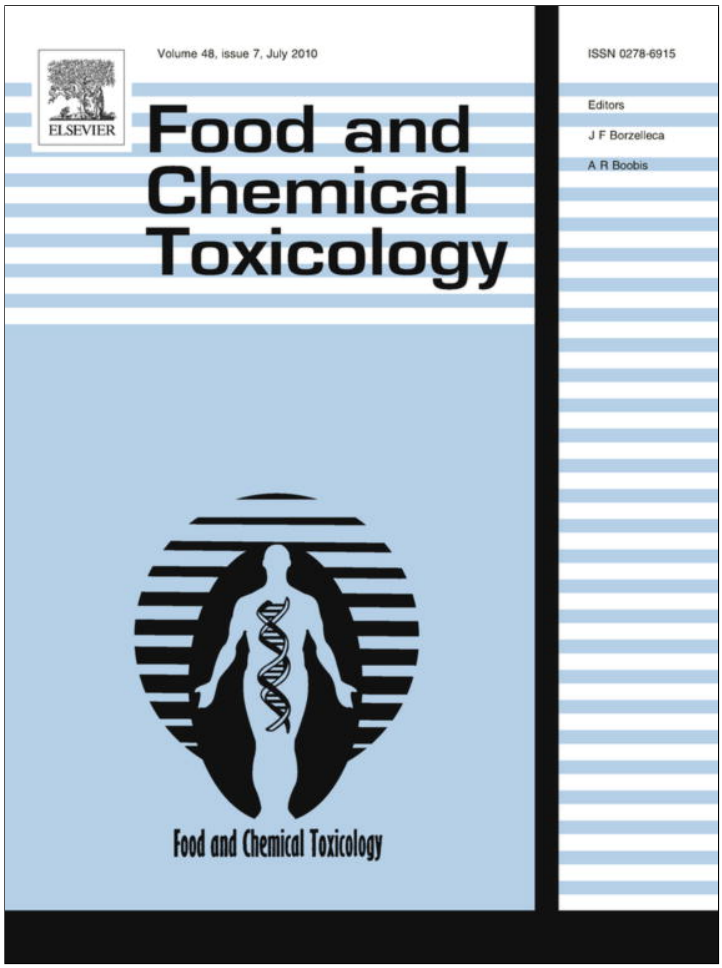

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Liv.52 attenuate copper induced toxicity by inhibiting glutathione depletion and increased antioxidant enzyme activity in HepG2 cells

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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 (Cu^{2+}) was evaluated in HepG2 cells. Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} induced cytotoxicity in HepG2 cells by inhibiting lipid peroxidation and increased GSH content and antioxidant enzyme activity.

1. Introduction

Copper (Cu^{2+}) 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 Cu^{2+} in cells (Luza and Speisky, 1996). Cu^{2+} induced toxicity involves its ability to catalyze the generation of free radicals and/or to directly interact with essential biomolecules, hence Cu^{2+} sequestering is of vital importance for maintaining cellular integrity. Reduced glutathione (GSH) plays a pivotal role in maintaining the intracellular Cu^{2+} 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 Cu^{2+}. GSH is likely to be one of the first molecules with which Cu^{2+} ions interact upon entering cells (Freedman and Peisach, 1989). During such interaction, the tripeptide reduces Cu^{2+} ions as a Cu–GSH adduct (Jimenez and Speisky, 2000). This adduct forms the principle carrier of Cu^{2+} to several metalloproteins. GSH on the other hand, via its interaction with Cu^{2+} ions is also considered to play a role in defining the susceptibility of the cells to excess Cu^{2+} (Freedman et al., 1986, 1989). To the extent that GSH sequesters redox-active Cu^{2+} ions, it would prevent these from catalyzing free radical generation, thus serving as a mechanism that protect cells against the deleterious consequences of excessive Cu^{2+} 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 Cu^{2+}-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 in animal models. It is shown that diets rich in fruit and vegetables and other plant foods (including tea and wine) are

Abbreviations: GSH, reduced glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; LPO, lipid peroxidation; BCS, bathocuproine disulfonate; BSO, buthionine sulfoximine.

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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 antigenotoxic effects of compounds (Knasmuller 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 ($CuSO_4 \cdot 7H_2O$), 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 (*Capparis spinosa*) 17 mg, Kasani (*Cichorium intybus*) 17 mg, Kakamachi (*Solanum nigrum*) 8 mg, Arjuna (*Terminalia arjuna*) 8 mg, Kasamarda (*Cassia occidentalis*) 4 mg, Biranjasipha (*Achillea millefolium*) 4 mg, Jhavuka (*Tamarix gallica*) 4 mg. The good agricultural and collection practices (GACP) were employed for plants used in the formulation. Plants 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 in 25 cm^2 polystyrene flasks (Tarsons) with DMEM containing 10% FBS, 1% antibiotic–antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO_2 at 37 °C with 95% humidity. Continuous cultures were maintained by subculturing flasks every 4 days at 2.2×10^6 cells/25cm^2 flask by trypsination.

2.4. Cu^{2+} induced cytotoxicity and protection by Liv.52

HepG2 cells were plated in 96-multiwell culture plates at 1×10^5 cells per well. To study Cu^{2+} cytotoxicity, 24 h after plating, the medium was discarded and fresh medium containing Cu^{2+} at various concentrations was added. At different time points, cellular viability was determined by the MTT assay (Mosmann, 1983). In order to determine the concentration of Liv.52 that protects 50% (IC_{50}) of the cells from damage induced by the toxicant, cells were incubated with Cu^{2+} for 24 h to induce significant cell death. Based on the dose–response curves of cell death protection by Liv.52 against the Cu^{2+} induced oxidative damage in HepG2 cells, the IC_{50} 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 Cu^{2+} 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 7.5×10^5 cells per well. Forty hours after plating, the medium was discarded and fresh medium containing Cu^{2+} 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 KH_2PO_4, 2 mM $MgCl_2$, 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 (Ohakawa et al., 1979) The results were expressed as nmol/mg of protein using a molar extinction coefficient of 1.56×10^5 Mcm^{-1}.

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 uncut DNA (resuspended in 200μ 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 quantified spectrophotometrically at 575 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatants 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 reductase recycling 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 1000*g* 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 H_2O_2 consumption spectrophotometrically at 240 nm and expressed as μmol H_2O_2 oxidized/min/mg protein (Aebi, 1984). Glutathione peroxidase activity was determined by following the enzymatic NADPH oxidation at 340 nm (Flohe 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 Cu^{2+} and Liv.52 in HepG2 cells was evaluated using MTT assay. Liv.52 did not present any cytotoxic effect at concentration ranging from 1% to 5% (Fig. 1A). On the other hand Cu^{2+} 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 Cu^{2+} (>750 μM) caused significant cytotoxicity to HepG2 cells. Hence 750 μM concentration of Cu^{2+} was used in all the subsequent experiments for testing hepatoprotective effect of Liv.52.

3.2. Effect of Liv.52 on Cu^{2+} induced toxicity on HepG2 cell viability

The Liv.52 protection against Cu^{2+} induced cytotoxicity was evaluated by MTT assay. The cells were incubated with Liv.52

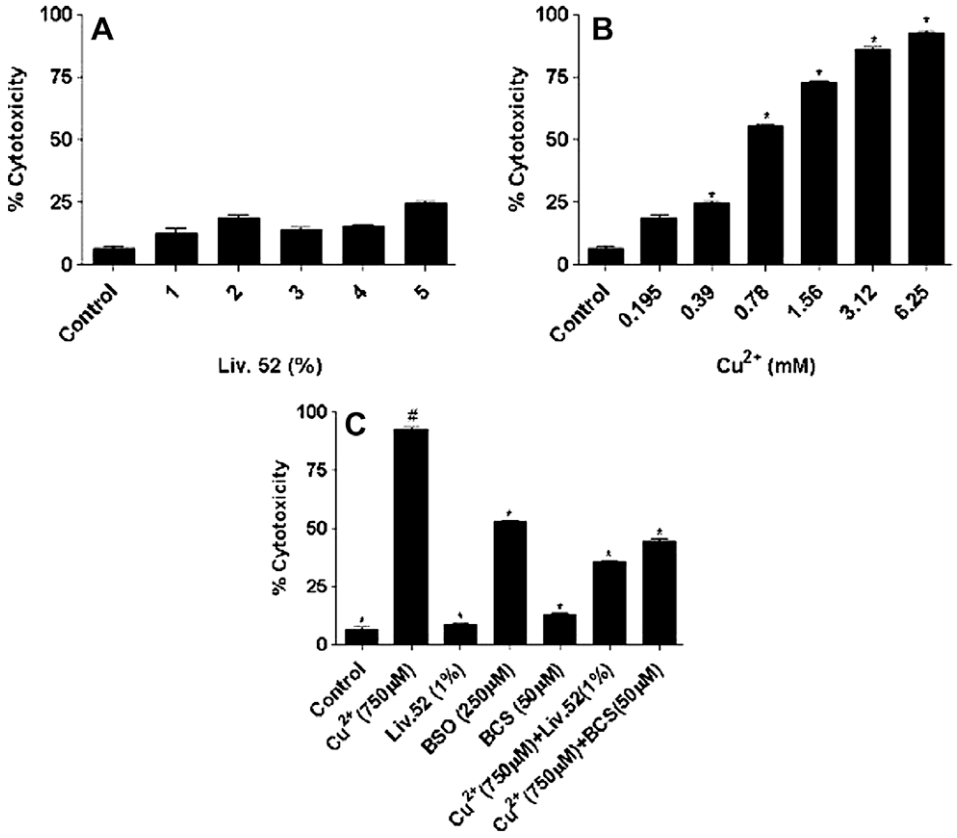

Fig. 1. (A) Dose dependent effect of Liv.52 in HepG2 cells. HepG2 cells were incubated with or without Liv.52 at different concentrations and cell viability was measured by MTT assay. (B) Dose dependent effect of Cu^{2+} toxicity in HepG2 cells. HepG2 cells were incubated with or without Cu^{2+} at different concentrations and cell viability was measured by MTT assay. (C) The hepatoprotective effect of Liv.52 in HepG2 cells. The HepG2 cells were treated with or without Liv.52 and Cu^{2+} (750 μM) in the presence or absence of BSO (250 μM) and BCS (50 μM) and the cytotoxicity was determined by MTT assay. 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.

(1%) and Cu^{2+} (750 μM) for 24 h and the cell viability was measured. Liv.52 at 1% effectively protected 62% of the cells against Cu^{2+} induced toxicity as shown in Fig. 1C. The BSO (an inhibitor of GSH synthesis) at 250 μM induced significant toxicity to HepG2 cells. But on the other hand BCS (Cu^{2+} chelating agent) had no effect on the cell viability. BCS when added with Cu^{2+} it significantly resulted in decreased cytotoxicity by 52%.

3.3. Effect of Liv.52 on GSH depletion in Cu^{2+} induced toxicity

The effect of Cu^{2+} on GSH and GSSG levels in the HepG2 cell was evaluated and the results are given in Table 1. The results showed that Cu^{2+} 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 Cu^{2+} induced toxicity. On the other hand, when cells were exposed to Cu^{2+} along with BCS (Cu^{2+} chelating agent) the GSH level was significantly increased by 42%. Liv.52 significantly replenished the GSH levels by 74% in Cu^{2+} induced toxic conditions.

Cu^{2+} 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 Cu^{2+}.

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 Cu^{2+} in HepG2 cells as shown in Fig. 2A. The cells treated with Cu^{2+} 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 Cu^{2+} treated cells.

The Cu^{2+} 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 Cu^{2+} the DNA damage was significantly increased by 49%. The DNA damage was inhibited by 37% in cells treated with Cu^{2+} by Liv.52. Similarly the cells treated Cu^{2+} along with BCS showed decreased DNA damage compared to toxicant group.

Table 1
Effect of Liv.52 on reduced glutathione (GSH) and oxidized glutathione (GSSG) in Cu^{2+} induced toxicity in HepG2 cells.

Groups	GSH (nmol/mg protein)	GSSG (nmol GSH equiv/mg protein)
Control	104.36 ± 8.35*	4.51 ± 1.11*
Cu^{2+} (750 μM)	14.40 ± 3.22#	16.84 ± 2.60#
Liv.52 (1%)	100.90 ± 17.85*	3.19 ± 0.64*
BSO (250 μM)	23.38 ± 2.63*	9.95 ± 1.92*
BCS (50 μM)	91.48 ± 11.13*	4.31 ± 0.48*
Cu^{2+} (750 μM) + Liv.52 (1%)	55.69 ± 2.28*	5.08 ± 0.52*
Cu^{2+} (750 μM) + BCS (50 μM)	24.72 ± 3.28*	8.59 ± 1.38*

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).
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.

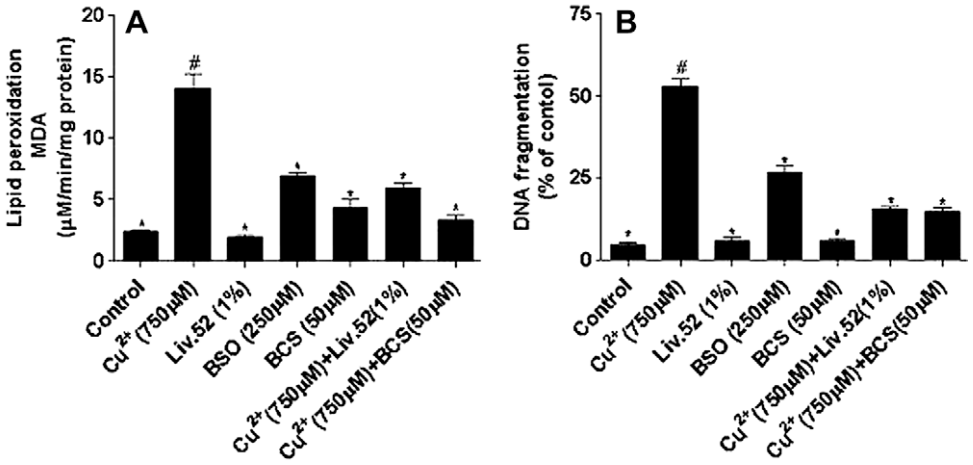

Fig. 2. (A) Effect of Liv.52 on lipid peroxidation in Cu^{2+} induced toxicity in HepG2 cells. HepG2 cells were incubated with Cu^{2+} (750 μM) with or without Liv.52 in the presence or absence of BSO (250 μM) and BCS (50 μM) and lipid peroxides was measured. (B) Effect of Liv.52 on DNA fragmentation 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 control. *Statistically significant at $P < 0.05$ compared to toxicant group.

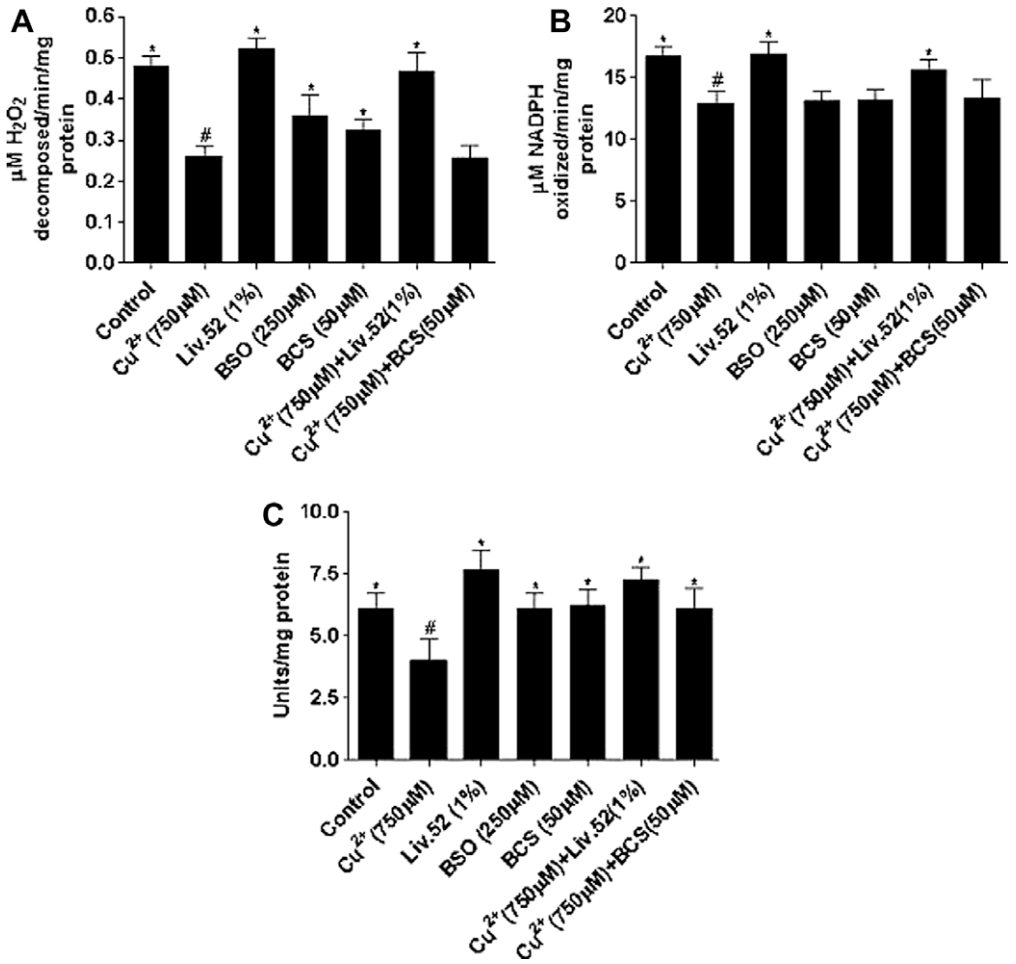

Fig. 3. Effect of Liv.52 on antioxidant enzymes (A) catalase (CAT), (B) glutathione peroxidase (Gpx) and (C) superoxide dismutase (SOD) activity in HepG2 cells. HepG2 cells were incubated with Cu^{2+} (750 μM) with or without Liv.52 in the presence or absence of BSO (250 μM) and BCS (50 μM) and enzyme activity was measured as described in materials and methods. [a]μmoles of H_2O_2 decomposed/min/mg protein, [b]μmoles of NADPH oxidized/min/mg protein, [c]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 Cu^{2+} induced toxicity

The antioxidant enzymes catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were evaluated and the results (Fig. 3) showed that Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} (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 Cu^{2+} 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 Cu^{2+} 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 IC_{50} concentration on markers of cellular oxidative stress, such as lipid peroxidation, DNA fragmentation and glutathione levels.

The susceptibility of HepG2 cells towards Cu^{2+} induced cytotoxicity shows good co-relation between initial cell viability and lipid peroxidation compared to control in the present study ($P < 0.05$). But Liv.52 significantly increased the cell viability (67%) and decreased the lipid peroxidation (58%) along with elevated GSH levels. Thus, showing that hepatoprotection by Liv.52 is brought about by preventing excessive lipid peroxidation. The ability of Liv.52 to chelate metal ions and/or to act as chain breaking antioxidants by scavenging (as hydrogen donors) lipid alkoxyl and peroxyl radicals could provide an explanation for the observed reduction in lipid peroxidation. Hence, from these findings it is speculated that besides inhibiting lipid peroxidation Liv.52 may have also involved in other mechanisms in abrogating the cytotoxicity in HepG2 cells.

Earlier we have shown that, cytotoxicity induced by tertiary butyl hydroperoxide in HepG2 cells has been shown to be proportional to the depletion of GSH (Vidyashankar et al., 2010). Therefore, low GSH levels in Cu^{2+} loaded cells could be considered a major intracellular determinant of their susceptibility to cytotoxicity. Another report also demonstrated that Cu^{2+} induced toxicity is modulated by depleted amounts of GSH content and increased lipid peroxidation (Freedman and Peisach, 1989). In concurrence to these in the present study, GSH levels were significantly depleted by Cu^{2+} causing cytoxicity in HepG2 cells. But upon treatment with Liv.52 the GSH levels was replenished and the cell viability was increased.

Increase in GSSG levels was not in the same range as the decrease in GSH levels as observed and part of the GSSG generated by the HepG2 cells is likely to be continuously pumped out (Lu et al., 1993), but to establish the exact contribution of such mechanisms to the loss of GSH is difficult to measure. Thus, it is inferred that Cu^{2+} reduced GSH levels mainly through formation of GSH conjugates rather than oxidation to GSSG. These findings are in accordance with studies reported earlier in liver cells (Sies and Summer, 1975; Jewell et al., 1986; Buc-Calderon et al., 1991; Alia et al., 2005, 2006). Within the scope of the present study if the adduct is assumed to have been formed, it is likely to have compromised the GSH pool to an extent comparable to that of Cu^{2+} needed to be intracellularly sequestered. Considering the role that GSH plays in sequestering copper and in carrying the metal into MT (Freedman and Peisach, 1989; Steinebach and Wolterbeek, 1994), the results addressed the possible changes in the GSH pool associated with Cu^{2+} induced toxicity.

The lower GSH levels seen in cells are in line with the low GSH levels occurring in the livers of individuals with Wilson's disease (Summer and Eisenburg, 1985). Although our observation may partially reflect the Wilson's disease hepatocytes, absence of a challenge other than continuous exposure to non-toxic amounts of copper has not been studied. However, Liv.52 treatment significantly replenished GSH levels depleted by Cu^{2+} and increased antioxidant enzyme activity.

It is known that, GSH plays an important role in hepatocyte defence against ROS, free radicals and electrophilic metabolites (Kedderis, 1996; Castell et al., 1997). Hence, severe GSH depletion leaves cells more vulnerable to oxidative damage by radicals and increases protein thiolation or oxidation of SH groups that may lead to alterations in cellular calcium homeostasis (Castell et al., 1997). A sustained increase in cytosolic calcium levels results in activation of enzymes (phospholipases, non-lysomal proteases, endonucleases) and cytoskeletal damage, which ultimately causes cell death (Castell et al., 1997). The decrease of GSH levels has indeed been suggested as one of the primary mechanisms of Cu^{2+} induced toxicity in liver cells (Jewell et al., 1986; Buc-Calderon et al., 1991; Martin et al., 2001) which is generally followed by an increase in the intracellular levels of calcium (Bellomo et al., 1982; Nicotera et al., 1988; Buc-Calderon et al., 1991). Thus, the potential of Liv.52 to maintain GSH at reasonably high levels is of importance against Cu^{2+} induced toxicity. Therefore, the ability of Liv.52 in preventing Cu^{2+} induced GSH depletion by about 74% is very significant in restoring the cell viability. The GSSG formation was inhibited by Liv.52 and this may be attributed to the formation of GSH conjugates rather than oxidation to GSSG in Cu^{2+} induced toxic conditions.

Beside these the antioxidant enzymes viz; superoxide dismutase, catalase and glutathione peroxidase activities were increased by Liv.52 during Cu^{2+} induced toxicity. It was also shown that DNA fragmentation was decreased by Liv.52 during Cu^{2+} induced toxicity.

In conclusion, the Liv.52 abrogated the Cu^{2+} 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 Wilsons's disease and in other various liver disorders where it is known that Cu^{2+} 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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