software (version 3.06). All data were expressed as Mean ± SD values. In all analyses, a p-value of <0.05 was considered statistically significant.

RESULTS:

Effect of Selected Plant Extracts on Viability of RINm5f Cells: Viability studies were performed using MTT assay to eliminate the cytotoxic doses of plant extracts from further study. The concentrations studied ranged from 2.5 to 200µg/ml.

As depicted in **Fig. 1**, the aqueous, hydroalcoholic, and alcoholic extracts of all 5 plants did not affect the viability of RIN cells when compared to the untreated, and DMSO-treated RIN cells.

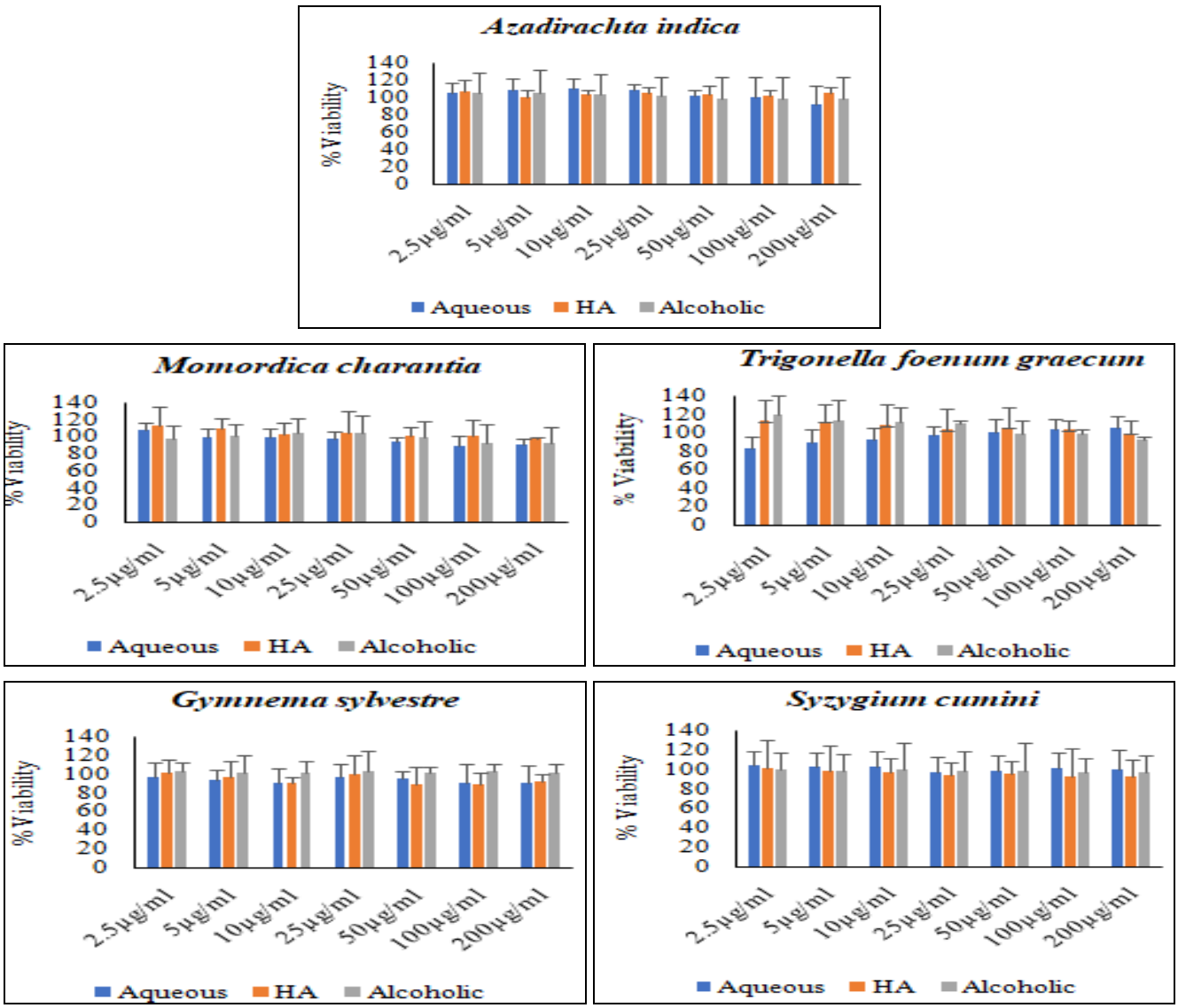
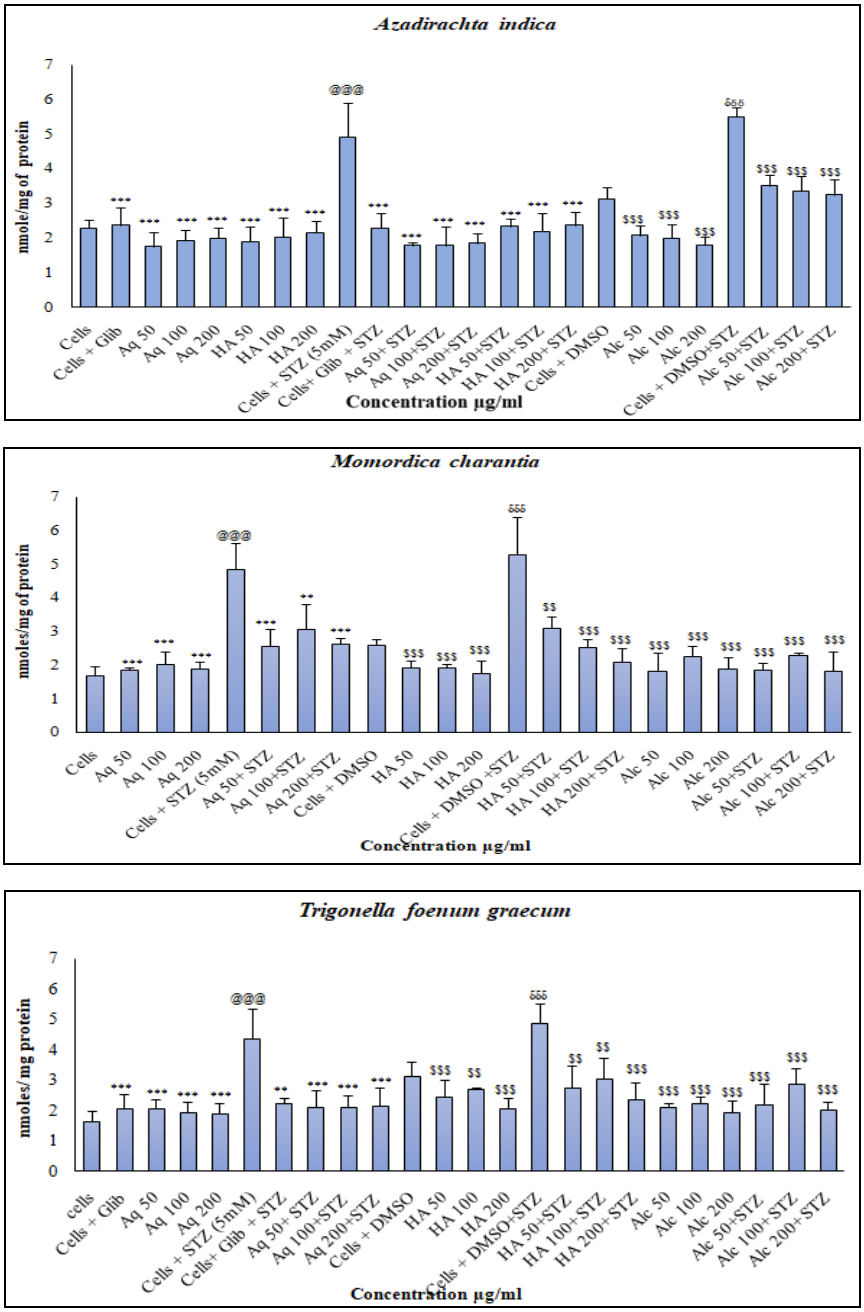


FIG. 1: EFFECT OF PLANT EXTRACTS ON VIABILITY OF RIN CELLS (N=3

Further, the anti-diabetic mechanisms of action of the selected plant extracts at 50, 100 and 200 µg/ml were elucidated against beta-cell damage induced by STZ by exploring their role on lipid peroxidation, intracellular DNA damage and insulin secretion. The effect of selected plant extracts was compared with glibenclamide, a standard anti-diabetic drug, and with control cells and STZ treated cells.

Effect of Select Plant Extracts on Lipid Peroxidation: The effect of selected plant extracts on anti-oxidant mechanism was explored by evaluating Malondialdehyde (MDA) levels, a marker used to assess lipid peroxidation. The results of MDA release from RINm5F cells in the presence or absence of STZ on the selected plant extracts are depicted in **Fig. 2**. As expected, RINm5f cells, when treated with STZ, caused a significant increase in MDA level compared to untreated or control cells. A significant decrease was observed when the cells were treated with glibenclamide alone and in the presence of STZ compared to STZ-treated cells. All the plant extracts in the presence of STZ inhibited the release of MDA as compared to STZ-treated cells or DMSO and STZ-treated cells.



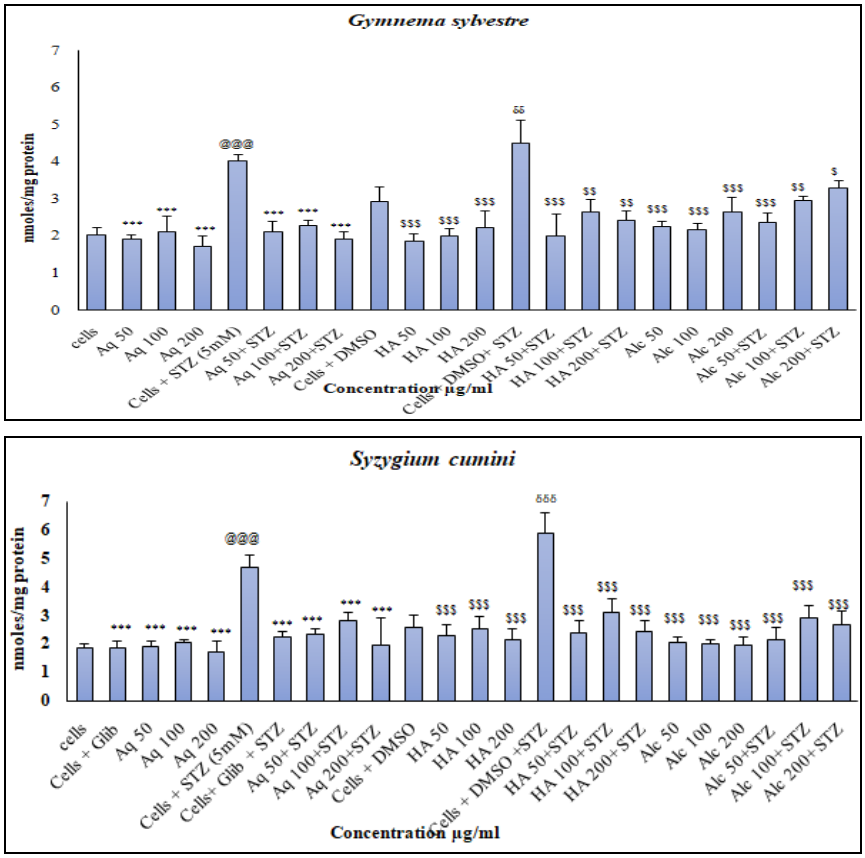
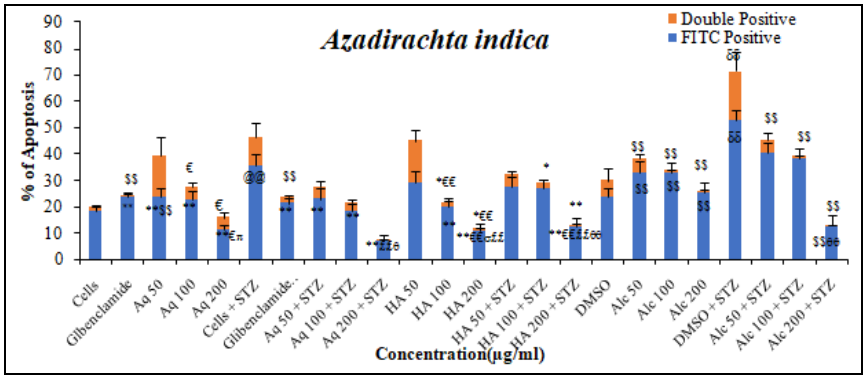


FIG. 2: FIG. 2: EFFECT OF SELECT PLANT EXTRACTSON MDA LEVELS. Results expressed as Mean ± **SD** $^{@@@}$p<0.001 as compared to cells; **p<0.01; ***p<0.001 as compared to cells + STZ; $^{\delta\delta}$p<0.01; $^{\delta\delta\delta}$p<0.001 as compared to Cells + DMSO; $^{\$}$p<0.05; $^{\$\$}$p<0.01; $^{\$\$\$}$p<0.001 as compared to Cells + DMSO + STZ

Effect of Selected Plant Extracts on Intracellular DNA Damage: Streptozotocin treatment causes pancreatic beta cells to enter apoptosis which represents an essential step in the molecular mechanism of the diabetogenic effect of STZ. Hence the effect of STZ on RIN cells was evaluated for apoptosis and cell cycle analysis using Flow cytometry.

Apoptosis: As shown in **Fig. 3**, a significant increase in the percentage of cells undergoing apoptosis was observed with 5mM STZ treatment for 24 h compared to control cells. Pre-treatment with the plant extracts at a 200 µg/ml concentration resulted in a significant reduction in apoptotic cell death in STZ-treated cells, suggesting protection from cell death by the extracts except for *Momordica charantia* and *Syzygium cumini*. Maximum reduction was observed with the plant extracts of *Azadirachta indica* and *Gymnema sylvestre* compared to STZ-treated cells. This reduction was greater than that seen in cells treated with standard drug *i.e.*, glibenclamide.

HA & alcoholic extracts of *Momordica charantia*, at lower concentrations of 100 µg/ml and 50 µg/ml, respectively, showed a decrease in apoptotic cells.



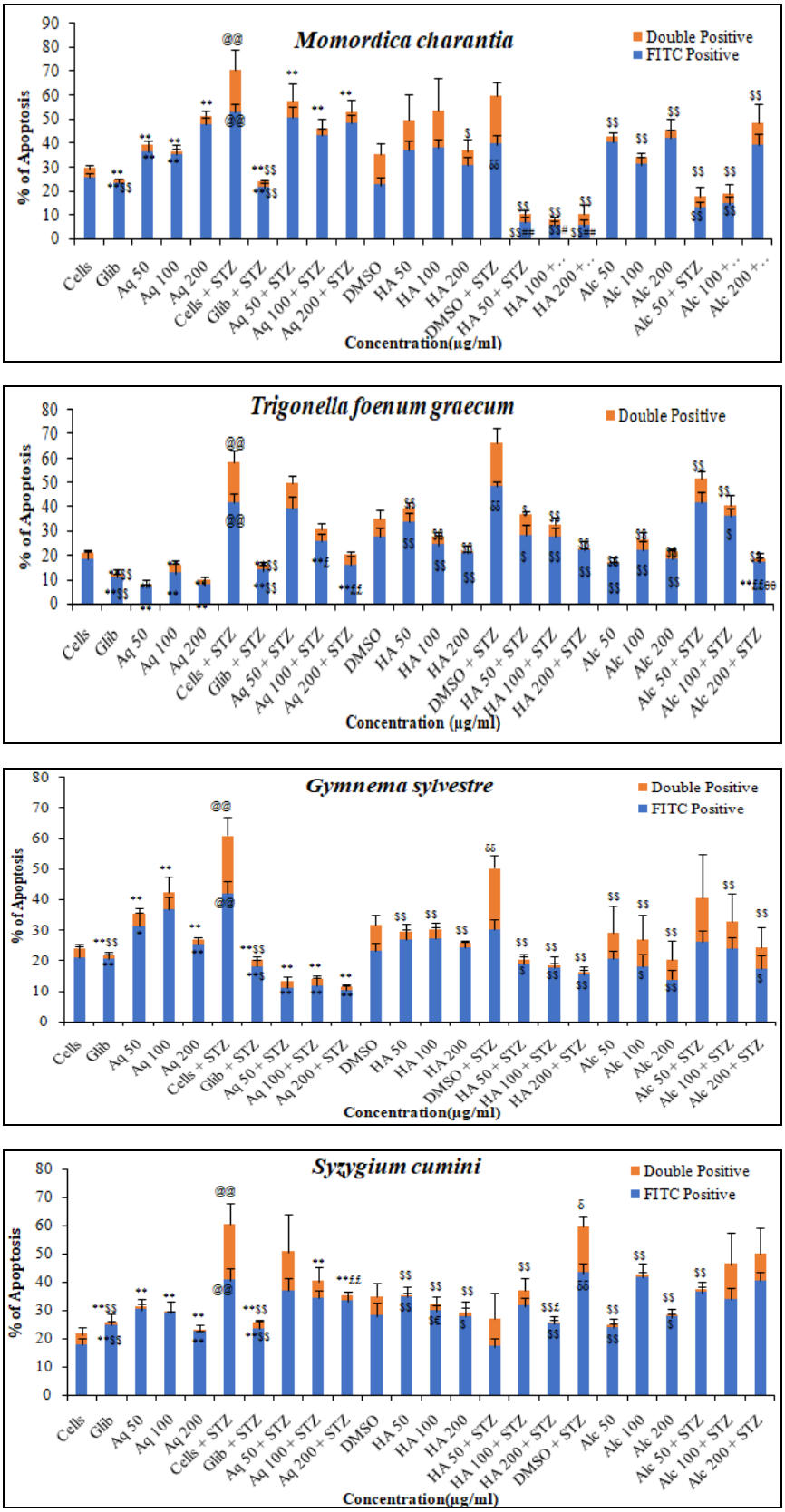
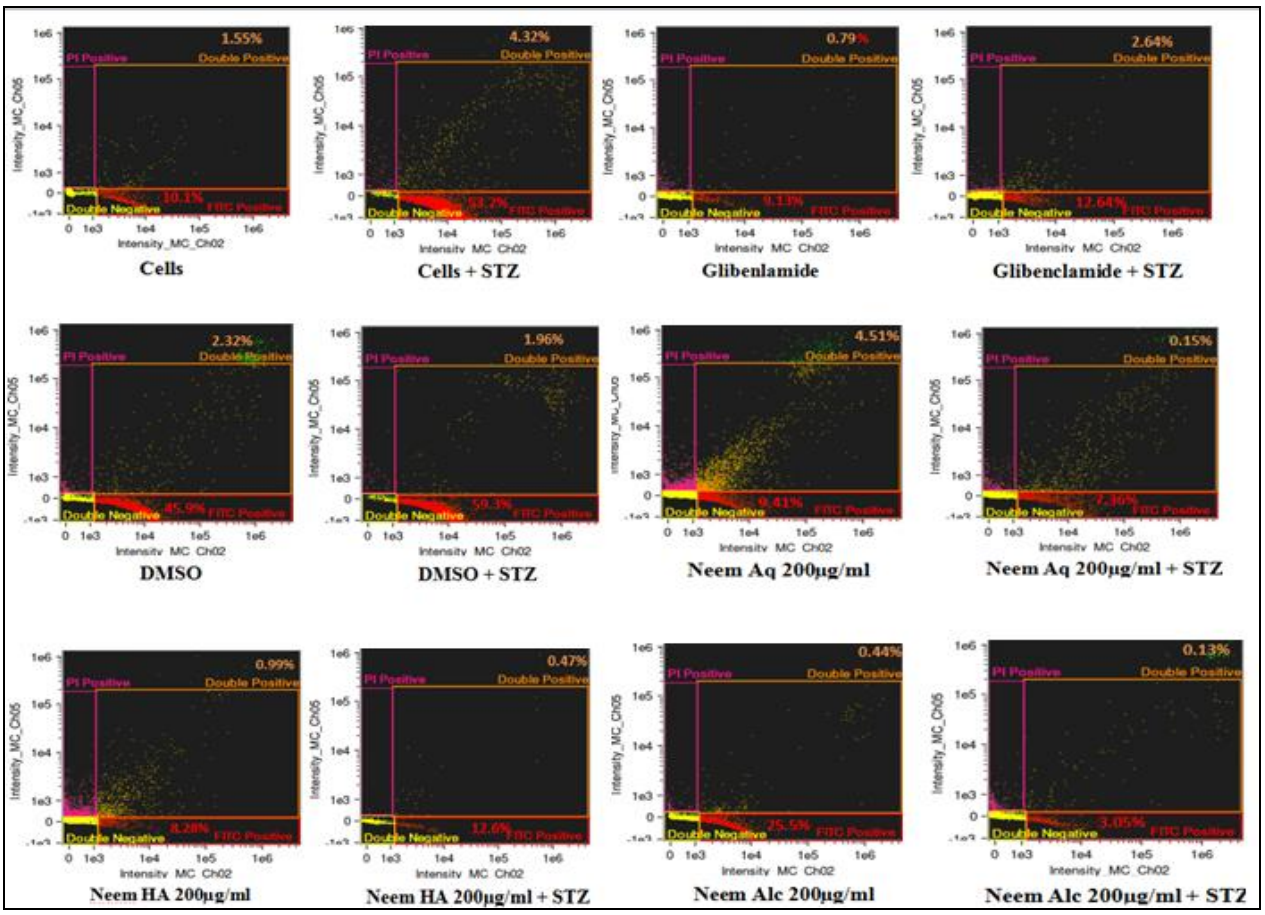
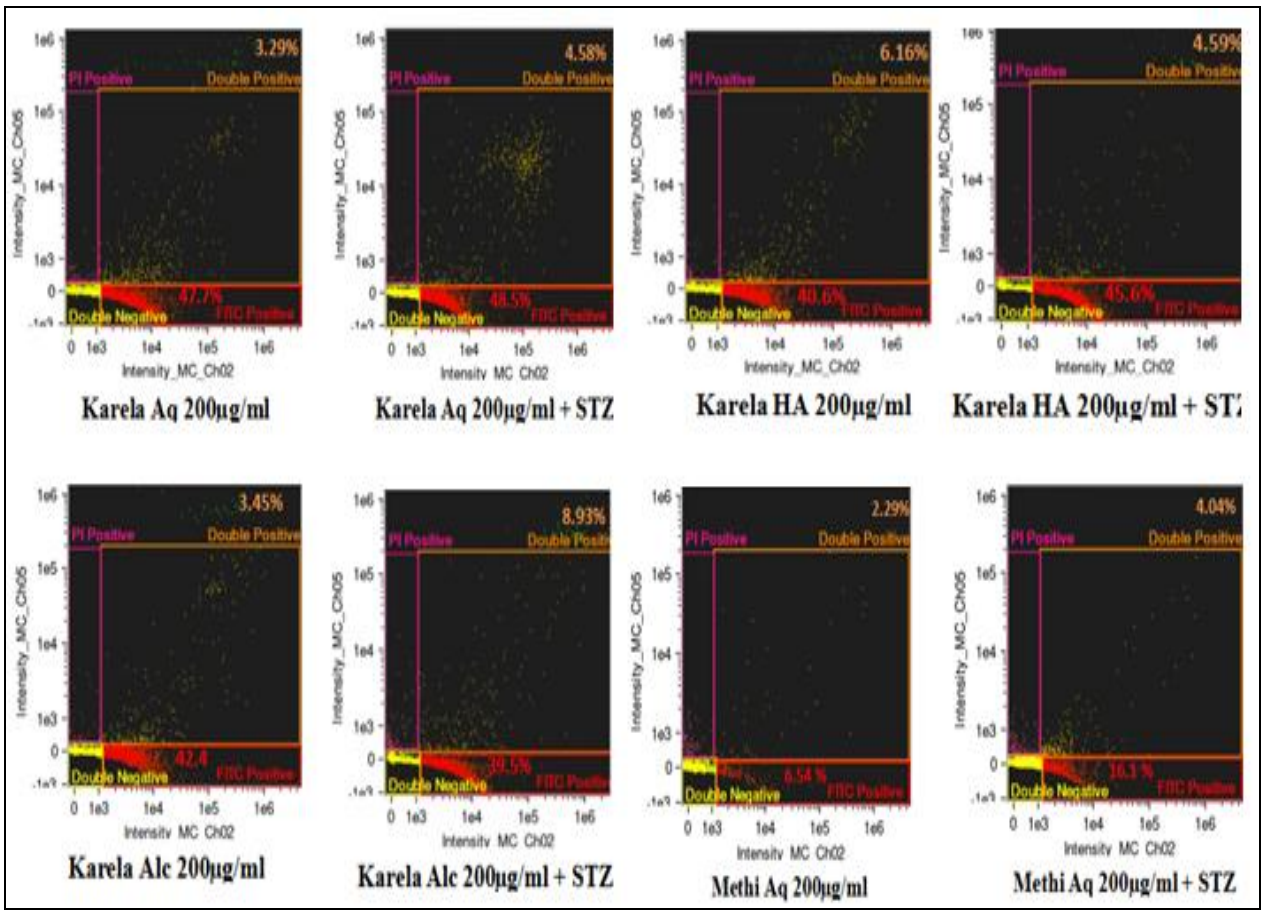


FIG. 3: EFFECT OF SELECT PLANT EXTRACTS ON APOPTOSIS INDUCED BY STZ IN RINM5F CELLS (N=3). Results are expressed as Mean ± SD $^{@}p<0.01$,$^{@@}p<0.001$ as compared to cell control; $^{*}p< 0.01$, $^{**}p< 0.001$ as compared to cells +STZ $^{\delta}p<0.01$, $^{\delta\delta}p<0.001$ as compared to cells +DMSO; $^{\$}p<0.01$, $^{\$\$}p<0.001$ as compared to cells + DMSO + STZ $^{\sigma}p<0.01$ as compared to cells + Glib; $^{\#\#}p<0.001$ as compared to cells + Glib + STZ $^{\epsilon}p<0.01$, $^{\epsilon\epsilon}p<0.001$ as compared to cells +50µg/ml; $^{£}p<0.01$, $^{££}p<0.001$ as compared to cells + 50µg/ml + STZ $^{\pi}p<0.01$ as compared to cells + 100µg/ml; $^{\theta}p<0.01$, $^{\theta\theta}p<0.001$ as compared to cells + 100µg/ml +STZ


1.55%
Cells
4.32%
Cells + STZ
0.79%
Glibenlamide
2.64%
Glibenclamide + STZ
2.32%
DMSO
1.96%
DMSO + STZ
4.51%
Neem Aq 200µg/ml
0.15%
Neem Aq 200µg/ml + STZ
0.99%
Neem HA 200µg/ml
0.47%
Neem HA 200µg/ml + STZ
0.44%
Neem Alc 200µg/ml
0.13%
Neem Alc 200µg/ml + STZ


3.29%
Karela Aq 200µg/ml
4.58%
Karela Aq 200µg/ml + STZ
6.16%
Karela HA 200µg/ml
4.59%
Karela HA 200µg/ml + STZ
3.45%
Karela Alc 200µg/ml
8.93%
Karela Alc 200µg/ml + STZ
2.29%
Methi Aq 200µg/ml
4.04%
Methi Aq 200µg/ml + STZ

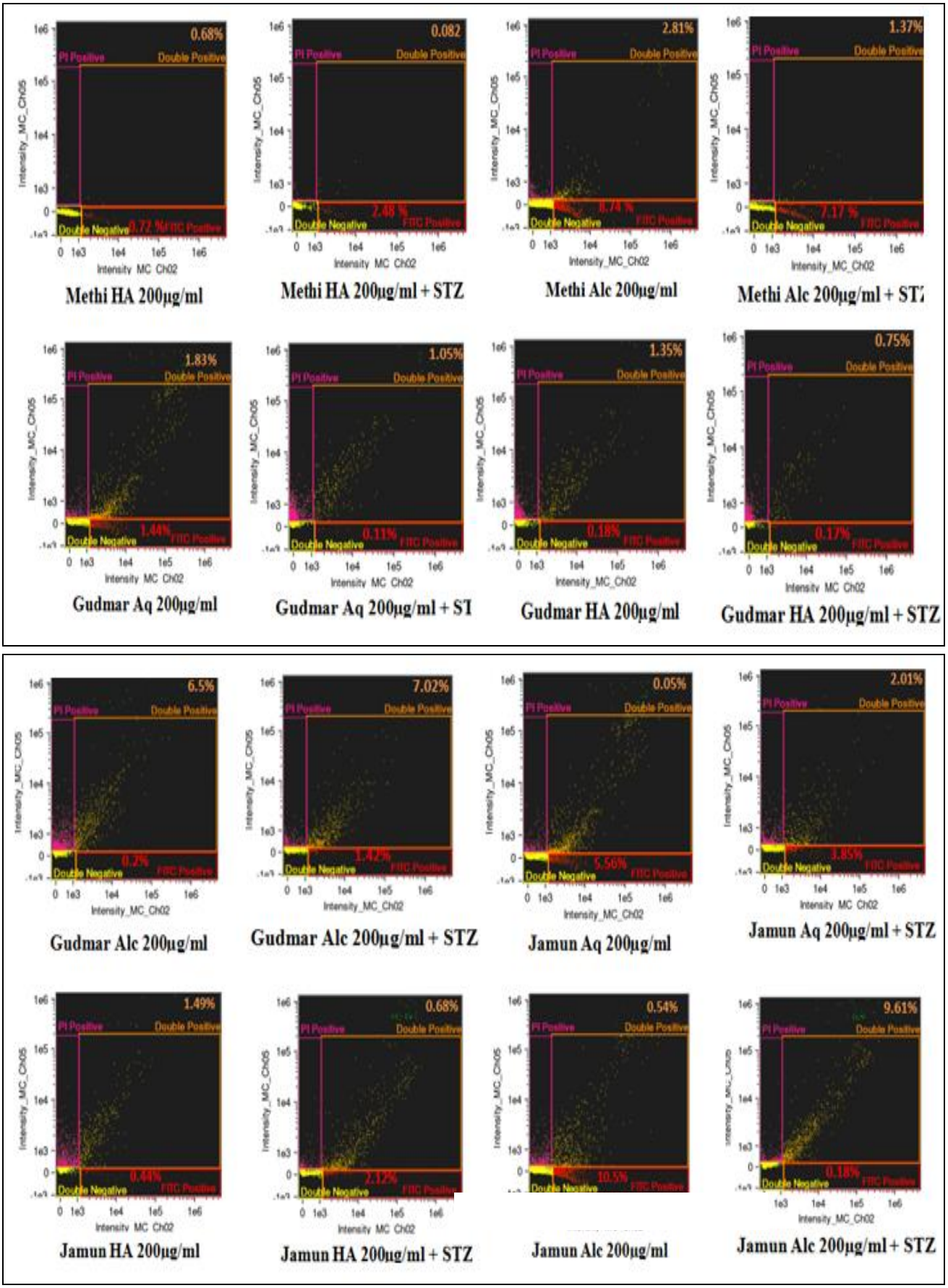
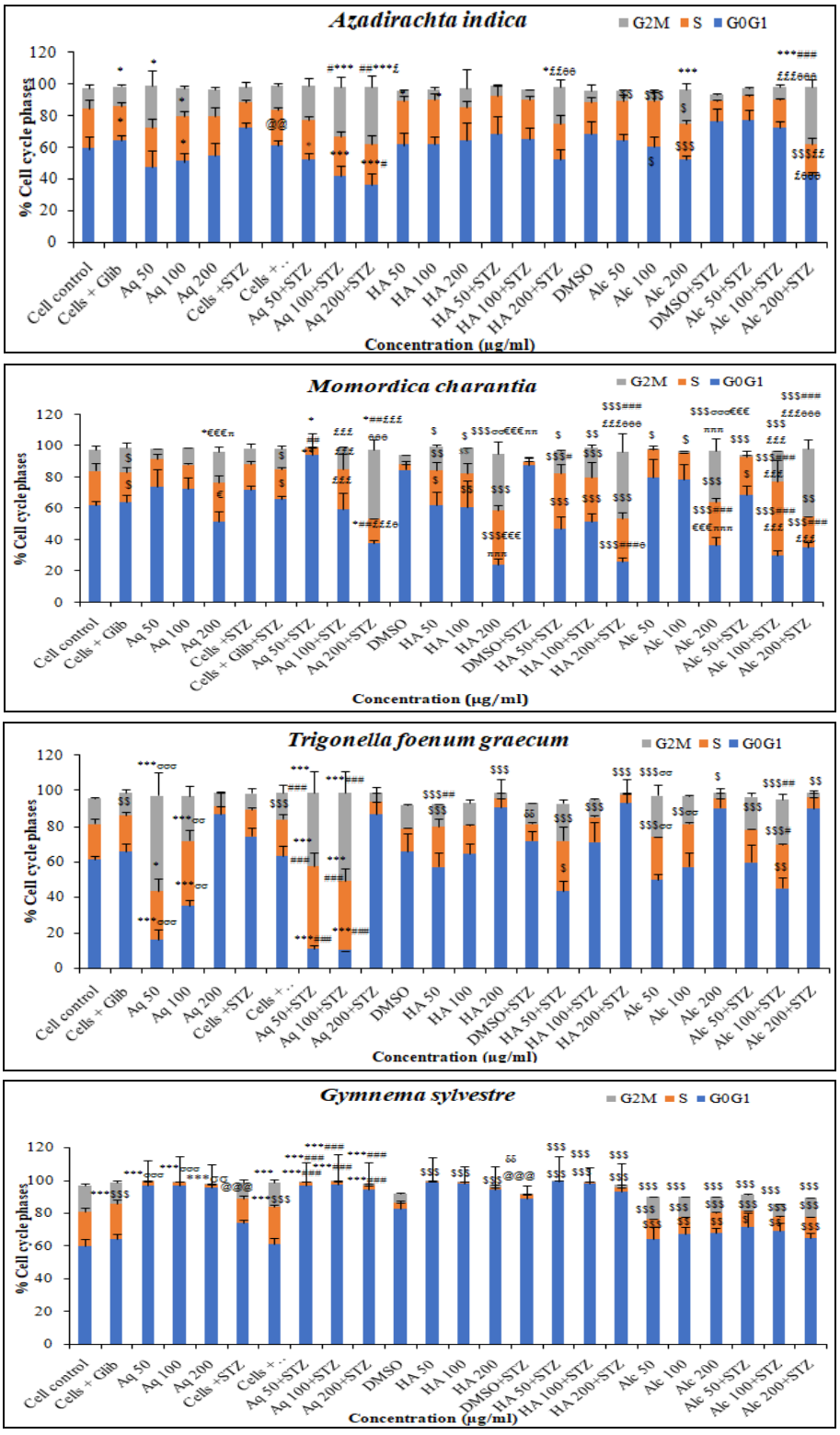


FIG. 4: REPRESENTATIVE IMAGES OF APOPTOSIS USING FLOW CYTOMETRY. Quadrant 1: PI positive represent Necrosis; Quadrant 2: Double positive (Annexin V+/PI+) represent late apoptosis; Quadrant 3: FITC positive (Annexin V+/ PI-) represents early apoptosis and Quadrant 4: Double Negative (Annexin V-/ PI-) represent viable cells.

Cell Cycle: The distribution of RIN cells in different cell cycle phases as depicted in Fig. 5 & 6 was analyzed using flow cytometry. STZ treated RIN cells showed a significant increase in the G0G1 phase indicating arrest at this phase with a simultaneous decrease in S and G2M phases. Thus, the accumulation of cells in G1 phase indicates that STZ delayed the progression of cells to the next phase of the cell cycle. However, when the cells were treated with plant extracts and glibenclamide, the percentage of cells in G0/G1, S, and G2M phases were restored similar to untreated cells compared to STZ treated cells.



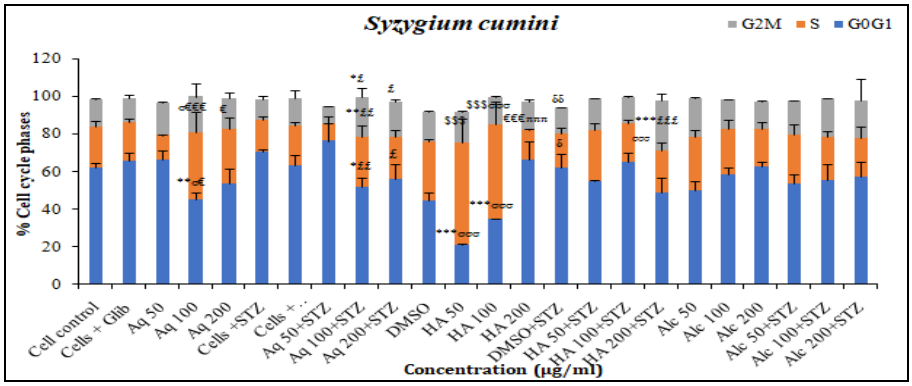
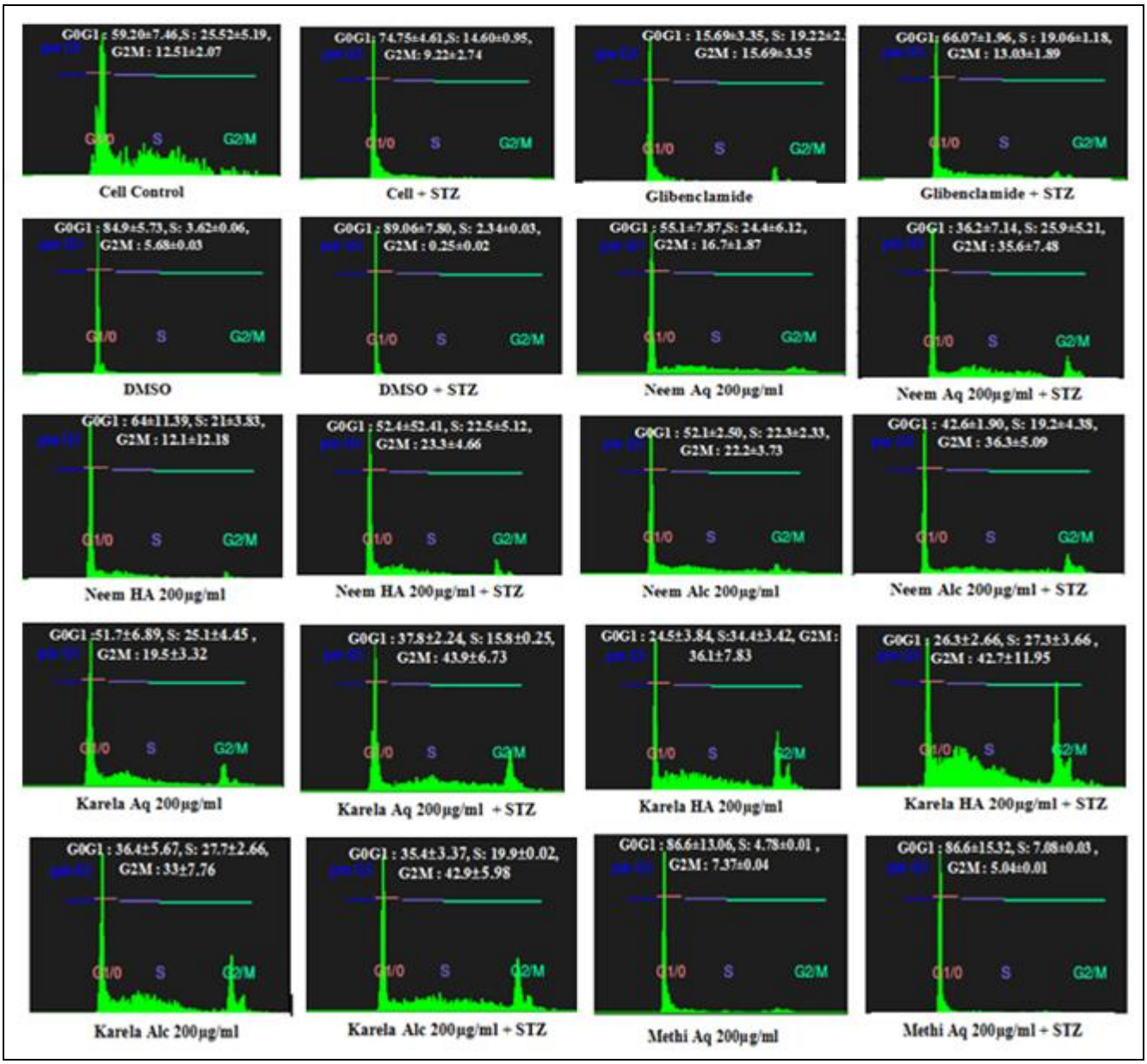


FIG. 5: EFFECT OF SELECTED PLANT EXTRACTS ON CELL CYCLE ANALYSIS USING FLOW CYTOMETRY. Results are expressed as Mean ± SD $^{@@}p<0.01$,$^{@@@}p<0.001$ as compared to cell control; $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ as compared to cells +STZ $^{\delta}p<0.05$, $^{\delta\delta}p<0.01$, $^{\delta\delta\delta}p<0.001$ as compared to cells +DMSO; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ as compared to cells + DMSO + STZ; $^{\sigma}p<0.05$, $^{\sigma\sigma}p<0.01$,$^{\sigma\sigma\sigma}p<0.001$ as compared to cells + Glib; $^{\#}p<0.05$, $^{\#\#}p<0.01$,$^{\#\#\#}p<0.001$ as compared to cells + Glib + STZ; $^{\epsilon\epsilon}p<0.01$, $^{\epsilon\epsilon\epsilon}p<0.001$ as compared to cells +50µg/ml; $^{£}p<0.05$, $^{££}p<0.01$, $^{£££}p<0.001$ as compared to cells + 50µg/ml + STZ; $^{\pi\pi}p<0.01$, $^{\pi\pi\pi}p<0.001$ as compared to cells + 100µg/ml; $^{\theta}p<0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared to cells + 100µg/ml +STZ



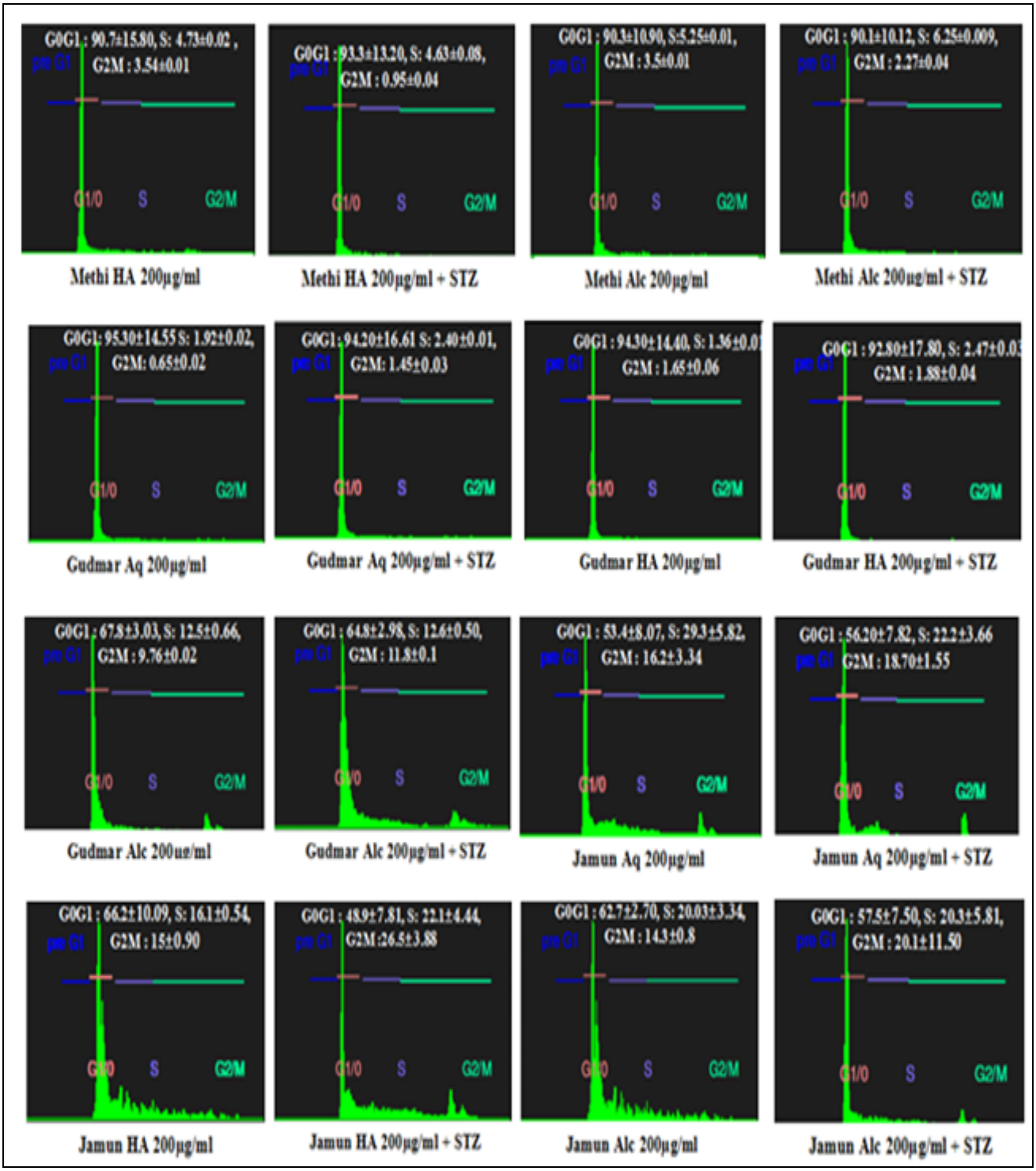


FIG. 6: REPRESENTATIVE IMAGES OF CELL CYCLE ANALYSIS USING FLOW CYTOMETRY

Effect of Insulin Secretion: Insulin levels were studied to assess the effect on functional damage and are summarised in **Table 1**.

RIN cells, when exposed to STZ, showed a decrease in insulin release in comparison to untreated control RIN cells. Glibenclamide *per se* and in the presence of STZ showed an increase in insulin release. However, all the 5 selected plant extracts showed an increase in insulin release *per se* and in the presence of STZ.

Maximal and significant insulin release was observed at a 200 µg/ml concentration of *Syzygium cumini* followed by *Gymnema sylvestre* and *Momordica charantia* as compared to cells treated with STZ; this increase was higher than that of glibenclamide.

TABLE 1: EFFECT OF SELECTED MEDICINAL PLANT EXTRACTS ON INSULIN LEVEL (N=3)

Group		**Insulin µIU/ml**	
Cell Control		6.24±0.23	
Cells + STZ		**3.18± 0.31$^{@@@}$**	
Cells + Glibenclamide(Glib)		5.38± 0.29***	
Cells + Glib + STZ		5.01± 0.41***	
Concentration of Plant extract	**Aqueous (Aq)**	**Hydroalcoholic (HA)**	**Alcoholic(Alc)**
		***Azadirachta indica* (*Ai*)**	
Cells + *Ai* 50 µg/ml	5.87 ± 0.05$^{*** \#}$	Cells + *Ai* 50 µg/ml	5.87 ± 0.05$^{*** \#}$
Cells + *Ai* 100 µg/ml	5.28 ± 0.12***	Cells + *Ai* 100 µg/ml	5.28 ± 0.12***
Cells + *Ai* 200 µg/ml	4.98 ± 0.07***	Cells + *Ai* 200 µg/ml	4.98 ± 0.07***
DMSO	-	DMSO	-
DMSO + STZ	-	DMSO + STZ	-
Cells + *Ai* 50 µg/ml + STZ	5.43 ± 0.15***	Cells + *Ai* 50 µg/ml + STZ	5.43 ± 0.15***
Cells + *Ai* 100 µg/ml + STZ	5.36 ± 0.23***	Cells + *Ai* 100 µg/ml + STZ	5.36 ± 0.23***
Cells + *Ai* 200 µg/ml + STZ	5.18 ± 0.48***	Cells + *Ai* 200 µg/ml + STZ	5.18 ± 0.48***
		***Momordica charantia* (*Mc*)**	
Cells + *Mc* 50 µg/ml	5.77± 0.02***	Cells + *Mc* 50 µg/ml	5.77± 0.02***
Cells + *Mc* 100 µg/ml	5.87± 0.41***	Cells + *Mc* 100 µg/ml	5.87± 0.41***
Cells + *Mc* 200 µg/ml	6.06± 0.68***	Cells + *Mc* 200 µg/ml	6.06± 0.68***
DMSO	-	DMSO	-
DMSO + STZ	-	DMSO + STZ	-
Cells + *Mc* 50 µg/ml + STZ	5.43± 0.34***	Cells + *Mc* 50 µg/ml + STZ	5.43± 0.34***
Cells + *Mc* 100 µg/ml + STZ	5.28± 0.56**	Cells + *Mc* 100 µg/ml + STZ	5.28± 0.56**
Cells + *Mc* 200 µg/ml + STZ	5.49± 0.34***	Cells + *Mc* 200 µg/ml + STZ	5.49± 0.34***
		***Trigonella foenum graecum* (*Tfg*)**	
Cells + *Tfg* 50 µg/ml	4.43± 0.05	Cells + *Tfg* 50 µg/ml	4.43± 0.05
Cells + *Tfg* 100 µg/ml	4.39± 0.13	Cells + *Tfg* 100 µg/ml	4.39± 0.13
Cells + *Tfg* 200 µg/ml	4.83± 0.54	Cells + *Tfg* 200 µg/ml	4.83± 0.54
DMSO	-	DMSO	-
DMSO + STZ	-	DMSO + STZ	-
Cells + *Tfg* 50 µg/ml + STZ	4.18± 0.32	Cells + *Tfg* 50 µg/ml + STZ	4.18± 0.32
Cells + *Tfg* 100 µg/ml + STZ	4.58± 0.64	Cells + *Tfg* 100 µg/ml + STZ	4.58± 0.64
Cells + *Tfg* 200 µg/ml + STZ	4.27± 0.38	Cells + *Tfg* 200 µg/ml + STZ	4.27± 0.38
		***Gymnema sylvestre* (*Gs*)**	
Cells + *Gs* 50 µg/ml	5.55 ±0.23	Cells + *Gs* 50 µg/ml	5.55 ±0.23
Cells + *Gs* 100 µg/ml	5.83 ± 0.02*	Cells + *Gs* 100 µg/ml	5.83 ± 0.02*
Cells + *Gs* 200 µg/ml	6.24 ± 0.43$^{** \#}$	Cells + *Gs* 200 µg/ml	6.24 ± 0.43$^{** \#}$
DMSO	-	DMSO	-
DMSO + STZ	-	DMSO + STZ	-
Cells + *Gs* 50 µg/ml + STZ	5.39 ± 0.05	Cells + *Gs* 50 µg/ml + STZ	5.39 ± 0.05
Cells + *Gs* 100 µg/ml + STZ	5.61 ± 0.18	Cells + *Gs* 100 µg/ml + STZ	5.61 ± 0.18
Cells + *Gs* 200 µg/ml + STZ	5.57 ± 0.33	Cells + *Gs* 200 µg/ml + STZ	5.57 ± 0.33
		***Syzygium cumini* (*Sc*)**	
Cells + *Sc* 50 µg/ml	5.88 ± 0.32	Cells + *Sc* 50 µg/ml	5.88 ± 0.32
Cells + *Sc* 100 µg/ml	6.03 ± 0.04*	Cells + *Sc* 100 µg/ml	6.03 ± 0.04*
Cells + *Sc* 200 µg/ml	6.44 ± 0.08$^{** \#}$	Cells + *Sc* 200 µg/ml	6.44 ± 0.08$^{** \#}$
DMSO	-	DMSO	-
DMSO + STZ	-	DMSO + STZ	-
Cells + *Sc* 50 µg/ml + STZ	5.52 ± 0.47	Cells + *Sc* 50 µg/ml + STZ	5.52 ± 0.47
Cells + *Sc* 100 µg/ml + STZ	5.66 ± 0.13	Cells + *Sc* 100 µg/ml + STZ	5.66 ± 0.13
Cells + *Sc* 200 µg/ml + STZ	5.87 ± 0.41	Cells + *Sc* 200 µg/ml + STZ	5.87 ± 0.41

Results are expressed as Mean ±SD $^{@@@}$p<0.001 as compared to control cells *p<0.05, **p<0.01, ***p<0.001 as compared to STZ treated cells $^{\delta}$p<0.05, $^{\delta\delta}$ p<0.01 as compared to DMSO $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$ p<0.001 as compared to DMSO +STZ treated cells $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 as compared to Glib + STZ using ANOVA followed by post hoc test

DISCUSSION: In the present study, RINm5F an insulin-secreting pancreatic beta-cell line, was used as a model to represent pancreatic beta cells. The choice of our model was based on the potential mechanisms by which the selected plants exert their anti-diabetic effect and the feasibility and ease

of setting up the model. Diabetic conditions were induced by streptozotocin, a chemical diabetogenic agent which induces experimental diabetes [24] leading to generation of reactive oxygen species. Several *in vitro* studies have demonstrated that STZ (up to 20mM) induces β cell death by alkylating DNA by activation of poly ADP-ribosylation (PARP) causing DNA damage. Activation of PARP leads to initiate the programmed cell death pathway, resulting in the depletion of cellular NAD^+ [25]. The present study was thus planned to elucidate the mechanisms of action of 5 medicinal plants selected on the basis of Ayurveda to assess their therapeutic potential in diabetes mellitus and its associated complications. The 5 plants selected are used extensively by traditional medicine practitioners to treat diabetes. However, their mechanism/s of action has not been fully elucidated with the three extracts *viz.*, aqueous, hydroalcoholic, and alcoholic.

The pancreatic beta-cell damage induced by STZ was assessed with respect to oxidative damage in terms of lipid peroxidation, intracellular DNA damage regarding apoptosis, and insulin secretion with respect to functional damage. Glibenclamide is a second-generation sulfonylurea that reduces blood glucose by increasing insulin secretion from pancreatic beta cells and was used as a standard control in the study. Viability studies were initially carried out to eliminate the cytotoxic doses of plant extracts and thereby find out the precise dose range of the extracts for further functional assays.

The MTT assay assayed the viability of RINm5F cells after treatment with the plant extracts. All the 5 plants did not affect the viability of RIN cells up to 200µg/ml. Hence, for all the 5 plants extracts, efficacy studies were carried out using 50, 100 and 200µg/ml concentrations. The effect of the selected plant extracts was evaluated on the selected variables based on therapeutic interest to assess the plants' anti-oxidant, anti-apoptotic and insulin secretagogue effects. Results of all the plants were compared with glibenclamide, the standard drug used to treat diabetes, at a concentration (1µg/ml) extrapolated from the median therapeutic human dose. Lipid peroxidation was evaluated in terms of MDA release to assess the antioxidant potential of the plant extracts. As expected, STZ-treated RINm5f cells showed a significant increase in MDA levels indicating increased oxidative stress. However, treatment with the extracts in the presence of STZ demonstrated that all the extracts of 5 plants inhibited MDA release, therefore, exhibiting antioxidant properties. The results suggested that these plant extracts could effectively protect pancreatic cells by reducing STZ-induced oxidative damage, thereby strengthening the evidence that these plants have antioxidant activity, which could delay the progression of diabetic complications. Based on this evidence, the anti-apoptotic action of all the extracts could be due to inhibition of oxidative stress, possibly due to phenolic phytochemicals present in the extracts [22, 26]. These results suggest that phenolic compounds play a significant role in protecting pancreatic cells against oxidative damage. Secondly, the anti-apoptotic potential of these 5 selected plant extracts was evaluated using flow cytometry.

All the plant extracts at a concentration of 200µg/ml *per se* and in the presence of STZ showed a significant reduction in apoptotic cells except for *Syzygium cumini.* Our results are in accordance with reported literature [27]. The results of all plants were comparable to glibenclamide which also exhibited protection against the apoptotic damage induced by STZ. Further cell cycle analysis results reported that aqueous extract of *Trigonella foenum graecum* and the hydroalcoholic extract of *Momordica charantia* showed a significant decrease in the G0/G1 (apoptosis) phase the rest of the extracts.

Hence, the antiapoptotic action of these extracts could be the inhibition of oxidative stress, possibly due to phenolic phytochemicals present in the extracts. The results revealed that the anti-apoptotic property of all the extracts plays a significant role in protecting and maintaining insulin secretion of pancreatic cells. Further, we evaluated the effect of drugs on insulin secretion as damaged pancreatic beta cells lose their capacity for insulin secretion. Significantly high insulin release was observed at a concentration of 200 µg/ml of *Syzygium cumini* followed by *Gymnema sylvestre* and *Momordica charantia* as compared to cells treated with STZ increase was greater than that seen with glibenclamide. Glibenclamide *per se* showed an increase in insulin secretion. Also, it stimulated insulin release from STZ treated cells, thus

confirming its mode of action which is mainly by stimulating the β pancreatic cells. Laha and Ezeigwe *et al* demonstrated that *Gymnema sylvestre* and extract of *A. indica* have pancreatic islet regeneration properties in diabetic mice [13, 19]. The potential effects observed when using these extracts can certainly be ascribed to cellular mechanisms, which include scavenging of free radical, DNA repair, cell cycle alteration, programmed cell death mitigation, and the ability to modulate various signaling pathways [28, 29].

CONCLUSION: The present study has exposed the path for research on Indian medicinal plants that have the potential to modulate diabetic conditions through their anti-oxidant, anti-apoptotic and cytoprotective actions. Although the study reveals the possible mechanisms of action of the selected plants, it is essential to confirm all the reported activities further using *in vivo* models of diabetes and its associated complications. Additionally, isolation and identification of active constituents from these plants will help develop these plants into potential anti-diabetic drugs which can be incorporated in the anti-diabetic drug armamentarium after clinical assessment.

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