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Glycyrrhizic acid modulates t-BHP induced apoptosis in primary rat hepatocytes

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ABSTRACT

Glycyrrhizic acid (GA) is the main bioactive ingredient of licorice (*Glycyrrhiza glabra*). The object of this study was to evaluate the protective effects of GA on tert-butyl hydroperoxide (t-BHP) induced oxidative injury leading to apoptosis in cultured primary rat hepatocytes. Throughout the study silymarin was used as positive control. Molecular mechanisms involved in apoptotic pathways induced in hepatocytes by t-BHP at 250 μM were explored in detail. DNA fragmentation, activation of caspases and cytochrome *c* release were demonstrated. In addition, changes in the mitochondrial membrane potential and ROS generation were detected confirming involvement of mitochondrial pathway. Pre-treatment with GA (4 μg) protected the hepatocytes against t-BHP induced oxidative injury and the results were comparable to the pre-treatment with positive control, i.e. silymarin. The protective potential against cell death was achieved mainly by preventing intracellular GSH depletion, decrease in ROS formation as well as inhibition of mitochondrial membrane depolarization. GA was found to modulate critical end points of oxidative stress induced apoptosis and could be beneficial against liver diseases where oxidative stress is known to play a crucial role.

1. Introduction

Hepatocytes make up 60–65% of the cells in the liver and play a pivotal role in the metabolism of exogenous chemicals and toxins, thus making liver a target for toxic substances. Reactive oxygen and nitrogen species (ROS/RNS) are generated during detoxification which leads to oxidative stress. These free radicals are considered critical molecules as they take part in a variety of cellular functions (Das et al., 2004). Imbalance in the cellular redox status, the increased levels of oxidants overwhelm the capacity of the antioxidant defense network resulting into oxidative stress. Involvement of ROS/RNS in the pathogenesis of certain human diseases, including cancer, diabetes, cataract, neuronal disorders and artherosclerosis is increasingly being recognized. Upsetting this balance causes oxidative stress, which can lead to cell death/injury. Natural antioxidants that can inhibit lipid peroxidation or are able to protect the system from the damage caused by free radicals are being explored for strengthening antioxidant defense. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases, used as a chemopreventive agent in cancers and neurodegenerative disorders (Di Matteo and Esposito, 2003).

Tert-butyl hydroperoxide (t-BHP) is an organic lipid hydroperoxide analogue, used as pro-oxidant to evaluate mechanisms involving oxidative stress in cells and tissues. t-BHP has been shown to induce cell death in a variety of cells via apoptosis including U937 macrophages, PC-12 cells, SH-SY5Y neuroblastoma cells and HepG2 cells (Amoroso et al., 2002; Kanupriya et al., 2007). Two distinctive pathways are involved in the metabolism of t-BHP in hepatocytes (Haidara et al., 2001). The first employs microsomal cytochrome P-450 system leading to the production of ROS such as peroxyl and alkoxyl radicals. These radicals initiate peroxidation of the cell membrane phospholipids and accumulation of lipid peroxides which are expected to alter the membrane fluidity and permeability, consequently leading to disruption of membrane structure and function. The second pathway involves

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; AIF, apoptosis inducing factor; APAF-1, apoptosis protease activating factor-1; CAD, caspases-activated DNase; CMF-DA, 5′-chloromethylfluorescein diacetate; DCF, dichlorofluorescein; $DCFH_2$, dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GA, glycyrrhizic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; JC-1, 5′,5′,6′,6′-tetrachloro-1′,1′,3′,3′-tetraethylbenzamidazolcarbocyanine iodide; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malonyldialdehyde; MFI, mean fluorescence intensity; MPT, mitochondrial permeability transition; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; *p*-NA, *p*-nitro aniline; PARP, poly ADP ribose polymerase; PBS, phosphate buffer saline; PVDF, polyvinylidene fluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, 2′-thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; t-BHP, tert-butyl hydroperoxide; TCA, trichloroacetic acid.

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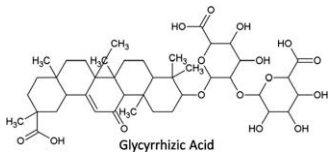
GSH peroxidase and its substrate GSH, which converts t-BHP to t-butanol and GSH to GSSG. The GSSG is then reduced to GSH by GSH reductase, resulting in NADPH oxidation. Decreased GSH and oxidized NADPH contribute to altered Ca^{2+} homeostasis, which is considered to be a major event in t-BHP-induced toxicity (Shimizu et al., 1998). It has been documented that GSH depletion and MDA increase, events observed frequently during oxidative damage, are inducers of mitochondrial permeability transition (MPT). Loss of $\Delta\Psi_m$ is directly associated with apoptosis. Apoptosis permits the removal of damaged, senescent or unwanted cells in the multicellular organisms, without damaging the microcellular environment and can be induced by various extracellular or intracellular factors. It results in several morphological changes which are characteristic feature of apoptosis such as cell shrinkage, membrane blebbing along with biochemical changes like DNA fragmentation, cleavage of wide variety of cellular proteins including PARP and lamin. In apoptosis, the $\Delta\Psi_m$ is lost, releasing small apoptogenic molecule, cytochrome *c*, leading to the formation of apoptosome. Apoptosome is high in molecular weight and consists of cytochrome *c*, dATP, apoptosis protease activating factor-1 (APAF-1) and procaspases-9. Once the signal is received the initiator caspase activates the downstream caspases, like caspase-3. Caspases are synthesized as pro-enzymes, which are cleaved at internal sites to form an active enzyme. These are group of cystein proteases which can be either initiator (caspase-2, -8, -9, -10) or effector (caspase-3, -6, -7).

Exogenous dietary antioxidants capable of scavenging free radicals are of great interest in combating oxidative stress induced cell damage. Plants containing high content of polyphenols, flavanoids are considered as potential antioxidants and can be used as adjuvant therapy. These plant polyphenols and flavanoids are multifunctional and can act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal ion chelators (Gassen and Youdim, 1999). Some hepatoprotective plants as well as formulations used in traditional medicine have been pharmacologically evaluated for their efficacy such as *Andrographis panniculata* (Singh et al., 2001) and *Eclipta alba* (Saxena et al., 1993) to name a few. Amongst all these medicinal plants *Glycyrrhiza glabra* (licorice) is a plant with a rich ethno-botanical history. The roots are used as a folk medicine both in Europe and in Eastern countries. The main components are the triterpene, saponins, glycyrrhizin/glyccyrrhizic acid and glycyrrhetic acid. Glycyrrhizic acid (GA), a biologically active constituent of licorice root with a structure of 20β-carboxy-11-oxo-30-norolean-12en-3-β-yl-2-o-β-D-glucopyranosiduronic acid, is believed to be partly responsible for anti-ulcer, anti-inflammatory, anti-diuretic, anti-epileptic, anti-allergic, anti-dote, anti-tumor, anti-viral, anti-hypotensive and several other properties of the plant (Baltina, 2003). Its hypocholesterolaemic and hypoglycemic activities have been reported (Sitohy et al., 1991).



Different polyphenols, which make up 1–5% of the root of *G. glabra*, have been evaluated for antioxidant and anticarcinogenic properties. GA is a powerful sweetener and 50 times as potent as sucrose. This is also used in herbal teas and in herbal formulations. A nutritive substance with additional properties such as antioxidant and hepatoprotective can be routinely taken as a food supplement. The aim of the present study was therefore to assess the anti-apoptotic property of GA against t-BHP induced oxidative stress in relation with mitochondria-mediated cell death process in primary hepatocytes.

Silymarin, a known hepatoprotectant, was used as a positive control throughout the study. It is known to have clinical applications in the treatment of toxic hepatitis, fatty liver, cirrhosis, ischemic injury, radiation toxicity, and viral hepatitis via its antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, immunomodulating, and liver regenerating effects.

2. Materials and methods

2.1. Test substances and reagents

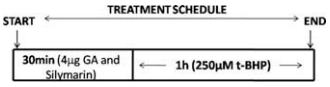
Tert-butyl hydroperoxide (t-BHP) (EC No. 200-915-7; CAS No. B2633-100ML) and glycyrrhizic acid (GA) (EC No. 258-887-7; CAS No. G2137-25G) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Calbiochem. DNA 50bp ladder was procured from Fermentas whereas agarose was obtained from GE. Mouse polyclonal antibody against cytochrome *c* and horse-radish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL kit for detection of western blot was purchased from Amersham Biosciences. All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise mentioned.

2.2. Animals

Sprague–Dawley male rats weighing 180 ± 20 g were taken from Indian Institute of Toxicology Research (IITR) animal colony and used for the experiment. Animals were kept under standard conditions of humidity (60–70%), temperature (25 ± 2 °C) and a controlled 12 h light/dark cycle. Rats were fed Ashirwad pellet diet and water *ad libitum*. All the guidelines of Institutional Animal Ethics Committee (ITRC/IAEC/20/2006) were followed while handling the animals and chloroform was used for euthanasia.

2.3. Primary cell-culture

Hepatocytes were isolated from liver of overnight fasted rat after subjecting it to two-stage collagenase perfusion with HEPES buffer (Seglen, 1976). Cell viability was checked by trypan blue dye exclusion test within an hour of cell isolation. Only preparations with cell viability greater than 95% were used for subsequent experiments. Hepatocytes were maintained in RPMI-1640 media supplemented with heat-inactivated 10% fetal bovine serum and 1% of 10,000 units Penicillin,10 mg Streptomycin and 25 μg Amphotericin-B at 37 °C in a 5% CO_2 −95% air incubator (Thermo-forma) with controlled humidity. The cells were seeded at a density of 1.0×10^4 cells in 0.1% collagen pre-coated 96-well plate, and used for the drug exposure experiments after being cultured overnight. Further treatment schedule was followed as described.



2.4. MTT assay

Cell viability was determined by a colorimetric MTT assay, as described by Mosmann (1983). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase, which demonstrates functional mitochondrial dehydrogenase, i.e. functional mitochondria. Twenty four hours old hepatocytes, were exposed to various concentrations of t-BHP and glycyrrhizic acid. At the end of the incubation period, the culture medium was removed and 0.1 ml of MTT (from a stock of 5 mg/ml) was added to each well. After 4 h incubation the medium was removed and to each well 0.2 ml DMSO was added. Optical density (OD) was measured at 530 nm using a Spectramax PLUS 384 microplate reader (Soft max pro version 5.1; Molecular Devices, USA). The linear relationship between OD and cell density was taken into account. The data are expressed as a percentage of control viability measurement in untreated cells.

2.5. GSH content

5′-Chloromethylfluoroscein diacetate was used to measure the total GSH in the cells. The dye passes freely through the cell membrane, but once inside the cells they are transformed into cell impermeant reaction products. CMF-DA is colourless and nonfluorescent until cytosolic esterases cleave off the acetates, and the bright fluorescence coloured product is formed which is detected by flowcytometer. The treated cells were incubated with the fluorescent dye (5 μg/ml) for 30 min in dark at 37 °C (Okada et al., 1996) and the analysis of treated/ control cells was carried out using flowcytometer (BD-LSR), cell quest software.

2.6. Measurement of intracellular ROS

ROS levels were determined using 2′7′-dichlorofluorescein diacetate (DCFH-DA), which is de-estrified to 2′7′-dichlorodihydrofluorescein ($DCFH_2$) by cellular esterases. This $DCFH_2$ is further oxidized to DCF by ROS and increase in fluorescence intensity is used to quantify the generation of intracellular ROS. Control cells as well as cells with treatment were incubated for 30 min with DCFH-DA (5 μg/ml) at 37 °C in dark. To evaluate ROS mediated oxidation of DCFH-DA to the fluorescent compound DCF, samples were analyzed at an excitation wavelength of 480 nm and an emission wavelength of 525 nm by flowcytometer (BD-LSR). Each determination is based on mean fluorescence intensity of 10,000 events (Mohammad et al., 2001).

2.7. SOD activity

SOD activity was determined spectrophotometrically by measuring inhibition of nicotinamide adenine dinucleotide (reduced)–phenazine methosulfate–nitroblue tetrazolium reaction system by the method of Kakkar in 1984 after adoption on microplate. Superoxide radical is produced *in situ*, which is involved in the NBT reduction leading to the formation of blue formazan, which is read at 560 nm. Fifty percent inhibition of formazan formation in 1 min is taken as 1 unit activity/min (Kakkar et al., 1984).

2.8. MDA determination

Thiobarbituric acid reactive substance (TBARS) formation as a product of lipid peroxidation was estimated in pre-treated hepatocytes by using the method of Wallin in 1993. In this method, oxidation of phospholipids and evaluation of TBARS is achieved in single 96-well microplate. Major oxidative product of phospholipids, i.e. malondialdehyde (MDA) was estimated by measuring the amount of MDA formed as a breakdown product at 530 nm. The lipids were isolated by precipitating the cell lysate with TCA and then indirectly the lipid peroxide concentration was measured with TBA reaction. The amount of MDA formed as a breakdown product was measured at 530 nm and 600 nm (Wallin et al., 1993).

2.9. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in the $\Delta\Psi_m$ in treated hepatocytes were monitored after staining them with JC-1. 5′,5′,6′,6′-Tetrachloro-1′,1′,3′,3′-tetraethylbenzamidazolcarbocyanine iodide commonly known as JC-1 which is specific for mitochondria. In cells not undergoing apoptosis, the $\Delta\Psi_m$ remains intact and the dye accumulates to form an aggregate that gives red fluorescence. In mitochondrial membrane where potential is compromised the formation of JC-1 aggregate is prevented and the fluorescence shifts from red to green. Treated hepatocytes were incubated with JC-1 for 15 min at 37 °C in a CO_2 incubator. After washing the hepatocytes with PBS, change in the $\Delta\Psi_m$ was assessed by comparing the two fluorescence 590 nm (red)/527 nm (green) using flowcytometer (BD-LSR) (Cossarizza et al., 1996).

2.10. Preparation of cytosolic fraction

Cytosolic fractions were prepared by the method described by Zhang et al. (1999) with some modification. Briefly, cells were harvested, washed and suspended in ice-cold buffer containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF and sonicated for 10 s (Sartorius, Labsonic M). The lysate were now centrifuged at 800*g* for 4 min at 4 °C under cold conditions. The supernatant was again centrifuged at 22,000*g* for 15 min at 4 °C in a refrigerated centrifuge (Sigma 3K18) and the resulting supernatant was used as cytosolic fraction.

2.11. Western blot analysis

The protein content corresponding to each treatment was quantified using Lowry's method (Lowry et al., 1951). Samples were boiled with Lammeli's buffer for 5 min and immediately kept in ice. 20 μg of each protein sample was separated by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gel and electroblotted on PVDF membrane (Amersham). After blocking non-specific sites washing was performed using PBS containing tween-20. The membrane was then incubated for 1 h with goat polyclonal IgG antibody in dilution 1:500 (Santa Cruz Biotechnology, Inc.). Then it was washed with PBS and incubated with horse-radish peroxidase-conjugated rabbit anti-goat IgG secondary antibody in dilution 1:1000 at 1 h for room temperature. Again it was rewashed and the immunoblot was revealed using ECL chemiluminescent detection kit according to the manufacturer's instructions. β-Actin was used as internal standard. The bands obtained were analyzed using NIH software Image J version 13.2.

2.12. Caspases activity

To investigate the role of mitochondria in the cytotoxicity caused by t-BHP caspase-3 and -9 activity were measured. Caspase activities were determined by a colorimetric assay based on the ability of caspases-3, -9 to change acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA) and acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-*p*NA) into yellow formazan product (*p*-Nitro aniline), respectively. An increase in absorbance at 405 nm was used to quantify the activation of caspase activities. In brief, isolated hepatocytes were pre-incubated with GA as well as t-BHP for specified time. After incubation, the medium was discarded and adherent cells were harvested in PBS and sedimented by centrifugation at 600g for 3 min. The pellets were then resuspended in lysis buffer for 20–30 min on ice. The lysed cells were centrifuged at 10,000g for 3 min and to the supernatant, reaction mixture and buffer were added. The concentration of the *p*-NA released from the substrate was calculated from the absorbance value at 405 nm using a calibration curve.

2.13. DNA fragmentation assay

DNA was isolated from control and treated hepatocytes. After incubation, cells were washed with PBS and lysed with lysis buffer containing 250 mM Sucrose, 5 mM Tris–HCl (pH 8.0), 1 mM EDTA, 20% SDS and incubated overnight at 37 °C. Subsequently, RNase A was added for 1 h at 37 °C followed by addition of 8 M potassium acetate. The lysate was extracted twice with an equal volume of chloroform/isoamylalcohol (24:1) and centrifuged at 1000g for 5 min. The upper aqueous layer was taken and two volumes of absolute ethanol was added to it and incubated at −20 °C in order to precipitate the DNA. The pellet obtained after centrifugation at 14,000*g* for 15 min was air dried and then dissolved in Tris–EDTA buffer. DNA quantification was done spectrophotometrically at 260/280 nm. DNA samples were finally separated on 1.8% agarose gel with Tris–Borate/EDTA buffer and analyzed on an Alfa-innotech image analyzer (Hermann et al., 1994).

2.14. Analysis of hepatocyte nuclear morphology

Changes in the nuclear morphology were observed using bisbenzimide (Hoechst 33258) fluorochrome that binds with DNA. Primary hepatocyte monolayers were fixed in ice cold methanol/acetic acid (3:1) for 5 min. Cells were stained with Hoechst 33258 (5 μg/ml) for 10 min and washed. Cells were mounted in a solution of 20 mM citric acid, 50 mM di sodium orthophosphate, and 50% glycerol (pH 5.5) and examined at a wavelength of 350–460 nm using a Nikon microscope with fluorescence attachment (Bayly et al., 1994).

2.15. LDH activity-based cytotoxicity assay

LDH (lactate dehydrogenase) was measured using standardized kit from Sigma–Aldrich. LDH activity was measured both in floating dead cells and viable adherent cells. The floating cells were collected from culture medium by centrifugation (240*g*) at 4 °C for 5 min and LDH content from the pellet was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (LDHe; extracellular LDH) was used as an index of necrotic cell death and the LDH present in the adherent viable cells as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\% \text{ Apoptosis} : \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$
$$\% \text{ Necrosis} : \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

2.16. Annexin V-FITC binding assay

Annexin is a 35 to 36 kDa Ca^{2+} dependent, phospholipid binding protein that has high affinity for phosphotidylserine and binds to cells with exposed phosphotidylserine. In the early stages of apoptosis membrane phosphotidylserine is translocated from inner side to outer side of the plasma membrane. The annexin V assay was carried out in conjugation with PI (propidium iodide) staining in order to distinguish between apoptosis and necrosis, because PI staining can detect DNA that has leaked from the necrotic cells. (a) Viable hepatocytes are negative for both annexin V and PI, (b) early apoptotic hepatocytes were labeled with annexin V while negative with PI, (c) late apoptotic cells were labeled with both annexin V and PI, and (d) necrotic cells were labeled with PI but negative with annexin (Liu et al., 2003).

2.17. Statistical analysis

Data are expressed as mean ± S.E. Data were analyzed on SPSS software version 14.0 using one way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were used as the criterion for significance.

3. Results

3.1. GA increased survival of t-BHP stressed primary hepatocytes

The preventive effect of GA was studied on the cytotoxicity of t-BHP, a chemical that generates alkoxyl/peroxyl radicals leading to oxidative stress in the cellular system. Inhibitory concentration (IC_{50}) was determined using MTT cytotoxicity assay. Only 95% viable primary hepatocyte population was used throughout the study as evident from the trypan blue dye exclusion assay (data not shown). It was observed that t-BHP at 250 μM, reduced the cell survival to 52% ± 0.12 ($P < 0.001$), i.e. the inhibitory concentration (IC_{50}) of t-BHP was found to be 250 μM (Fig. 1a). This concentration (250 μM) of t-BHP was used for further experiments in the presence of GA. Isolated primary rat hepatocytes were also treated with varying concentrations of GA (2–12 μg) as shown in Fig. 1b to study its effect on cell viability. At 4 μg of GA the increase in survival was 58.18% ± 0.02 ($P < 0.001$) with respect to control, so for further experiments 4 μg of GA was taken up as the selected dose for treating cells. At the same concentration (4 μg) of silymarin, a positive control, cell survival rate was found to increase by 43.36% ± 0.05 ($P < 0.001$). During the pre-treatment schedule, 24 h cultivated hepatocytes were incubated with GA (4 μg) for 30 min before subjecting them to oxidative stress of t-BHP (250 μM) for an hour. A positive correlation between dose-response in terms of viability was seen. The cells which were pre-incubated with GA showed significant increase in survival by 44.89% ± 0.03 ($P < 0.001$), when compared to control cells whereas cells treated with silymarin showed increase by 38% ± 0.08 ($P < 0.001$) (Fig. 1c).

3.2. Restoration of antioxidant status in stressed rat hepatocytes by GA treatment

3.2.1. GSH content

The fluorescence intensity of CMF dye was captured using flow-cytometer, reflecting GSH content in the cellular system and the results mentioned here are the mean fluorescence intensities (MFI). Cells when stressed with t-BHP (250 μM), showed decrease in GSH content by 0.6-folds ($P < 0.05$). Cells pre-treated with GA at the selected dose (4 μg) were found to restore the GSH content by 1.58-folds ($P < 0.01$) or inhibited the depletion of GSH which was comparable to control cells. Silymarin (4 μg) was also found to inhibit GSH depletion in the stressed cells (1.56-folds) as shown in Table 1. The data indicates that GA treatment is effective in abrogating oxidative damage that further results in GSH depletion by t-BHP.

3.2.2. ROS generation

The extent of ROS generation during t-BHP induced stress in hepatocytes, was monitored by flow cytometry using DCFH-DA dye. Hepatocytes stressed with t-BHP showed an increase in the ROS generation by 2.3-folds as compared to untreated cells. Whereas in the cells that were pre-treated with GA 0.51-folds decline in the ROS generation was observed as compared to t-BHP stressed cells. The decline in ROS generation (Table 1) in pre-treated cells was very much comparable with silymarin, i.e. 0.5-folds (positive control).

3.2.3. SOD activity

Cultured hepatocytes subjected to t-BHP stress showed SOD activity 4.6 units/min/10^4 cells which was 0.7-folds less than the

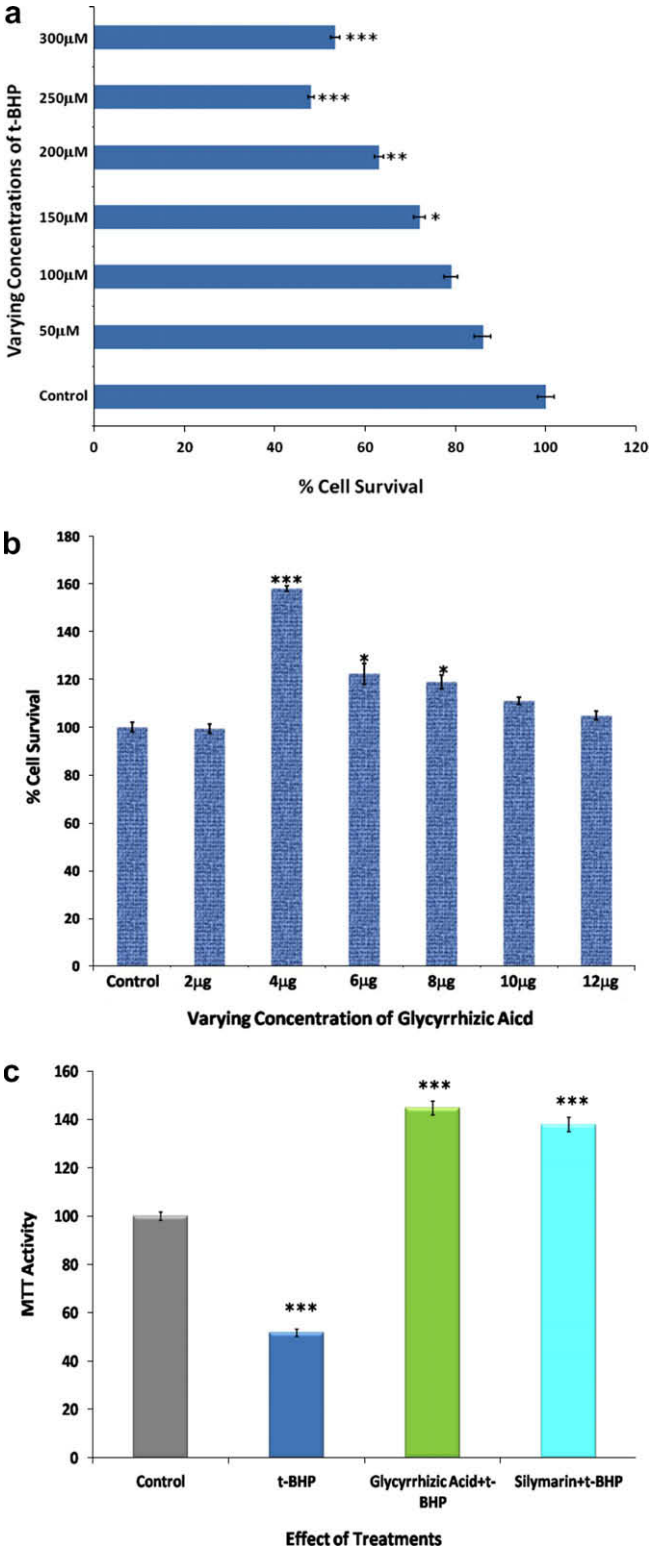


Fig. 1. Effect of treatments on the viability of cultured primary rat hepatocytes: (a, b) Different concentrations of (50–300 μM) t-BHP and glycyrrhizic acid (2–12 μg) were administered to primary rat hepatocytes (1×10^4 cells/well of collagen coated 96-well cell-culture plate in 100 μl of assay medium) for 1 h and 30 min, respectively. At 250 μM concentration of t-BHP the viability of cells decreased to 51%. Viability of hepatocytes was maximum in the presence of 4 μg GA. (c) Hepatocytes were pre-treated with GA (4 μg) and silymarin (4 μg), respectively, for 30 min and then were treated with t-BHP (250 μM) for 1 h. Values are mean ± S.E. of 5 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1
Antioxidant status and ROS generation under oxidative stress in hepatocytes: hepatocytes were treated with the selected concentration of t-BHP (250 μM); glycyrrhizic acid (4 μg); silymarin (4 μg); glycyrrhizic acid + t-BHP and silymarin + t-BHP. Total cellular GSH content and intracellular ROS generation was measured by CMF-DA and DCFH-DA fluoroprobes, respectively, using flow cytometry whereas antioxidant potential (SOD activity and lipid peroxidation) was measured using biochemical assays. Cells treated with glycyrrhizic acid and silymarin alone were compared to control cells, whereas cells pre-treated with GA and silymarin followed by t-BHP treatment were compared to cells treated with t-BHP alone. Values are mean ± S.E. of 5 determinations in each case.

Treatments	ROS (DCF mean fluorescence intensity)	GSH content (CMF mean fluorescence intensity)	SOD activity (unit/min/10^4 cells)	Lipid peroxidation (nMMDA formation/10^4 cells)
Control	100.8 ± 1.7	228.0 ± 3.1	12.4 ± 0.64	0.23 ± 0.02
t-BHP	232.9 ± 4.9**	139.7 ± 2.6*	4.6 ± 0.91***	0.41 ± 0.04**
Glycyrrhizic acid	82.0 ± 1.4**	310.0 ± 4.9***	20.4 ± 0.63**	0.14 ± 0.05**
Silymarin	94.5 ± 1.6*	340.0 ± 3.1***	21.3 ± 0.44**	0.11 ± 0.01**
Glycyrrhizic acid + t-BHP	119.3 ± 1.9***	221.0 ± 5.1**	15.6 ± 0.24***	0.19 ± 0.04***
Silymarin + t-BHP	119.8 ± 1.5***	219.0 ± 4.5**	18.0 ± 0.92***	0.15 ± 0.01***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

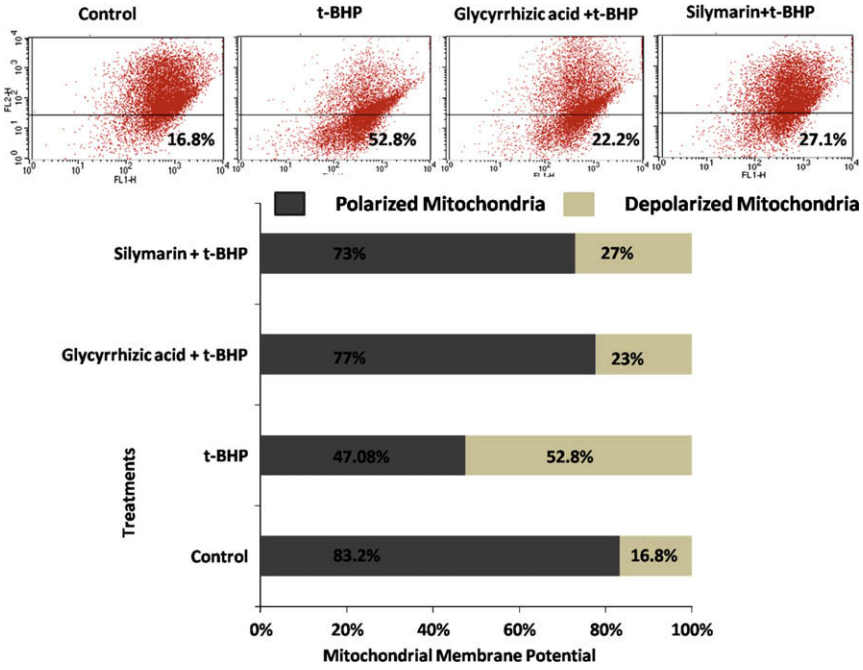


Fig. 2. Mitochondrial membrane potential: changes in mitochondrial membrane potential ($\Delta\Psi_m$) observed using mitochondria specific fluorescent probe JC-1. Monomer green fluorescence increased as MMP dropped. Graph indicates percentage mitochondrial population differentiated by flow cytometer, whereas, in dotplot, quadrant shows the two populations having green fluorescence (depolarized mitochondria) and red fluorescence (polarized mitochondria). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

untreated cells. Cells that were pre-incubated with GA (4 μg) showed 3.4-folds increase in superoxide dismutase activity (table 1) whereas cells pre-incubated with silymarin at the same concentration showed, 3.91-folds increase in superoxide quenching capacity. Thus, effect of GA compared well with a known hepatoprotectant, i.e. silymarin.

3.2.4. LPO inhibitory potential

Oxidative stress induced in the hepatocytes by free radical generation due to t-BHP caused 0.71 ± 0.005 ($P < 0.01$) nM MDA formation per 1.0×10^4 cells. In hepatocytes pre-treated with GA, the peroxidative decomposition of phospholipids was reduced to 0.198 ± 0.036/4 μg ($P < 0.001$). Treatment with silymarin reduced MDA formation to 0.15 ± 0.02/4 μg ($P < 0.001$), indicating strong antioxidant action of both GA and silymarin (Table 1).

3.3. t-BHP induced loss of $\Delta\Psi_m$ which is recovered by GA treatment

Generation of ROS and alteration in mitochondrial functions are well correlated by Kakkar and Singh (2007). Disruption of the mitochondrial membrane potential is one of the earliest indicator of induction of cellular damage. Under experimental conditions if the hepatocytes are not undergoing apoptosis, the mitochondrial membrane remains polarized ($\Delta\Psi_m$) and JC-1 dye gets accumulated and j-aggregates are formed due to which red fluorescence occurs. When the mitochondrial $\Delta\Psi_m$ is lowered the JC-1 aggregate dissipates into monomers and lead to shift from red to green fluorescence captured on flow cytometer. Cultivated hepatocytes when treated with 250 μM of t-BHP showed 52.80% green fluorescence as compared to control or untreated cells with 16.80% green fluorescence. In the cells pre-treated with GA, there was considerable abolition of t-BHP

induced lowering in $\Delta\Psi_m$ and the hepatocytes containing mitochondria with green fluorescence were found to be only 22.22%. GA alone did not alter $\Delta\Psi_m$, confirming the protective effect of GA pre-treatment. Hence, the results indicate that mitochondrial membrane is depolarized when treated with t-BHP and GA accords protection by preventing the mitochondrial depolarization (Fig. 2).

3.4. GA reduced cytochrome c released by t-BHP stress

Cytochrome *c* is located between the inter-membrane spaces of mitochondria where it assists in the production of life sustaining ATP by participating in electron transport. Several studies have shown that release of cytochrome *c* from mitochondria is associated with opening of mitochondrial permeability transition pores (Kroemer et al., 2007). Following the exposure of cells to apoptosis stimuli, cytochrome *c* is rapidly released from the mitochondria to cytosol which further activates cell death proteases (caspases). Thus, release of cytochrome *c* from mitochondria to cytosol is a trigger in the induction of apoptosis. Level of cytochrome *c* release was not so significant in the cytosolic fraction of cells treated with GA and silymarin (Fig. 3a). When stressed cells were analyzed there was an increase in the level of cytochrome *c* release by 4.75-folds as compared to unstressed cells, whereas GA pre-treated cells were observed the level decreased to 0.51-folds. The decrease in the cytochrome *c* level of cells pre-treated with GA was comparable to the positive control, i.e. 0.49-folds.

3.5. GA treatment lowered caspase-activation

To investigate the involvement of different caspases in t-BHP-induced apoptosis, we focused on initiator caspase-9, in the light of prior information that t-BHP induced pro-apoptotic events at

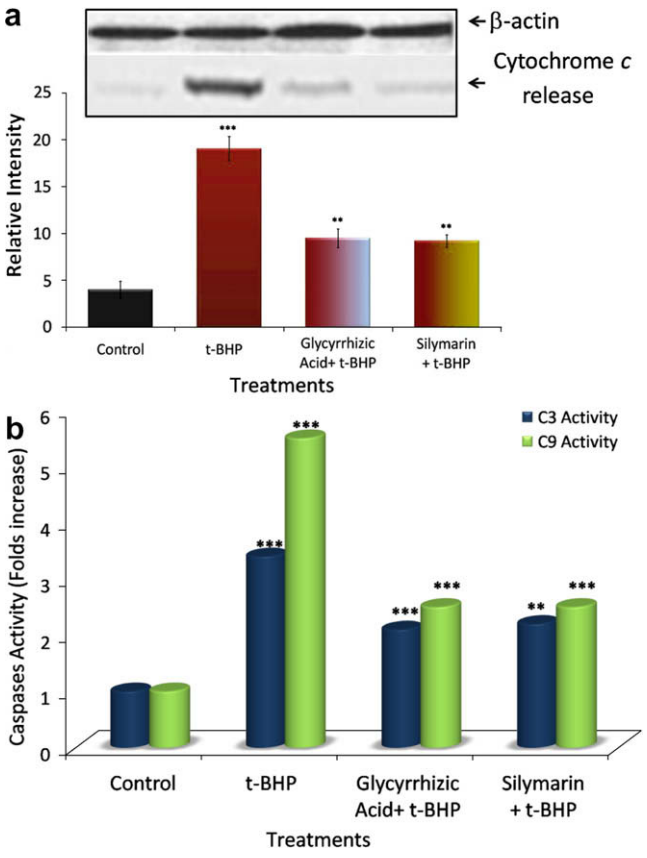


Fig. 3. Effect of treatments on cytochrome *c* release and caspases activation: (a) cytochrome *c* release was assessed from oxidatively stressed hepatocytes which were well protected by pre-treatment of glycyrrhizic acid and silymarin. The sequence of samples in cytochrome *c* blot is (from left to right) control, t-BHP, glycyrrhizic acid + t-BHP and silymarin + t-BHP. β-Actin was used as internal control. Values are mean ± S.E. of 3 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (b) Caspase-3 and Caspase-9 activity in primary hepatocytes exposed to t-BHP, glycyrrhizic acid + t-BHP and silymarin + t-BHP. Values are mean ± S.E. of 5 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

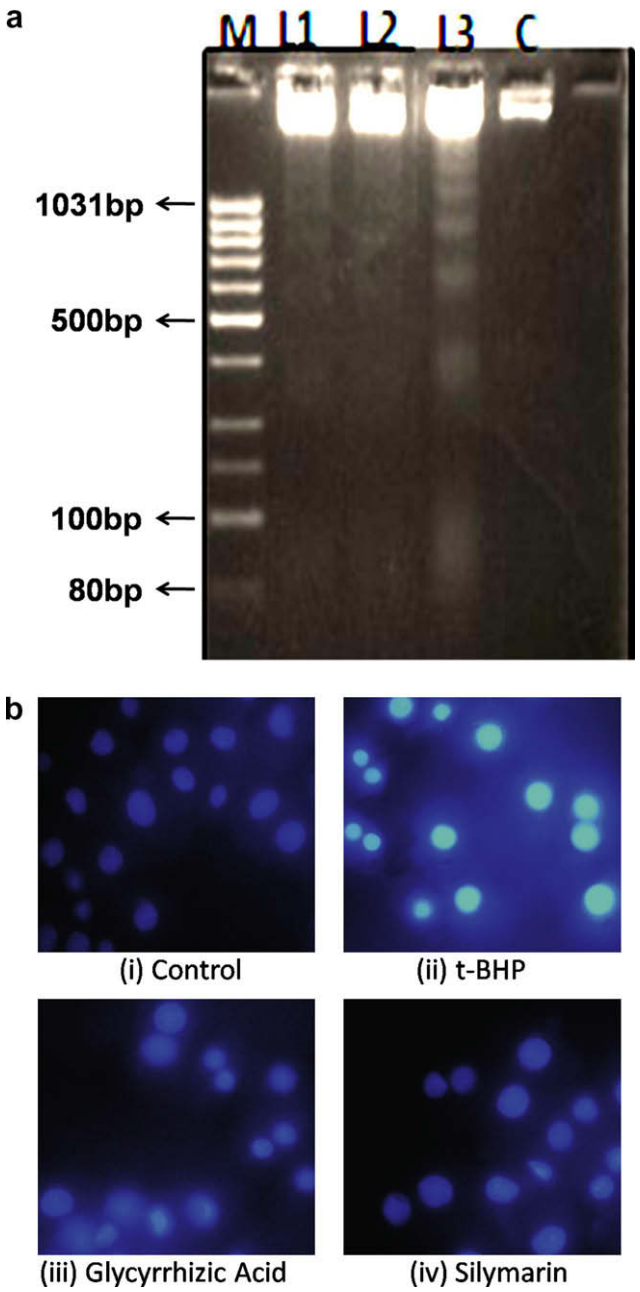


Fig. 4. DNA damage: (a) glycyrrhizic acid inhibits t-BHP-induced apoptosis in primary rat hepatocytes. Cells were incubated with 250 μM t-BHP and 4 μg of glycyrrhizic acid DNA fragmentation was measured by agarose gel electrophoresis (M – 100bp marker, L1 – glycyrrhizic acid pre-treated, L2 – silymarin pre-treated and C – control.). (b) Glycyrrhizic acid inhibits t-BHP-induced apoptosis in primary rat hepatocytes. Cells were incubated with 250 μM t-BHP and 4 μg of glycyrrhizic acid and nuclear morphology was assessed using Hoechst 33258. (i) Control hepatocytes; (ii) cells treated with 250 μM of t-BHP for an hour; (iii) Cells pre-treated with glycyrrhizic acid; (iv) cells pre-treated with silymarin.

the mitochondrial level (Haidara et al., 2001). Caspase-3 is also an important effector protease, activated by cleavage as a step in certain apoptosis-signaling pathway. t-BHP caused significant increase in caspase-3 and caspase-9 activity which was 3.4- and 5.5- ($P < 0.001$) folds higher when compared to control. These enzyme activities were found to decrease significantly when GA and silymarin treated hepatocytes were assessed, i.e. with the pre-treatment of GA, caspase-3 and -9 activity was found to be lowered by 2.6- and 2.2-folds, respectively. Pre-treatment with silymarin showed results comparable with GA, i.e. 2.5- and 2.2-folds lowering in activity, respectively (Fig. 3b).

3.6. t-BHP caused apoptosis in hepatocytes which is prevented by GA

Morphological assessment of rat hepatocytes was done using two methods: DNA fragmentation and condensation of nuclear chromatin to assess the cell damage. DNA fragmentation, is an important hallmark for apoptosis, where DNA is degraded by caspases-activated DNase (CAD), a nuclease enzyme showing characteristic ladder pattern on agarose gel. No fragmentation was observed in control cells. Ladder pattern was observed when cells were treated with 250 μM of t-BHP (Fig. 4a). On pre-treatment of cells with GA no such pattern was observed.

Nuclear chromatin condensation is a morphological assessment, which is characteristic of apoptosis and can be visualized using Hoechst 33258. Condensation of nuclear chromatin was found in the cells treated with t-BHP. This was observed by an increase in fluorescence intensity as compared to untreated cells. Cells which were pre-treated with GA showed low intensity of fluorescence as compared to stressed cells. This indicates that apoptosis is involved in the molecular mechanism of action of t-BHP induced toxicity in rat hepatocytes which can be prevented by glycyrrhizic acid (Fig. 4b).

3.7. LDH release in cells treated with t-BHP is prevented by GA

Cell death through oxidative stress may be accomplished by two distinct mechanisms, necrosis or apoptosis. To further characterize the possible mechanism involved in t-BHP induced cell death and efficacy of GA as protective agent, the ratios of necrosis and apoptosis in primary hepatocytes was analyzed using LDH activity-based assay. Intracellular LDH release was evaluated as a result of the breakdown of plasma membrane and alteration of its permeability. The cells were exposed to t-BHP for 1 h -and LDH leakage was taken as the cell death indicator. As shown in Fig. 5a, LDH leakage increased significantly in the presence of 250 μM t-BHP and showed 51.04% ($P < 0.001$) apoptotic and 29.68% ($P < 0.001$) necrotic cells indicating apoptosis as a predominant mechanism responsible for cell death. Cells pre-incubated with GA showed decrease in the number of apoptotic (20.23%) ($P < 0.001$) as well as necrotic cells (10.68%) ($P < 0.001$) whereas silymarin decreased the number of apoptotic cells by (28.99%) ($P < 0.01$) and necrotic (18.99%) ($P < 0.01$). Results indicate that 4 μg of GA/10^4 cells showed significant decrease in the number of apoptotic and necrotic cells induced by t-BHP.

3.8. Annexin V/PI staining and flow cytometric analysis

t-BHP treated cells were monitored for expression of phosphotidylserine in the outer cell membrane, and percentage of viable, early and late apoptotic and necrotic population was assessed as shown in Fig. 5b. From the dot plots (divided in four quadrants), viable hepatocytes were negative for both annexin V and PI (lower left quadrant). The cells that were annexin V positive and PI negative represent the population in early apoptosis (upper left quadrant). The cells that showed annexin V positive and PI positive represent population with late apoptosis (upper right quadrant) and the cells that were negative for annexin and positive for PI are shown in lower right quadrant. The viable hepatocytes in oxidatively challenged cells were 59.66% that was 35% less than untreated cells. Whereas when GA and silymarin, pre-treated cells were taken into consideration the viability observed was 89.16% and 87.78%. The number of apoptotic population also decreased from 42% (t-BHP treated) to 7.45% and 8.71%, respectively, in GA and silymarin pre-treated cells.

4. Discussion

In this study, we demonstrated increase in ROS generation, GSH depletion, increased MDA formation and apoptosis initiated by mitochondria in t-BHP treated rat primary hepatocytes. Our results collectively indicate that t-BHP induced apoptosis is dependent on ROS production as well as GSH depletion. Involvement of mitochondrial pathway in t-BHP induced apoptosis was also investigated by measuring caspases-3, caspase-9 and cytochrome *c* release along with DNA fragmentation and chromatin condensation. Rat hepatocytes, treated with t-BHP caused DNA fragmentation (laddering) confirming the presence of apoptotic phenomenon. Cytochrome *c* release was detected in the cytosol after 1h treatment of t-BHP (250 μM) in rat hepatocytes. This corresponds to an early response in apoptosis. The upstream caspase-9 was activated in t-BHP induced apoptosis. These finding demonstrate that t-BHP activates the mitochondrial pathway in hepatocytes.

Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living system. GA exhibits a number of pharmacological effects including anti-inflammatory and is used in hepatoprotective formulations. Pre-treatment with GA has been reported to show protective action against carbon tetrachloride (CCl_4)-induced liver injury in rats (Wang and Han, 1993). GA protects against aflatoxin-induced oxidative stress (Chan et al., 2003). GA is also a potent inhibitor of bile acid-induced apoptosis and necrosis (Gumpricht et al., 2005).The results of the present study suggest that GA is capable of ameliorating hepatocyte lipid peroxidation caused by t-BHP. It is well established that intracellular GSH, the most important biomolecule protecting against chemically induced oxidative stress, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction. These metabolic pathways could increase cellular reactive metabolites, which may attack membrane phospholipids, proteins, and nucleic acids. Thus, antioxidants which can inhibit free radical generation are important in terms of protecting the liver from chemical-induced damage by stabilizing antioxidant systems in the cell. The present study showed that treatment with t-BHP stimulates ROS overproduction, mitochondrial membrane depolarization (loss of $\Delta\Psi_m$), which in turn activates caspase cascade and cause reduction in the level of GSH. The data further showed that cells treated with GA displayed a reduction of t-BHP-induced ROS generation. Moreover, GSH depletion was inhibited when the cells were pre-incubated with GA. The results strongly suggest that the protection accorded by GA against t-BHP-induced hepatotoxicity might be related to its ability to reduce oxidative stress.

In summary, the present study indicates the protective effect of GA on cytotoxicity induced by t-BHP in cultured hepatocytes. t-BHP toxicity is associated with depletion of GSH levels, elevated ROS level, lipid peroxidation, disruption of intracellular antioxidant defense, depolarization of mitochondrial membrane, DNA fragmentation, chromatin condensation as well as release

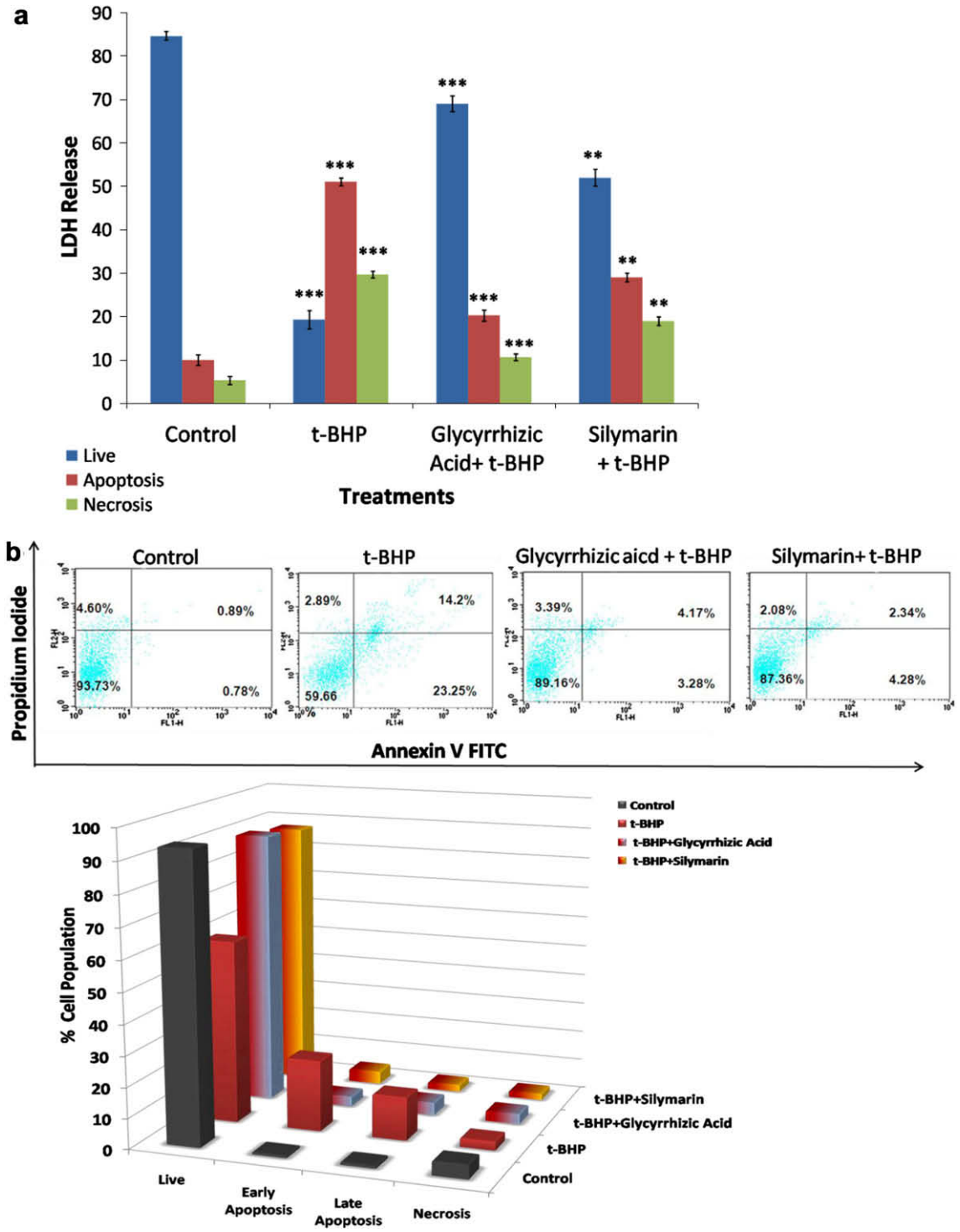


Fig. 5. (a) Effect of glycyrrhizic acid on t-BHP induced cell death: monolayer cultures of rat hepatocytes were exposed to t-BHP (250 μM) and 4 μg of Glycyrrhizic acid (37 °C; in cell-culture medium). The occurrence of apoptosis was assessed by the release of lactate dehydrogenase (LDH). Values are mean ± S.E. of 5 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (b) Apoptosis determination: Dual-parameter flow cytograms of FITC-labeled annexin V vs. PI staining of the control as well as treated cells. Viable hepatocytes were negative for both annexin V and PI (lower left quadrant); early apoptotic hepatocytes were labeled by annexin V, while negative for PI (upper left quadrant); late apoptotic hepatocytes were positive for both annexin V and PI (upper right quadrant); necrotic hepatocytes were labeled by PI, while negative for annexin V (lower right quadrant).

of cytochrome *c* and caspase-activation as evident from this study. Pre-treatment of GA protected against all these alterations induced by t-BHP. Quenching the radical species could be one of the mechanisms involved in its protective action. This effect was comparable to silymarin, which was used as a positive control. The present findings suggest that GA may be used as a natural antioxidant to protect against oxidative cellular damage caused by toxic chemicals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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