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Evaluation of cytoprotective impact of Asiatic acid during hyperglycemia in Hep G2 Cell line

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Abstract
Plant metabolites are promising sources for the development of safe hypoglycemic drugs mainly due to their enhanced compatibility in biological systems. The present study was designed to investigate the antidiabetic activity of a plant derived triterpene; Asiatic acid obtained from the derived from *Centella asiatica*. *In vitro* results showed that Asiatic acid exhibited the potential to reverse the cytotoxic manifestations caused by hyperglycemia and significantly increased extracellular glucose consumption in HepG2 cells. We have demonstrated that Asiatic acid brings all diabetic parameters including GSH level and expression of different caspases to the comparable level of untreated group. Our results signify the antidiabetic potential of Asiatic acid and provide evidence to support the application of the substance in the treatment of diabetes.

Keywords: HepG2, asiatic acid, hypoglycemic drugs, diabetes

1. Introduction
Diabetes is a major health complication which is spreading fast across the globe (Zimmet *et al*., 2001) [12]. It is a chronic disease which is characterized by impaired glucose metabolism, leading to a high concentration of glucose in blood plasma (Giugliano *et al*., 2008) [4]. Oxidative stress in the cell occurs due to the increased production of reactive oxygen species (ROS) and decreased levels of antioxidants (Wu and Cederbaum, 2003) [5]. High concentration of glucose generates ROS which often plays a major role in glucose induced cellular dysfunction in diabetes (Kaneto *et al*., 2007; Goh and Cooper, 2008) [7, 13]. Liver is the primary organ for glucose metabolism and regulation; therefore, it is interesting to elucidate the effects of hyperglycemia in cultured liver cells. The human hepatoma cell line, HepG2 has been used extensively to study hyperglycemia *in vitro* as evidenced by several reports. The mRNA and protein expression of Scavenger Receptor B1 and the uptake of cholesterol from high density lipoprotein were suppressed in HepG2 cells exposed to high glucose (Murao *et al*., 2008) [11]. HepG2 cells treated with glucose exhibited an upregulation of the transcription of the human apolipoprotein A-II gene which causes a rise in plasma triglyceride level and glucose intolerance, resulting in hyperglycemia (Sauvaget *et al*., 2004) [6]. Hyperglycemia induced an insulin resistant state in HepG2 cells as indicated by decreased phosphorylation. All the above studies suggest that hyperglycemia elicits detrimental changes in liver cells. The role of high glucose induced cell death in liver has not been studied. The present study aims to investigate the mechanisms and mode of cell death due to exposure of HepG2 cells to a high concentration of glucose and this study suggests the role of hyperglycemia induced oxidative stress as an important agent for initiating apoptosis in liver, using an *in vitro* model of diabetes.

2. Materials and Methods
2.1 Maintenance of Cell Line and Sub Culture
Cell lines obtained, cultured in DMEM F12 (Dulbecco's Modified Eagle Medium) with 10% Foetal Bovine Serum (FBS) and antibiotic solution. Culture flasks were kept in CO_2 incubator (5% CO_2) at 37 °C.

2.2 Induction of Hyperglycaemia and Treatments
Hyperglycaemic conditions were induced by treating cells with 5 mm, 10 mm, 20 mm and 40 mm glucose solutions. Control cells were not treated with any additional glucose concentration.

2.3 MTT Assay

Cytotoxicity evaluation was done through MTT assay as described by Mossaman (1983) [14]. MTT assay assesses mitochondrial function by measuring the ability of cells to reduce MTT into purple coloured formazone due to succinate dehydrogenase activity as dead cells are not able to reduce it into farmazone. Approximately 104 cells/100 µl/well were seeded in 96 well flat bottom plate. The plate was incubated in CO_2 incubator (5% CO_2, 37 ˚C) for 24 hours. Media was discarded after incubation, then different dilutions of glucose and/or Asiatic acid were added to the wells in triplets while in control wells 100 µl of complete media was added. The plate incubated for 20 hours and 10 µl of MTT solution (5 mg/ml) was added then plate further incubated for 4 hours. At 24th hour (after 4 hours of MTT treatment) plate was removed from CO_2 incubator and media was discarded then 200 µl of DMSO was added to dissolve formazone crystals. Plate was read at 570 nm. DMSO alone was used as blank well.

2.4 LDH Assay

Lactate dehydrogenase assay was done to determine LDH release by cells after exposing to different concentrations of Asiatic Acids and/or glucose. It was done according to manufacturer's instruction (TAKARA). Lactate dehydrogenase is cytosolic enzyme that convert lactate into pyruvate, when cell membrane ruptured it leaks to surrounding medium therefore it can be used as marker to measure cytotoxity. In the experiment 100 cells/100 µl/well were seeded in 96 well flat bottom transparent plate. After 24 hours cells were treated with 100 µl different dilutions of glucose and/or Asiatic acid then incubated further for 72 hours. After incubation 100 µl of reaction mixture was injected in wells and incubate for 1 hour. Plate was read at 490nm.

2.5 Estimation of Cellular GSH Level

Reduced glutathione protects the cell from oxidative damage. The available glutathione measured according to manufacturer's instruction (Sigma Aldrich) by using CMFDA (5-chloromethylfluorescein diacetate) dye.

2.6 Mitochondrial Membrane Potential Assay (JC-1 dye)

Cell death can also be confirmed by using mitochondrial permeability as an indicator. Normally mitochondria remain in a polarized state however it loses the state when cell furthers toward cell death. JC-1 is a nontoxic fluorescent probe used to monitor membrane potential. The electrochemical gradient is responsible for this JC-1 aggregation. At low transmembrane potentials, JC-1 maintains its monomeric form, thus the cells emit green fluorescence. In contrast, at high transmembrane potentials, JC-1 forms more aggregates and red fluorescence. The green-to-red (G/R) ratio is an indicator of $\Delta\Psi$. Green-to-red fluorescence ratios of cells. Stained by JC-1 were detected in merged images by a confocal imaging. The cell groups (Control, Asiatic Acid treated hyperglycaemia and hyperglycaemia with Asiatic acid) were treated with JC-1 dye andfluorescence was recorded in merged images by a confocal imaging technique as described elsewhere.

2.7 Propidium Iodide (PI) Uptake Assay

To measure leaky cells Propidium Iodide assay was performed. Propidium Iodide tends to bind double stranded DNA as it passes through ruptured membrane thus it is used as a probe to mark cell death. HepG2 cells (5×105 cells/well), grown in 6-well plates for 24 h were harvested by trypsinization and fixed in 70% ethanol. Following treatment with RNase (1 mg/ml in 0.1 M phosphate buffer, pH 7.0), the cells were stained in propidium iodide (PI) solution (4 mg/ ml in 0.1 M phosphate buffer, pH 7.0, 0.1% Triton X-100) and then analyzed using a Nikon TE2000-U fluorescence microscope. Plate was read at excitation 530nm and emission 620nm.

2.8 Western Blotting

Cells were washed with PBS solution and centrifuged at 1000×g for 5 min. Cell pellets was lysed for 15 min at 40C in whole cell extraction buffer containing 50 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonylfloride, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mg/ml pepstatin A, 40 mM a-glycerophosphate, 0.1 mM DTT. Lysates were centrifuged at 20 000×g for 10 min at 40C, and supernatant proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England) at 30 V for overnight. The membrane was blocked with 5% skim milk in Tween-20 containing Tris buffered saline (TTBS) (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) and incubated with primary anti-human Caspase 3, Caspase 8 or Caspase 9 antibody in TTBS containing 5% skim milk. GAPDH expression was measured as constitutive expressing control model. After incubation with horseradish peroxidase-conjugated anti-IgG antibody, immunodetected proteins were visualized by using enhanced chemiluminescence assay kit (Amersharm Life Science, Buckinghamshire, England).

3. Results

3.1 MTT Assay

As shown in Fig. 1, 5 mM glucose which replicates normoglycemic conditions did not cause toxicity to HepG2 cells. Cell viability was not affected when HepG2 cells were incubated with up to 20 mM glucose for 48 h or 72 h (about 98-99% viability). Further treatment of HepG2 cells with 30 mM glucose for 48 h or 72 h did not affect the viability of HepG2 cells significantly (92 to 88% viability). However, incubation with 40 mM for 48 h or 72 h glucose resulted in a significant decrease (p<0.05) in viability (80% or 63% viability). Treatment with different concentrations of Asiatic acid to Hep G2 cell is depicted in figure (6). Cells treated with up to 20 µM concentration for 48 h or 72 h did not show any significant impact on their viability (Survival 96% to 98.54%). However, significant percentage of cell death was observed (61.55% to 68.61% survival) when treated with 30 µM Asiatic acid for 48 h or 72 h. Further, treatment of the cells with 40 µM Asiatic acid led to survival of 32.54% to 39.54% of cells (Fig 7). Therefore, subsequent experiments were carried out using the 40 mM glucose as hyperglycemic concentration whereas 20 µM of Asiatic acid was used as cytoprotective concentration. All the experiments carried out having a treatment period of 72 hours.

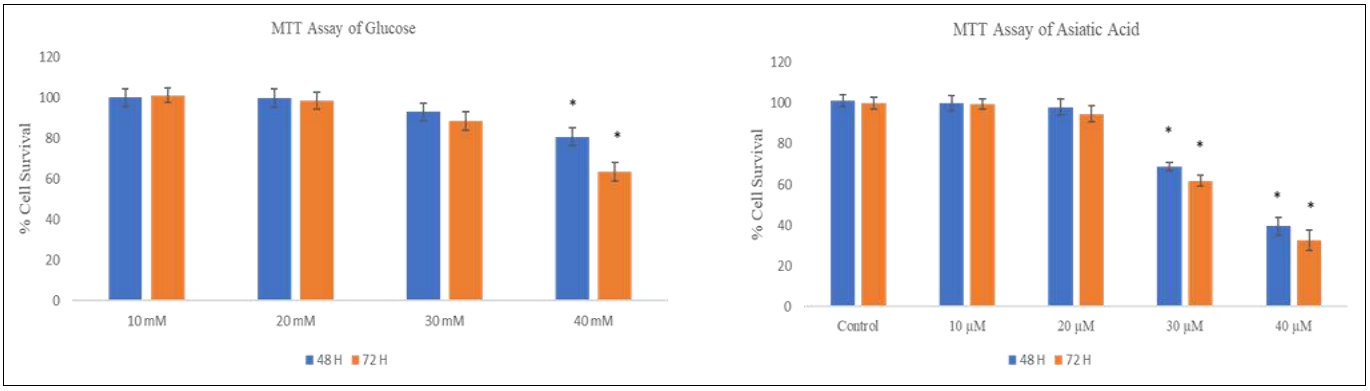

Fig 1: Cell viability was measured by MTT assay. In case of glucose a concentration of 30mM and 40mM causes for 48 or 72 h caused significant cell death whereas a concentration of 30µM and 40µM causes for 48 or 72 h caused significant cell death.Here */** shows significant/highly significant changes with respect to control anddiabetic group (p<0.05).

3.2 LDH Assay

LDH assay was performed to validate the results of cytotoxic dose obtained from MTT assay. No significant rise in LDH release was found when treated with 10mM glucose for 72 h while a 1.4-fold increase in LDH release was observed upon 20mM concentration treatment. 30mM and 40 mM concentrations, with same treatment period, significantly elevated LDH activity with 3.43-and 5.89-fold increase respectively (Fig. 2).

Up to 20 µM Asiatic acid concentration, no significant LDH release was detected. When Hep G2 cells were treated with 30 µM and 40 µM for 48 h, 2.36-fold and 4.86-fold increase respectively was observed.

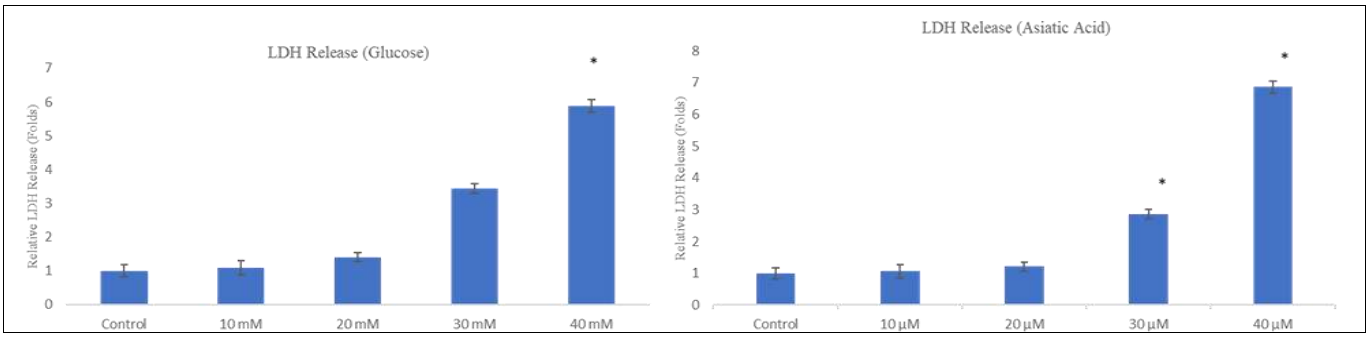

Fig 2: LDH assay was performed to validate the results of MTT. A concentration of 30 µM and 40µM causes for 72 h caused significant LDH release and a concentration of 30 µM and 40 µM causes for 72 h caused significant LDH release. Here */** and # shows significant/highly significant changes with respect to control anddiabetic group (p<0.05).

3.3 Morphological Changes in Cells

The morphology of untreated HepG2 cells or HepG2 cells treated with 20µM Asiatic acid or 40 mM glucose or 40 mM glucose plus 20 µM for 72 h was recorded by visualizing the cells under a light microscope (Nikon, Japan). Treatment of HepG2 cells with 20 µM Asiatic acid for 72 h did not cause any morphological alterations (data not shown). At 72 h, 40 mM glucose caused marked morphological changes when added to the HepG2 cells, such as shrinkage of cells, decreased confluence and detachment of cells as visualized by greater number of floating cells (Fig. 3)

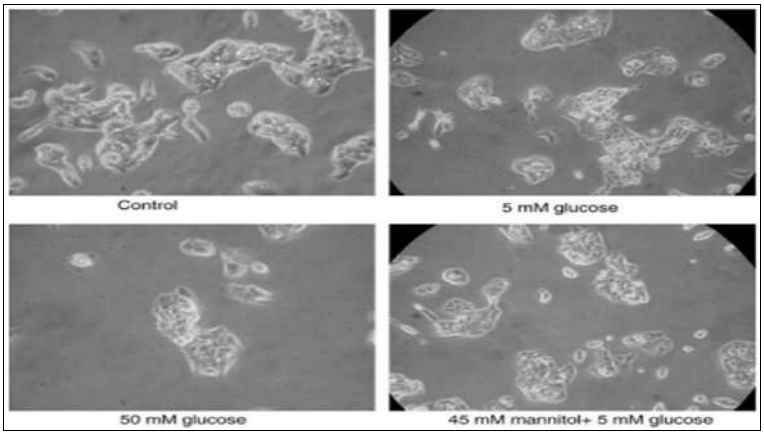

Fig 3: Morphology of HepG2 cells treated with high glucose. HepG2 cells were treated with 5 mM glucose or 40 mM glucose or 40 mM glucose plus 20 µM Asiatic Acid for 48 h and the morphology of the cells were examined under inverted microscope at 20 magnifications. One representative experiment of three is shown.

3.4 Cellular GSH Level

Incubation of HepG2 cells with 40 mM glucose for 72 h caused a decrease in the level of GSH roughly by half, a major intracellular antioxidant (Fig. 4). No significant changes in the levels of GSH in 20 µM Asiatic acid was observed. The level of reduced glutathione was restored to normal when treated with 40 mM glucose plus 20 µM Asiatic acid.

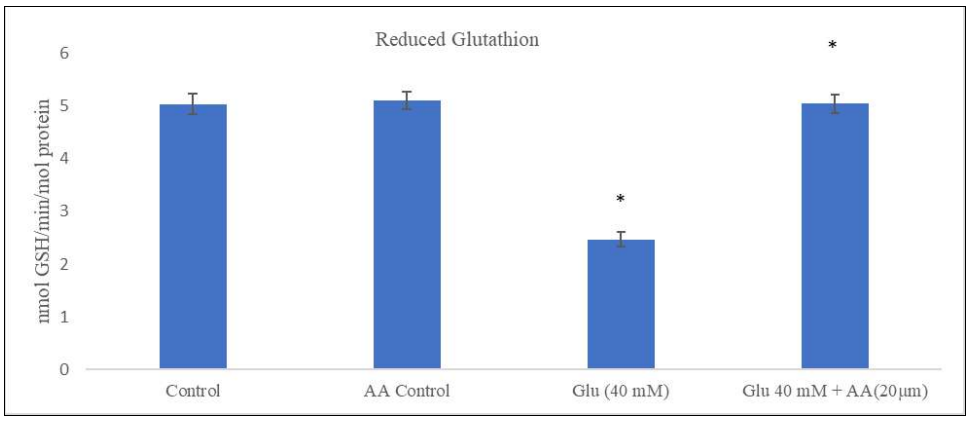

Fig 4: The amount of reduced glutathione is inversely proportional to the amount of oxidative stress of present in the cell. A hyperglycemic concentration of glucose caused reduction of reduced glutathione to half which was re-established to normal when non-toxic concentration of Asiatic acid was given along with 40mM Glucose. Here */** and # shows significant/highly significant changes with respect to control anddiabetic group (p<0.05). Diabetic group (p<0.05).

3.5 Mitochondrial Membrane Potential Assay (JC-1 dye)

Collapse of mitochondrial membrane potential is one of the earliest events in apoptosis. We proceeded to measure mitochondrial membrane potential (ΔΨ) using JC-1 as a probe. In comparison, ΔΨ as reflected by the G/R ratio was highest in hyperglycemic glucose concentration treated Hep G2 cells whereas in 40 mM glucose plus 20 µM treated cells the ratio was comparable with rest of 2 groups (Control and 20 µM Asiatic acid treated cells). This data demonstrated that Asiatic is indeed capable of maintaining mitochondrial membrane potential (Fig 5).

3.6 Propidium Iodide (PI) Uptake Assay

Propidium iodide (PI) binds to DNA by intercalating between the bases which results in an increase in its fluorescence. As compared to untreated or 20 µM Asiatic acid a hyperglycaemic glucose concentration for 72 hour causes an increased amount of PI to bind to DNA resulted in increased red fluorescent, whereas cells treated with hyperglycemic concentration of glucose along with Asiatic acid emits fluorescence comparable with control group (6).

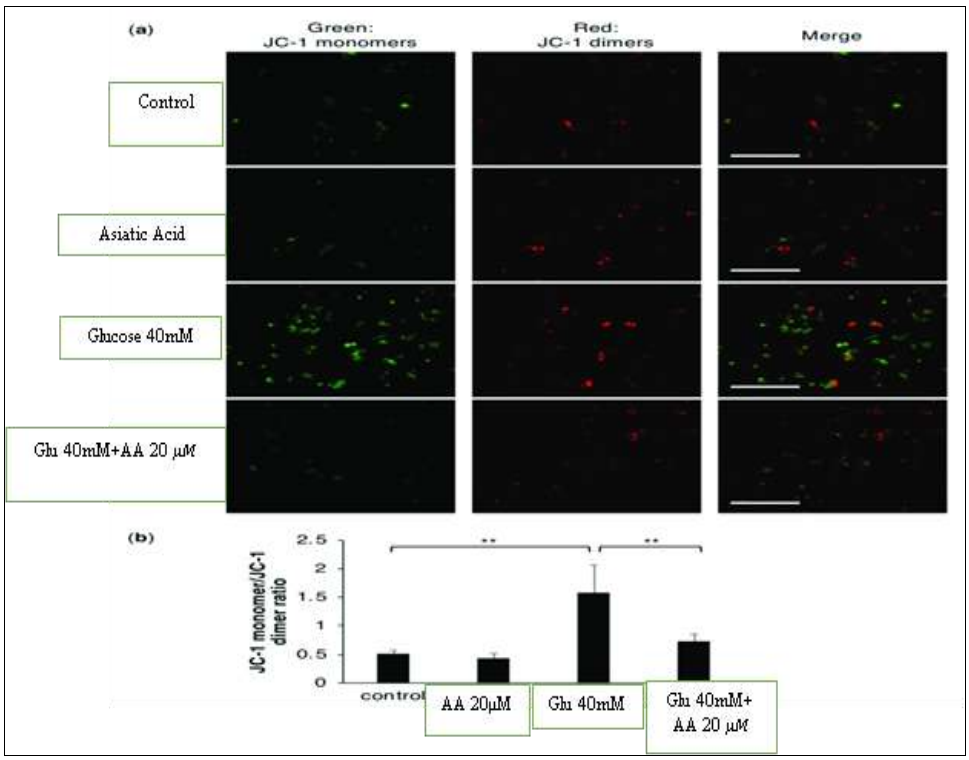

Fig 5: Mitochondrial membrane depolarization in HepG2. (a) Depolarization of the mitochondrial membrane potential was observed (the magnification for the figure is ×20). Scale bar = 200 µm. (b) The relative fluorescence intensities of JC-1 monomer/JC-1 dimer. Results are mean ± S.D. ** p< 0.01 (n = 3) based on a one-way ANOVA followed by Tukey's test.

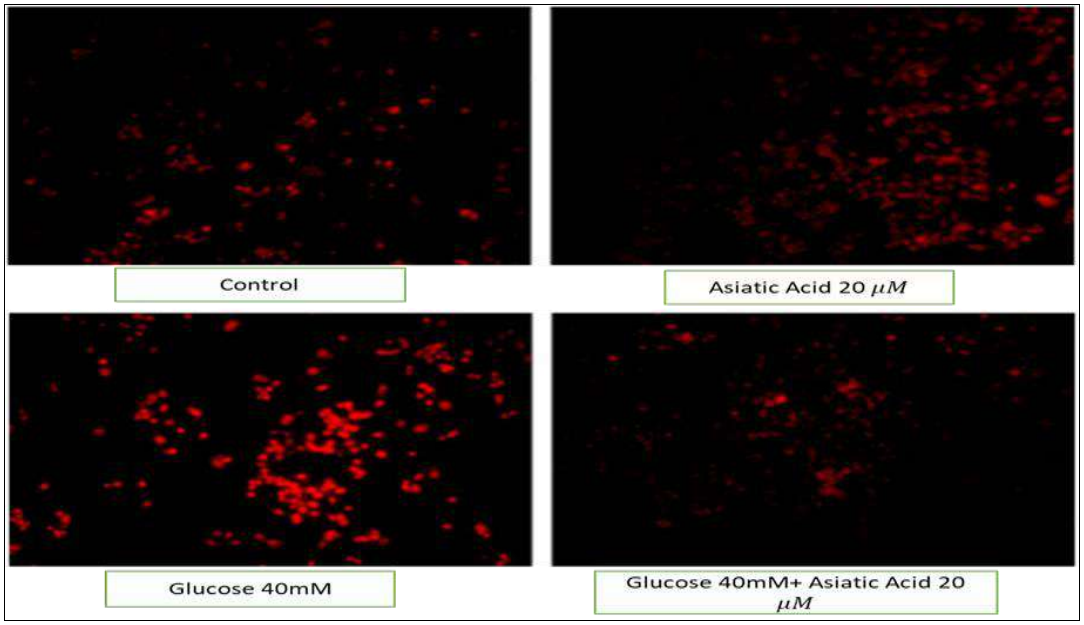

Fig 6: Cytoprotective role of Asiatic Acid on DNA fragmentation in HepG2 cells. HepG2 cells were untreated or 40 mM glucose or 40 mM Glucose plus Asiatic Acid (20 µM) for 72 h and the cells were stained with PI and observed under a fluorescence microscope at 10x magnification.

3.7 Western Blotting

Expression of caspases was recorded in order to establish the fact that Asiatic acid exert cytoprotectivity by preventing cell from leading to apoptosis. We observed that a significant rise in expression of caspase 3, 8 and 9 was taking place when cells were treated with hyperglycemic concentration of glucose (40 mM) for 72 hours. However, this expression was significantly subsided when 20 µM of Asiatic acid was added along with 40 mM of glucose for same time period in the medium and bring it to similar level as it was in either control group or Asiatic acid treated group. Results of SDS-PAGE are depicted in figure 7.

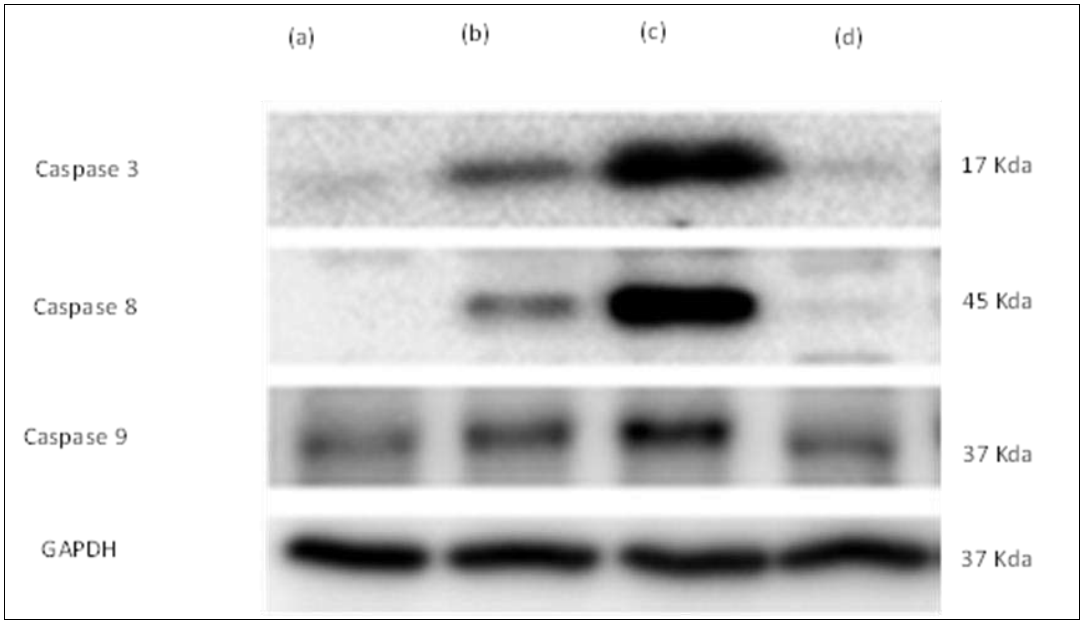

Fig 7: Expression of caspases to monitor the involvement of apoptosis. The expression of caspases was increased significantly in hyperglycemic group which was succefully down regulated by Asiatic acid treatment.

4. Discussion

Hyperglycemia affect liver in negative manner i.e., dysregulation of glucose, lipid, bile acid and triglyceride metabolism and disruption of several cellular functions (Evans *et al*., 2002) [8]. In humans, nonalcoholic fatty liver disease (NAFLD) characterized by steatosis has been consistently associated with hepatic insulin resistance generated due to hyperglycemia (Younossi *et al*., 2004) [15]. Liver injury in various stages of its development is of common occurrence in type 2 diabetes and diabetic predisposes individuals to the development of NAFLD (Cusi, 2009) [9]. Thus, hyperglycemia plays a major role in the onset of liver damage in humans and as exemplified by the use of various animal and cell culture models in the literature, hyperglycemia alters the normal cellular environment of the liver. Here, in this study using HepG2 cells as an *in vitro* model aims to provide insights into the mechanisms and mode of cell death induced due to glucose

toxicity in liver, also elucidates cytoprotective role of Asiatic acid. A decrease in viability was observed at 72 h when the cells were incubated with 30 or 40 mM glucose. Since high cell death was observed at 40mM concentrations, we used the higher concentration i.e., 40 mM glucose to treat HepG2 cells and a time period of 72 h for subsequent experiments as it is causing significant toxicity. High concentration of glucose (40 mM) has been used to study hyperglycemia induced injury in various tissues, as also closely replicating *in vivo* diabetic ketoacidosis observed in acute or untreated diabetes (Ramana *et al*., 2003) [10]. Treatment of HepG2 cells with 10 mM or 20 mM glucose for the same duration did not affect the viability of the cells significantly. Addition of glucose at a higher concentration i.e., 40 mM for a same time period caused significant alterations in morphology i.e. presence of more rounded, dispersed and detached floating cells. Similar observations were made when different Hep G2 cells treated with varied concentrations of Asiatic acid. Cell viability was not altered significantly when treated with 10µM or 20µM of Asiatic acid for 48 h. However, cell death start manifesting when 30µM or 40µM concentrations of Asiatic acid were administered to Hep G2 cells.

Significant increases in LDH activity indicators of hepatic injury were observed in HepG2 cells treated with 40 mM glucose. Therefore, a high concentration of glucose for a long duration i.e.72 h causes significant toxicity in HepG2 cells. Caspase-3, 8 and 9 have been implicated in initiating apoptosis (Nicholson and Thornberry, 1997). We observed an increase in caspase-3 activity in 40 mM glucose treated HepG2 cells, suggesting apoptosis as the principal mode of cell death. PI is taken up by cells with lysed plasma membrane and specifically stains DNA. When observed under the microscope, high glucose treated HepG2 cells showed a much greater number of apoptotic cells which appeared as red fluorescent cells as compared to the extremely low distribution of red fluorescent cells in untreated or 20 µM g Asiatic acid or 40 mM glucose plus 20µM Asiatic acid treated groups. High glucose treatment resulted in apoptosis rather than necrosis as characterized by the presence of PI-stained red fluorescent cells having DNA breakage. All these parameters in Asiatic acid treated diabetic induced group were similar to control group suggesting cytoprotective activity of Asiatic acid in hyperglycemia induced Hep G2 cell line. To investigate the mechanism by which Asiatic acid prevents cell to move into apoptotic pathway caused by hyperglycemia in HepG2 cells, several oxidative stress parameters were studied. The ROS level as indicated by increased DCF fluorescence was about 2-fold greater in the 40 mM glucose treated group whereas the untreated or 20 µM Asiatic acid or 40 µM glucose plus 20 µM Asiatic acid treated HepG2 cells showed values comparable with control group. Several lines of evidence suggest that diabetes increases oxidative stress, including increased O^{2-} production, lipid peroxides, and DNA damage (Kaneto *et al*., 2007) [7].

GSH helps to maintain the cellular redox balance and decreased intracellular levels of GSH reflect oxidative stress in the cellular environment (Hall, 1999) [1]. Altered levels of GSH have been observed in liver of animals or liver cells treated with different hepatotoxins (Dey and Cederbaum, 2006) [3]. A decrease in GSH level roughly to half was observed in the high glucose treated HepG2 cells, which was reversed when Hep G2 cells were treated with Asiatic acid along with 40 mM glucose. A fall in GSH level may be explained as its interactions with reactive oxygen species produced as a result of hyperglycemia and subsequent conversion of reduced glutathione into GSSG i.e., Oxidized Glutathione. Asiatic acid treatment, in our studies restore reduced glutathione level comparable to control groups. Thus, our findings suggest that oxidative stress plays a major role in promoting hyperglycemia mediated apoptosis in HepG2 cells and the antioxidant Asiatic acid protects against high glucose induced oxidative stress and toxicity in HepG2 cells. In conclusion, HepG2 cells treated with 40 mM glucose for 72 h show decreased viability and significant changes in morphology. High glucose caused a significant increase in caspase-3 activity and apoptosis. The cell death observed in high glucose treated HepG2 cells is a consequence of increased oxidative stress as evidenced by increases in intracellular ROS level. The antioxidant GSH was decreased in high glucose treated HepG2 cells, as it reacts with reactive oxygen species produced by glucose induced toxicity. Level of GSH was normalized when treated with Asiatic acid as it rescues cells from apoptosis. Further, hyperglycemia induced oxidative stress was confirmed with the reversal to normal morphology, increased viability and decrease in ROS level, in Asiatic acid treated HepG2 cells. Thus, glucose promotes apoptosis in HepG2 cells through increased oxidative stress which was ameliorated to normal by Asiatic acid also, the findings of this study elucidate the mechanisms by which the compound exerts cytoprotectivity.

5. Conclusion

From this study it is evident that Asiatic acid successfully prevent the cells from leading into cell death pathway.

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