Liv.52 protects HepG2 cells from oxidative damage induced by tert-butyl hydroperoxide

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Abstract Oxidative stress induced by toxicants is known to cause various complications in the liver. Herbal drug such as Liv.52 is found to have hepatoprotective effect. However, the biochemical mechanism involved in the Liv.52 mediated protection against toxicity is not well elucidated using suitable in vitro models. Hence, in the present study, the hepatoprotective effect of Liv.52 against oxidative damage induced by tert-butyl hydroperoxide (t-BHP) in HepG2 cells was evaluated in order to relate in vitro antioxidant activity with cytoprotective effects. Cytotoxicity was measured by MTT assay. Antioxidant effect of Liv.52 was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ferric-reducing antioxidant power (FRAP) assay, and lipid peroxidation and measurement of non-enzymic and antioxidant enzymes in HepG2 cells exposed to t-BHP over a period of 24 h. The results obtained indicate that t-BHP induced cell damage in HepG2 cells as shown by significant increase in lipid peroxidation as well as decreased levels of reduced glutathione (GSH). Liv.52 significantly decreased toxicity induced by t-BHP in HepG2 cells. Liv.52 was also significantly decreased lipid peroxidation and prevented GSH depletion in HepG2 cells induced by t-BHP. Therefore, Liv.52 appeared to be important for cell survival when exposed to t-BHP. The protective effect of Liv.52 against cell death evoked by t-BHP was probably achieved by preventing intracellular GSH depletion and lipid peroxidation. The results showed protective effect of Liv.52 against oxidative damage induced in HepG2 cells. Hence, taken together, these findings derived from the present study suggest the beneficial effect of Liv.52 in regulating oxidative stress induced in liver by toxicants.

Keywords Liv.52 · Liver · Oxidative stress · HepG2 cells · tert-butyl hydroperoxide · Hepatoprotection

Introduction

Oxidative stress may contribute to the development of several age-related and chronic diseases such as cancer, diabetes, neurodegenerative, and cardiovascular diseases [1–3]. In particular, with respect to liver diseases such as hepatocellular carcinoma, viral and alcoholic hepatitis and non-alcoholic steatosis, it is known that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a crucial role in disease induction and progression [4, 5]. The liver is particularly susceptible to toxicants. The absorbed drugs and xenobiotics can cause ROS- and free radical-mediated inflammatory and fibrotic liver disorders [6]. Since oxidative stress plays a central role in liver diseases pathology, herbal therapeutic agents to counteract liver damage will be of immense importance [5]. Several epidemiological and toxicological studies using in vivo and in vitro models suggest that Liv.52 plays a pivotal role in detoxification of xenobiotics from liver [7–13]. Several epidemiological studies have shown that diets rich in fruits and vegetables and other plant foods (including tea and wine) tend to reduce the risk of premature death and mortality from chronic diseases, such as cardiovascular diseases and some types of cancer [14, 15]. Liv.52, which is rich in phenolic compounds, and in particular polyphenols, is believed to be, at least in part, responsible for such beneficial effects. Few studies, however, address the biological effects of Liv.52, and the ones performed using

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cellular and in vivo models indicate a poor correlation between the antioxidant potency of Liv.52 measured in vitro and the biological activity of the drug. The biological effect of active constituents of Liv.52 and their in vivo circulating metabolites will ultimately depend on their cellular uptake. HepG2 cells, a human hepatoma cell line, are considered a good model to study in vitro xenobiotic metabolism and toxicity to the liver, since they retain many of the specialized functions, which characterize normal human hepatocytes. In particular, HepG2 cells retain the activity of many phase I, phase II, and antioxidant enzymes ensuring that they constitute a good tool to study cytoprotective, genotoxic, and antigenotoxic effects of compounds [16, 17].

In this study, we evaluated hepatoprotective effect of Liv.52 against t-BHP induced oxidative damage in HepG2 cells by measuring anti-oxidant and cytoprotective activities of Liv.52. The concentration of Liv.52 that protects 50% (IC_{50}) cells from tert-butyl hydroperoxide (t-BHP)-induced cell death was determined. Subsequently, IC_{50} values of Liv.52 were used to evaluate the effects of Liv.52 on several markers of oxidative damage, such as intracellular glutathione, lipid peroxidation, and glutathione-related enzyme such as glutathione peroxidase (GPox) in HepG2 cells treated with and without t-BHP.

Materials and methods

Materials

Bradford reagent, cytochrome-C, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dubellco’s Minimum Essential Medium Eagle (DMEM), ferric chloride (anhydrous), Fetal bovine serum (FBS), glutathione, hydrogen peroxide, MTT, and NADPH were from Sigma-Aldrich (St. Louis, MO, USA). t-BHP, thiobarbituric acid, xanthine and xanthine oxidase were purchased from BDH, India. All other reagents were of analytical grade. Liv.52 was received from the Distribution Unit, Himalaya Drug Company, Bangalore, India. The composition of the drug has been described elsewhere [10, 13].

Determination of total phenolic content

The amount of total phenolic content (TPC) was determined and used in the subsequent experiments to evaluate the protective effect of the Liv.52. HepG2 cells treated with and without t-BHP and Liv.52 were fixed with

Antioxidant activity

The free radical scavenging (antiradical) activity of Liv.52 was studied with the stable free radical DPPH. For DPPH scavenging activity, after addition of Liv.52 to DPPH (90 µM), the percentage of remaining DPPH was determined at different con of Liv.52 at 515 nm using a 96-well plate reader. As suggested by Sanchez-Moreno et al. [19], the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration (IC_{50}) was expressed in terms of initial concentration of DPPH.

FRAP assay

The ferric-reducing antioxidant power (FRAP) of Liv.52 was determined as described previously [20] with slight modifications. one milliliter of different con of Liv.52 was added to 2.5 ml PBS and 2.5 ml of potassium ferriyanide (1% w/v). The mixture was incubated at 50°C for 20 min. 2.5 ml trichloroacetic acid solution (10% w/v) was added to the mixture to stop the reaction. The mixture was then separated into 2.5 ml aliquots and each was diluted with 2.5 ml water. To each diluted aliquot, 500 µl ferric chloride solution (0.1% w/v) was added and allowed to stand for 30 min for color development. Absorbance was measured at 700 nm in triplicate and was used to calculate the gallic acid equivalents (GAE) and expressed as mg GAE.

Cell culture

HepG2 cells were obtained from the National Center for Cell Science (NCCS) Pune, India. Cells were cultured in DMEM containing 10% FBS and maintained at 5% CO₂ at 37°C as described previously [13].

Assay for t-BHP cytotoxicity and protection by Liv. 52

HepG2 cells were cultured in 96-well plates at 1 × 10^5 cells per well over night. In order to study t-BHP cytotoxicity, the medium was discarded and fresh medium containing t-BHP at various concentrations was added. Cells were treated with different con of t-BHP and incubated for 24 h to determine the cytotoxicity by MTT assay as described by us previously [13, 21]. In order to determine the concentration of Liv.52 that protects 50% (IC_{50}) of the cells from damage induced by the t-BHP, cells were incubated with 200 µM of t-BHP for 24 h to induce cell significant death. Based on the dose-response curves of cell death protection by Liv.52 against the t-BHP-induced damage in HepG2 cells, the IC_{50} concentrations were determined and used in the subsequent experiments to evaluate the protective effect of the Liv.52. HepG2 cells treated with and without t-BHP and Liv.52 were fixed with
alcohol, Giemsa (5%) stained and observed under microscope (Leica, Germany).

Effects of t-BHP and Liv.52 on lipid peroxidation, glutathione levels, and antioxidant enzyme activities in HepG2 cells

HepG2 cells were plated in 60 mm culture plates at $7.5 \times 10^5$ cells/well. Forty hours after plating, the medium was discarded and fresh medium containing 200 $\mu$M t-BHP and Liv.52 was added. After 24 h incubation, cell culture medium and cell scrapings were harvested and kept at $-80^\circ$C for quantification of several parameters. After rinsing cell layer with phosphate buffered saline (PBS, pH 7.4), cells were scrapped and harvested in lysis buffer containing 25 mM KH$_2$PO$_4$, 2 mM MgCl$_2$, 5 mM KCl, 1 mM EDTA, 1 mM EGTA, and 100 $\mu$M PMSF (pH 7.5).

Lipid peroxidation

The extent of lipid peroxidation was estimated by measuring malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm as described previously [22]. The results are expressed as nmol/mg of protein using a molar extinction coefficient of $1.56 \times 10^5$ M$^{-1}$Cm$^{-1}$.

Measurement of non-enzymic antioxidants

Cells were homogenized in trichloroacetic acid (5% w/v), and the supernatants were used for determining reduced glutathione (GSH). The glutathione levels from the cell homogenates were determined by the DTNB-GSSG reductase-recycling assay as previously described [23], with some modifications. The results are expressed as nmol GSH/mg of protein.

Measurement of antioxidant enzymes

The activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) were assayed in supernatants of cell homogenates. Total SOD activity was determined by monitoring the inhibition of the reduction of ferricytochrome C at 550 nm, using the xanthine–xanthine oxidase system as a source of superoxide. One unit of the SOD is defined as the amount of the enzyme required to inhibit 50% of the rate of cytochrome-C reduction [24]. Catalase activity was measured by following the rate of H$_2$O$_2$ consumption spectrophotometrically at 240 nm [25] and expressed as $\mu$mol H$_2$O$_2$ oxidized/min/mg protein. Glutathione peroxidase activity was determined by following the enzymatic NADPH oxidation at 340 nm [26].

Statistical analysis

Statistical significances were determined by one-way ANOVA and employing Tukey Kramer post test using Graphpad Prism 4. Data are presented as Mean $\pm$ SEM. $P < 0.5$ was taken as significant.

Results

Total phenolic content (TPC) and antioxidant activity of Liv.52

Figure 1 shows the level of TPC in Liv.52. It is evident that phenolic content showed linearity with the concentration of Liv.52 tested. The free radical scavenging activity (DPPH) was determined and the results showed linear antiradical activity at the lower concentration of Liv.52 (1 and 2%) and at higher concentration the activity did not show linearity (Fig. 2A). The results also showed that Liv.52 at 2% could effectively scavenge 50% of the original DPPH content (Fig. 2B) and the same concentration was effective in bringing about cytoprotective effect in HepG2 cells as observed from the cytotoxicity experiments performed in this study. The FRAP assay showed that at lower concentrations of Liv.52 there was no significant difference in the antioxidant activity (Fig. 2C). However at higher con of Liv.52 the activity was increased by 2 folds (Fig. 2C).

Cytotoxicity

Cytotoxicity of t-BHP and Liv.52 on HepG2 cells was evaluated by MTT assay. Liv.52 did not present any cytotoxic effect at concentration ranging from 1–5% (data not shown). On the other hand t-BHP was tested for its...
Cytotoxicity with wide range of concentration for 24 h. t-BHP has evoked a concentration dependent toxicity in HepG2 cells and ≥95% toxicity was attained at 200 μM (Fig. 3A). Photomicrography analysis showed some morphological changes in HepG2 cells due to the toxic effect of t-BHP. Such morphological changes observed in these cells were reduced by Liv.52 and also by Vitamin C treatment (Fig. 3B). Liv.52 at all the concentrations tested (1–5%) effectively protected 50% of the cells from t-BHP (200 μM) induced toxicity as shown in Figs. 3B and 4A. Thus, 200 μM of t-BHP was used for all subsequent experiments for testing hepatoprotective effect of Liv.52.

Cytoprotective effect of Liv.52

The possible cytoprotection of cells by Liv.52 against t-BHP induced loss of cell viability was evaluated by
pre-incubating the cells with Liv.52 for 30 min, followed by treatment with 200 μM of t-BHP for 24 h. Similarly, cells were co-incubated with Liv.52 and t-BHP together for 24 h. Liv.52 at the different concentrations used to pre-treat the cells significantly abrogated the toxicity induced in the cells by t-BHP ($P < 0.05$, Fig. 4A) Results obtained from the co-incubation procedure also significantly abolished the t-BHP induced cell death ($P < 0.05$, Fig. 4B). However, incubation of both Liv.52 and t-BHP together (co-incubation) with HepG2 showed more effective inhibition of t-BHP mediated cell death than that observed with pre-incubation. We have taken Vitamin C as a control and when cells were treated with Vitamin C (50 μM) showed higher cytoprotection as compared to Liv.52 in t-BHP mediated toxicity (Fig. 4A, B).

**Lipid peroxidation and glutathione content**

Increased lipid peroxidation was observed in HepG2 cells by t-BHP treatment ($P < 0.05$, Fig. 5). Liv.52 restored the increased level of MDA at all the concentrations of Liv.52 tested by inhibiting lipid peroxidation ($P < 0.05$, Fig. 5). Vitamin C significantly reduced the lipid peroxide content in HepG2 cells treated with t-BHP. Liv.52 was as effective as Vitamin C in inhibiting the formation of lipid peroxides (Fig. 5). Similarly, the GSH levels in HepG2 cells were significantly depleted by t-BHP treatment, which was rescued by Liv.52 ($P < 0.05$, Table 1). On the other hand, the cellular GSSG content in HepG2 cells was increased to 4.8 folds by t-BHP and Liv.52 brought down the GSSG levels in t-BHP treated cells as given in Table 1 ($P < 0.05$).

**Antioxidant enzymes**

The effect of Liv.52 on t-BHP treated cells on antioxidant enzymes was evaluated and the results are given in Table 2. Catalase and SOD activities were increased to 1.6 and 12.6 folds in HepG2 cells when exposed to t-BHP. Liv.52 was capable of decreasing the t-BHP mediated increase of catalase and SOD enzymes in these cells by 50% ($P < 0.05$, Table 2). t-BHP partially increased the GPx activity and Liv.52 could only marginally modulate the t-BHP induced GPx levels in HepG2 cells which was not statistically significant (Table 2).
P| Values are Mean ± C176 Liv.52 at 37°C.

HepG2 cells were incubated with 200 µM t-BHP with or without Liv.52 at 37°C for 24 h.

Values are Mean ± SEM of three samples

*P < 0.05 compared to t-BHP treated cells.

Table 1
<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (nmol/mg protein)</th>
<th>GSSG (nmol GSH equiv/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.30 ± 3.76*</td>
<td>1.41 ± 0.21*</td>
</tr>
<tr>
<td>t-BHP</td>
<td>8.56 ± 0.81</td>
<td>6.84 ± 0.46</td>
</tr>
<tr>
<td>Liv.52 (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20.85 ± 2.62*</td>
<td>3.19 ± 0.64*</td>
</tr>
<tr>
<td>2</td>
<td>20.75 ± 2.63*</td>
<td>3.69 ± 0.19*</td>
</tr>
<tr>
<td>3</td>
<td>24.50 ± 3.25*</td>
<td>4.31 ± 0.48*</td>
</tr>
<tr>
<td>4</td>
<td>25.48 ± 2.20*</td>
<td>5.08 ± 0.52*</td>
</tr>
<tr>
<td>5</td>
<td>26.82 ± 5.38*</td>
<td>5.61 ± 0.38</td>
</tr>
</tbody>
</table>

HepG2 cells were incubated with 200 µM t-BHP with or without Liv.52 and the antioxidant enzyme activity was determined as described in “Materials and methods” section.

Values are Mean ± SEM of three samples

*P < 0.05 compared to t-BHP treated cells.

Table 2
<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase a (µmol H2O2 decomposed/min/mg protein)</th>
<th>Gpx b (µmol of NADPH oxidized/min/mg protein)</th>
<th>SOD c (Units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.36 ± 0.11*</td>
<td>11.54 ± 0.74*</td>
<td>7.32 ± 0.60*</td>
</tr>
<tr>
<td>t-BHP</td>
<td>0.60 ± 0.23</td>
<td>13.18 ± 1.10*</td>
<td>92.44 ± 9.29</td>
</tr>
<tr>
<td>Liv.52 (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.47 ± 0.04*</td>
<td>12.42 ± 0.64*</td>
<td>38.20 ± 0.89*</td>
</tr>
<tr>
<td>2</td>
<td>0.52 ± 0.05</td>
<td>11.83 ± 0.36*</td>
<td>34.61 ± 0.96*</td>
</tr>
<tr>
<td>3</td>
<td>0.43 ± 0.11*</td>
<td>12.08 ± 0.38*</td>
<td>24.49 ± 0.98*</td>
</tr>
<tr>
<td>4</td>
<td>0.50 ± 0.06</td>
<td>11.55 ± 0.90*</td>
<td>25.46 ± 1.32*</td>
</tr>
<tr>
<td>5</td>
<td>0.58 ± 0.03</td>
<td>14.37 ± 1.14*</td>
<td>19.0 ± 1.54*</td>
</tr>
</tbody>
</table>

HepG2 cells were incubated with 200 µM t-BHP with or without Liv.52 and the antioxidant enzyme activity was determined as described in “Materials and methods” section.

Values are Mean ± SEM of three samples

*P < 0.05 compared to cells treated with t-BHP.

Rice-Evans suggested that the antioxidant biological activity of biologically active compounds will depend more heavily on the extent to which they associate, interact, and permeate cell membranes than on its antiradical activity alone [27]. In agreement with this, Liv.52 liquid concentrate has a direct correlation between biological and antioxidant activity. The results observed for Liv.52 implicate hydrophilicity as an important factor for this cytoprotective and antioxidant effect. Results showed that in co-incubations of HepG2 cells with t-BHP and Liv.52 showed better protection than when pre-incubated. This difference in the activity of Liv.52 as noticed from the pre-incubation and co-incubation experiments may be due to the varied action of Liv.52 on several cellular components involved in mediating cytotoxicity. Among this, one reason could be the ability of Liv.52 to chelate metal ions as previously described by Rice-Evans on the antioxidant properties of phenolic compounds [27].

Iron chelation appears to be important for the protection against t-BHP induced toxicity, and this could be mediated by intracellular iron. Liv.52 may also indirectly act as antioxidants in cells by modulating the activity of antioxidant, detoxifying, and repairing enzymes as well as enzymes involved in the bioactivation of xenobiotics [28]. In the present study, Liv.52 mediated protection of HepG2 cells exposed to t-BHP toxicant is evident through increased activity of glutathione-related enzymes. On the other hand long-term pre-incubations would still provide opportunity for the drug to interact with antioxidant response elements in the cells resulting in the induction of proteins and enzymes such as antioxidant enzymes.

t-BHP induced cell death was accompanied by increased lipid peroxidation and GSSG levels as well as decreased GSH levels and glutathione-related enzyme activity. The increase in GSSG levels was not in the same range as the decrease in GSH levels. This indicates that t-BHP reduced GSH levels mainly through formation of GSH conjugates rather than oxidation to GSSG. These t-BHP mediated effects on HepG2 cells as observed in our study are in accordance with findings from the previous studies with liver cells [29–41]. However, with regard to HepG2 cells, t-BHP exposure conditions were different among different studies carried out previously [35–40]. Previous reports pointed out that different origins of HepG2 clones, culture medium composition, and cultivation time (age of cells) may affect the experimental outcome based on the differences in the sensitivity of HepG2 cells to drugs [16, 17]. Therefore, it becomes imperative to consider the response(s) of the cells to the toxicant as well as the reaction conditions used for the detection of protective effects of test drugs. In order to explain the mechanisms underlying the observed cytoprotective effects of the tested drug, we further looked at the effects of Liv.52 at IC50 on several
markers of cellular oxidative stress, such as lipid peroxidation, and glutathione levels. It was observed from the present study that t-BHP-induced lipid peroxidation in HepG2 cells was attenuated by Liv.52 at all the tested concentrations and the average inhibitory effect was found to be 60% (65–85%) as par with Vitamin C. A good correlation appears to exist between the hepatoprotective effects and the prevention of lipid peroxidation by Liv.52 in these cells. Hence, from these findings derived from our study it can be speculated that Liv.52 mediated protection of HepG2 cells against toxicity by t-BHP may be modulated by other factors besides the inhibition of lipid peroxidation. In order to support this view, previous reports suggest that t-BHP-induced toxicity might not be mediated by lipid peroxidation [31, 32, 35, 42]. In this regard, Liv.52 treatment significantly replenished GSH levels depleted by t-BHP. It is known that, GSH plays an important role in hepatocytes defence against ROS, free radicals, and electrophilic metabolites [43, 44]. Hence, a severe GSH depletion can make the cells more vulnerable to oxidative damage by oxygen radicals and increases protein thiolation or oxidation of SH groups that may lead to alterations in cellular calcium homeostasis [44]. A sustained increase in cytosolic calcium levels results in activation of enzymes (phospholipases, non-lysosomal proteases, endonucleases) and cytoskeleton damage, which ultimately causes cell death [44]. The decrease in GSH levels has indeed been suggested as one of the primary mechanisms of t-BHP-induced toxicity in liver cells [32, 35, 42] that is generally followed by an increase in the intracellular levels of calcium [30, 33, 35]. Thus, the potential of Liv.52 to maintain GSH at reasonably high levels could be of great importance against t-BHP induced toxicity in HepG2 as observed in the present study. Therefore, the ability of the tested concentrations of Liv.52 in preventing t-BHP-induced GSH depletion by about 40% may be a major contribution to the cytoprotective effect of Liv.52. The protection by Liv.52 against increased GSSG levels in HepG2 cells induced by t-BHP was found effective and the Liv.52 inhibited GSH levels in the cells may be attributed to the formation of GSH conjugates rather than oxidation to GSSG.

In conclusion, in the present study Liv.52 showed protective effects against oxidative damage in HepG2 cells induced by t-BHP. This protective effect of Liv.52 in liver cells as observed in the study could be beneficial against liver diseases where it is known that oxidative stress plays a crucial role. Moreover, protective potential of Liv.52 appears to be dependent on the nature of compounds present in Liv.52 in conjunction with its antioxidant activities. The effect of Liv.52 on protection against t-BHP-induced GSH depletion seems to be an important factor for preserving cell viability. Therefore, taken together, these findings derived from the present study suggest the beneficial effect of Liv.52 in regulating the oxidative stress and damage in liver induced by toxicants.

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References