Liv.52 attenuate copper induced toxicity by inhibiting glutathione depletion and increased antioxidant enzyme activity in HepG2 cells

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ABSTRACT

Altered copper metabolism plays a pivotal role in the onset of several hepatic disorders and glutathione (GSH) plays an important role in its homeostasis. Hepatic diseases are often implicated with decreased content of intracellular GSH. GSH depleted cells are prone to increased oxidative damage eventually leading to its death. Liv.52 is used to treat hepatic ailments since long time. Hence, in the present study the potential cytoprotective effect of Liv.52 against toxicity induced by copper (Cu2+) was evaluated in HepG2 cells. Cu2+ at 750 μM induced cytotoxicity to HepG2 cells as determined by MTT assay. The toxicity was brought about by increased lipid peroxidation, DNA fragmentation and decreased GSH content. But, upon treatment with Liv.52 cell death induced by Cu2+ was significantly abrogated by inhibition of lipid peroxidation by 58% and DNA fragmentation by 37%. Liv.52 increased the GSH content by 74%. Activities of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase were increased by 46%, 22% and 81% respectively in Liv.52 treated cells. Thus, it is apparent from these results that Liv.52 abrogates Cu2+ induced cytotoxicity in HepG2 cells by inhibiting lipid peroxidation and increased GSH content and antioxidant enzyme activity.

1. Introduction

Copper (Cu2+) is an essential trace element in human nutrition. Physiologically it is controlled by well-established homeostatic mechanisms (Linder et al., 1993; Pena et al., 1999). However under certain environmental or genetic conditions, such mechanisms can be altered, leading to accumulation of toxic concentration of Cu2+ in cells (Luza and Speisky, 1996). Cu2+ induced toxicity involves its ability to catalyze the generation of free radicals and/or to directly interact with essential biomolecules, hence Cu2+ sequestering is of vital importance for maintaining cellular integrity. Reduced glutathione (GSH) plays a pivotal role in maintaining the intracellular Cu2+ homeostasis. It is due to the fact that cysteine residues constitute one-third of GSH and is a key structural feature for its ability to bind Cu2+. GSH is likely to be one of the first molecules with which Cu2+ ions interact upon entering cells (Freedman and Peisach, 1989). During such interaction, the tripeptide reduces Cu2+ ions as a Cu–GSH adduct (Jimenez and Speisky, 2000). This adduct forms the principle carrier of Cu2+ to several metalloproteins. GSH on the other hand, via its interaction with Cu2+ ions is also considered to play a role in defining the susceptibility of the cells to excess Cu2+ (Freedman et al., 1986, 1989). To the extent that GSH sequesters redox-active Cu2+ ions, it would prevent these from catalyzing free radical generation, thus serving as a mechanism that protect cells against the deleterious consequences of excessive Cu2+ accumulation. GSH is a potent antioxidant molecule and protects cells by acting directly as a scavenger of free radicals generated during cellular metabolism and serves as cofactor in the GSH-peroxidase-dependent removal of peroxides generated in Cu2+-overloaded cells (Dillard and Tappel, 1984; Meister, 1988; Sokol et al., 1990).

The liver is particularly susceptible to metal induced toxicity since it is the seat of diverse biochemical reactions. The absorbed metal ions in a concentrated form can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic processes (Jaeschke et al., 2002). Liv.52 is used since long time to combat liver disorders claimed for its potential hepatoprotective effects. Several epidemiological and toxicological studies suggest that Liv.52 formulation plays a pivotal role in detoxification of xenobiotics from liver (Husseini et al., 2005; Mitra et al., 2008; Vidyashankar et al., 2010) in humans as well as in animal models. It is shown that diets rich in fruit and vegetables and other plant foods (including tea and wine) are
associated with a decreased risk of chronic diseases, such as cardiovascular diseases and some types of cancer (Stanner et al., 2004; Scalbert et al., 2005). Polyphenols have immense beneficial effects on certain diseases through their potential to scavenge free radicals and neutralize the reactive oxygen species and act as metal ion chelators (Rice-Evans et al., 1997). Liv.52 is rich in phenolic compounds, and in particular polyphenols, are believed to be, at least in part, responsible for such effects (Vidyashankar et al., 2010). Few studies, however, address the biological effects of Liv.52, and the ones performed using cellular and in vivo models indicate a poor correlation between the antioxidant potency and biological activity.

HepG2 cells is considered as 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 antimutagenic effects of compounds (Kossmuller et al., 2004; Mersch-Sundermann et al., 2004). Thus, it is postulated that Liv.52 may rescue liver from metal induced toxicity. Hence, we evaluated hepatoprotective effect of Liv.52 against copper induced toxicity in HepG2 cells.

2. Materials and methods
2.1. Chemicals
Bradford reagent, BCS, BSO, copper sulphate (CuSO4·7H2O), cytochrome-C, 2,2-Diphenyl-1-picryl hydrazyl (DPPH), diphenylamine (DPA), Dulbecco’s Minimum Essential Medium (DMEM), ferric chloride (anhydrous), Fetal bovine serum (FBS), glutathione (GSH), hydrogen peroxide, 3(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), β-Nicotinamide adenine dinucleotide phosphate (β-NADPH), perchloric acid, thiobarbituric acid, xanthine and xanthine oxidase were purchased from Sigma–Aldrich (St. Louis, MO, USA), All other reagents were of analytical grade.

2.2. Composition of Liv.52
The Liv.52 is the approved ayurvedic proprietary medicine by drug regulatory authority Department of AYUSH, Ministry of Health and Family Welfare, Government of India. The each mL of Liv.52 liquid concentrate contains extracts of the following medicinal plants in definite proportions – Himsra (Cichorium intybus) 8 mg, Kasamarda (Teramnus cataphylla) 4 mg, Maqabara (Solanum nigrum) 8 mg, Kasamarda (Casia occidentalis) 4 mg, Bisanjapushpi (Achillea millefolium) 4 mg, Jaivakra (Tamoxis gillicus) 4 mg. The good agricultural and collection practices (GACP) were employed for plants used in the formulation. Plant samples were identified and certified by Botanist and a voucher specimen of each constituent plant has been archived in the herbarium of R&D, The Himalaya Drug Company, Bangalore, India.

2.3. Cell culture
All the experiments were performed using HepG2 cells on 10 passages after thawing. The HepG2 cells (hepatocellular carcinoma cell line) was obtained from the National Center for Cell Science (NCCS) Pune, India, were maintained in culture thawing. The HepG2 cells (hepatocellular carcinoma cell line) was obtained from Bangalore, India.

2.4. Cu2+ induced cytotoxicity and protection by Liv.52
HepG2 cells were plated in 96-multiwell culture plates at 1 x 10^3 cells per well. To study Cu2+ cytotoxicity, 24 h after plating, the medium was discarded and fresh medium containing Cu2+ at various concentrations was added. At different time points, cellular viability was determined by the MIT assay (Mosmann, 1983). In order to determine the concentration of Liv.52 that protects 50% (IC50) of the cells from damage induced by the toxicant, cells were incubated with Cu2+ for 24 h to induce significant cell death. Based on the dose-response curves of cell death protection by Liv.52 against the Cu2+ induced oxidative damage in HepG2 cells, the IC50 concentrations were determined and used in the following experiments to evaluate the protective potential of the Liv.52 on several cellular parameters.

2.5. Effect of Cu2+ and Liv.52 on lipid peroxidation, glutathione levels, DNA fragmentation and antioxidant enzyme activities in HepG2 cells
HepG2 cells were plated in 60 mm culture plates at 7.5 x 10^3 cells per well. Forty hours after plating, the medium was discarded and fresh medium containing Cu2+ and Liv.52 was added. Twenty-four hours later, cell culture medium and cell scrapings were harvested and kept at –80 °C for following quantification of several parameters. Cell scrapings were harvested in lysis buffer (25 mM KH2PO4, 2 mM MgCl2, 5 mM KCL, 1 mM EDTA, 1 mM EGTA, 100 μM PMSF, pH 7.5) after rinsing the cells with PBS, (pH 7.4).

2.6. Lipid peroxidation
The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm (Ohkawa et al., 1979). The results were expressed as nmol/mg of protein using a molar extinction coefficient of 1.56 x 10^5 Mcm⁻¹. nmol.

2.7. DPA assay for DNA fragmentation
The diphenylamine (DPA) reaction was performed by the method of (Paradones et al., 1993). Perchloric acid (0.5 M) was added to the cell pellets containing unct DNA (resuspended in 200 μl of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then two volumes of a solution consisting of 0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v) sulphuric acid, and a 0.5% (v/v) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4 °C for 48 h. The reaction was quantitated spectrophotometrically at 575 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA.

2.8. Non-enzymic antioxidants
Cells were homogenized in trichloroacetic acid (5% w/v), and deproteinized supernatant was used for GSH assay. The glutathione levels from the cell homogenates was determined by the DTNB-GSSG redox cycling assay as previously described (Anderson, 1985) with some modifications. The results are expressed as nmol GSH/mg of protein.

2.9. Antioxidant enzymes
The activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were assayed in 1000g 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 the 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 (Flohe and Otting, 1984). Catalase activity was measured by following the rate of H2O2 consumption spectrophotometrically at 240 nm and expressed as μmol H2O2 oxidized/min/mg protein (Aebi, 1984). Glutathione peroxidase activity was determined by following the enzymatic NADPH oxidation at 340 nm (Ueber and Gunzler, 1984).

2.10. Statistical analysis
Results were expressed as Mean ± SEM. Statistical significances were determined by one-way ANOVA by employing Tukey Kramer post test using Graph Pad prism 4. Results were considered to be significant at P < 0.05.

3. Results
3.1. Cytotoxicity
Cytotoxicity of Cu2+ and Liv.52 in HepG2 cells was evaluated using MIT assay. Liv.52 did not present any cytotoxic effect at concentration ranging from 1% to 5% (Fig. 1A). On the other hand Cu2+ was tested for its cytotoxicity with wide range of concentration for 24 h and the results are given in Fig. 1B. The results showed that Cu2+ (>750 μM) caused significant cytotoxicity to HepG2 cells. Hence 750 μM concentration of Cu2+ was used in all the subsequent experiments for testing hepatoprotective effect of Liv.52.

3.2. Effect of Liv.52 on Cu2+ induced toxicity on HepG2 cell viability
The Liv.52 protection against Cu2+ induced cytotoxicity was evaluated by MITT assay. The cells were incubated with Liv.52
(1%) and Cu²⁺ (750 lM) for 24 h and the cell viability was measured. Liv.52 at 1% effectively protected 62% of the cells against Cu²⁺ induced toxicity as shown in Fig. 1C. The BSO (an inhibitor of GSH synthesis) at 250 lM induced significant toxicity to HepG2 cells. But on the other hand BCS (Cu²⁺ chelating agent) had no effect on the cell viability. BCS when added with Cu²⁺ it significantly resulted in decreased cytotoxicity by 52%.

### 3.3. Effect of Liv.52 on GSH depletion in Cu²⁺ induced toxicity

The effect of Cu²⁺ on GSH and GSSG levels in the HepG2 cell was evaluated and the results are given in Table 1. The results showed that Cu²⁺ significantly depleted the GSH levels by 86% in the cells. The BSO (an inhibitor of GSH synthesis) treated cells resulted in 78% lowered GSH content and the cells were found to be more vulnerable for Cu²⁺ induced toxicity. On the other hand, when cells were exposed to Cu²⁺ along with BCS (Cu²⁺ chelating agent) the GSH level was significantly increased by 42%. Liv.52 significantly replenished the GSH levels by 74% in Cu²⁺ induced toxic conditions. Cu²⁺ significantly increased the GSSG content but the increase in the GSSG content was not proportionate with the depleted GSH. On the other hand Liv.52 effectively reduced the GSSG content in the cells treated with Cu²⁺.

### 3.4. Effect of Liv.52 on lipid peroxidation and DNA fragmentation

The lipid peroxidation was significantly increased by 5.6 folds upon addition of Cu²⁺ in HepG2 cells as shown in Fig. 2A. The cells treated with Cu²⁺ along with BSO showed 5.5 folds higher MDA levels. The Liv.52 addition significantly reduced the lipid peroxidation by 58% compared to toxicant group. Similarly BCS significantly inhibited the lipid peroxidation by 76% in Cu²⁺ treated cells.

The Cu²⁺ significantly increased the DNA fragmentation by 48% compared to control cells as shown in Fig. 2B. The results showed that BSO alone did not cause significant DNA damage to cells, but cells upon incubation with Cu²⁺ the DNA damage was significantly increased by 49%. The DNA damage was inhibited by 37% in cells treated with Cu²⁺ by Liv.52. Similarly the cells treated Cu²⁺ along with BCS showed decreased DNA damage compared to toxicant group.

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (nmol/mg protein)</th>
<th>GSSG (nmol GSH equiv/mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>104.36 ± 8.35*</td>
<td>4.51 ± 1.11*</td>
</tr>
<tr>
<td>Cu²⁺ (750 lM)</td>
<td>14.40 ± 3.22#</td>
<td>16.84 ± 2.60#</td>
</tr>
<tr>
<td>Liv.52 (1%)</td>
<td>100.90 ± 17.85*</td>
<td>3.19 ± 0.64*</td>
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<tr>
<td>BSO (250 lM)</td>
<td>23.38 ± 2.63*</td>
<td>9.95 ± 1.92*</td>
</tr>
<tr>
<td>BCS (50 lM)</td>
<td>91.48 ± 11.13*</td>
<td>4.31 ± 0.48*</td>
</tr>
<tr>
<td>Cu²⁺ (750 lM) + Liv.52 (1%)</td>
<td>55.69 ± 2.28*</td>
<td>5.08 ± 0.52*</td>
</tr>
<tr>
<td>Cu²⁺ (750 lM) + BCS (50 lM)</td>
<td>24.72 ± 3.28*</td>
<td>8.59 ± 1.38*</td>
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</table>

HepG2 cells were incubated with Cu²⁺ (750 lM) with or without Liv.52 in the presence or absence of BSO (250 lM) and BCS (50 lM). Values are Mean ± SEM of three samples.

* Statistically significant at P < 0.05 compared to control.  † Statistically significant at P < 0.05 compared to toxicant.
Fig. 2. (A) Effect of Liv.52 on lipid peroxidation in Cu\(^{2+}\) induced toxicity in HepG2 cells. HepG2 cells were incubated with Cu\(^{2+}\) (750 μM) with or without Liv.52 in the presence or absence of BSO (250 μM) and BCS (50 μM) and lipid peroxides was measured. (B) Effect of Liv.52 on DNA fragmentation in Cu\(^{2+}\) induced toxicity in HepG2 cells. HepG2 cells were incubated with 750 μM Cu\(^{2+}\) with or without Liv.52 in the presence or absence of BSO (250 μM) and BCS (50 μM) and DNA fragmentation was measured as described in materials and methods. Values are Mean ± SEM of three independent experiments carried out in triplicates. *Statistically significant at \(P < 0.05\) compared to toxicant group.

Fig. 3. Effect of Liv.52 on antioxidant enzymes (A) catalase (CAT), (B) glutathione peroxidase (Gpx) and (C) superoxide dismutase (SOD) activity in HepG2 cells. HepG2 cells were incubated with Cu\(^{2+}\) (750 μM) with or without Liv.52 in the presence or absence of BSO (250 μM) and BCS (50 μM) and enzyme activity was measured as described in materials and methods. \(\mu\)moles of H\(_2\)O\(_2\) decomposed/min/mg protein, \(\mu\)moles of NADPH oxidized/min/mg protein, Units/mg protein. Values are Mean ± SEM of three independent experiments carried out in triplicates. *Statistically significant at \(P < 0.05\) compared to control. Statistically significant at \(P < 0.05\) compared to toxicant group.
3.5. Effect of Liv.52 on antioxidant enzyme activity in Cu2+ induced toxicity

The antioxidant enzymes catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were evaluated and the results (Fig. 3) showed that Cu2+ inhibited the catalase activity by 47% (Fig. 3A) whereas, the GPx and SOD activity was inhibited by 23% and 34% (Fig. 3B and C) respectively in Cu2+ induced toxic conditions. The catalase, GPx and SOD activity were increased upon addition of Liv.52 by 46%, 22% and 81% respectively compared to toxicant group.

4. Discussion

The present study demonstrates the antioxidant and cytoprotective effects of Liv.52 against Cu2+ induced toxicity in HepG2 cells which is attributed to the inhibition of GSH depletion. Liv.52 liquid concentrate is used in the present study, since the biological activity of the drug depends on its association and permeability. The antioxidant and biological activity of biologically active compounds will depend more heavily on the extent to which they associate, interact and permeate cell membranes (Rice-Evans et al., 1997). The mechanisms by which cells handle an excess of copper is limited; however existing evidence supports the participation of GSH and metallothionein (MT) as two of the major molecules involved in the intracellular sequestering and storing of Cu2+ (Freedman and Peisach, 1989). Earlier, it is shown that, Liv.52 rich in polyphenols act as hepatoprotective agent by modulating the antioxidant molecules and xenobiotic enzymes in cells (Vidyashankar et al., 2010). It was also reported that phenolic compounds activates enzymes involved in the biodegradation of xenobiotics (Ferguson 2001).

The results showed that, cytotoxicity of Cu2+ is mediated by increased lipid peroxidation (5.6-folds) and GSSG levels (3.7-folds) as well as decreased GSH levels (86%) and glutathione-related enzyme activity in HepG2 cells. But, Liv.52 attenuated the cytotoxicity in HepG2 cells (Fig. 3) when co-incubated with Cu2+ by increased glutathione content and activity of glutathione-related enzymes.

Previous reports indeed alert to the fact that different origins of HepG2, culture medium composition and cultivation time (age of cells) may affect the experimental outcome through differences in sensitivity towards drugs (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004). It therefore becomes imperative to characterize the cells response to the toxicant as well as the experimental conditions used for the detection of protective effects of test compounds. In an attempt to explain the observed cytoprotective effects of Liv.52, we looked at its effects at IC50 concentration on markers of cellular oxidative stress, such as lipid peroxidation, DNA fragmentation and glutathione levels. Thus, Liv.52 may be beneficial in

In conclusion, the Liv.52 abrogated the Cu2+ induced toxicity in HepG2 cells by inhibition of glutathione depletion, lipid peroxidation and DNA fragmentation and on the other hand increased antioxidant enzyme activity. Thus, Liv.52 may be beneficial in...
treatment of Wilson’s disease and in other various liver disorders where it is known that Cu²⁺ induced oxidative stress and homeostasis play a crucial role.

Conflict of interest

The authors declare that there are no conflicts of interest.

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