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# Hepatoprotective properties of kombucha tea against TBHP-induced oxidative stress via suppression of mitochondria dependent apoptosis

Semantee Bhattacharya [a], Ratan Gachhui [a], Parames C. Sil [b,*]

[a] *Department of Life Sciences & Biotechnology, Jadavpur University, 188 Raja S C Mullick Road, Kolkata 700032, India*
[b] *Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M, Kolkata 700054, West Bengal, India*

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**Abstract**

Kombucha, a fermented tea (KT) is claimed to possess many beneficial properties. Recent studies have suggested that KT prevents paracetamol and carbon tetrachloride-induced hepatotoxicity. We investigated the beneficial role of KT was against tertiary butyl hydroperoxide (TBHP) induced cytotoxicity and cell death in murine hepatocytes. TBHP is a well known reactive oxygen species (ROS) inducer, and it induces oxidative stress in organ pathophysiology. In our experiments, TBHP caused a reduction in cell viability, enhanced the membrane leakage and disturbed the intra-cellular antioxidant machineries while simultaneous treatment of the cells with KT and this ROS inducer maintained membrane integrity and prevented the alterations in the cellular antioxidant status. These findings led us to explore the detailed molecular mechanisms involved in the protective effect of KT. TBHP introduced apoptosis as the primary phenomena of cell death as evidenced by flow cytometric analyses. In addition, ROS generation, changes in the mitochondrial membrane potential, cytochrome *c* release, activation of caspases (3 and 9) and Apaf-1 were detected confirming involvement of mitochondrial pathway in this pathophysiology. Simultaneous treatment of KT with TBHP, on the other hand, protected the cells against oxidative injury and maintained their normal physiology.

In conclusion, KT was found to modulate the oxidative stress induced apoptosis in murine hepatocytes probably due to its antioxidant activity and functioning via mitochondria dependent pathways and could be beneficial against liver diseases, where oxidative stress is known to play a crucial role.


*Keywords:* Tertiary butyl hydroperoxide; Reactive oxygen species; Murine hepatocytes; Cyto-toxicity and apoptosis; Kombucha tea; Antioxidant and cytoprotection

## 1. Introduction

Kombucha, a fermented tea (KT) is generated by a consortium of yeast and bacteria. A jelly-like membrane floats in the nutrient solution of tea and sugar exposed to oxygen. At the right temperature, it multiplies continuously. At first it spreads over the entire surface of the tea, and then thickens. KT was subcultured every 7–14 days by mixing 10% of old soup with 10% sucrose dissolved in brewed black tea [1]. KT contains many different cultures along with several organic acids, active enzymes, amino acids, polyphenols, and other compounds formed during the fermentation [2]. This tea is claimed to have many beneficial effects as it possesses antioxidant and anti-microbial activities [3,4]. Recent studies have suggested that KT prevents paracetamol and carbon tetrachloride-induced hepatotoxicity [5,6] and chromate (VI)-induced oxidative stress in albino rats [7].

Although therapeutic benefits have been attributed to this tea, some side effects of KT have also been reported. Srinivasan et al. [8] reported some complications in four patients consuming KT. However, they could not explain the

*Abbreviations:* KT, kombucha tea; TBHP, tertiary butyl hydroperoxide; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethyl sulphoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing/antioxidant power; MDA, malonaldehyde; NADH, nicotinamide adenine dinucleotide reduced disodium salt; NBT, nitro blue tetrazolium chloride; PMT, phenazine methosulphate; ROS, reactive oxygen species; $NaN_3$, sodium azide; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid.

* Corresponding author. Tel.: +91 33 2569 3243; fax: +91 33 2355 3886.
*E-mail addresses:* parames@bosemain.boseinst.ac.in, parames_95@yahoo.co.in (P.C. Sil).

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mechanism of its side effects as they were not sure about whether the complications were because of KT or any contaminant present in its home-brewed version. Moreover, the FDA, surveying commercial producers, has found no pathogenic bacteria or hygiene violations [9] in KT.

TBHP is an organic hydroperoxidant that can be metabolized to free radical intermediates and by exposing primary cultures of murine hepatocytes to TBHP it is possible to mimic several aspects related to liver pathology characterized by increased lipid peroxidation and cytotoxicity due to oxidative stress [10]. TBHP has been shown to induce cell death in a variety of cells via apoptosis [11,12]. Decreased GSH and increased MDA level contribute to the loss of mitochondrial membrane potential which is directly associated with apoptosis.

Although the hepatoprotective property of KT has been studied by a number of researchers [5,6], the mode of its protective action in TBHP-induced pathophysiology is yet to be studied. In the present study we aimed to investigate the beneficial role of KT against TBHP induced oxidative damage and cell death in mouse hepatocytes. The molecular mechanisms underlying the protective action of KT were also assessed by evaluating mitochondrial membrane potential, alterations of the Bcl-2 family proteins, cytosolic cytochrome *c*, activities of Apaf-1, caspase-9 and caspase-3.

## 2. Materials and methods

### 2.1. Animals

Male adult albino mice of Swiss strain, weighing between 20 and 25 g were acclimatized under laboratory condition for a fortnight before starting experiments. The animals were maintained on a standard diet and water ad libitum. They were housed in polypropylene cages and exposed to 10–12 h of daylight under standard conditions of temperature (30 °C) and humidity (50%). All studies were performed in conformity with the Guidance for Care and Standard Experimental Animals Study Ethical Protocols.

### 2.2. Chemicals

Black tea was purchased from market. Collagenase type I, Dulbecco's modified eagle's medium (DMEM), fetal bovine sera (FBS), bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Folin-Ciocalteu reagent, gallic acid, quercetin, aluminium chloride, potassium acetate, acetic acid, gluconic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), isobutyl methyl ketone (IBMK), nicotinamide adenine dinucleotide reduced (NADH), nitro blue tetrazolium (NBT), phenazine methosulphate (PMT), potassium dihydrogen phosphate ($KH_2PO_4$), sodium dihydrogen phosphate ($NaH_2PO_4$), tertiary butyl hydrogen peroxide (TBHP), sodium pyrophosphate, trichloro acetic acid (TCA), thiobarbituric acid (TBA), Tris buffer, L-ascorbic acid, butanol, ethanol and other reagents were bought from Sisco Research Laboratory, India. The antibodies were purchased from Sigma–Aldrich Chemical Co.

### 2.3. Preparation of kombucha tea

Eight grams of black tea was added to 300 ml water and allowed to boil for 5 min. Then it was filtered through a sterile sieve and cooled to room temperature. It was then adjusted to 1600 ml with water. Ten percent (w/v) sucrose was added to it. The cooled tea was poured into 3 l glass beaker that has been previously sterilized at 121 °C for 20 min. It was then inoculated with freshly grown kombucha mat that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth. The beaker was covered with clean cheese cloth and fixed with rubber bands. The fermentation was carried out under room temperature (25 °C) for 21 days. New kombucha mat developed over the mother culture. Sampling was performed periodically (for days 3, 5, 7, 10, 14, 17 and 21) for determination of total phenolic compounds and flavonoids. The fermented tea was centrifuged at 10,000 rpm for 15 min and the supernatant was taken for the analysis. Finally the kombucha tea fermented for 14 days was selected and neutralized (pH 6.9). This was then lyophilized to powdered form and used further for the investigation against TBHP induced cytotoxicity of murine hepatocytes.

### 2.4. Determination of total phenolic compounds and flavonoids

The total phenolic compounds were measured by Folin-Ciocalteu method [13]. 100 μl of kombucha tea was mixed with 0.2 ml of Folin-Ciocalteu reagent, 2 ml of purified water and 1 ml of 15% $Na_2CO_3$. The mixture was incubated for 2 h at room temperature (25 °C) and then the absorbance was measured at 765 nm. Gallic acid was used as a standard and the total phenolic compounds were expressed as gallic acid equivalents (GAE).

Flavonoid content was determined by the following calorimetric method [14]. 0.5 ml solution of KT was mixed with 1.5 ml methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and incubated at room temperature (25 °C) for 30 min. The absorbance of the reaction mixture was measured at 415 nm. Quercetin was used as standard and the flavonoid content was expressed as quercetin equivalents.

### 2.5. Composition of kombucha tea

#### 2.5.1. Analysis of organic acids

The major organic acids were determined by high performance liquid chromatography (HPLC). The tea broth samples were filtered through 0.22 μm sterile microfilter and 20 μl of filtrate was injected into the HPLC system. Phenomenex

Luna C-18 column (4.6 mm ID × 25 cm, 5 μm) was used for the analysis. The mobile phase was 20 mM potassium dihydrogen phosphate, pH 2.4 with a flow rate of 1.0 ml/min and running time of 40 min. The column temperature was maintained at 28 °C and the detection was carried out at 220 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of each compound. The concentrations of organic acids were quantified from standard curves.

#### 2.5.2. Analysis of D-saccharic acid-1,4 lactone

DSL was determined in KT by HPLC analysis using a $C_{18}$ column (8 nm × 10 cm). The column was eluted with a mobile phase of 40 mmol/l borax buffer ($Na_2B_4O_7$–$KH_2PO_4$), pH 6.5 and 15% methanol (v/v) at a flow rate of 1 ml/min and the elution profile was monitored at 190 nm. Standard DSL was run under the same conditions and its elution pattern was compared with that of KT.

#### 2.5.3. Analysis of black tea polyphenols

The main black tea polyphenols theaflavin and thearubigins were estimated in fermented tea broth by the method of Takeo and Oosawa [15] as modified by Ramaswamy [16] and Thanaraj and Seshadri [17]. Twenty five milliliters of fermented tea broth was extracted with 25 ml of isobutyl methyl ketone (IBMK) at room temperature. After phase separation, 1 ml of upper IBMK phase was mixed with 9 ml of 45% ethanol and absorbance was measured at 380 nm (A) and the aqueous phase was retained for thearubigins estimation. Ten milliliters of IBMK phase was extracted with 10 ml of 2.5% disodium hydrogen phosphate. After extraction and phase separation, 1 ml of IBMK phase was mixed with 9 ml of 45% ethanol and absorbance was measured at 380 nm (B). Ten milliliters of aqueous phase from the first step was extracted with 10 ml of n-butanol. After phase separation, 1 ml of n-butanol layer was mixed with 9 ml of 45% ethanol and absorbance was measured at 380 nm (C). Concentration of theaflavin and thearubigin was calculated from the absorbance values as given below:

$$\text{TF} \quad (\%) = 4.313 \times \text{B}$$

$$\text{TR} \quad (\%) = 13.643 \quad (\text{A} + \text{C} - \text{B})$$

Multiplication factors for calculation of TF and TR were derived from molar extinction coefficients of pure compounds and dilution factors [18].

### 2.6. Antioxidant activity of kombucha tea in cell free system

#### 2.6.1. DPPH radical quenching activity

The antioxidant activity of KT was measured using the DPPH radical as described by Blois [19]. Two milliliters of DPPH solutions (125 μM) in methanol and 2 ml of tested samples with different concentrations (2, 5, 10, 20, 30, 40, 50, 60, 70 and 80 μg) of KT were mixed in the tubes. The solution was shaken and incubated at 37 °C for 30 min in dark. The decrease in absorbance at 517 nm was measured against methanol blank using a UV/visible spectrophotometer. Percent inhibition was calculated by comparing the absorbance values of control and the sample:

$$\text{Percentage inhibition} = \frac{A_1 - A_2}{A_1} \times 100$$

$A_1$ is the absorbance of the blank and $A_2$ is the absorbance in the presence of KT.

#### 2.6.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of KT has been investigated following the method of Nash [20] using the same concentrations of KT as mentioned above. In vitro hydroxyl radicals were generated by $Fe^{3+}$/ascorbic acid system. The detection of hydroxyl radicals was carried out by measuring the amount of formaldehyde produced from the oxidation of dimethyl sulphoxide (DMSO). The formaldehyde produced was detected spectrophotometrically at 412 nm.

#### 2.6.3. Superoxide radical scavenging activity

The superoxide radical scavenging activity was measured following the method of Siddhuraju and Becker [21]. The reaction mixture contained 0.1 M phosphate buffer, pH 7.4, 150 μM nitroblue tetrazolium (NBT), 60 μM phenazine methosulphate (PMT), 468 μM NADH and different concentrations of KT (as mentioned earlier). The mixture was incubated in the dark for 10 min at 25 °C and the absorbance was read at 560 nm. Results were expressed as percentage inhibition of the superoxide radicals.

### 2.7. Hepatocyte isolation

For the present study, hepatocytes were aseptically isolated from mice livers following the modified method as described previously by Sarkar and Sil [22]. Briefly, the livers were isolated under aseptic conditions, placed in phosphate buffer saline, irrigated in buffer A (10 mM HEPES, 3 mM KCl, 130 mM NaCl, 1 mM $NaH_2PO_4$–$H_2O$ and 10 mM glucose, pH 7.4) and then incubated in buffer B (5 mM $CaCl_2$, 0.03% collagenase type I) for about 45 min at 37 °C. The tissue was passed through wide bore syringe and 80 μm decron mesh respectively. The dissociated cells were centrifuged at 500 × *g* and the pellet was suspended in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum and 5 μg/ml insulin, 5 μM hydrocortisone, 100 U/ml penicillin and 100 μg/ml streptomycin. The suspension was adjusted to obtain ~$2 \times 10^6$ cells/ml.

### 2.8. Determination of time and dose-dependent effect of TBHP by FRAP assay

Time and dose-dependent effect of TBHP was determined by FRAP assay [23]. Briefly, for dose-dependent study,

eight different sets of hepatocytes, each containing about $2 \times 10^6$ cells were incubated with eight different doses of TBHP (50, 100, 200, 300, 400, 500, 600 and 700 μM) for 180 min to determine the maximum damage caused by TBHP treatment. For time-dependent study, six different sets of hepatocytes (1 ml cell suspension $\sim 2 \times 10^6$ in each) were exposed to TBHP (500 μM) for different times (30 min, 1 h, 1.5 h, 2 h, 2.5 h and 3 h). Briefly, after performing the experiments (as described in Section 2.10) 50 μl of hepatocytes (normal as well as experimental cells) suspension was added to 1.5 ml freshly prepared and pre-warmed (37 °C) FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1) and incubated at 37 °C for 10 min. The absorbance of the sample was read against reagent blank (1.5 ml FRAP reagent + 50 μl distilled water) at 593 nm.

### 2.9. Assessment of dose and time dependent activity of KT by FRAP assay

To determine the optimum dose of KT needed for the cytoprotection against TBHP-induced cyto-pathophysiology, six different sets of hepatocytes, each containing about $2 \times 10^6$ cells were exposed to 500 μM TBHP along with KT (10, 20, 40, 60, 80 and 100 μg) for 2 h. For time-dependent study, six different sets of hepatocytes (1 ml cell suspension $\sim 2 \times 10^6$ in each) were incubated with KT (60 μg) for different times (30 min, 1 h, 1.5 h, 2 h, 2.5 h and 3 h). The FRAP level was then determined in both the normal and experimental cells as described above.

### 2.10. Experimental setup

Based on the results of the dose and time dependent effects of both TBHP and KT, we designed the in vitro experiments with different sets of hepatocytes containing 1 ml suspension ($\sim 2 \times 10^6$ cells) in each. Cells were incubated in a $CO_2$ incubator at 37 °C throughout the experiment with gentle shaking. The hepatocytes kept in culture medium only, served as normal control (marked as "Cont"). Hepatocytes ($\sim 2 \times 10^6$ cells) incubated with KT (60 μg) alone for 2 h served as a group showing the effect of KT on hepatocytes (marked as "KT"). Hepatocytes ($\sim 2 \times 10^6$ cells) incubated with 500 μM TBHP for 2 h served as toxin control (marked as "TBHP"). The combined effect of KT and TBHP was studied by incubating the cells with these agents together for 2 h (marked as "TBHP + KT"). Positive control cells were prepared by incubating hepatocytes with vitamin C (100 μg) instead of KT (marked as "TBHP + VitC"). For all the subsequent experiments (results, figures, tables, etc.) the above notations would be used if not mentioned otherwise.

### 2.11. Determination of cell viability by MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of viable cells to reduce MTT from a yellow water-soluble dye to a dark blue insoluble formazan product. After appropriate experimental procedure as described earlier, the resulting cells were rinsed and incubated with 0.5% FBS–DMEM medium containing MTT (5 mg/ml) for 4 h. At the end of the incubation period the medium was removed and the converted dye was solublized with DMSO (0.3 ml). Absorbance was measured at 570/620 nm and cell viability was expressed as a percentage of the corresponding control as described by Madesh and Balasubramanian [24].

### 2.12. Determination of ALT and LDH leakage

The leakage of the enzymes, ALT and LDH is associated with cell viability and is considered as an important indicator of cellular membrane damage. After appropriate experimental procedure as described earlier, hepatocyte suspensions were centrifuged at $60 \times g$. The leakage of ALT and LDH (secreted outside the cells) was determined from the supernatant using a kit and following the methods of Sinha et al. [23].

### 2.13. Determination of protein content

The protein content was measured by the method of Bradford [25] using crystalline BSA as standard.

### 2.14. Measurement of intracellular ROS production

ROS levels were determined using 2,7-dichlorofluorescein diacetate (DCFHDA), following the method of Manna et al. [26]. This is then 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. After performing the experiments (as described in Section 2.10) hepatocytes were incubated with the assay media (20 mM Tris–HCl, 130 mM KCl, 5 mM $MgCl_2$, 20 mM $NaH_2PO_4$, 30 mM glucose and 5 μM DCFDA) at 37 °C for 15 min. The formation of DCF was measured at the excitation wavelength of 488 nm and emission wavelength of 510 nm for 10 min by using fluorescence spectrometer (HITACHI, Model No F4500) equipped with a FITC filter.

### 2.15. Estimation of lipid peroxidation end products

Lipid peroxidation in all the experimental sets (as described in Section 2.10) was assessed by a colorimetric reaction with thiobarbituric acid (TBA) as described by Sinha et al. [23]. Briefly, hepatocytes were treated with TBA (0.67%) and then with TCA (20%) solutions. The mixture was heated for 30 min at 100 °C. The precipitate was removed by centrifugation and absorbance of thiobarbituric acid reactive substance (TBARS) formed was measured at 532 nm. TBARS concentration of the samples was cal-

culated using the extinction coefficient of MDA which is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (since 99% of TBARS exists as MDA). All the experiments were done in triplicates under the same conditions.

### 2.16. Determination of GSH level

Glutathione (GSH) levels in all the experimental sets of hepatocytes were measured according to the method of Sinha et al. [23]. Hepatocyte suspension (720 μl) was double diluted and 5% TCA was added to precipitate the protein content of the suspension. After centrifugation (at 10,000 × *g* for 5 min) the supernatant was taken, DTNB solution (Ellman's reagent) was added to it and the absorbance was measured at 412 nm. A standard curve was drawn using different known concentrations of GSH solution. With the help of this standard curve, GSH contents were calculated.

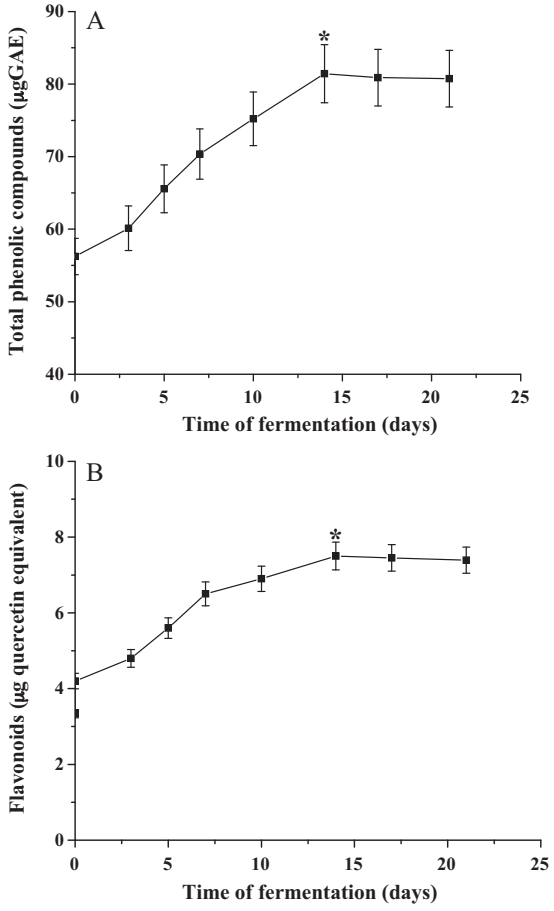


Fig. 1. Concentrations of phenolic compounds (A) and flavonoids (B) during the course of fermentation of KT. Values are represented as the means of six different experiments in each set. Data represent the average ± SD of six separate experiments. "*" sign indicates the optimum fermentation day of KT when the phenolic compounds and flavonoids, respectively, were found to be maximum.

### 2.17. Determination of SOD activity

SOD activity was measured following the method of Sinha et al. [23]. The hepatocyte suspension containing 5 μg protein was mixed with sodium pyrophosphate buffer, PMT and NBT. The reaction was started by the addition of NADH. Reaction mixture was then incubated at 30 °C for 90 s and stopped by the addition of 1 ml of glacial acetic acid. The absorbance was measured at 560 nm. One unit of SOD activity is defined as the enzyme concentration required inhibiting chromogen production by 50% in 1 min under the assay conditions.

### 2.18. Detection of the nature of cell death by flow cytometry (FACS)

Dual parameter FACS analysis allows for the discrimination between viable, apoptotic and necrotic cells. After appropriate treatments (as described in Section 2.10), hepatocytes were washed with PBS, centrifuged at 800 × *g* for 6 min, resuspended in ice-cold 70% ethanol/PBS, centrifuged at 800 × *g* for a further 6 min, and resuspended in PBS. Cells were then incubated with PI and FITC-labelled Annexin V for 30 min at 37 °C. Excess PI and Annexin V were then washed off; cells were fixed and then stained cells were analyzed by flow cytometry using FACS Calibur (Becton Dickinson, Mountain View, CA) equipped with 488 nm argon laser light source; 515 nm band pass filter for FITC-fluorescence and 623 nm band pass filter for PI-fluorescence using Cell Quest software. A dot plot of PI-fluorescence (*y*-axis) versus FITC-fluorescence (*x*-axis) has been prepared.

### 2.19. Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was estimated on the basis of cell retention of the fluorescent cationic probe rhodamine 123 [26]. Briefly, the mitochondrial suspension was incubated with 1 μM rhodamine 123 for 10 min, centrifuged at 50 × *g* for 5 min at 4 °C, washed and resuspended in 1 ml of 0.1% Triton X-100. After centrifugation at 2000 × *g* for 5 min, fluorescence of rhodamine 123 was determined using BD-LSR flow cytometer. Cell debris, characterized by a low FSC/SSC was excluded from analysis. The data was analyzed by Cell Quest software. In this assay carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a positive control.

### 2.20. Western blot analysis

For western blot analysis, the samples containing 50 μg proteins were subjected to 10% SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% nonfat dry milk to prevent nonspecific binding. The membrane was then incubated in (anti-Bcl-2, anti-Bax, anti-

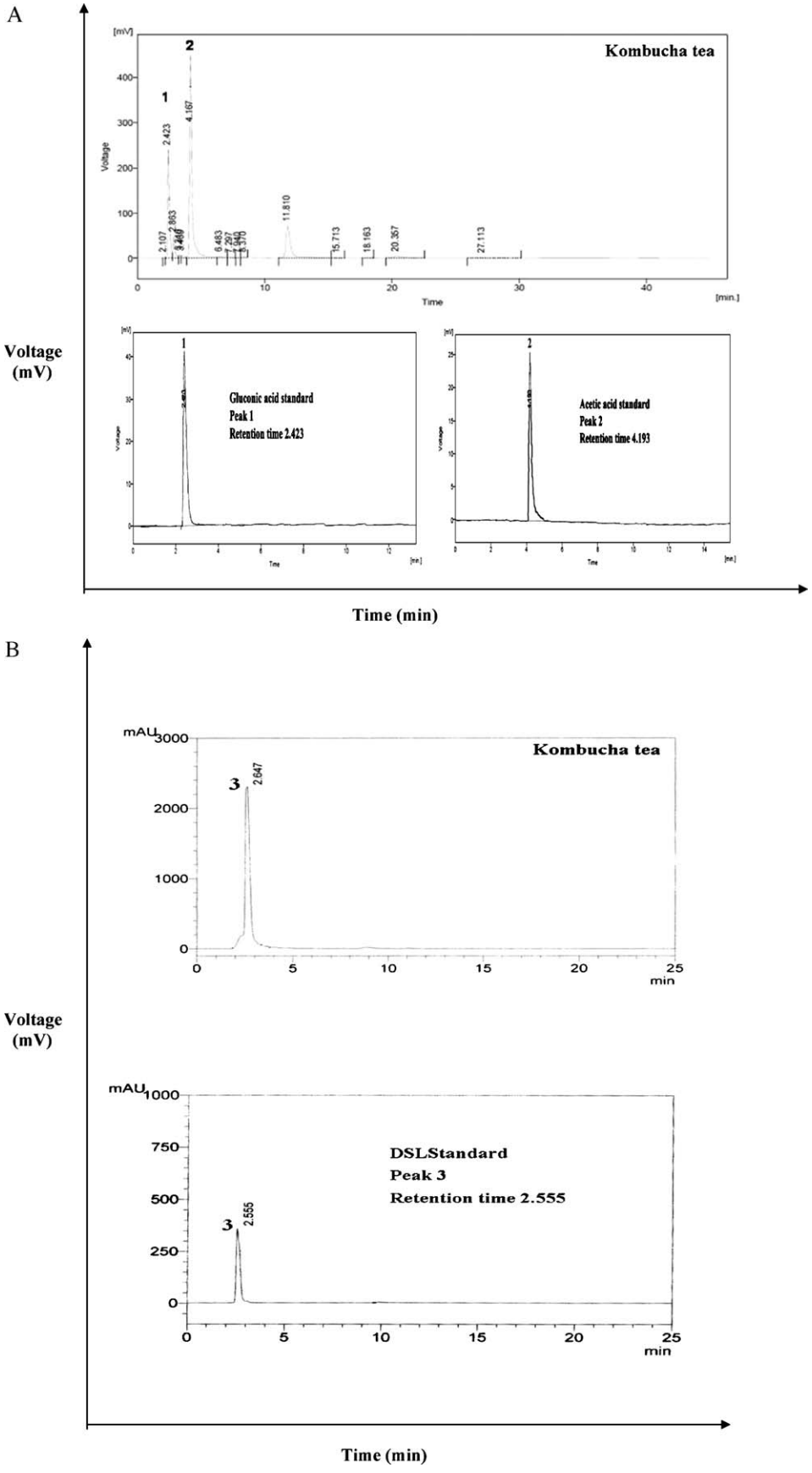


Fig. 2. Reverse phase HPLC analysis of KT. Peak 1: gluconic acid; peak 2: acetic acid; peak 3: D-saccharic acid 1,4-lactone (respective standard given below).

cytochrome *c* (1:1000 dilution), anti-caspase-9, anti-Apaf-1 (1:500 dilution) and anti-caspase-3 (1:100 dilution)) primary antibodies separately at 4 °C overnight under mild rocking condition. The membranes were washed in TBST (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate HRP conjugated secondary antibody (1:2000 dilution) for 2 h at room temperature (25 °C) and developed by the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB) system.

### 2.21. Statistical analysis

All the values are expressed as mean ± SD ($n = 6$). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) for Windows using one-way analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). A difference was considered significant at the $p < 0.05$ level.

## 3. Results

### 3.1. Time-dependent compositions of the total phenolic compounds and flavonoids in KT

Changes in concentration of phenolic compounds and flavonoids in KT during the course of fermentation were shown in Fig. 1A and B, respectively. From the results it is clear that the concentrations of total phenolic compounds and flavonoids increased progressively and linearly up to day 14 probably because the enzymes liberated by the bacteria and yeast during fermentation degrade the complex polyphenols to small molecules and that in turn results in the increase of total phenolic compounds and flavonoids. As phenolic compounds and flavonoids are potent antioxidants, KT fermented for 14 days, was used for all the experiments in the present study because of the presence of maximum level of these compounds.

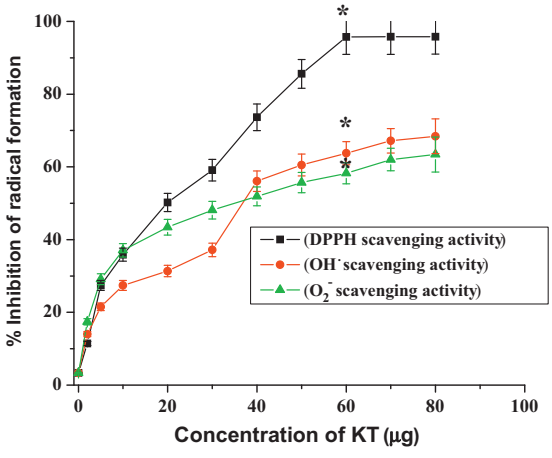


Fig. 3. Free radical scavenging activities of KT in cell free system. Values are represented as the mean of six different experiments in each set. Data represent the average ± SD of six separate experiments. "*" sign indicates the optimum dose of KT at which it shows its significant radical scavenging activity.

Table 1
Major components of KT.

| Serial no. | Component | Concentration |
|---|---|---|
| 1 | Acetic acid (mg/ml) | 13.43 ± 0.6 |
| 2 | Gluconic acid (mg/ml) | 6.45 ± 0.3 |
| 3 | Theaflavins (%) | 0.175 ± 0.01 |
| 4 | Thearubigins (%) | 1.98 ± 0.095 |
| 5 | D-Saccharic acid-1,4-lactone (mg/ml) | 1.32 ± 0.06 |

### 3.2. Composition of kombucha tea

The chemical composition of KT has been widely studied by a number of investigators based on their needs. But the results varied widely because of the different microbial composition arose due to varying time length and the source of kombucha mat used for fermentation. So, to have a clear picture of the responsible antioxidant active ingredients in our preparation of KT, we analyzed our experimental samples using HPLC and observed that the main organic acids present in kombucha tea (fermented for 14 days) were acetic acid and gluconic acid. In addition, the black tea polyphenols, theaflavins and thearubigins were also found to be present as determined by the analytical techniques described above. In addition D-saccharic acid 1,4-lactone was also detected in KT (Fig. 2 and Table 1). Generally tea polyphenols, organic acids, glucose, fructose, and ethanol were the primary constituents of KT and that is in good agreement with our analysis.

### 3.3. Radical scavenging activity of KT in cell free system

To begin with the evaluation of the antioxidant nature of KT, we looked for the free radical scavenging activities of KT in cell free systems. The inhibition for the production of free radicals (DPPH, super oxide, hydroxyl), in cell free system was carried out using different concentrations of KT (Fig. 3). Results suggest that KT shows its maximum radical scavenging property when used at a concentration of 60 µg. Thus, it can be anticipated that KT possesses potent free radical scavenging activity and could be used as a free radical quencher.

### 3.4. Dose and time dependent effects of KT by FRAP assay

Dose and time dependent effects of KT against TBHP toxicity has been represented in Fig. 4. TBHP exposure decreased the intracellular ferric reducing antioxidant power and that could be well inhibited by the treatment with KT at dose of 60 µg incubated for 2 h. Therefore, these were the selected dose and time of KT for treating the murine hepatocytes.

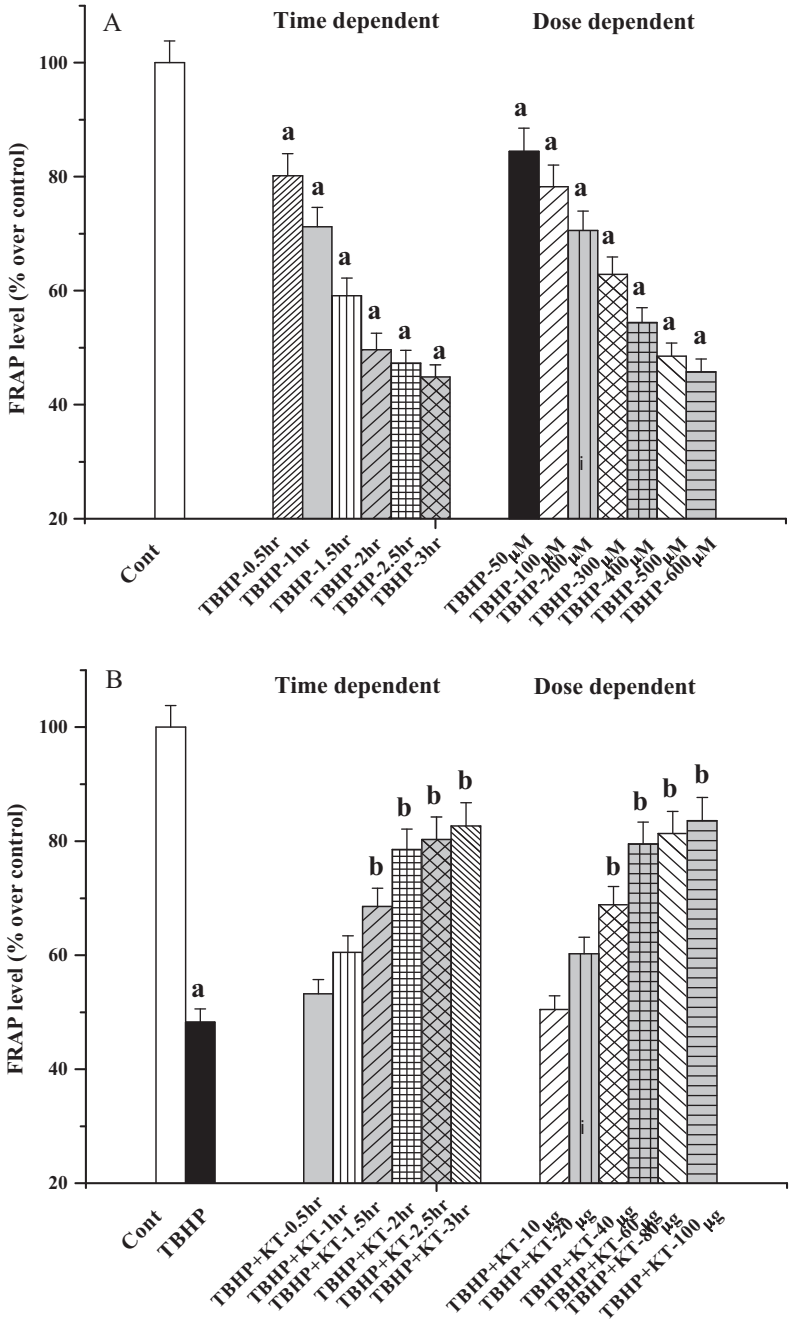


Fig. 4. (A) Time and dose-dependent effect of the TBHP on intracellular antioxidant power of the hepatocytes. Values are expressed as percent over control. Cont: antioxidant power in normal hepatocytes; TBHP-0.5hr, TBHP-1hr, TBHP-1.5hr, TBHP-2hr, TBHP-2.5hr, TBHP-3hr: antioxidant power in TBHP-treated hepatocytes for the respective time at a dose of 500 μM; TBHP-50 μM, TBHP-100 μM, TBHP-200 μM, TBHP-300 μM, TBHP-400 μM, TBHP-500 μM, TBHP-600 μM: antioxidant power in TBHP-treated hepatocytes at the respective doses for 2 h. Data are mean ± SD, for six sets of experiments per group. "a" values indicate significant differences between normal control and TBHP treated groups ($p^a < 0.05$). (B) Time and dose-dependent effect of KT on intracellular antioxidant power of the TBHP-exposed hepatocytes. Values are expressed as percent over control. Cont: antioxidant power in normal hepatocytes; TBHP: antioxidant power in TBHP-treated hepatocytes; TBHP + KT-0.5hr, TBHP + KT-1hr, TBHP + KT-1.5hr, TBHP + KT-2hr, TBHP + KT-2.5hr, TBHP + KT-3hr: antioxidant power in KT-treated hepatocytes for the respective time at a dose of 60 μg; TBHP + KT-10 μg, TBHP + KT-20 μg, TBHP + KT-40 μg, TBHP + KT-60 μg, TBHP + KT-80 μg, TBHP + KT-100 μg: antioxidant power in KT-treated hepatocytes at the respective doses for 2 h. Data are mean ± SD, for six sets of experiments per group. "b" values indicate significant differences between TBHP treated groups and KT treated groups ($p^b < 0.05$).

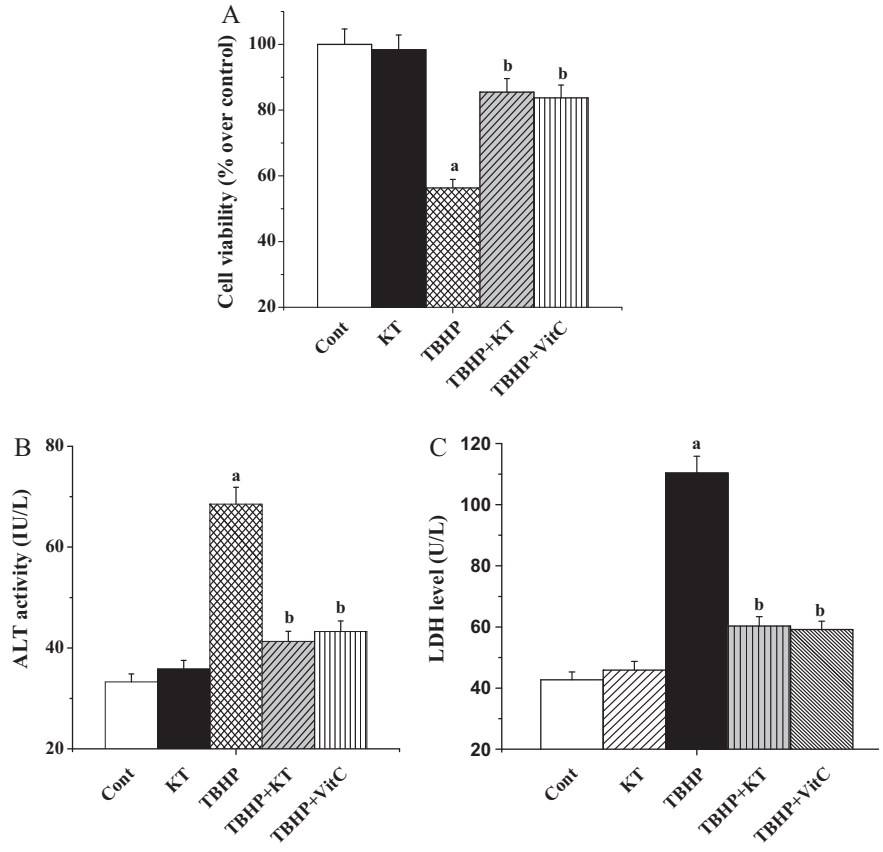


Fig. 5. (A) Effect of KT on TBHP-induced loss of cell viability of murine hepatocytes. Cont: levels in normal control; KT: levels in KT treated hepatocytes; TBHP: levels in only TBHP exposed hepatocytes; TBHP + KT: levels in hepatocytes after simultaneous exposure with KT and TBHP; TBHP + VitC: levels in hepatocytes after simultaneous exposure with VitC and TBHP. Data are mean ± SD, for six sets of experiments per group. "a" values differ significantly from normal control ($p^a < 0.05$); "b" values indicate significant differences between TBHP treated groups and KT, and VitC treated groups ($p^b < 0.05$). (B and C) Effect of KT on TBHP-induced ALT activity and LDH leakage in murine hepatocytes. (B) Represents the effect on ALT activity and (C) represents LDH leakage. Cont: levels in normal control; KT: levels in KT treated hepatocytes; TBHP: levels in only TBHP exposed hepatocytes; TBHP + KT: levels in hepatocytes after simultaneous exposure with KT and TBHP; TBHP + VitC: levels in hepatocytes after simultaneous exposure with VitC and TBHP. Data are mean ± SD, for six sets of experiments per group. "a" values differ significantly from control ($p^a < 0.05$); "b" values indicate significant differences between TBHP treated groups and KT, and VitC treated groups ($p^b < 0.05$).

### 3.5. Effect on cell viability

The effect of KT on cell viability in isolated hepatocytes has been represented in Fig. 5A. Cell viability was reduced in TBHP treated hepatocytes compared to normal cells. KT, on the other hand, prevented the toxin-induced lose in cell viability.

### 3.6. Effect on ALT and LDH

Oxidative stress is known to cause the production of ROS which in turn can disrupt the cell membrane, initiate the release of ALT, LDH and other intracellular enzymes and reduces cell viability. Severe ALT and LDH leakage was detected in the TBHP treated hepatocytes indicating a loss of cell membrane integrity and cytotoxicity in hepatocytes (Fig. 5B and C). KT effectively inhibited the membrane disruption caused by the toxin as revealed from the less ALT and LDH levels outside the cells.

### 3.7. Restoration of antioxidant status in TBHP-stressed murine hepatocytes by KT

#### 3.7.1. Effect on ROS production

TBHP causes a significant increase in the formation of ROS (like superoxide anion radical ($O_2^-$), hydroxyl radical ($OH^\bullet$) and hydrogen peroxide ($H_2O_2$), etc. The changes in the production of intracellular ROS under oxidative stress in the hepatocytes were assayed using DCFDA by fluorescence spectrophotometer. Hepatocytes stressed with TBHP showed an increase in the ROS generation by 2.1-fold as compared to untreated cells. Whereas in the cells treated with KT, a 0.5-fold decline in the ROS generation was observed as compared to TBHP stressed cells (Table 2). This suggests the intracellular ROS scavenging nature of KT.

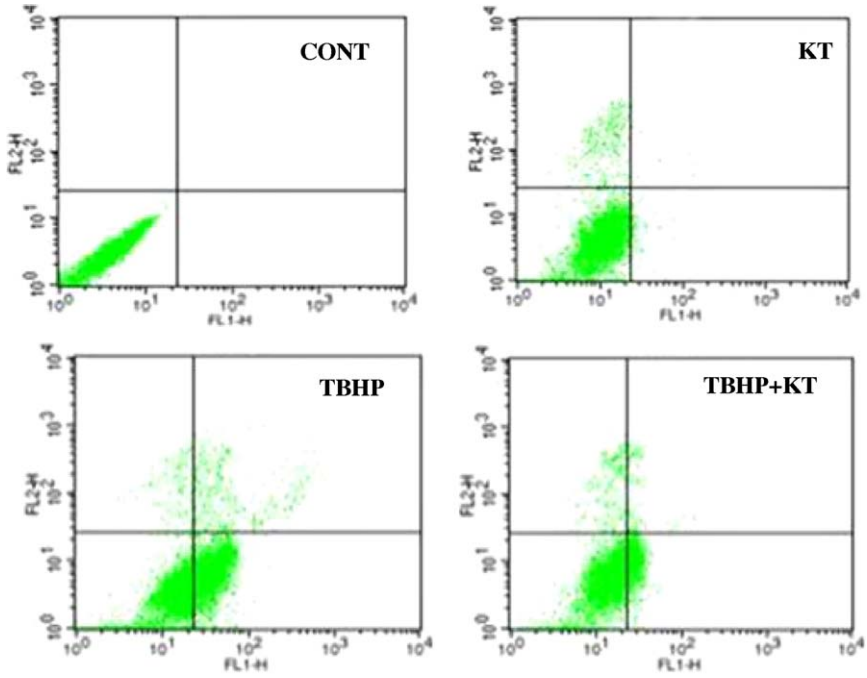


Fig. 6. Representative flow cytometric analysis of hepatocytes from experimental animals. Cont: normal hepatocytes; KT: hepatocytes treated with KT only; TBHP: hepatocytes treated with TBHP only; TBHP + KT: hepatocytes treated with KT and TBHP simultaneously. Dual parameter dot plot of FITC-lebelled Annexin V fluorescence (*x*-axis) versus PI-fluorescence (*y*-axis) has been shown in logarithmic fluorescence intensity. Quadrants: lower left, live cells; lower right, apoptotic cells; upper left, necrotic cells. Data are representative of three independent experiments.

### *3.7.2. Effect on lipid peroxidation, GSH content and SOD activity*

Cellular lipid peroxidation, GSH content and activities of the antioxidant enzyme (like SOD) are some important parameters related to oxidative stress induced cellular pathophysiology. In the present study, increased levels of MDA have been observed in the hepatocytes incubated with TBHP. The activities of the SOD and GSH content were also depleted under the same exposure. However, KT could protect the cellular defense against TBHP induced oxidative damage and maintain the normalcy in hepatocytes (Table 2).

The effect of a known antioxidant, vitamin C was included in the above experiments as positive control. Being an antioxidant, vitamin C could prevent TBHP-induced ROS mediated cellular oxidative dysfunction.

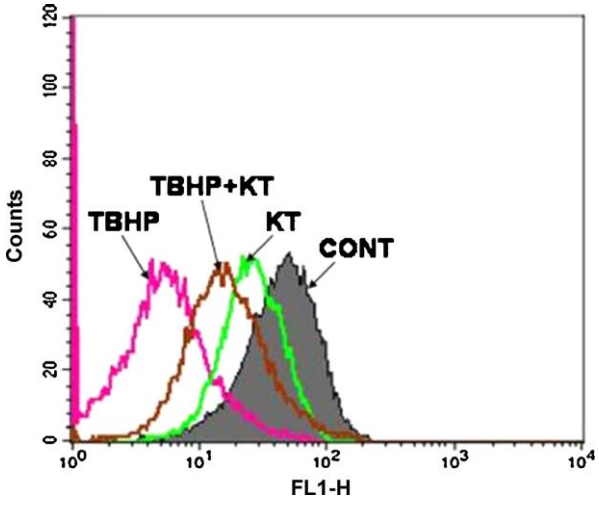


Fig. 7. Study on the mitochondrial membrane potential in the hepatocytes by flow cytometry analysis. Figure represents the histogram plot. Cont: mitochondrial membrane potential in normal control; KT: mitochondrial membrane potential in KT treated hepatocytes; TBHP: mitochondrial membrane potential in only TBHP exposed hepatocytes; TBHP + KT: mitochondrial membrane potential in hepatocytes treated with KT and TBHP simultaneously. The measurements were made in six times.

### *3.8. Protective role of KT in TBHP-induced cell death*

Loss of mitochondrial membrane potential ($\Delta\Psi_m$) causes the release of cytochrome *c* and other pro-apoptotic factors into the cytosol and that can lead to subsequent cell death. We, therefore, studied the effect of KT on TBHP induced mitochondrial events and observed that TBHP reduced the mitochondrial membrane potential (Fig. 7), increased the expression of cytosolic versus mitochondrial cytochrome *c* ratio (Fig. 8B) in association with up-regulating caspase-9, Apaf-1 and caspase-3 (Fig. 8C–E), indicating the involvement of the mitochondrial apoptotic pathway in this pathophysiology. In addition, Bcl-2 family proteins are upstream regulator of mitochondrial membrane potential and play critical roles in mitochondrial-mediated cell

death. The Bcl-2:Bax ratio drew particular interest due to their significance in the mitochondrial mediated events. We observed that TBHP caused a marked decrease in Bcl-2 expression in hepatocytes and increased the expression of Bax which led to decreased ratio of Bcl-2 over Bax (Fig. 8A). KT treatment, on the other hand, inhibited TBHP-induced mitochondrial events and reciprocal regulation of Bax and Bcl-2.

We also conducted FACS analysis to characterize the nature of TBHP induced cell death and protective role of KT by a double labeling technique using Annexin V/PI to distinguish between apoptotic and necrotic cells.

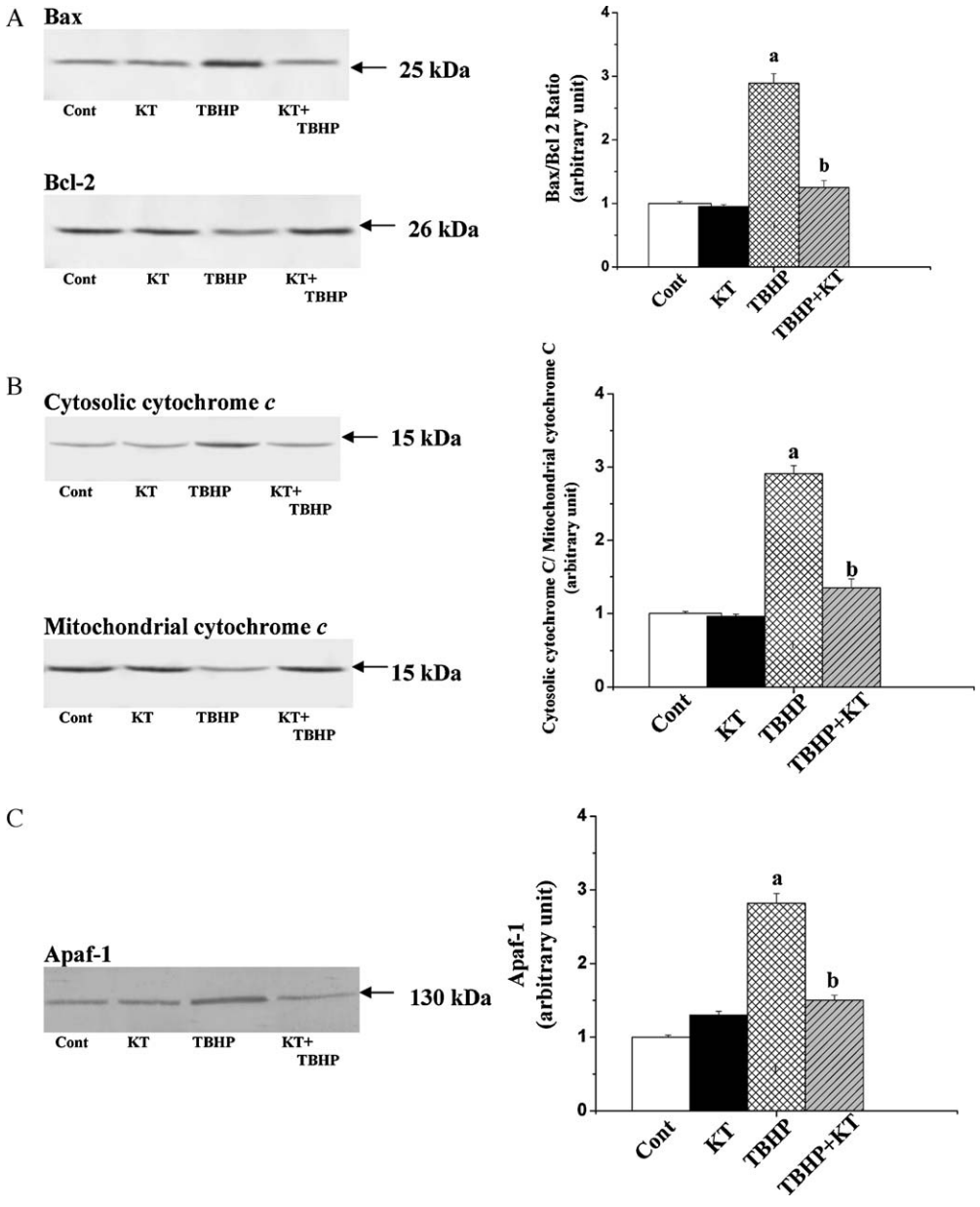


Fig. 8. (A–E) Western blot analysis and study on mitochondrion-dependent cell death pathway in TBHP exposed hepatocytes in the absence (TBHP treated) and presence of KT (TBHP + KT). (A) Left panel represents the protein level of Bax (top) and Bcl-2 (bottom) and right panel represents quantitative analysis of different bands. (B) Left panel represents the protein level of cytosolic (top) and mitochondrial (bottom) cytochrome *c* and right panel represents quantitative analysis of different bands. (C) Left panel represents the protein level of Apaf-1 and right panel represents quantitative analysis of different bands. (D) Left panel represents the protein level of caspase-9 and right panel represents quantitative analysis of different bands. (E) Left panel represents the protein level of caspase-3 and right panel represents quantitative analysis of different bands. Cont: levels in normal control; KT: levels in KT treated hepatocytes; TBHP: levels in only TBHP exposed hepatocytes; TBHP + KT: levels in hepatocytes after simultaneous exposure with KT and TBHP. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. Data are mean ± SD, for six sets of experiments per group. "a" values differ significantly from normal control ($p^a < 0.05$); "b" values indicate significant differences between TBHP treated groups and KT treated groups ($p^b < 0.05$).

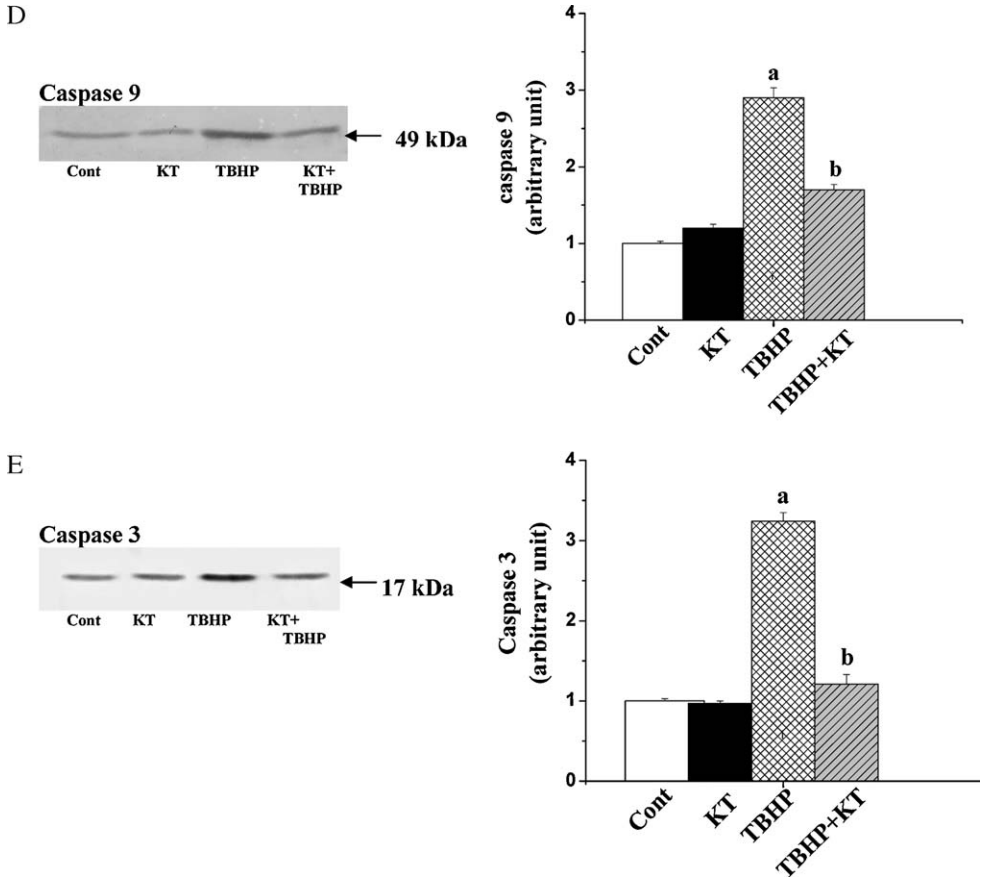


Fig. 8. (*Continued*).

Table 2
Antioxidant markers in normal and experimental hepatocytes.

| Treatment groups | ROS[a] | Lipid peroxidation[b] | SOD activity[c] | GSH[d] |
|---|---|---|---|---|
| Normal control | 100 ± 4.52 | 10.21 ± 0.48 | 114.5 ± 5.4 | 11.35 ± 0.5 |
| KT | 94.63 ± 4.38 | 12.46 ± 0.53 | 113.26 ± 5.3 | 13.25 ± 0.6 |
| TBHP | 231.56 ± 10.6a | 35.45 ± 1.57a | 72.36 ± 3.01a | 5.07 ± 0.23a |
| TBHP + KT | 116.32 ± 5.4b | 19.75 ± 0.8b | 110.72 ± 5.2b | 8.7 ± 0.4b |
| TBHP + VitC | 128.39 ± 6.2b | 20.65 ± 0.96b | 92.45 ± 4.3b | 8.36 ± 0.39b |

Normal control: normal hepatocytes; KT treated: only KT treated hepatocytes; TBHP treated: only TBHP exposed hepatocytes; TBHP + KT: hepatocytes exposed to simultaneous KT and TBHP administration; TBHP + VitC: hepatocytes exposed to simultaneous VitC and TBHP administration. Data are mean ± SD, for six sets of experiments per group. "a" values differ significantly from normal control ($p^a < 0.05$); "*b*" values indicate significant differences between TBHP treated groups and KT and VitC treated groups ($p^b < 0.05$).

[a] % over control.
[b] nM MDA formed/mg protein.
[c] Unit/mg protein.
[d] nmol/mg protein.

Annexin V binds specifically to phosphatidylserine and FITC-conjugated Annexin V can, therefore, be used as a fluorescent probe to label apoptotic cells. Propidium iodide (PI) is used in conjunction with Annexin V–FITC. The cell membrane integrity excludes PI in viable and apoptotic cells, whereas necrotic cells are permeable to PI. Results suggest that KT treatment reduced the cell numbers to 90% viability (approximately) whereas 500 μM TBHP alone caused apoptosis to nearly 60% of the total cell populations (Fig. 6).

## 4. Discussion

Present study described the protective role of KT against TBHP-induced cytotoxicity and cell death in murine hepatocytes. Incubation of hepatocytes with TBHP caused a significant increase in ROS generation, depletion of GSH content, increase in MDA formation, and decrease in SOD activity leading to apoptotic cell death initiated by mitochondrial pathway. KT treatment in combination with TBHP,

however, could protect the cells by preventing the alterations caused by this ROS inducer.

In the present study, the antioxidant activity of KT in cell free system was determined by its DPPH radical scavenging activity. In addition, the hydroxyl and superoxide radicals scavenging activities of KT were also investigated in cell free system. Results of these studies clearly established KT as a potent radical scavenger. Moreover, the results of the present study suggest that KT contains more polyphenols and flavonoids than black tea itself (Fig. 1A and B). It also contains black tea polyphenols like theaflavin and thearubigins and antioxidants like D-saccharic acid 1,4-lactone. It is, therefore, likely to believe that KT might alter the toxic effects of TBHP by quenching the excessive free radicals produced in hepatocytes by this free radical inducer.

TBHP is a well known ROS inducer. The results of the present study showed that treatment with TBHP stimulates ROS overproduction, induces the production of MDA, and causes reduction in the level of GSH. 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 data showed that cells treated with KT displayed a reduction of TBHP-induced ROS generation, attenuation of MDA levels to a considerable extent comparable to toxin control and inhibition of GSH depletion.

Mitochondria play a major role in regulating cell death pathways [27,28]. ROS are predominantly produced in mitochondria and play an important role in apoptosis. These reactive species attack membrane phospholipids and cause loss of mitochondrial membrane potential. Moreover, the proapoptotic proteins belonging to the Bcl-2 family, such as Bad and Bax are also activated. These trigger the release of cytochrome *c* from the mitochondria to the cytosol. In the cytosol, Apaf-1, procaspase-9 and released cytochrome *c* from the mitochondria interact to form the apoptosome that drives the activation of caspase-3. In the cytosol the cytochrome *c* forms a macromolecular complex with the apoptotic protease activating factor 1 (Apaf-1). The complex cleaves and activates procaspase-9, which in turn causes cleavage and activation of procaspase-3. Caspase-3 is responsible for the breakdown of a number of cytosolic/nuclear proteins resulting in cellular morphological and biochemical changes that are hallmarks of apoptosis, such as cell shrinkage, DNA fragmentation, and chromatin condensation. Antiapoptotic proteins also belonging to the Bcl-2 gene family, such as Bcl-2 itself and Bcl-xL, inhibit the release of cytochrome *c* from mitochondria and thereby prevent the occurrence of apoptosis [29–31]. To confirm TBHP-induced mitochondria-dependent apoptosis, we determined

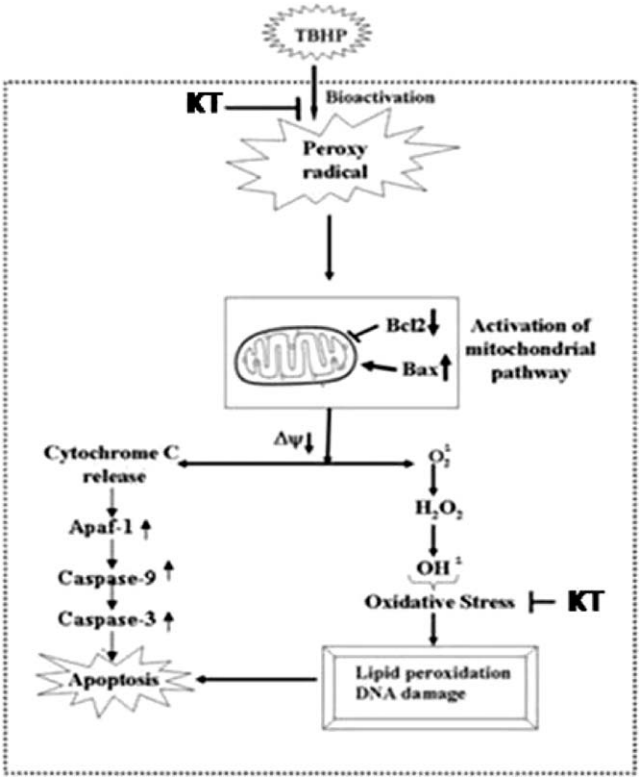


Fig. 9. Schematic diagram of TBHP induced hepatotoxicity and its prevention by KT.

the mitochondrial membrane potential as well as studied the immunoblot analyses of Bcl-2 family proteins (Bax and Bcl-2), cytochrome c, caspase-9, Apaf-1 and caspase-3 in TBHP-induced hepatic pathophysiology. Results showed that TBHP reduced mitochondrial membrane potential, elevated the concentration of cytosolic cytochrome *c* and simultaneously increased the activities of caspase-9, Apaf-1 and caspase-3 in experimental hepatocytes. Immunoblotting studies also demonstrated that TBHP up regulated the proapoptotic (Bax) and downregulated the anti-apoptotic (Bcl-2) Bcl-2 family proteins in hepatocytes (Fig. 8). Treatment with KT could, however, effectively suppress these alterations of mitochondrial events induced by TBHP and kept the cells in their normal physiological status. Fig. 9 shows the possible mechanistic pathways about the beneficial role of KT against TBHP induced hepatic pathophysiology.

In conclusion, incubation of murine hepatocytes with TBHP caused an alteration in the cellular antioxidant status and induced apoptotic cell death by disrupting natural mitochondrial events. Treatment of KT in combination with the toxin, on the other hand, kept that antioxidant status quite similar to that of normal hepatocytes, prevented the disruption of mitochondrial membrane potential and blocked the activation of mitochondria dependent apoptotic signaling pathways (Fig. 9). Quenching the radical species could be one of the mechanisms involved in its protective action. In addition, the presence of glucaric acid and its derivatives like

D-saccharic acid 1,4-lactone (DSL) in KT as potent detoxifying agents may be one of the reasons for the hepatoprotective and curative effect of KT against hepatic pathophysiology [7]. The present findings suggest that KT may be used as a natural antioxidant for the protection of hepatocytes in TBHP-induced oxidative insult and death.

## Conflict of interest

The authors have declared that no conflict of interest exists.

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