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Protective effect of flavanone – naringenin on high glucose-induced hepatotoxicity to Hep G2 cells

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Protektivni učinak flavanona naringenina na glukozom izazvanu hepatotoksičnost u Hep G2 stanicama

S a ž e t a k – Naringenin je flavanon, vrsta flavonoida. Opisan je aktívni učinak flavonoida na ljudsko zdravlje. Djeluju kao antioksidansi i čistači slobodnih radikala, imaju protuupalni učinak, potiču metabolizam ugljikohidrata te aktivnost imunološkog sustava.

Cilj je ovog rada bio utvrditi koja od ispitivanih koncentracija naringenina u rasponu 1–1000 μM ima protektivni učinak na Hep G2 stanice koje se nalaze u hiperglikemijskim uvjetima.

Hiperglikemija je u šećernoj bolesti primarni uzrok oksidacijskom stresu koji kasnije dovodi do komplikacija. U Hep G2 stanicama koje se nalaze u hiperglikemijskim uvjetima dolazi do značajnog porasta jetrenih funkcijskih markera kao što su AST, ALT, LDH i GGT, smanjene vijabilnosti stanica (MTT test) i smanjene aktivnosti antioksidativnih enzima: glutation-peroksidaze (GPX) i superoksid-dizmutaze (SOD). Naringenin pri koncentracijskom rasponu od 1–100 μM ima hepatoprotektivni učinak na Hep G2 stanice u hiperglikemijskim uvjetima, dok više koncentracije naringenina (1000 μM) ne pokazuju više taj učinak.

INTRODUCTION

The liver plays an important role in maintaining glucose homeostasis during fasting as well as the postprandial state (1). Several studies have shown that high levels of ALT and GGT are independent predictors of type 2 diabetes incidence (2,3). The authors Lee *et al.* 2004., investigated hepatoprotective effects of naringenin in rats (4). Citrus fruits (including naringenin) and their potential benefits in the management

of diabetes is described in study of the authors Aruoma *et al.* 2012 (5). Furthermore, naringenin potentiates intracellular signaling responses to low insulin doses, suggesting that naringenin sensitizes hepatocytes to insulin (6). So far no study of the naringenin effect in the hyperglycemic condition *in vitro* has been performed.

Naringenin, the predominant flavanone in grapefruits, oranges and tomatoes (skin), is a type of flavonoid that is considered to have a bioactive effect on human health as antioxidant, free-radical scavenger, anti-inflammatory, carbohydrate metabolism promoter, immune system modulator (7) and has been shown to reduce oxidative damage to DNA *in vivo* (8).

The results of the authors Tsai *et al.* 2012 indicate that naringenin could attenuate diabetic nephropathy via its anti-inflammatory and antifibrotic activities (9). It has also been reported that high consumption of grapefruit juice improved blood lipid profile in hyperlipidemic humans (10,11). Naringenin has an inhibitory effect on the human cytochrome P450 isoform (CYP1A2) (12,13).

In the hyperglycemia environment the high level of reactive oxygen species (ROS) exist (14). Oxidative stress is the principal mechanism in the progression of diabetes and actively leads to cellular injury that can precede the onset of many diabetic complications (15). The development of insulin resistance is also closely related to the presence of cellular oxidative stress (16). Diabetes is associated with the generation of reactive oxygen species, which cause oxidative damage, particularly to heart, liver, eyes, nerves and kidney (17). Hyperglycemia can degrade antioxidant enzyme defenses (18), thereby allowing ROS to damage other enzymes and also structural proteins. Antioxidants in foods, such as vitamin C, vitamin E, selenium and many phytochemicals can eliminate these free radicals (19). The ethnomedical approach to plant drug discovery is practical, cost-effective, and logical. Phytochemicals with antioxidant activity are: allyl sulfides (onions, leeks, garlic), carotenoids (fruit, carrots), flavonoids (fruit, vegetables), polyphenols (tea, grapes).

The present study was planned to elucidate whether naringenin, when administered, can ameliorate toxic effects of glucose in Hep G2 cells.

Materials and Methods

Materials and chemicals

Naringenin and D-glucose were from Sigma (St. Louis, MO, USA). All other chemicals used in these measurements were of the highest purity commercially available.

Cell culture

Human Caucasian hepatocyte carcinoma (Hep G2) cells from European Collection of Cell Cultures (ECACC) were maintained in an incubator at 37 °C with a

humidified atmosphere of 5 % CO₂ and cultured in Minimum Essential Media (MEM) (Gibco*) supplemented with 10 % (v/v) fetal bovine serum (FBS) and 20 IU/mL penicillin, 20 µg/mL streptomycin. The medium was refreshed twice a week. For the experiments, cells were seeded into six-well plates (or in 96-well plates for mitochondrial dehydrogen activity assay and LDH activity). Medium was changed to FBS-free medium 24 h before the assay. The Hep G2 cells were treated another 24 h with 30 mM glucose and naringenin in following concentrations: 1000 µmol/L, 100 µmol/L, 10 µmol/L, and 1 µmol/L. The control cells were grown in the medium which contained 5.56 mM D-glucose.

Treatment of the Hep G2 cells

Before treatment Hep G2 cells were cultured in 6-well Nunc plates in complete MEM medium. On the day of treatment complete MEM medium is removed, and Hep G2 cells were divided in six groups:

C – negative control

Hep G2 cells were incubated in FBS- free MEM medium, containing 5.56 mM glucose

D – positive control

In this group, Hep G2 cells were cultured in FBS- free MEM, supplemented with D-glucose (Sigma) in concentration of 30 mM

D/N_{1-1000 µmol/L} – four treated groups with naringenin

Hep G2 cells were cultured in FBS- free MEM, supplemented with D-glucose (Sigma) in concentration of 30 mM + different concentrations of naringenin:

D/N_{1-1000 µmol/L}

For statistical comparison is used D – positive control vs. D/N_{1-1000 µmol/L} (*) and C vs. D (**)

Cell viability assay

Cell viability was assessed by MTT test (20). The cytotoxicity was carried out by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay for investigating changes in mitochondrial/non-mitochondrial dehydrogenase activity. After overnight growth, cells were treated with different concentrations of naringenin described above for 24 h. At the end of the treatment the media were removed from wells (for LDH activity). Cells were washed twice with sterile phosphate buffered saline (PBS). MTT plus medium was added. After 4 h incubation at 37 °C, the supernatant was removed and formazan crystals were dissolved by DMSO (100 %). Finally, the absorbance at a wavelength of 595 nm was recorded using a plate reader (Victor). The absorbance at 595 nm was taken as an index of the activity of mitochondria.

Cell membrane damage

Activity of lactate dehydrogenase (LDH) in the cell medium was measured as an index of cell membrane damage. Hep G2 cells were cultured in 96-well plate at a density of 1×10^5 cells / 200 μ L. Cells were treated 24 h with naringenin. At the end of the treatment the media were collected. The LDH activity was measured with spectrophotometric method using lactate as substrate (21). For this, 200 μ L aliquots of culture medium were added to the LDH assay system, and the absorbance at 340 nm was recorded.

Determination of glutathione peroxidase (GPx) activity

The cells were seeded in six-well plates at concentration of 1×10^6 cells/mL and 24 h after plating, were treated with naringenin (in the concentration range 1 to 1000 μ mol/L) in the serum free media. After additional 24 h, the medium was removed, the cells were washed twice with sterile PBS solution, and scraped. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by ultrasonication for 15 sec. Then, Triton X-100 (1 %) was added to the lysates and incubated for 10 min on ice. The lysates were clarified by centrifugation at 14 000 rpm for 20 min at + 4 °C to remove cellular debris.

We used Glutathione Peroxidase Assay Kit (Cayman Chemical Company) for GPx activity in the cell lysate (22, 23). Cayman's GPx Assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx was recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP⁺ was accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} nm is directly proportional to the GPx activity in the sample. The A_{340} nm was recorded using VictorTM multilabel reader (Perkin Elmer). GPx activity was expressed as units/mg protein.

Determination of superoxide dismutase (SOD) activity

We used Superoxide Dismutase Assay Kit (Cayman Chemical Company), (24,25) for SOD activity in the cell lysate. Cayman's utilizes tetrazolium salt for detection of superoxide radicals generated by xantine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical. The SOD assay measures all three types of SOD (Cu/Zn, Mn and Fe SOD). The A_{450} nm was recorded using VictorTM multilabel reader (Perkin Elmer). Total SOD activity was expressed as units/mg protein.

The protein content in Hep G2 cells by QuibitTM Quantitation System

This is a fluorescence-based quantitation assay. The kit provides concentrated assay reagent, dilution buffer, and pre-diluted BSA standards. The QuibitTM fluorometer

generates concentration data based on the relationship between the three standards used in calibration and gives values for the proteins in cells in $\mu\text{g/mL}$.

Biochemical assays of liver enzymes

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured spectrophotometrically using the α -ketoglutarate reaction according to the standard procedures for automatic analyzer (Olympus, AU 1000). γ -glutamyl transferase (GGT) activity was determined by the method of Rosalki *et al.* 1970 using γ -glutamyl-p-nitroanilide as substrate (26). All enzymes were analyzed in Hep G2 cell lysate.

Statistical analysis

All analyses were performed using SigmaStat 2.0 Software programme. Results were expressed as means \pm SEM. For multiple comparisons, one-way analysis of variance was followed by the Holm-Sidak method. P-value < 0.05 was considered to be statistically significant (27).

Results

Effects of naringenin on the cell viability

The exposure of Hep G2 cells to 30 mM glucose led to a loss of cell viability ($p < 0.05$) but the treatment with naringenin in concentration 1 $\mu\text{mol/L}$ protected cells against damage induced by 30 mM glucose ($p < 0.05$) (Fig. 1).

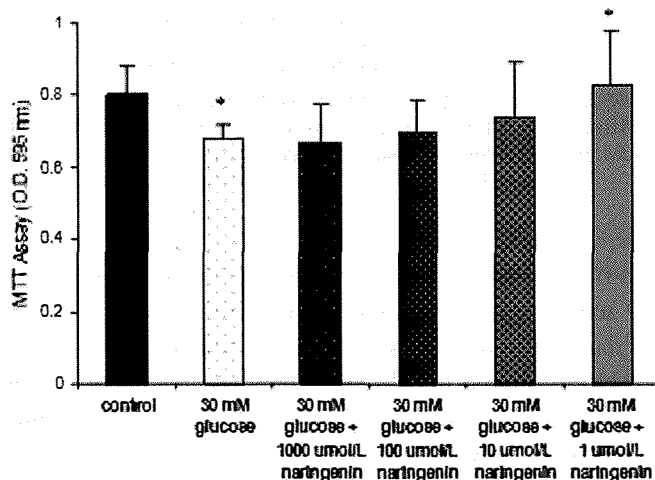


Fig. 1. EFFECT OF NARINGENIN AGAINST 30 mM GLUCOSE-INDUCED CYTOTOXICITY IN HEP G2 CELLS. The data were expressed at the mean \pm SEM of three independent experiments. * $p < 0.05$ was considered to be statistically significant.

Effects of naringenin on the cell membrane damage

Cell membrane integrity was determined using the activity of LDH in the medium of Hep G2 cells treated with different concentrations of naringenin. In cells treated only with 30 mM glucose, LDH activity in medium was significantly increased ($p < 0.05$) (Fig.2.). Supplementation of Hep G2 cells with 100, 10 and 1 $\mu\text{mol/L}$ naringenin significantly decreased ($p < 0.05$) LDH release, when compared with cells treated only with 30 mM glucose.

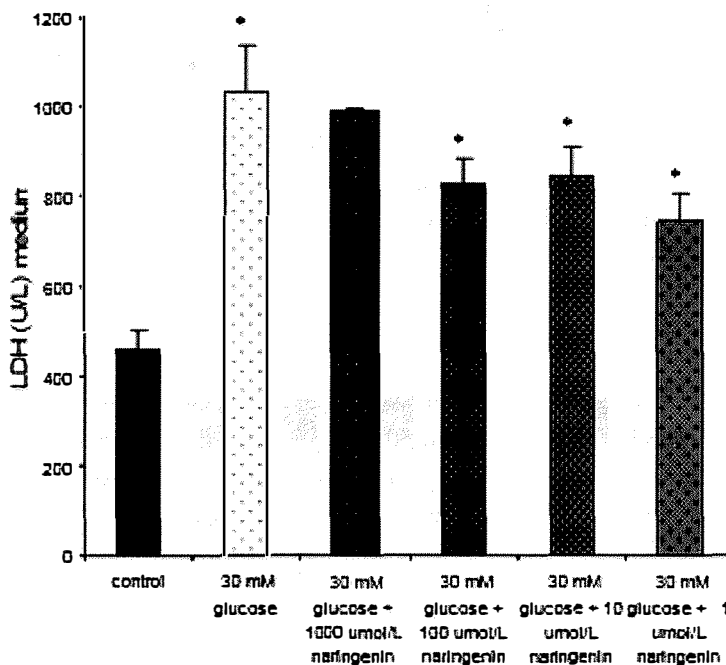


Fig. 2. EFFECT OF NARINGENIN ON GLUCOSE-INDUCED MEMBRANE INSTABILITY. HEP G2 CELLS WERE INCUBATED 24 h WITH NARINGENIN PRIOR TO LDH ASSAY. LDH RELEASE REPRESENTS LDH IN U/L IN THE CULTURE MEDIUM. The data were expressed at the mean \pm SEM of three independent experiments.

*P-value < 0.05 was considered to be statistically significant.

Effects of naringenin on the antioxidant status of Hep G2 cells

Gpx, SOD activities and protein levels were measured to investigate the antioxidative effect of naringenin on glucose-induced damage of Hep G2 cells. The activity was expressed as U/mg protein in cells (Fig.3. and Fig.4.). Compared with the control, cells in hyperglycemic conditions showed significantly decreasing specific activity of GPx ($p < 0.05$). GPx specific activity was significantly increased after 24 h of incubation with 1 and 100 mmol/L naringenin ($p < 0.05$). There was no significant difference in the specific SOD activity between cells treated with glucose and non-treated cells ($p > 0.05$). Fig. 4. depicts the effect of naringenin on SOD specific activity

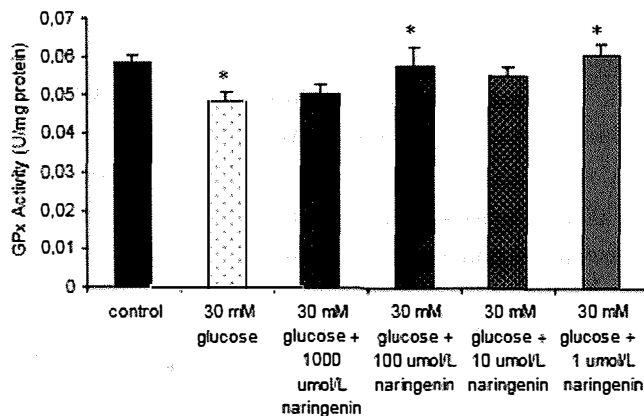


Fig. 3. SPECIFIC ACTIVITY OF GLUTATHIONE PEROXIDASE (GPx) IN HEP G2 CELLS INCUBATED FOR 24 h WITH NARINGENIN AND GLUCOSE. The data were expressed at the mean \pm SEM of three independent experiments.

*P-value < 0.05 was considered to be statistically significant.

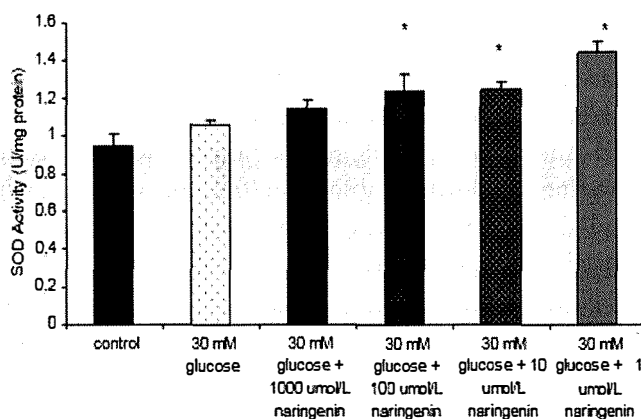


Fig. 4. SPECIFIC ACTIVITY OF SUPEROXIDE DISMUTASE (SOD) IN HEP G2 CELLS INCUBATED FOR 24 h WITH NARINGENIN AND GLUCOSE. The data were expressed at the mean \pm SEM of three independent experiments.

*P-value < 0.05 was considered to be statistically significant

upon the exposure of 30 mM glucose. There was a significant increase in SOD activity after the treatment with 100 and 10 and 1 μ mol/L naringenin ($p < 0.05$).

Hepatoprotective effect of naringenin on Hep G2 cells

Table 1. shows the hepatic functional markers of the control and treated Hep G2 cells. The treatment of Hep G2 cells with 30 mM glucose significantly increased the levels of hepatic functional markers such as AST, ALT and GGT when compared with the control, untreated cells ($p < 0.05$). The administration of naringenin in concentration from 1 to 100 μ mol/L, significantly decreased the activities of hepatic biochemical markers when compared Hep G2 cells treated only with 30 mM glucose ($P < 0.05$).

Table 1. ACTIVITY OF AST, ALT AND GGT IN HEP G2 CELLS LYSATE AFTER NARINGENIN TREATMENT UNDER HYPERGLYCEMIC CONDITION (30 mM GLUCOSE)

Sample	AST (U/L)	ALT (U/L)	GGT (U/L)
Control	63,67±4,6	16,67±1,5	15,00±5,0
30 mM glucose	133,67±8,5 ^a	38,33±3,1 ^a	30,00±5,0 ^a
30 mM glucose+1000 µmol/L naringenin	154,67±26,6	32,33±2,1 ^b	38,00±1,2
30 mM glucose+100 µmol/L naringenin	108,67±6,6 ^b	24,67±0,6 ^b	33,67±1,4
30 mM glucose+10 µmol/L naringenin	122,33±9,3	19,00±0,1 ^b	45,00±7,7 ^b
30 mM glucose+1 µmol/L naringenin	102,00±5,2 ^b	13,33±1,5 ^b	31,00±2,0

Data expressed as mean ± S.E.M.

^a control vs. 30 mM glucose, P<0,05

^b naringenin vs. 30 mM glucose, P<0,05

Discussion

In the present study, we showed that naringenin in concentrations of 1–100 µmol/L had hepatoprotective effect on Hep G2 cells under hyperglycemic condition. Naringenin is a natural flavonoid responsible for the bitter taste in several plant foods. The study of Ortiz-Andrade *et al.* 2008. revealed that naringenin exerted its antidiabetic effect by lowering carbohydrate absorption from the intestine, which alleviated postprandial blood glucose level (28). Kannapan and Anuradha, 2010 reported that naringenin enhanced insulin sensitivity in fructose-fed animals (29).

Diabetes mellitus has been shown to be a state of increased free radical formation (30). As the level of ROS was high in the hyperglycemia environment, oxidative stress may be increased.

Flavonoids have free radical scavenging effect with the formation of less reactive phenoxyl radicals by donation of electron or hydrogen, the ability to chelate a transition metal like iron, thereby suppressing the hydrogen peroxide-driven Fenton reaction (31).

Iron chelating properties and radical scavenging activity of flavonoid are closely related: iron is chelated by the flavonoid and the reactive oxygen species which are formed in its vicinity are subsequently scavenged by the flavonoids (32).

LDH is a cytoplasmic enzyme present in all cells. Its release into the cell culture medium is a sensitive indicator of cell membrane injury. Glucose in concentration of 30 mM caused LDH leakage from the cells into the medium and naringenin in low concentrations decreased this leakage. The glucose-mediated increase in LDH leakage observed here (Fig.2.) indicates that cell membranes were damaged, possibly due to oxidative injury. Naringenin in concentrations 100, 10 and 1 µmol/L reduced LDH leakage, possibly by lowering oxidative stress.

To elucidate the protective effects on the cell against a high glucose level, we measured cell viability and demonstrated that the exposure of Hep G2 cells to high glucose resulted in the loss of cell viability. However, naringenin in concentration of 1 μM protected Hep G2 cells from high glucose-induced cytotoxicity (Fig. 1.).

The cellular antioxidative enzyme system plays a crucial role in the defense against liver injury with high glucose level. The cytoprotective mechanism of naringenin against glucose-induced toxicity in Hep G2 cells was investigated by assessing the status of various antioxidant enzymes including GPx and SOD. In the study of Jain et al., 2011, antioxidant potential of naringenin and silymarin in the dose of 50 mg/kg each was evaluated in rats (33). They showed that naringenin and silymarin significantly protected SOD, CAT and GPx activities by directly scavenging ROS as well as by inhibiting lipid peroxidation, suggesting antioxidant properties of both flavonoids.

Glutathione peroxidase (GPx) catalyses the reduction of hydroperoxides, including hydrogen peroxide, by reducing glutathione and thus protects the cell from oxidative damage. (22). In our study, specific GPx activity was significantly increased ($p < 0.05$) after 24 h of incubation with 1–100 μM of naringenin, except of the naringenin in the highest concentration (Fig. 3).

In recent years, antioxidants and prooxidants have been extensively studied and it seems that most of the dietary antioxidants can behave as prooxidants; it all depends on their concentration and the nature of neighbouring molecules (34). In work of the authors Martin and Appel 2009 described that high concentrations of phenolic antioxidants, high pH, presence of iron phenolic antioxidants can initiate an auto-oxidation process and behave like pro-oxidants (35). Flavonoids are an antioxidant group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups, attached to ring structures and they can act as reducing agents, hydrogen donors, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators (36). In work of the authors Yen et al. 2003, the human lymphocytes were incubated with different concentrations of flavonoids included naringenin (up to a final concentrations of 25–200 μM) for 30 min at 37 °C. Conclusion of this study was that all the flavonoids (included naringenin) above 100 μM were highly cytotoxic toward the lymphocyte cells (37). In our experiments, naringenin in a final concentration of 1000 $\mu\text{mol/L}$ in all experiments did not have a positive effect on hyperglycemia induced changes and this finding can be explained with a prooxidative effect of high concentration of naringenin on Hep G2 cells.

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism. The reaction catalyzed

by SOD is extremely fast, having a turnover of $2 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ and the presence of sufficient amount of the enzyme in cells and tissues typically keeps the concentration of superoxide very low (38). Despite no differences in SOD activity between control (C) cells and glucose-treated cells (D), naringenin in lower concentrations (1–100 μM) significantly increased the activity of SOD in Hep G2 cells showing the protective, antioxidative role (Fig. 4).

These observations supported the idea that naringenin did protect Hep G2 cells from glucose-induced cytotoxicity by its hepatoprotective property. In our study we also measured the activities of two aminotransferases: AST and ALT. These enzymes are widely used in clinical practice as a sensitive, albeit non-specific index of acute damage to hepatocytes irrespective of its etiology. GGT is a microsomal enzyme that is widely distributed in tissues including liver and renal tubules, and it is a very sensitive index of liver pathology. In acute hepatic damage, changes in GGT activity parallel those of the aminotransferases. An increase in these enzymatic activities reflects active liver damage.

The liver plays an important role in maintaining glucose homeostasis during fasting as well as the postprandial state. The term non-alcoholic fatty liver disease (NAFLD) refers to a spectrum of conditions that ranges from simple hepatic steatosis to more severe disorders, including non alcoholic steatohepatitis (NASH), which can progress to fibrosis and cirrhosis. The NAFLD is associated with obesity, insulin resistance and Type 2 diabetes. The NAFLD is characterised by elevated levels of liver enzymes, including ALT, AST and GGT (39) Several prospective studies have shown that high levels of ALT and GGT are independent predictors of incident Type 2 diabetes. It remains undefined whether subjects with 1-h post-load glucose level are at increased risk of having elevated of liver enzymes (40).

Glucose treatment of Hep G2 cells, in our study, has a significant role in the alteration of liver functions (Table 1) because the activities of AST, ALT and GGT were significantly higher ($p < 0.05$) than those of normal value. Table 1 shows that naringenin decreased the activities of hepatic enzymes.

As a result, it may be concluded that naringenin in low concentrations (1–100 $\mu\text{mol/L}$) possesses hepatoprotective activities in hyperglycemic conditions. The results of the study could serve as a step towards the development of a mechanism-based therapeutic approach for the management of diabetes.

However, further experimentation needs to be done to find a possible mechanism by which naringenin could have a positive effect in hyperglycemia.

Conflict of interest statement

The authors of the present manuscript declare that there are no conflicts of interest.

Acknowledgments

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ABSTRACT

Naringenin is a flavanone, a type of flavonoid that is considered to have a bioactive effect on human health as antioxidant, free-radical scavenger, anti-inflammatory, carbohydrate metabolism promoter, and immune system modulator. This study was initiated to determine in which of the examined concentrations at the range 1–1000 μM of the naringenin has the protective effect on high glucose-induced toxicity in Hep G2 cells. The treatment of Hep G2 cells with 30 mM glucose significantly increased the levels of hepatic functional markers such as AST, ALT, LDH and GGT, decreased cell viability (MTT test), and decreased the activity of hepatic antioxidative enzyme: glutathione peroxidase (GPx), when compared with normal glucose level ($p < 0.05$).

We showed, that naringenin at concentration 1 to 100 $\mu\text{mol/L}$ had hepatoprotective effect on Hep G2 cells under hyperglycemic condition, but at a concentration of 1000 $\mu\text{mol/L}$ this effect is lost and naringenin showed toxic effect.

Keywords: naringenin, hepatoprotective, Hep G2 cells, MTT test, liver enzymes

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