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Evaluating The Effects Of *Phyllanthus Emblica* Extract, *Tinospora Cordifolia* Extract And Their Combination On Rat Kidney Cell Injury Model

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Abstract

Oxidative stress induced by hyperglycemia is thought to play an important role in the pathogenesis of diabetic nephropathy (DN). NRK-52E mouse renal epithelial cells (NRK-52E) retained the properties of normal proximal renal tubule cells, when NRK-52E cells were cultured in glucose-enriched medium, renal cells were Apoptotic death causes high oxidative stress. Treatment of hyperglycemic (HG) proximal tubule damage has emerged as a potential treatment option to reduce the onset and progression of DN. The present study aimed to investigate the regenerative effects of *Phyllanthus emblica* and *Tinospora cordifolia* on HG-induced

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

cytotoxicity in NRK-52E cells. Aqueous extracts of *Phyllanthus emblica* and *Tinospora cordifolia* were prepared using a rotary vacuum evaporator. Cell viability was assessed using the MTT assay kit (Hi-media) and the flow cytometric assay kit (Sigma) used to detect apoptosis. Treatment of cells with HG significantly reduced cell viability while significantly increasing reactive oxygen species (ROS) content. In this study, we hypothesized that the excessive induction of ROS by increased glucose plays a key role in the apoptosis of NRK-52E cells. Cell treatment with *Phyllanthus emblica* and *Tinospora cordifolia* ameliorated these HG-induced changes. Thus, *Phyllanthus emblica*, *Tinospora cordifolia* and their combination exhibited cytoprotective effects against HG culture-induced renal cell damage by inhibiting oxidative stress and mitochondrial apoptosis.

Keywords: NRK-52E cell, Hyperglycemia, Diabetic nephropathy, *Phyllanthus emblica, Tinospora cordifolia,* Oxidative stress, apoptosis

1. INTRODUCTION

Diabetic nephropathy is a kidney disease that occurs as a result of diabetes (1). The International Diabetes Federation (IDF) predicts that 425 million adults are suffering from diabetes worldwide which is expected to reach 629 million by 2045 (2). According to the World Health Organization (WHO) data 2015, India had 69.2 million (8.7%) diabetic people which is expected to rise to 109 million by 2035 (3). Approximately, 40 percent of diabetic individuals may develop diabetic nephropathy (4). This dramatic rise is largely due to lack of physical activity, hostile work environments, sedentary lifestyle, rapidly growing urbanization, industrialization, obesity, greater duration of diabetes, hypertension, poor metabolic control, smoking, and hyperlipidemia. According to the WHO data, about 65-80% of the world's population in developing countries depends essentially on plants for their primary health care, because herbal medicines offer a promising alternative due to their safety, efficacy, diverse potential of antioxidant activities, and very fewer side effects as compared to synthetic drugs (5). In the Ayurvedic Pharmacopoeia of India, *Phyllanthus emblica* and *Tinospora cordifolia* are categorized as antidiabetic herbal drugs

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

and used as "Rasayana" (rejuvenating) therapy. A single antidiabetic herb contains various phytochemicals such as polyphenols, alkaloids, flavonoids, glycosides, polysaccharides, tannin, and the combination of antidiabetic herbs might work synergistically with each other to induce beneficial therapeutic efficacy. There are numerous reports that have suggested the anti-oxidant (6), (7) and anti-diabetic properties of various types of *Phyllanthus emblica* (Amla extract) in the *in vivo* models and clinical studies (8), (9), (10). But there is only one report suggesting the p38 MAPK induced anti-oxidant and anti-inflammatory effects of Gallic Acid (one of the components of Amla) in both Type II Diabetic rat model as well as cultured rat renal proximal tubular cells, namely NRK-52E, cultured in high glucose milieu of DN (11). Several *in vitro* and *in vivo* studies have also shown the hypoglycemic effect of *Tinospora cordifolia* stem extract *via* a mechanism of insulin releasing, insulin-mimicking and gluconeogenesis inhibition (12), (13), (14), (15). But there is no report till date about the anti-oxidative stress effect, anti-apoptotic effect of *Phyllanthus emblica* (Amla extract), *Tinospora cordifolia* stem aqueous extract and their combination on Diabetic Nephritis.

In this study, we evaluated the therapeutic efficacy of *Phyllanthus emblica* extract, *Tinospora cordifolia* stem aqueous extract and their combination (*Phyllanthus emblica* + *Tinospora cordifolia)* through inhibition of renal cell apoptosis which could be a promising therapeutic target against hyperglycemia induced Diabetic nephropathy.

2. Objectives

1. Establishment of *in vitro* model of Diabetic Nephritis (DN) by culturing NRK-52E cells at high glucose concentration (30 mM) i.e. by mimicking high glucose milieu of DN.
2. Preparation of *Phyllanthus emblica* extract, *Tinospora cordifolia* stem aqueous extract and study the effects on cell viability at different concentrations of glucose.
3. Flow cytometric analysis of apoptosis.

3. Material and methods

Media DMEM (AL007A), Trypsin-EDTA Sol^{n} (TCL007), DPBS (TS1006), Antibiotic 100X (A001), DMSO (TC185), D-Glucose (MB037), D-Mannitol, MTT Kit (CCK003-1000) were purchased from Hi-media and fetal bovine serum (16000044) were purchased from Gibco United

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

states. Flow cytometry tubes Polystyrene round bottom tubes 5ml (352054) Falcon corning and FITC kit (APOAF-20TST) was obtained from Sigma.

3.1 Establishment of *in vitro* model of Diabetic Nephritis (DN) by culturing NRK 52E cells at high glucose concentration (30 mmol/l) i.e. by mimicking high glucose milieu of DN.

Culturing of NRK-52 E Cells

NRK-52E rat kidney epithelial cell line was procured from National Centre for Cell Science, Pune, India, and maintained as per supplier's protocol. The cells were cultured in Dulbecco's Modified Eagle Medium with 5% fetal bovine serum, 1% penicillin/streptomycin at 37 °C in a 5% CO2 incubator and media was replaced to new medium after every 2–3 days. The cells were grown till 80-90% confluency; after which they were used for *in vitro* experimental studies. The monolayer was cultured in FBS free DMEM for 24 hours so as to obtain the growth arrested cells. Subsequently the medium was replaced with DMEM supplemented with Normal Glucose (NG, 5.5mmol/l glucose) or with High Glucose (HG, 30 mmol/l glucose) followed by incubation for different time points at 5% CO_2 and 37ºC, for studying the time dependent effect (16), (17), (18).

The Time-dependent cytotoxic effects of high glucose (30 mmol/l glucose) on kidney cells- In this study, the time-dependent cytotoxic effects of high glucose (30 mmol/l glucose) on kidney cells was estimated by incubating the NRK-52E cells with 30 mM of D-glucose for 6, 12, 24, 48h (19).

The concentration dependent cytotoxic effects of D-glucose on kidney cells - The concentration dependent cytotoxic effects of D-glucose on kidney cells was estimated to optimize the glucose concentration for an *in vitro* diabetic nephropathy model. The normal rat kidney (NRK) cells were exposed to different concentrations of D-glucose (5, 10, 15, 20, 30 mM) for 48 h (19).

Preparation of *Phyllanthus Emblica* extract, *Tinospora cordifolia* aqueous extract and study their effects on cell viability at different concentrations of glucose.

3.2 Plant material collection and extraction

***Phyllanthus Emblica* aqueous extract preparation**

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

Fruits of Amla (*Phyllanthus Emblica*) and Stems of *Tinospora cordifolia* were collected from the Botanical garden of Panjab University Chandigarh, India. The pulp *Phyllanthus Emblica* was taken out and blended, followed by filtration through Whatman paper no. 1 in order to collect the filtered aqueous extract. Then this filtered aqueous extract was evaporated at reduced pressure using Rotary vacuum evaporator at 50ºC and lastly freeze dried with the help of freeze-drier. A stock solution (5mg/mL) of *Phyllanthus Emblica* was prepared in DMEM medium (Gibco, New York, NY, USA) and sterilized by filtration through a 0.22-µm pore size hydrophilic polyethersulfone membrane (Merck Millipore, MA, USA). The stock solution was stored at −20°C and thawed immediately before use (20), (21).

Tinospora cordifolia aqueous extract preparation

The Stems of *Tinospora cordifolia* will be dried in the oven at 60°C till almost 90% dry. Then dried stems ground to fine powder using laboratory blender on medium speed and mixed with distilled water in the ration 1:10 (5g powder + 50ml ddwater). This solution kept for 2.5 hours with shaking at regular interval. Filtered with whatman paper no. 1 and evaporate through rotary vaccum evaporator at 40°C for 30 minutes. At -20°C Crude extract was kept for further use (22), (23), (24). A stock solution (5 mg/mL) of *Tinospora cordifolia* was prepared in DMEM medium and sterilized by filtration. The solution will be filtered through a 0.22-µm pore size hydrophilic polyethersulfone membrane and stored at -20 °C and thawed immediately before use.

3.3 Measurement of cell viability- The viability of NRK-52E cells was determined by using commercially available 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cell proliferation assay. Briefly, cells were seeded in 96-well plates at a density of 8×10^4cells per well and incubated for 24 h in FBS free DMEM for 24 hours so as to obtain the growth arrested cells. NRK-52E cells were treated with normal glucose (NG, 5.5mM glucose), high glucose (HG, 30mM glucose) and high glucose with *Phyllanthus emblica* Extract, *Tinospora cordifolia* aqueous extract at different concentration (50, 100, 200µg/ml) for 48 h. After that cell viability also done by using their combination (*Phyllanthus emblica* Extract and *Tinospora cordifolia)* on concentration at which > 95% cell viability obtained, Then cells in each well were washed with PBS and 100 µl (0.5 mg/mL) of MTT solution was added to each well and incubated for 4 h at 37°C. The supernatants were then aspirated, 100 µl of DMSO was added to solubilize the

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

formazan crystals with shaking for 5 min. Cell viability was measured by reading absorbance at 570 nm in a plate reader. In control experiments, mannitol (25mM) was added to NG to bring total osmolality to values equivalent to high glucose (30mM) medium.

3.4 Flow cytometric analysis of NRK-52E Cells by using the Annexin V-FITC detection kit

Cell apoptosis was analyzed by using the Annexin V-fluorescein isothiocyanate/propidium iodide staining (FITC) Apoptosis Detection Kit Sigma (APOAF) according to the manufacturer's instructions.

Experimental Groups

NRK-52E cells were randomly divided into the following six groups:

1. The normal glucose (NG) medium contained 5.5 mmol/L d-glucose.
2. High glucose (HG) medium contained 30 mmol/L d-glucose.
3. Normal glucose DMEM media 5.5 mmol/L d-glucose supplemented with 24.5 mM mannitol (MG) was served as osmotic control in the experiment.
4. Cells cultured in HG medium followed treatment with *Phyllanthus Emblica* (100 µg/ml) for 48 h.
5. Cells cultured in HG medium followed treatment with *Tinospora cordifolia* TC (200 µg/ml) for 48 h.
6. Cells cultured in HG medium followed treatment with combination of *Phyllanthus emblica* (100 µg/ml) and *Tinospora cordifolia* (200 µg/ml) for 48 h.

Briefly, After the treatments, cells were detached by trypsinization and collected by centrifugation at 4000 × g for 4 min, washed with cold phosphate-buffered saline (PBS), and then resuspended in binding buffer at a density of 1×10^6 cells/ml. Each tube of the cell suspension was mixed gently with 1 µL of Annexin V-FITC and 1 µL of propidium iodide (PI), followed by incubation in the dark at room temperature for 15 min. Finally, the mixture was analyzed with a FACS flow cytometer (17), (25), (26), (27), (28).

4. RESULTS

4.1 The Time and concentration dependent cytotoxic effects of high glucose (30 mmol/l glucose) on kidney cells

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

The Time-dependent cytotoxic effects of high glucose (30 mmol/l glucose) on kidney cells- D-glucose at 5.5 mM served as the control (HG −), and the cell viability was assigned to 100%. As shown in Fig 1,2, a gradual decrease in the viability of NRK-52E cells was observed over time, with substantial differences between 6 to 24 h (88.44%) and a maximum reduction of cell viability at 48 h (50.49%). On the basis these observed effects, the concentration of D-glucose of 30 mM and an incubation time of 48 h were chosen as optimum dose and exposure time, respectively, for the *in vitro* model of diabetic nephropathy.

The concentration dependent cytotoxic effects of D-glucose on kidney cells - D-glucose at 5.5 mM served as the control (HG−), and the cell viability was assigned to 100%. D-glucose caused the loss of cell viability in a concentration-dependent manner with a maximum reduction (50.39%) at 30 mM of D-glucose. On the basis of the observed effects, the concentration of D-glucose of 30 mM and an incubation time of 48 h were chosen as optimum dose and exposure time, respectively, for *in vitro* model of diabetic nephropathy (Fig 1,2).

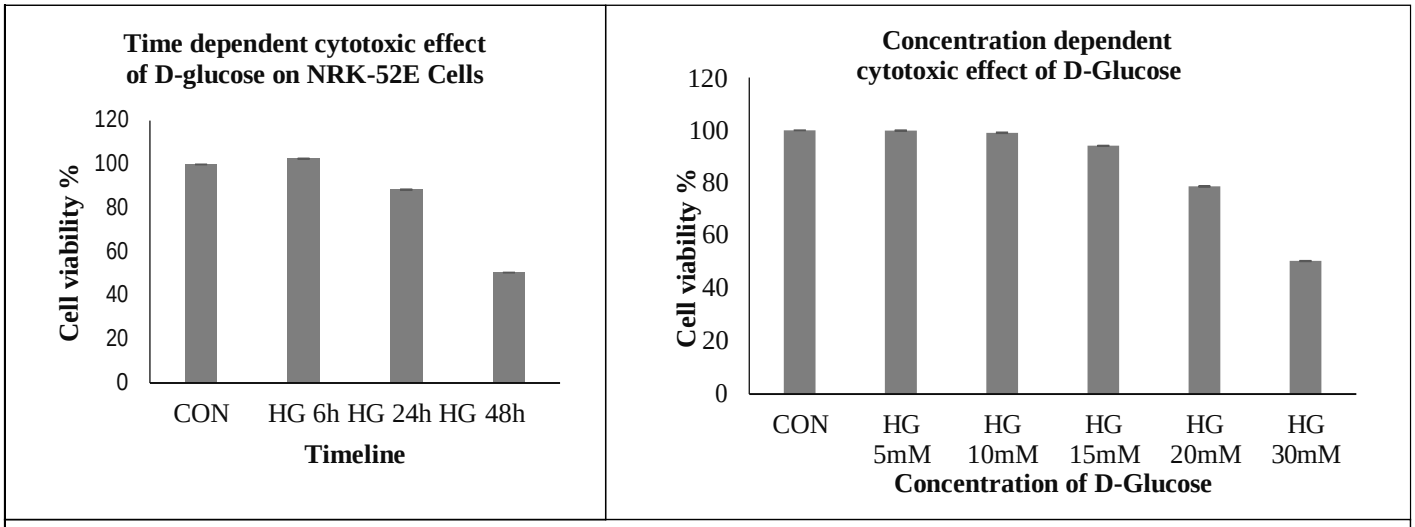

Fig 1,2 - Time dependent and Concentration dependent cytotoxic effect of D-glucose on NRK-52E Cells

4.2 Evaluating the effects of *Phyllanthus Emblica* extract, *Tinospora cordifolia* aqueous extract and their combination at high concentrations of glucose 30mM.

The normal rat renal (NRK-52E) cells maintain the characteristics of normal renal proximal tubular cells, when NRK-52E cells were cultured in high glucose (HG) medium, cell viability

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

was approximately 56.92% in HG-cultured cells, whereas the treatment along with the *Phyllanthus emblica* extract at a dose of (100 µg/ml), *Tinospora cordifolia extract at a dose of* (200 µg/ml) *and their combination (Phyllanthus emblica* 100 µg/ml and *Tinospora cordifolia* 200 µg/ml restored the cell viability in HG-treated NRK-52E cells. Therefore, these doses *Phyllanthus emblica* 100 µg/ml and *Tinospora cordifolia extract 2*00 µg/ml concentrations were selected for the further experiments as shown in Fig 3.

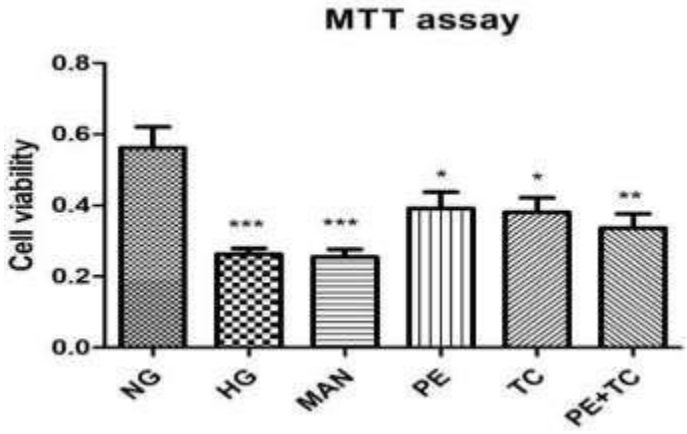

Figure 3. Effect of Phyllanthus emblica extract (PE), Tinospora cordifolia extract (TC) and their combination (PE +TC) to protect HG-induced cytotoxicity in NRK-52E cells was measured by MTT assay.

4.3 Effect of *Phyllanthus emblica* (100 µg/ml) and *Tinospora cordifolia* (200 µg/ml) on apoptosis in HG- treated NRK-52E cells by Annexin V-fluorescein isothiocyanate/propidium iodide staining –

Flow cytometry was used to assess the effect of *Phyllanthus emblica* (100 µg/ml) and *Tinospora cordifolia* (200 µg/ml) on high glucose induced apoptosis in NRK-52E cells. The HG group showed significantly higher apoptotic rate as compared to control NG group cells. The apoptotic rate of HG treated cells was significantly reduced by the treatment with *Phyllanthus emblica* (100 µg/ml), *Tinospora cordifolia* TC (200 µg/ml) and the combination of *Phyllanthus emblica* (100 µg/ml) and *Tinospora cordifolia* (200 µg/ml) as compared to the HG group (Fig 4-14).

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group-I) Journal Volume 11, SIss 1, Nov 202

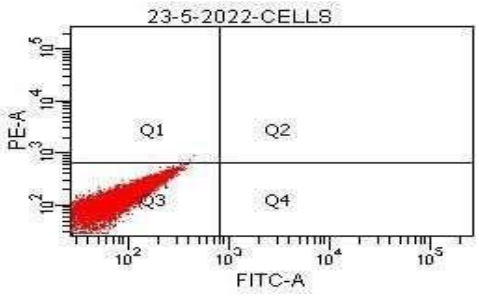

Figure 4 Cells only

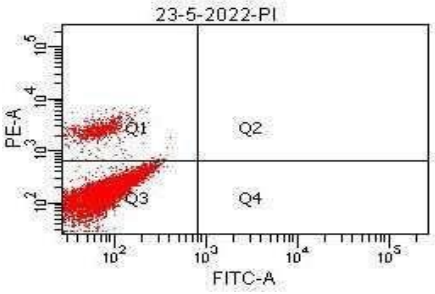

Figure 5 Propidium iodide

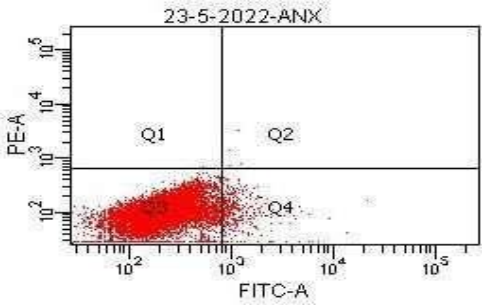

Figure 6 Annexin

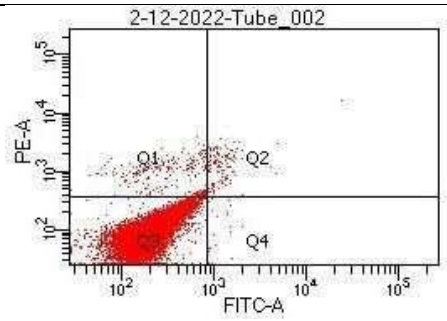

Figure 7 Dual (Propidium iodide + Annexin)

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group-I) Journal Volume 11, SIss 1, Nov 202

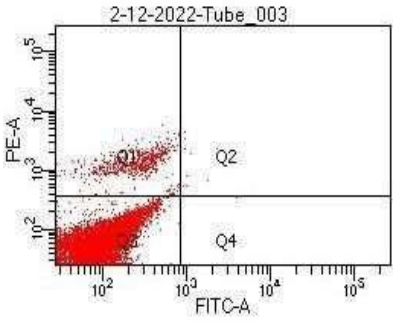

Figure 8 Normal glucose

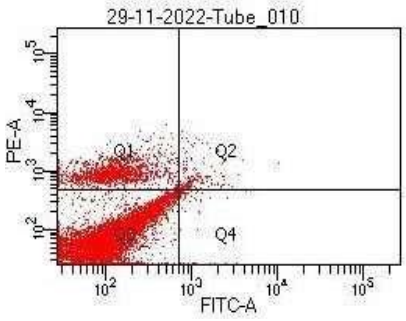

Figure 9 High glucose 30mM

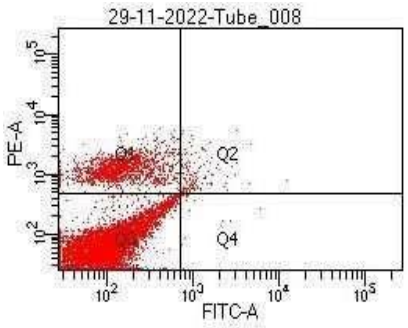

Figure 10 Mannitol 30mM

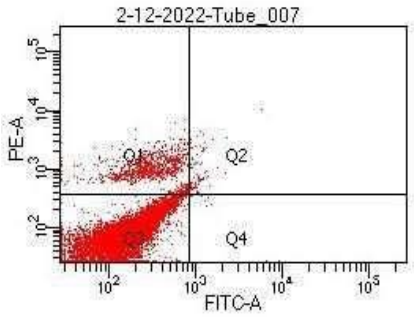

Figure 11 *Phyllanthus emblica* (100ug/ml)

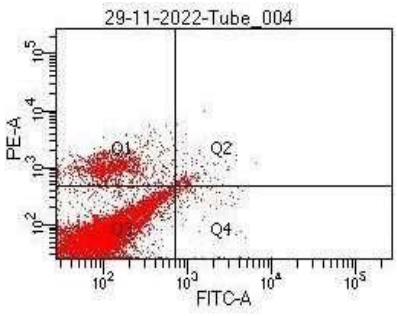

Figure 12 *Tinospora cordifolia* (200ug/ml)

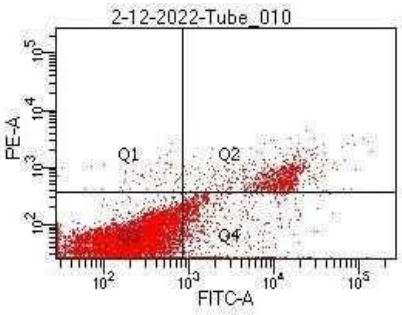

Figure 13 *Phyllanthus emblica* (100ug/ml) + *Tinospora cordifolia* (200ug/ml)

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

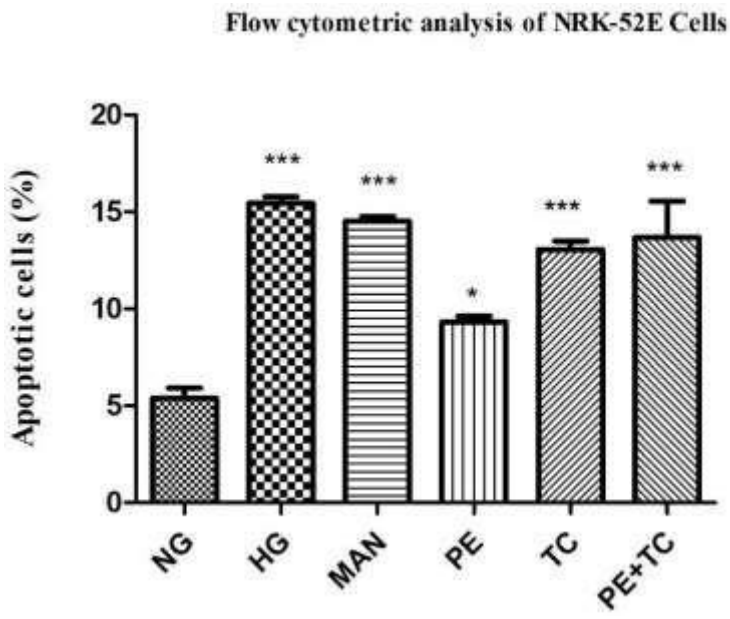

Figure 14. *Phyllanthus emblica extract (PE), Tinospora cordifolia extract (TC)* and their combination (*PE +TC*) reduced apoptosis against HG-induced cytotoxicity in NRK-52E cells.

Discussion

NRK-52E renal tubular epithelial cells are well known for their exceptional features in studies targeting the kidney system and diabetic nephropathy (29). Use of these cells is previously reported in investigating the renal toxicity and nephropathy effects of compound.

In hyperglycemia, the epithelial cells require a large amount of energy (ATP) to reabsorb excessive glucose (30) but the excessive presence of glucose increase the mitochondrial membrane permeability along with the release of cytochrome c from the mitochondrial intermembrane space by the activation of the intrinsic pathway of apoptosis. The loss of cytochrome c from the mitochondrial intermembrane space reduces the formation of ATP, which leads to renal cell apoptosis, especially in renal tubular epithelial cells. These tubular epithelial cells account for 90% of the total kidney volume. Therefore, by regulating the hyperglycemia-induced oxidative stress, mitochondrial dysfunction, and renal cell apoptosis could serve as a key target in improving the progression of DN.

In the current study, *Phyllanthus emblica* extract, *Tinospora cordifolia* extract and their combination was explored for its anti-apoptotic effect against diabetic nephropathy by virtue of *in vitro* studies. *Emblica officinalis* belongs to the family of *Euphorbiaceae*, there are various

ISSN PRINT 2319 1775 Online 2320 7876

Researchpaper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 1, Nov 202

names given to it, including Amla, Phyllanthus Emblica, and Indian gooseberry (31), (32) . The fruits of the plant are used for the formation of various traditional and Ayurvedic medicines. It is one of the most extensively studied plants in the Ayurvedic system of India. But Fruit is the most important part which is extensively used in the various traditional and folk medicines (44). The fruits of *Emblica officinalis* are sweet, sour, astringent, bitter, and pungent and are widely used in ayurana (drugs that delay aging and promote longevity). *Tinospora cordifolia* belongs to the family of *Menispermaceae*, used as a Rasayana therapy in the Ayurvedic system of India (45). Various scientific studies have revealed that the therapeutic efficacy of *T. cordifolia* significantly increased by interspecific interactions with other medicinal plants. The Extensive literature survey has reported that *T. cordifolia* also exhibited antidiabetic, antioxidation, anti-inflammation, antimicrobial, antiosteoporosis, anticancer, and immunostimulation activities (46). The stem of T. cordifolia contains various active phytochemicals such as alkaloids (magniflorine, berberine, palmatine), glycosides, sesquiterpenoids, saponins, flavonoids, steroids, and tannins (47) (Tiwari *et al.*, 2018).

The present study includes incubation of renal tubular epithelial cells in conditions of high glucose and studying their effect on diabetic nephropathy. Incubation in high glucose conditions showed significant reduction on cell viability. The fact was supported by numerous studies. When the ROS level exceeds the capacity of the antioxidant defense system, ROS initiates chain reactions by oxidizing cellular macromolecules like lipids and proteins, which in turn interrupts cellular activities, ultimately causing renal cell apoptosis (48). Our data showed that *Phyllanthus emblica extract, Tinospora cordifolia extract and their combination* protected NRK- 52E cells against HG-induced damage by ameliorating all of these events, resulting in reduced apoptosis and increased cell viability. Thus, our findings support a protective role of *Phyllanthus emblica extract, Tinospora cordifolia extract and their combination* in NRK-52E cells against high glucose induced disbetic nephropathy.

Conclusion

Hence our study has confirmed that *Phyllanthus emblica extract, Tinospora cordifolia extract and* their combination showed promising protective activity by preventing hyperglycaemia and renal tissue damage against the high glucose induced apoptosis in cultured rat renal proximal tubular cells (NRK-52E cell line).

Declaration of Competing Interest

None

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