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# Protective effect of kombucha tea against tertiary butyl hydroperoxide induced cytotoxicity and cell death in murine hepatocytes

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Kombucha (KT), a fermented black tea (BT), is known to have many beneficial properties. In the present study, antioxidant property of KT has been investigated against tertiary butyl hydroperoxide (TBHP) induced cytotoxicity using murine hepatocytes. TBHP, a reactive oxygen species inducer, causes oxidative stress resulting in organ pathophysiology. Exposure to TBHP caused a reduction in cell viability, increased membrane leakage and disturbed the intra-cellular antioxidant machineries in hepatocytes. TBHP exposure disrupted mitochondrial membrane potential and induced apoptosis as evidenced by flow cytometric analyses. KT treatment, however, counteracted the changes in mitochondrial membrane potential and prevented apoptotic cell death of the hepatocytes. BT treatment also reverted TBHP induced hepatotoxicity, however KT was found to be more efficient. This may be due to the formation of antioxidant molecules like D-saccharic acid-1,4-lactone (DSL) during fermentation process and are absent in BT. Moreover, the radical scavenging activities of KT were found to be higher than BT. Results of the study showed that KT has the potential to ameliorate TBHP induced oxidative insult and cell death in murine hepatocytes more effectively than BT.

**Keywords:** Antioxidant, Black tea, Cell death¸ Cytoprotection, Cyto-toxicity, Hepatocytes, Kombucha tea, Reactive oxygen species, Tertiary butyl hydroperoxide

Tertiary butyl hydroperoxide (TBHP) has been reported to induce oxidative stress in different organ systems including liver[1], testes[2], oocytes[3], retina[4]. It acts mainly by mobilization of arachidonic acid (AA) from membrane phospholipids under cytotoxic conditions leading to an increase in intracellular AA and malondialdehyde formation resulting in cell death. Although the exact mechanism of toxicity of this oxidative stress inducer is not known, studies suggest the involvement of cellular lipids peroxidation, alkylation of cellular macromolecules like protein and DNA[5] and alterations in cellular calcium and glutathione levels[6]. Dietary antioxidants capable of scavenging free radicals are of great interest in combating oxidative stress induced cell damage. Several studies have shown that antioxidants prevent TBHP toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation and increasing antioxidant enzyme activities[7-9].

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Kombucha tea (KT) is a sugared black tea fermented by a symbiotic culture of yeast and acetic acid bacteria. A jelly-like membrane floats in the nutrient solution of tea and sugar exposed to oxygen. At the right temperature, it multiplies continuously. It first 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[10]. KT is claimed to have many beneficial effects to human health. The US Food and Drug Administration has evaluated the practices of several commercial producers of the starter (kombucha mushroom or tea fungus) and found no pathogenic organisms or other hygienic violations in KT[11]. Some of the therapeutic effects of KT have been reported earlier[12-15].

In the present study, protective role of KT against TBHP induced oxidative damage and cell death in mouse hepatocytes has been reported. Cell viability, membrane leakage, activities of antioxidant enzymes, levels of cellular metabolites, mitochondrial membrane potential and flow cytometric analyses have been analysed. Further, antioxidative and hepatoprotective activities of KT were compared to black tea for the same pathophysiology.

## Materials and Methods

*Chemicals*—Black tea (BT) was purchased from local market. Collagenase type I, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine sera (FBS) were purchased from Sigma-Aldrich Chemical Company, (St. Louis, MO) USA. Bradford reagent were purchased from Sigma-Aldrich Chemical Company, (St. Louis) USA. Folin-Ciocalteu reagnt, gallic acid, quercetin, aluminium chloride, potassium acetate, acetic acid, gluconic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isobutyl methyl ketone (IBMK), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB, (Ellman's reagent)], disodium hydrogen phosphate ($Na_2HPO_4$), ethylene diamine tetraacetic acid (EDTA), glacial acetic acid, hydrogen peroxide ($H_2O_2$), N-ethylmaleimide (NEM), nicotinamide adenine dinucleotide reduced (NADH), nitro blue tetrazolium (NBT), oxidized glutathione (GSSG), phenazine methosulphate (PMT), reduced glutathione (GSH), Tertiary butyl hydrogen peroxide (TBHP), sodium pyrophosphate, thiobarbituric acid (TBA), tris buffer, ascorbic acid, butanol, ethanol were bought from Sisco research laboratory, India. The antibodies were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

*Animals*—Male adult albino mice of Swiss strain, weighing between 20-25 g were acclimatized under laboratory conditions for a fortnight before starting experiments. 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 (30ºC) and humidity (50%). All the studies were performed in conformity with the guidance for care and standard experimental animals study ethical protocols.

*Preparation of sweetened black tea and kombucha tea*—Black tea (8 gm) was added to water (300 ml) and allowed to boil for 5 min. Then it was filtered through a sterile sieve and cooled to room temperature, adjusted to 1600 ml with water and sucrose (10% w/v) was added to it. The cooled tea was poured into 3 L glass beaker that has been previously sterilized at 121ºC for 20 min. The sweetened black tea 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 to prepare kombucha tea. The beaker was covered with clean cheese cloths and fixed with rubber bands. The fermentation was carried out under room temperature 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 phenolics and flavonoids. The fermented tea was centrifuged at 10000 rpm for 15 min and analysed. KT fermented for 14 days was selected for the investigation against TBHP induced cytotoxicity of murine hepatocytes.

*Determination of total phenolic compounds and flavonoids*—Total phenolic compounds were measured by Folin-Ciocalteu method[16]. Kombucha (100 µL) tea was mixed with 0.2 ml of Folin-Ciocalteu reagnt, 2 ml of purified water and 1 ml of 15% $Na_2CO_3$. The mixture was measured at 765 nm after 2 h at room temperature. Gallic acid was used as a standard and the total phenolics were expressed as gallic acid equivalents.

Flavonoid content was determined by following the calorimetric method[17]. KT (0.5 ml) was mixed with methanol (1.5 ml), 0.1 ml of aluminium chloride (10%), 0.1 ml of 1 *M* potassium acetate, and of distilled water (2.8 ml), and incubated at room temperature for 30 min. Absorbance of reaction mixture was measured at 415 nm. Quercetin was used as standard and the flavonoid content was expressed as quercetin equivalents.

### Antioxidant activity of black tea and kombucha tea in cell free system

*DPPH radical quenching activity*—Antioxidant activity of kombucha tea was measured using the DPPH radical as described by Blos[18]. DPPH solutions (2 ml, 125 µ*M*) in methanol and sample at different volume (2 µl, 4 µl, 6 µl, 8 µl, 10 µl, 20 µl, 30 µl, 40 µl, 50 µl and 60 µl) were mixed in the tubes. The solution was shaken and incubated at 37ºC for 30 min in dark. Decrease in absorbance at 517 nm was measured against methanol blank using a UV/Visible spectrophotometer. Inhibition (%) was calculated by comparing the absorbance values of control and the sample.

$$\% \text{ inhibition} = \frac{A1 - A2}{A1} \times 100$$

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

*Hydroxyl radical scavenging activity*—Hydroxyl radical scavenging activity of KT has been

investigated following the method of Nash[19] using the same volume of KT and black tea used in the DPPH radical scavenging assay. *In vitro* hydroxyl radicals were generated by $Fe^{3+}$/ascorbic acid system. Detection of hydroxyl radicals was carried out by measuring the amount of formaldehyde produced from oxidation of dimethyl sulfoxide (DMSO). The formaldehyde produced was detected spectrophotometrically at 412 nm.

*Superoxide radical scavenging activity*—The superoxide radical scavenging activity was measured following the method of Siddhuraju and Becker[20]. The reaction mixture contained 0.1 *M* phosphate buffer, *p*H 7.4, 150 μ*M* nitroblue tetrazolium (NBT), 60 μ*M* phenazine methosulphate (PMT), 468 μ*M* NADH and different volume of KT same as above. The mixture was incubated in the dark for 10 min at 25ºC and the absorbance was read at 560 nm. Results were expressed as percentage inhibition of the superoxide radicals.

*Analysis of organic acids*—Major organic acids were determined by high performance liquid chromatography (HPLC). 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 m*M* potassium dihydrogen phosphate, *p*H 2.4 with a flow rate of 1.0 ml/min and running time of 40 min. Column temperature was maintained at 28ºC and the detection was carried out at 220 nm. Resolution peaks were recorded on the HPLC chart according to retention time of each compound. Concentrations of organic acids were quantified from standard curves.

*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 ($Na_2B_4O_7$–$KH_2PO_4$), *p*H 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. One separate experiment was done in which BT was applied under the same conditions.

*Analysis of black tea polyphenols*—Main black tea polyphenols theaflavin and thearubigins were estimated in fermented tea broth by the method of Takeo and Oosawa[21] as modified by Ramaswamy[22] and Thanaraj and Seshadri[23]. Concentration of theaflavin and thearubigin was calculated following the method of Roberts and Smith[24].

*Hepatocyte isolation*—Hepatocytes were aseptically isolated in a laminar flow hood of tissue culture laboratory from mice livers following the method of Sarkar and Sil[25] with some modifications. Animals were anaesthetized in ether, sacrificed and livers were collected. After collection the organs were extensively perfused *in situ* in phosphate buffer saline to get rid of blood and irrigated in a buffer containing Hepes (10 m*M*), KCl (3 m*M*), NaCl (130 m*M*), $NaH_2PO_4$-$H_2O$ (1 m*M*) and glucose (10 m*M*) *p*H 7.4 and incubated with a second buffer containing $CaCl_2$ (5 m*M*), 0.05% collagenase type I mixed with the buffer previously described for about 45 min at 37ºC. Liver sample was then passed through wide bore syringe, filtered, centrifuged and the pellet was suspended in DMEM containing FBS (10%) and the suspension was adjusted to obtain ~$2 \times 10^6$ cells/ml.

*Determination of time and dose-dependent effect of TBHP*—Time and dose-dependent effect of TBHP was determined by cell viability assessment. 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 μ*M*) for 180 min to determine the maximum damage caused by TBHP exposure. For time-dependent study, 6 different sets of hepatocytes (1 ml cell suspension ~$2 \times 10^6$ cells in each) were exposed to TBHP (500 μ*M*) for different times (30 min, 1, 1.5, 2, 2.5 and 3 h). The cell viability was determined and expressed as a percentage of the corresponding control as described by Madesh and Balasubramanian[26].

*Assessment of dose and time dependent activity of KT*—Cell viability assessment has been carried out to determine the optimum dose and time of KT needed for the cytoprotection against TBHP-induced cyto-pathophysiology. Briefly, for dose-dependent study, 6 different sets of hepatocytes, each containing about $2 \times 10^6$ cells were exposed to 500 μ*M* TBHP along with KT (10, 20, 30, 40, 50 and 60 μl) for 2 h. For time-dependent study, 6 different sets of hepatocytes (1 ml cell suspension ~$2 \times 10^6$ in each) were incubated with KT (40 μl) for different times (30 min, 1, 1.5, 2, 2.5 and 3 h). The cell viability was determined and expressed as described above.

*Experimental setup*—Based on the results of the dose and time dependent effects of both TBHP and

KT, *in vitro* experiments were designed with different sets of hepatocytes containing 1 ml suspension (~2 × $10^6$ cells) in each. Cells were incubated in a $CO_2$ incubator at 37ºC throughout the experiment with gentle shaking. The hepatocytes kept in culture medium only, served as normal control (marked as "Cont"). Hepatocytes (~2 × $10^6$ cells) incubated with KT (40 µl) alone for 2 h served as a group showing the effect of KT on hepatocytes (marked as "KT"). Hepatocytes (~2 × $10^6$ cells) incubated with 500 µ*M* 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 "KT+TBHP"). Hepatocytes (~2 × $10^6$ cells) were incubated with BT (40 µl) and TBHP simultaneously to study the effect of BT (marked as "BT+TBHP").

*Determination of ALT and LDH Leakage*—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 g. Secretion of ALT and LDH outside the cells was determined from the supernatant using a kit and following the methods as described elsewhere[27].

*Determination of protein content*—Protein content was measured by the method of Bradford[28] using crystalline BSA as standard.

*Determination of intracellular ROS production*—Intracellular ROS production was estimated by using 2,7-dichlorofluorescein diacetate (DCFDA) as a probe following the method of Manna *et al*.[29]. The formation of DCF was measured at the excitation wavelength of 488 nm and emission wavelength of 610 nm for 10 min by using fluorescence spectrometer (HITACHI, Model No F4500) equipped with a FITC filter.

*Assay of antioxidant power of hepatocytes: Ferric Reducing/Antioxidant Power (FRAP) assay*—Antioxidant power of hepatocytes under various experimental conditions has been evaluated by FRAP assay[30]. Basically, it measures the change in absorbance at 593 nm due to the formation of a blue colored $Fe^{II}$-tripyridyltriazine compound from the colorless oxidized $Fe^{III}$ form by the action of electron donating antioxidants.

*Estimation of lipid peroxidation end products*—Lipid peroxidation in terms of malondialdehyde (MDA) formation was assessed by a colorimetric reaction as described by Sinha *et al*.[31]. Absorbance was measured at 535 nm and MDA content was calculated using extinction coefficient of MDA which is 1.56 × $10^5$ $M^{-1}cm^{-1}$. All experiments were done in triplicates under the same conditions. For positive control, hepatocytes were incubated with vitamin C instead of KT.

*Estimation of protein carbonyl content*—Protein carbonyl contents were determined according to the methods of Sinha *et al*.[32]. Results were expressed as nmol of DNPH incorporated/mg protein based on molar extinction coefficient of 22000 $M^{-1}cm^{-1}$ for aliphatic hydrazones.

*Assay of antioxidant enzymes*—Activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase ($GP_x$) were measured following the method of Sinha *et al.*[33].

**Assay of cellular metabolites**

*GSH assay*—Glutathione (GSH) levels were measured according to the method of Ellman[34] by using DTNB (Ellman's reagent) as the key reagent and the absorbance was measured at 412 nm. A standard curve was drawn using different known concentrations of GSH solution and GSH contents of the experimental samples were calculated from the curve.

*GSSG assay*—Glutathione disulfide (GSSG) was assessed by measuring its level using a kit from Calbiochem, USA following the method of Manna *et al*.[35]. The absorbance of the sample was measured at 412 nm.

**Detection of the nature of cell death by flowcytometry (FACS)**

Dual parameter FACS analysis allows for the discrimination between viable, apoptotic and necrotic cells. After appropriate treatments, 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 CellQuest software. A dot plot of PI-fluorescence (y-axis) versus FITC- fluorescence (x-axis) has been prepared.

**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 as described by Das *et al.*[36]. Briefly, the mitochondrial suspension was incubated with 1 μ*M* 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 analysed by Cell Quest software.

*Statistical Analysis*—Values are expressed as mean ± S.D. (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.

## Results

*Time-dependent compositons 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 Figs 1A and 1B respectively. 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.

**Radical scavenging activity of KT and black tea in cell free system**

*DPPH radical scavenging activity*—Results of radical scavenging effect of KT and BT have been summarized in Fig. 2A. Inhibition of 85.73% of free radical formation occurred when 40 μl KT was incubated with DPPH solution whereas only 43.22% inhibition was observed when same volume of BT was used.

*Hydroxyl ($OH^{\cdot}$) and super oxide ($O_2^{\cdot-}$) radicals scavenging power*—The quenching activity of other free radicals (like hydroxyl, superoxide radicals, etc.,) by KT in cell free systems were determined and compared the results with those of BT. Fig. 2B and 2C show that 40 μl KT more effectively scavenged $OH^{\cdot}$ and $O_2^{\cdot-}$ radicals than BT.

*Composition of kombucha tea compared to black tea*—Kombucha tea was analysed using HPLC and the results showed that the main organic acids present in kombucha tea (fermented for 14 days) were acetic acid (peak 2, Fig. 3A) and gluconic acid (peak 1, Fig. 3A) as is evident from the retention time of standard gluconic acid and acetic acid eluting under

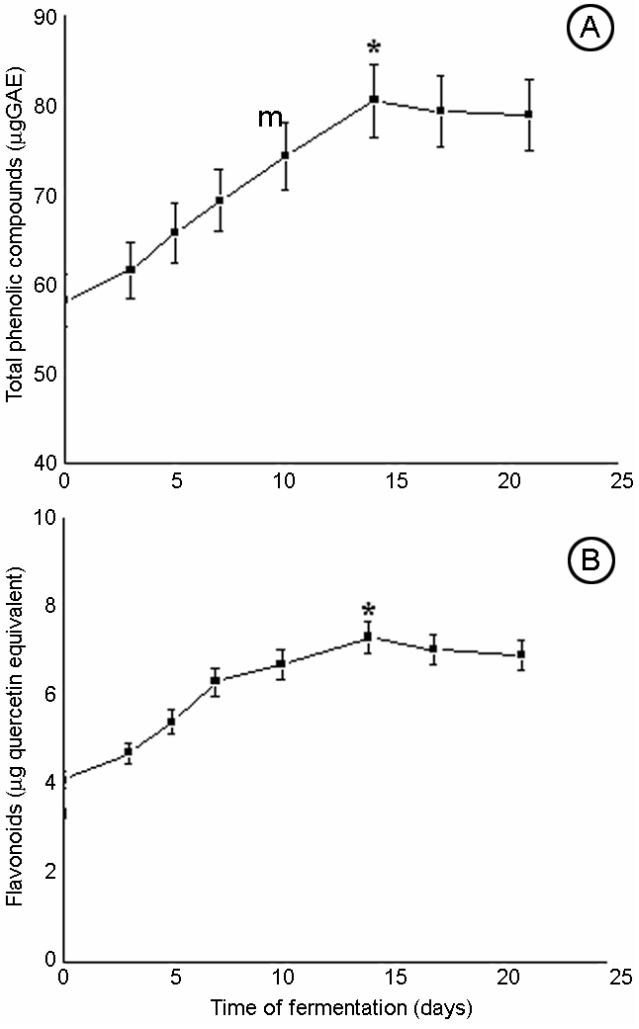


Fig. 1—Concentrations of (A)-phenolic compounds; and (B)-flavonoids during the course of fermentation of KT. [Values are mean ± SD of 6 experiments].

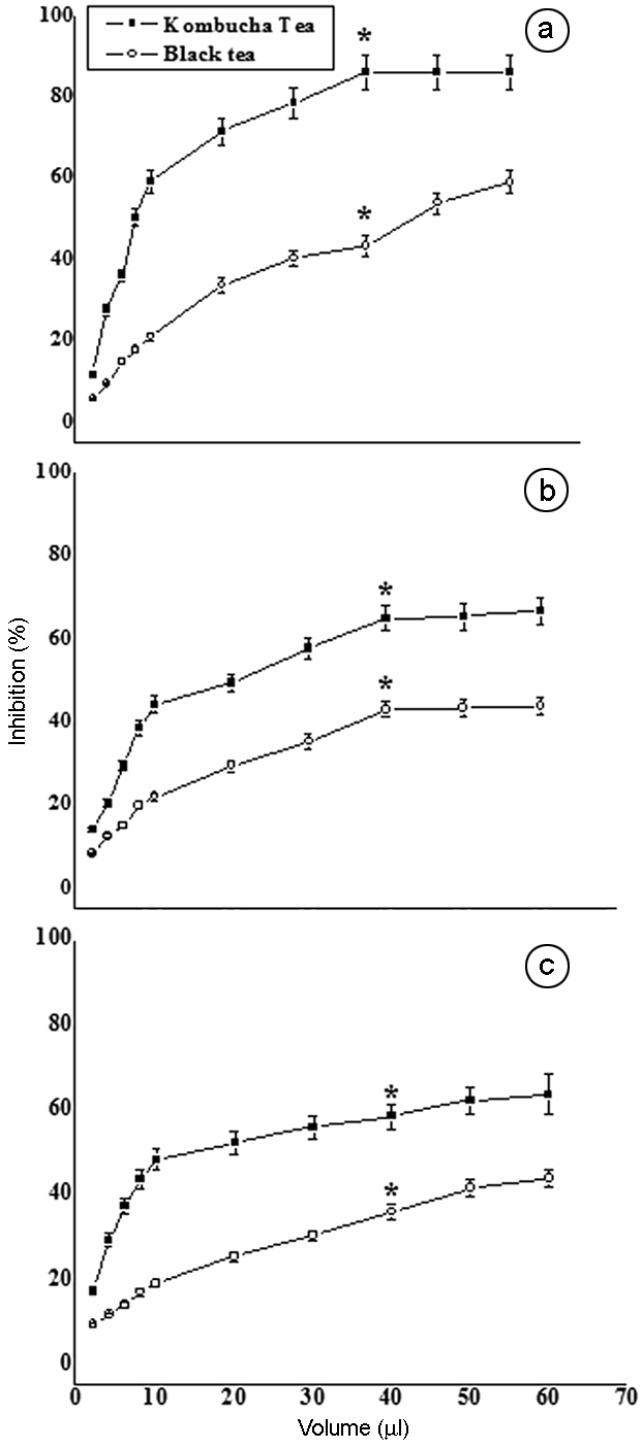


Fig. 2—Free radical scavenging activities of KT and BT in cell free system: (A)-DPPH; (B)-hydroxyl; and (C)-superoxide radical. [Values are mean ± SD of 6 experiments].

the same experimental conditions. In addition, the black tea polyphenols, theaflavins and thearubigins were present in KT (Table 1). DSL (peak 3) was also detected in KT by HPLC analysis at a concentration of 1.34 mg/ml (Fig. 3B). Presence of DSL and the acids was not observed in BT. Generally tea polyphenols, organic acids, glucose, fructose, ethanol and DSL were the primary constituents of KT and that is in good agreement with our analysis.

**Protective action of KT compared to black tea in TBHP-induced oxidative stress mediated cytotoxicity**

*Effect on cell viability*—Effect of KT on cell viability in isolated hepatocytes has been represented in Fig. 4. 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 in a dose and time dependent manner (Fig. 4A and 4B).

*Effect on ALT and LDH*—Significant ALT and LDH leakage was detected in the TBHP treated hepatocytes indicating a loss of cell membrane integrity and cytotoxicity in hepatocytes (Table 2). KT effectively inhibited membrane disruption caused by ROS inducer as revealed from the less ALT and LDH levels outside the cells. BT can also protect cellular membrane damage but not as effectively as KT.

*Effect on ROS production*—Oxidative stress inducers (like TBHP) may cause a significant increase in the formation of ROS (like superoxide anion radical ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide ($H_2O_2$) etc. In order to asses the changes in the production of intracellular ROS under oxidative stress, hepatocytes were isolated from the experimental animals and studied the ROS production assay using DCFDA under a fluorescence microscope and by a fluorescence spectrophotometer as well. Levels of intracellular ROS in normal and experimental hepatocytes are represented in Fig. 5A. It has been observed that TBHP exposure caused increased production of intracellular ROS and that could be prevented by the treatment with KT more efficiently than BT.

*Effect on ferric reducing/antioxidant power (FRAP)*—Antioxidant power of normal and experimental hepatocytes are represented in Fig. 5B. TBHP caused a significant reduction in FRAP value in hepatocytes compared to that level in normal cells. KT increased the cellular antioxidant power more

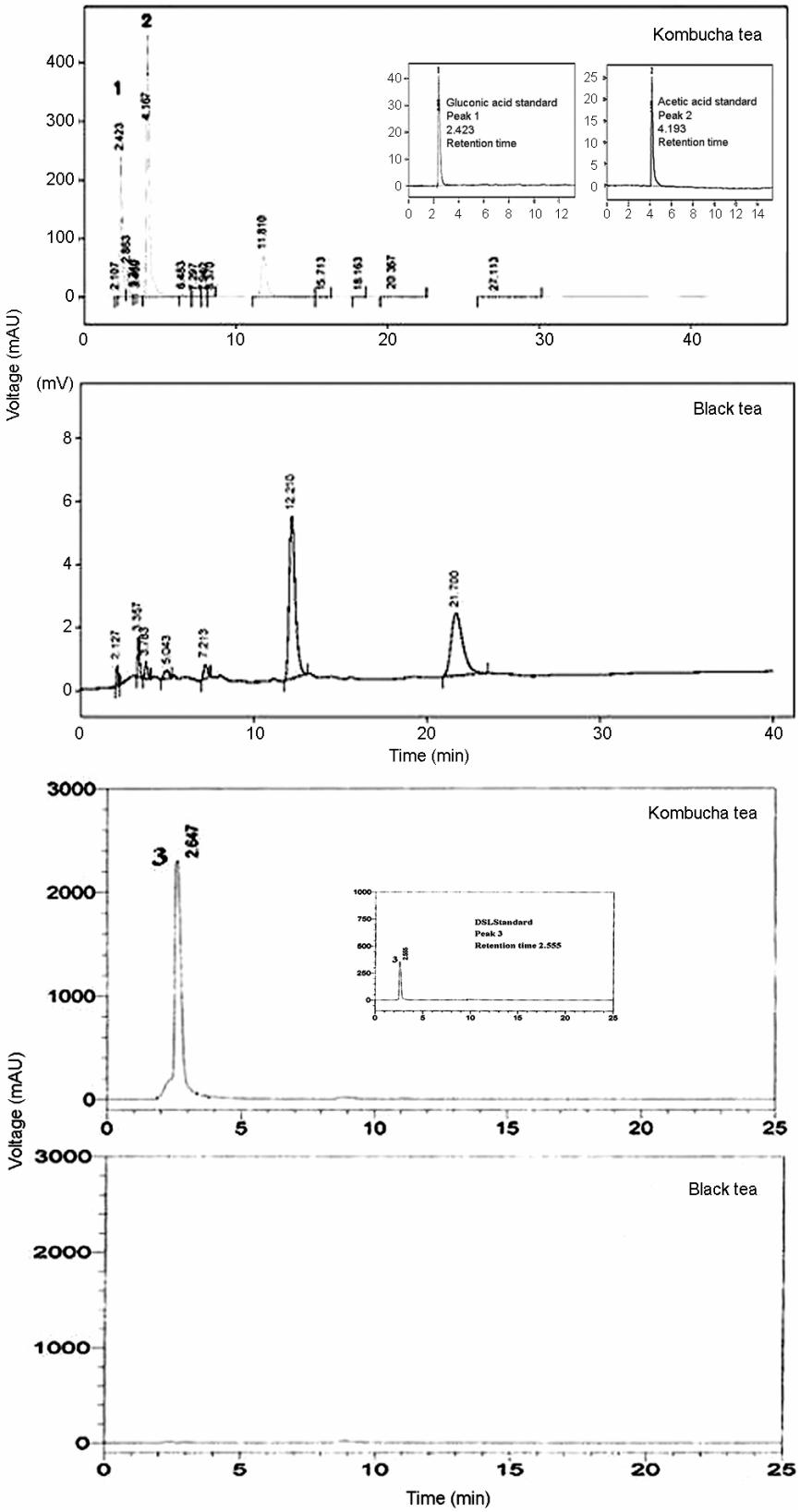


Fig. 3—Reverse phase HPLC analysis of KT and BT. Peak (1)- gluconic acid; Peak (2)- acetic acid; and Peak (3): DSL [respective standard are given as insert].

efficiently than BT when administered in combination with TBHP.

**Enhanced antioxidant properties of KT over black tea**

*Effects on lipid peroxidation, protein carbonyl content*—Increased levels of MDA and protein carbonylation have been observed in TBHP exposed hepatocytes (Table 2). KT was more effective than BT in preventing the TBHP induced alterations of both these parameters.

*Effect on antioxidant enzymes*—Activities of all the antioxidant enzymes were significantly reduced in TBHP exposed experimental hepatocytes and treatment with KT may provide more protection to the first line of cellular defense in oxidative damage induced by TBHP than BT (Table 3).

*Effect on cellular metabolites alterations*—The second line of cellular defense against reactive free radicals and other oxidant species mediated oxidative damage was offered by thiol based antioxidant system. GSH with its –SH group functions as a catalyst in disulfide exchange reaction. It functions by scavenging free radicals as well as detoxifying various xenobiotics and consequently converted to its oxidized form, glutathione disulfide (GSSG). Levels of cellular metabolites like GSH, GSSG and GSH/GSSG ratio have been described in Table 4. GSH level significantly decreased due to TBHP exposure along with the increased level of GSSG. KT could prevent the toxin induced alterations in the intracellular thiol status suggesting its protective role for the second line of cellular defense against free radicals and other oxidant species originated due to TBHP exposure. Moreover, the protective effect of KT was higher than BT. The effect of a known antioxidant vitamin C was included in the above experiments as positive control. Being an antioxidant,

Table 1—Composition of kombucha tea

| Component | Concentration |
|---|---|
| Acetic acid (mg/ml) | 12.53 ± 0.6 |
| Gluconic acid (mg/ml) | 6.38 ± 0.3 |
| Theaflavins (%) | 0.1746 ± 0.01 |
| Thearubigins (%) | 1.9 ± 0.095 |
| D-saccharic acid-1,4-lactone (mg/ml) | 1.34 ± 0.06 |

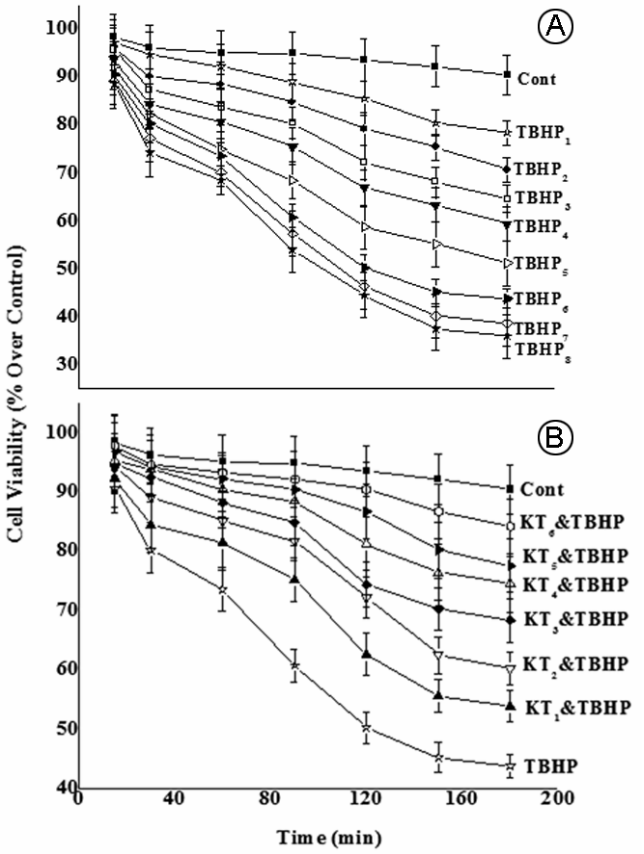


Fig. 4—(A)-Effect of the TBHP-induced loss of cell viability. [$TBHP_1$-$TBHP_8$ indicate TBHP concentration (50, 100, 200, 300, 400, 500, 600 and 700 μ*M*) in DMEM up to 180 min]. (B)-Prevention of the TBHP-induced loss of cell viability by KT. $KT_1$-$KT_6$+TBHP indicate concentration of KT (10, 20, 30, 40, 50 and 60 μl) and TBHP. [Cont- represents control (normal hepatocytes incubated in DMEM up to 180 min). TBHP: represents that hepatocytes were incubated with TBHP (500 μ*M*) in DMEM up to 180 min. Values are expressed as per cent over control. Data are mean ± SD, for 6 experiments per group. Data was analyzed by one-way ANOVA].

Table 2—Effect of TBHP and KT on ALT, LDH, MDA and protein carbonyl levels in normal and experimental hepatocytes

| Treatment groups | ALT* | LDH** | MDA# | Protein carbonyl# |
|---|---|---|---|---|
| Normal Control | 38.37 ± 2.45 | 42.74 ± 2.57 | 10.12 ± 0.5 | 35.12 ± 3.33 |
| KT | 40.41 ± 2.55 | 45.89 ± 2.85 | 12.54 ± 0.6 | 38.54 ± 2.55 |
| TBHP | 70.12 ± 3.55[a] | 110.33± 5.51[a] | 40.55 ± 1.98[a] | 65.78 ± 3.55[a] |
| KT+ TBHP | 49.14 ± 2.81[b] | 60.33 ± 3.01[b] | 16.75 ± 0.8[b] | 44.22 ± 2.81[b] |
| BT+ TBHP | 59.37 ± 2.95[b] | 75.29 ± 3.05[b] | 23.15 ± 1.1[b] | 54.15 ± 2.95[b] |
| Vit C+ TBHP | 45.89 ± 2.68[b] | 59.14 ± 2.75[b] | 15.57 ± 0.7[b] | 40.27 ± 2.68[b] |

* IU/L; ** U/L; # nmol/mg protein.

[Values are mean ± SD for 6 sets of experiments per group. [a]differ significantly from normal control ($P^a$<0.05); [b]significant differences between TBHP treated groups and KT, BT and Vit C treated groups ($P^b$<0.05)].

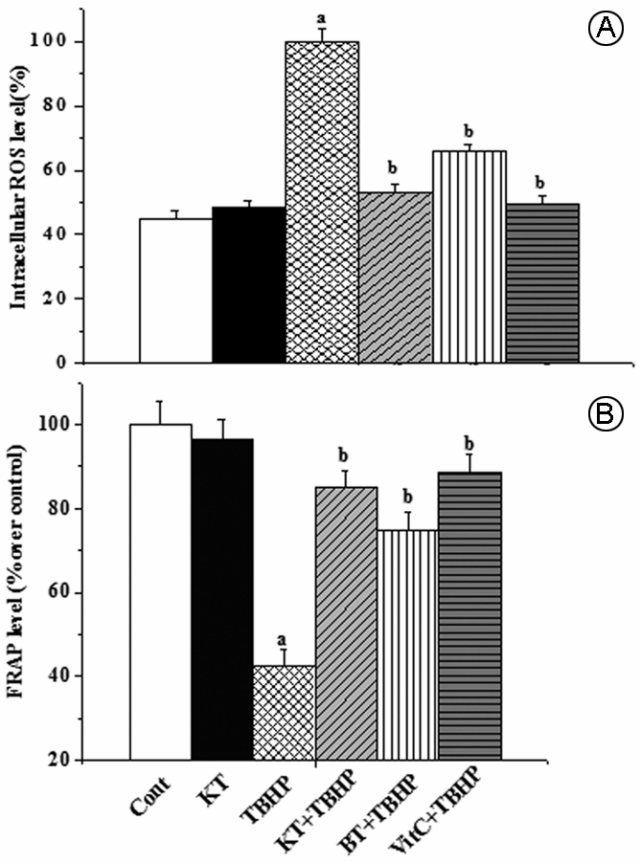


Fig. 5—(A)-Effect of KT and BT on TBHP-induced intracellular ROS production in hepatocytes (Average fluorescence intensity as calculated using Metamorph software); and (B)- Effect of KT and BT on intracellular antioxidant activity (FRAP) in hepatocytes treated with TBHP. [Cont-normal hepatocytes; KT-hepatocytes treated with KT; TBHP-hepatocytes exposed to TBHP; KT+TBHP-hepatocytes exposed to KT and TBHP; BT+TBHP-hepatocytes exposed to BT and TBHP; and VitC+TBHP-hepatocytes treated with vitamin C along with TBHP. Data are mean ± SD for 6 experiments per group. [(a)]indicates significantly different (at $P<0.05$). from normal control; and [(b)]indicate significant differences(at $P<0.05$) between TBHP treated groups and KT, BT and Vit C treated groups].

Table 3—Antioxidant enzyme activities in normal and experimental hepatocytes

| Treatment Groups | SOD* | CAT** | GST[#] | GR** | GPx** |
|---|---|---|---|---|---|
| Normal Control | 112.7 ± 5.5 | 180.15 ± 8.4 | 3.33 ± 0.15 | 190.35 ± 9.2 | 94.68 ± 4.5 |
| KT | 111.49 ± 5.4 | 179.49 ± 8.2 | 3.23 ± 0.14 | 188.25 ± 9.13 | 93.45 ± 4.46 |
| TBHP | 66.6 ± 3.16[a] | 48.78 ± 2.1[a] | 1.74 ± 0.07[a] | 125.07 ± 6.2[a] | 39.31 ± 1.8[a] |
| KT+ TBHP | 105.69 ± 5.12[b] | 174.87 ± 7.9[b] | 3.01 ± 0.13[b] | 188.7 ± 9.15[b] | 89.54 ± 4.2[b] |
| BT+ TBHP | 80.4 ± 3.9[b] | 125.84 ± 5.84[b] | 2.33 ± 0.11[b] | 175.45 ± 8.5[b] | 64.21 ± 3.12[b] |
| Vit C+ TBHP | 84.39 ± 4.17[b] | 155.39 ± 7.3[b] | 2.41 ± 0.12[b] | 183.36 ± 9.06[b] | 80.84 ± 3.98s[b] |

*Unit/mg protein; [#]μmol/min/mg protein; **nmol/min/mg protein. Further details are as represented in table 2.

vitamin C may prevent TBHP-induced ROS mediated cellular oxidative dysfunction**.**

*TBHP-induced cell death and protective role of KT*—Mode of hepatocytes death using flow cytometric analysis was investigated by a double labeling technique using Annexin V/PI. 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. Flowcytometric data (Fig. 6) revealed that, in comparison with control untreated hepatocytes, TBHP increased the number of Annexin V staining hepatocytes but very little PI binding, indicating majority of cells death via apoptotic pathway. Treatment with KT shows less number of apoptotic cells, indicating that KT treatment protected the TBHP-induced apoptotic cell death. On the other hand, BT could also protect TBHP-induced apoptotic cell death but to a lesser extent.

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed in the liver mitochondria of TBHP exposed hepatocytes. $\Delta\Psi_m$ was decreased in mitochondria isolated from the hepatocytes exposed with TBHP (Fig. 7). Administration of KT prevented this TBHP-induced loss in $\Delta\Psi_m$ more efficiently than BT. This indicates better membrane stabilizing effect of KT than BT.

## Discussion

The protective effect of tea polyphenolic compounds against various organ pathophysiology has already been reported[37-40]. Most of the beneficial

Table 4—Status of the thiol-based antioxidant in normal and experimental hepatocytes

| Treatment Groups | GSH* | GSSG* | Redox ratio (GSH/GSSG) |
|---|---|---|---|
| Normal Control | 10.21 ± 0.51 | 0.19 ± 0.012 | 53.73 ± 2.69 |
| KT | 10.11 ± 0.5 | 0.188 ± 0.01 | 56.16 ± 2.8 |
| TBHP | 5.09 ± 0.25[a] | 0.32 ± 0.016[a] | 15.9 ± 0.8[a] |
| KT+TBHP | 8.28 ± 0.4[b] | 0.18 ± 0.01[b] | 48.7 ± 2.4[b] |
| BT+TBHP | 7.12 ± 0.39[b] | 0.22 ± 0.013[b] | 40.76 ± 2.3[b] |
| VitC+ TBHP | 8.89 ± 0.44[b] | 0.171 ± 0.01[b] | 52.29 ± 2.63[b] |

*nmol/mg protein. Further details are as represented in table 2.

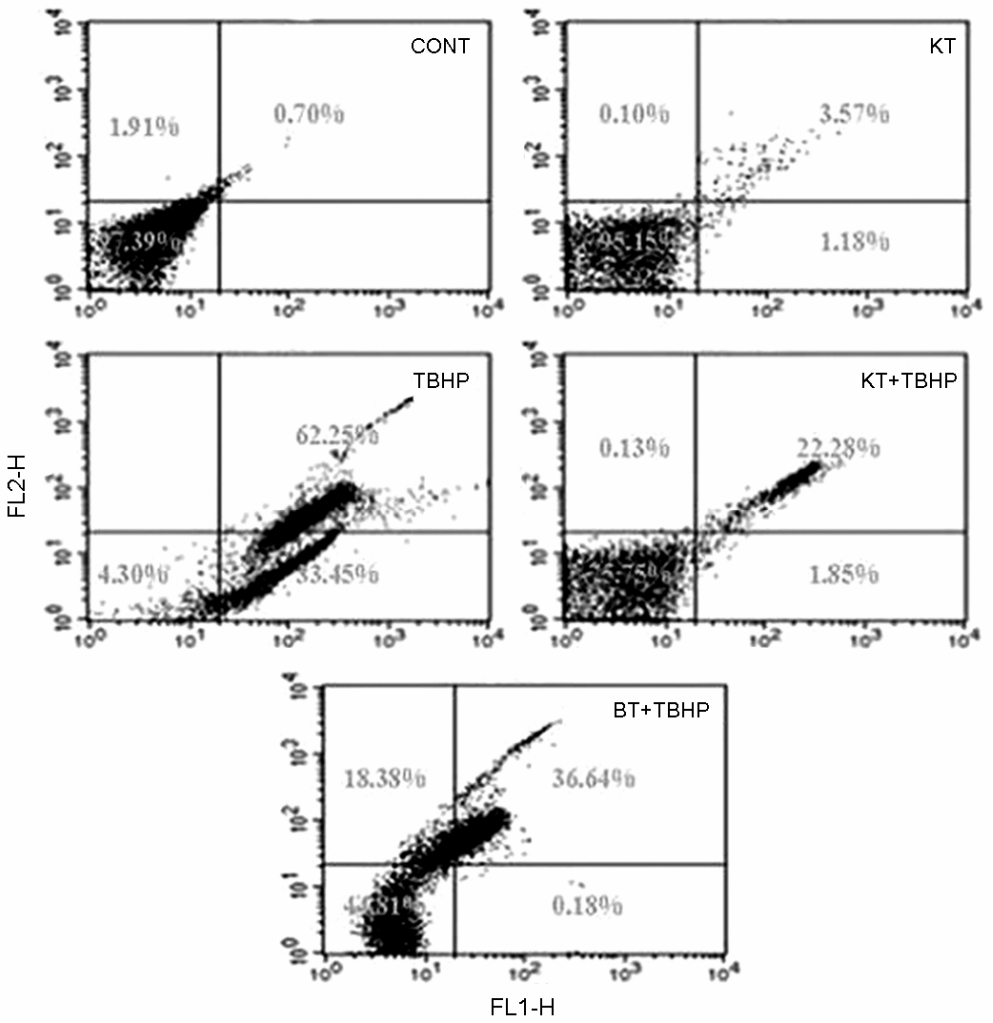


Fig. 6—Flow cytometric analysis of hepatocytes. [Cont-hepatocytes from normal animals; KT-hepatocytes from the animals treated with KT; TBHP-hepatocytes from TBHP intoxicated animals; KT+TBHP-hepatocytes from the animals after exposure to KT and TBHP; BT+TBHP-hepatocytes from the animals after exposure to BT and TBHP. [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 represents value of three independent experiments].

effects of tea have been attributed to the antioxidant and free-radical scavenging properties of its components like polyphenols and flavonoids[41]. KT is a sugared black tea fermented with a symbiotic association of acetic acid bacteria and yeasts for about 14 days. Recent studies suggest that KT prevents carbon tetrachloride-induced hepatotoxicity[42] and exerts hypocholesterolaemic effects in high cholesterol fed mice[43] because of its antioxidant properties. Moreover, the results of the present study suggest that KT contains more polyphenols and flavonoids than black tea itself (Fig. 1A & 1B). The chemical composition of KT has been already reported[44,45] and in this study KT has been shown to contain organic acids like acetic acid, gluconic acid and tea polyphenols like theaflavins, thearubigins. Yoshino *et al*. reported the antioxidative effects of theaflavins and thearubigins against TBHP-induced lipid peroxidation in rat liver homogenates[7]. In this study the presence of D-saccharic acid-1,4-lactone has been detected in KT which is absent in BT. This compound is considered to be the most healthful and crucial functional component found in KT[43,46-47]. According to Olas *et al.*[48] DSL possesses protective effects against oxidative/nitrative modifications of plasma proteins and blood platelets[49]. Moreover, a

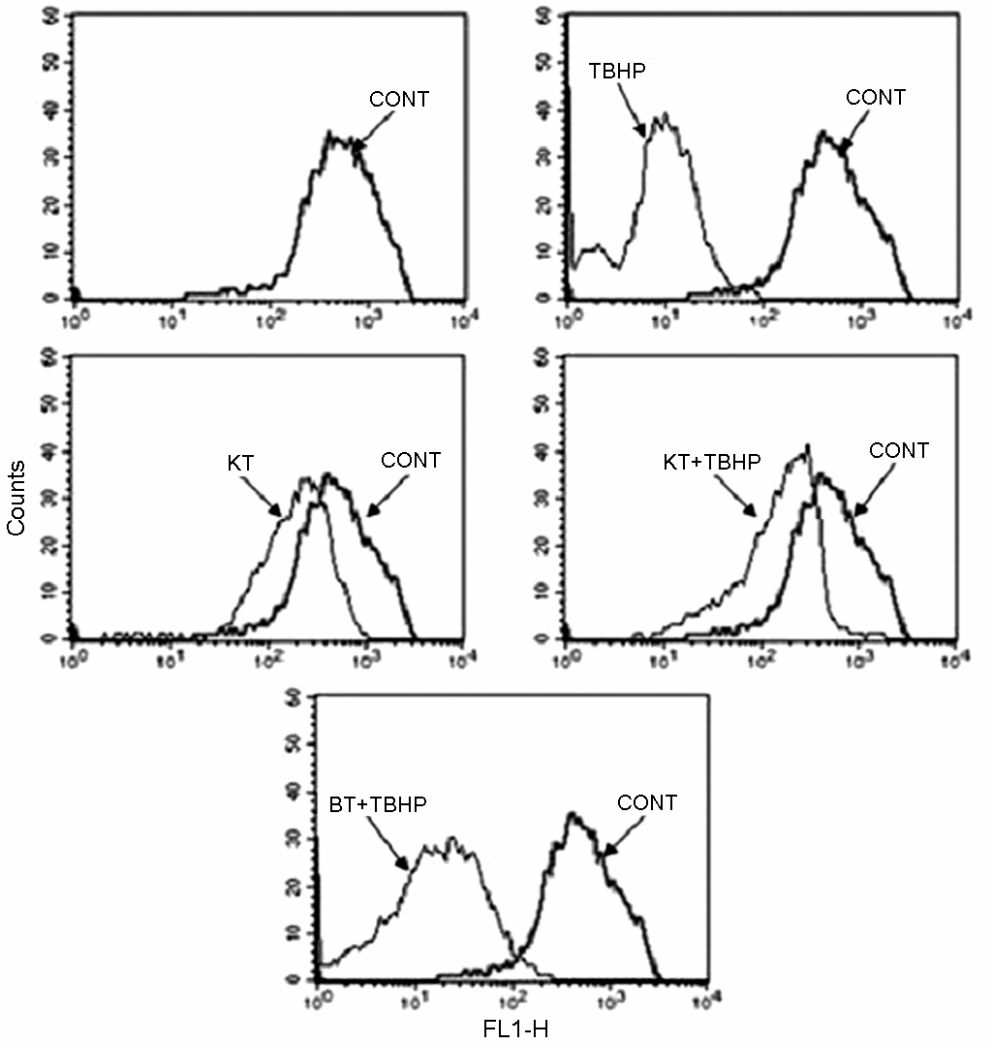


Fig. 7—Mitochondrial membrane potential in the hepatocytes by flow cytometry analysis. Figure represents the histogram plot. [Cont-normal control; KT-KT treated hepatocytes; TBHP-hepatocytes exposed to TBHP; KT+TBHP- hepatocytes exposed to KT and TBHP; and BT+TBHP-hepatocytes exposed to BT and TBHP. [Measurements were taken 6 times].

combination of DSL and phenolic compounds augments the antioxidative property of DSL[50]. This might be one of the reasons for the enhanced antioxidative and cytoprotective activity of KT over BT.

In the present study, antioxidant activity of KT in cell free system was compared with BT by DPPH radical scavenging assay. In addition, hydroxyl and superoxide radicals scavenging activity of KT were also compared with black tea in cell free system. Results of these studies clearly established KT as a better radical scavenger than BT due to the presence of high content of phenolic compounds. Therefore, KT may alter the toxic effects of TBHP by quenching the excessive free radicals produced in hepatocytes by this free radical inducer.

Generation of large amount of reactive oxygen species (ROS) due to TBHP toxicity can overwhelm the antioxidant defense mechanism and damage cellular ingredients such as lipids, proteins and DNA; this in turn can impair cellular structure and function. Evidence suggests that various enzymatic and non-enzymatic systems have been developed by the cell to cope up with the ROS and other free radicals. Antioxidant enzymes like SOD, CAT, GST, GR and GPx are considered to be the first line of cellular defense that prevents cellular ingredients from oxidative damage. Among them SOD and CAT are the most important enzymes against the toxic effects of oxygen metabolism. SOD quenches $O_2^-$ into $H_2O_2$

and $H_2O$[51]. CAT accelerates the dismutation reaction of $H_2O_2$ followed by the formation of $H_2O$ and $O_2$[52]. GR and GPx also maintain the intracellular redox status[53]. Thus, to eliminate free radicals, these cellular antioxidants play an important role and an equilibrium exists between these enzymes under normal physiological conditions. When excess free radicals are produced due to some toxin exposure, this equilibrium is lost and consequently oxidative insult is established. In the present study we observed that TBHP exposure increased the rate of DCF formation, an indicator of intracellular ROS production. There is also a greater degree of lipid peroxidation and protein carbonylation in the TBHP-exposed hepatocytes. The loss of membrane integrity is evident from the study of ALT and LDH leakage. We observed that incubation of hepatocytes with TBHP caused enhancement of ALT and LDH leakage with a concomitant reduction in the percent of cell viability compared to the normal cells. In addition, this potent ROS inducer caused significant decrease in SOD, CAT, GST, GR and GPx activities. On the other hand, present investigation showed that hepatocytes treated with KT in combination with the toxin displayed a reduction in ROS generation, prevented the alterations in the activities of antioxidant enzymes and attenuated MDA and protein carbonyl levels more efficiently than BT.

Thiol-based antioxidant system plays second line of cellular defense against reactive free radicals and other oxidant species mediated oxidative damage. GSH with its –SH group functions as a catalyst in disulfide exchange reaction. It functions by scavenging the free radicals as well as detoxifying various xenobiotics and consequently converts to its oxidized form, glutathione disulfide (GSSG)[54]. Thus depletion of GSH is associated with an increase in GSSG concentration thereby reducing the GSH/GSSG ratio. Results of this study suggest that administration of either KT or BT along with the toxin inhibited GSH depletion, decreased the level of enhanced GSSG due to TBHP and thus normalized the GSH/GSSG ratio. But the effect of KT was higher than that of BT.

Further evidence in TBHP-induced oxidative stress and the protective role of KT (as an antioxidant in this pathophysiology) came from the results of FRAP assay. Cellular antioxidant or reducing potential using FRAP assay showed that incubation of hepatocytes with TBHP reduced the cellular antioxidant power, as indicated by lower FRAP value compared to that in the normal hepatocytes. Treatment of hepatocytes with KT or BT increased the cellular antioxidant power by enhancing the FRAP value compared to the toxin control. The protective effect of KT is, however, more than that of BT.

Results from FACS analysis suggested that TBHP induced cell death occurs mostly via the apoptotic pathway which can be prevented by KT supplementation. Moreover, the reduction in mitochondrial membrane potential due to overproduction of ROS was also normalized when KT was administered along with TBHP to the hepatocytes.

In conclusion, incubation of hepatocytes with TBHP caused an alteration in the cellular antioxidant status. Treatment of KT in combination with this ROS inducer kept the antioxidant status quite similar to that of normal hepatocytes and protected them from TBHP induced damage. BT may also ameliorate the toxic effect of TBHP but to a lesser extent. Therefore, KT provides significant protection of hepatocytes in TBHP-induced oxidative insult and cell death compared to BT. Further work is required to fully characterize the active principle(s) present in KT and elucidate its possible mode of action.

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