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Identification of lead-produced lipid hydroperoxides in human HepG2 cells and protection using rosmarinic and ascorbic acids with a reference to their regulatory roles on Nrf2-Keap1 antioxidant pathway

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Running Head:

Lead produced lipid hydroperoxides in human liver cells

20 **Highlights**

- 21 • Lead (Pb) induced cytotoxicity and oxidative stress production in HepG2 cells
- 22 • Lead produced 20 lipid hydroperoxides including TGOOH, CEOOH and PCOOH
- 23 • Lead downregulated antioxidant enzymes
- 24 • Ascorbic and Rosmarinic acids reduced Pb-induced cytotoxicity and oxidative stress
- 25 • Ascorbic and Rosmarinic acids enhanced antioxidant enzymes via Nrf2 activation

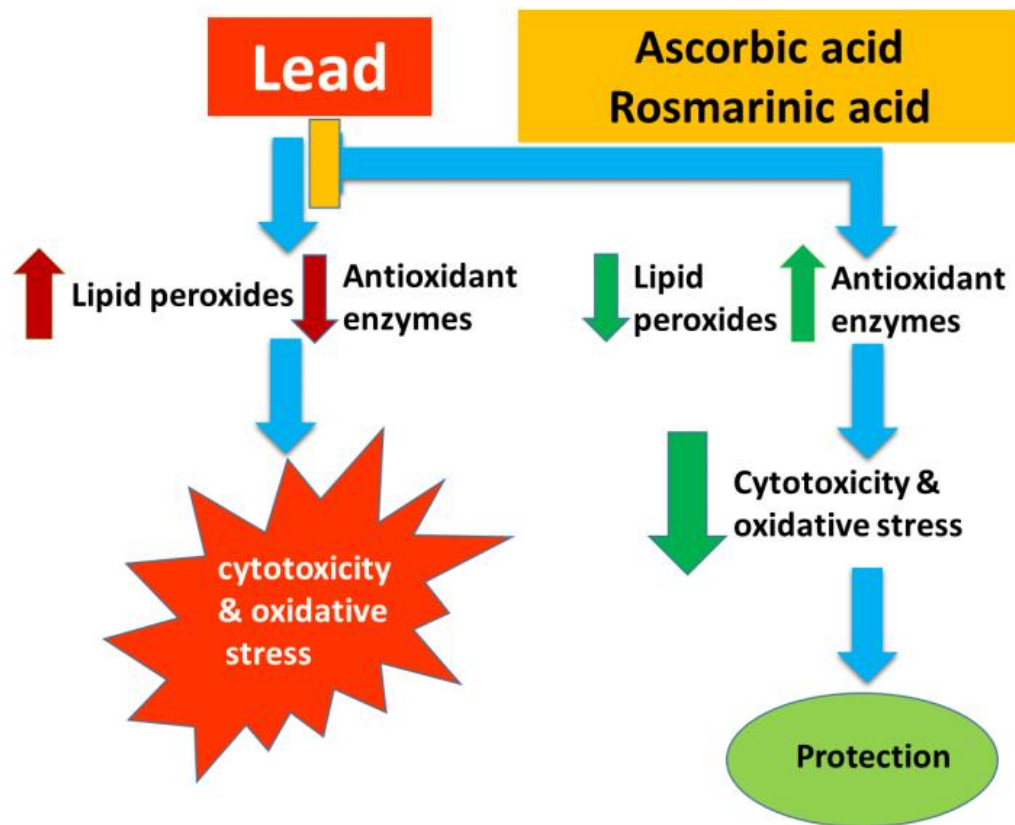
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Abstract

Lead (Pb) is one of the toxic heavy metals that have several toxicological implications including cytotoxicities and oxidative stress. The release of reactive oxygen species (ROS) usually initiates lipid peroxidation and resulting in inflammation and tissue injury. However, the detailed identification of the Pb-produced lipid hydroperoxides has received little attention. Furthermore, the mechanisms behind such effects are less informed. Therefore, this study firstly investigated Pb-produced lipid hydroperoxides in human HepG2 cells using LC/MS. The effects of Pb on the antioxidant enzymes were additionally examined using qPCR and their dependent activities. As a protection trial, the ameliorative effects of rosmarinic (RMA) and ascorbic (ASA) acids on Pb-induced cytotoxicity and oxidative stress and their regulatory effects on Nrf2/Keap1 pathway were investigated. The achieved results confirmed cytotoxicity and oxidative damage of Pb on HepG2 cells. In addition, 20 lipid hydroperoxides (LOOH) were identified including 11 phosphatidylcholine hydroperoxides (PCOOH), 5 triacylglycerol hydroperoxides (TGOOH) and 4 cholesteryl ester hydroperoxides (CEOOH). The most dominant LOOH species were PCOOH 34:2, PCOOH 34:3, PCOOH 38:7, TGOOH 60:14, TGOOH 60:15, CEOOH 18:3 and CEOOH 20:4. Pb significantly downregulated Nrf2-regulated antioxidant enzymes at both the pretranscriptional and functional levels. Co-exposure of HepG2 cells to RMA and ASA significantly reduced Pb-produced adverse outcomes. This protection occurred via activation Nrf2-Keap1 antioxidant pathway.

Keywords: Lead; HepG2 cells; lipid hydroperoxides; rosmarinic acid; ascorbic acid; Nrf2



52 **Abbreviations**

53 Lead (Pb); rosmarinic acid (RMA); ascorbic acid(ASA); Human liver hepatoma cell lines
54 (HepG2); Phosphatidylcholine hydroperoxides (PCOOH);
55 Triacylglycerol hydroperoxides (TGOOH); Cholesteryl ester hydroperoxides (CEOOH);
56 Dulbecco's modified Eagle's medium (DMEM); 2',7'-dichlorofluorescein diacetate (DCF-DA); 1-
57 chloro-2,4-dinitrobenzene (CDNB), glutathione reduced form (GSH); bovine serum albumin
58 (BSA); quantitative real-time PCR (qPCR); glutathione-S-transferases (GST); NAD(P)H
59 dehydrogenase [quinone] 1 (NQO1); heme oxygenase (HO)1; superoxide dismutase (SOD);
60 catalase (CAT); glutathione peroxidase 1 (GPx1); Erythroid 2-related factor 2 (Nrf2); kelch-like
61 ECH-associated protein-1 (Keap1);

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1. Introduction

Lead (Pb) is one of the toxic heavy metals that can get reach to human body via ingestion of contaminated foods and drinks, inhalation and occupational exposure. In our previous studies, Pb was detected at high levels in the fish, cattle, sheep and poultry meat and offal [1-3]. Consumption of such contaminated food may lead to several toxicological effects including behavioral changes, immune dysfunction, multiple organ damage and even deaths [4]. Liver is considered the target organ for accumulation and detoxification of heavy metals such as Pb. Human liver hepatoma cell lines (HepG2) are considered as an ideal *in vitro* tool for studying the toxic effects of Pb as they are widely used as alternative cell system to primary hepatocytes as they retain many metabolic functions of normal liver cells [5].

Induction of oxidative stress is suggested as one possible mechanism for Pb-induced cytotoxicity in several reports [6-8]. The release of reactive oxygen species (ROS) usually initiates lipid peroxidation and resulting in inflammation and tissue injury. However, the detailed identification of the Pb-produced lipid hydroperoxides had not so far been done.

Erythroid 2-related factor 2 (Nrf2) is a major regulator of cellular redox balance as it binds to kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm at normal physiological conditions. However, upon exposure to oxidative stress, Nrf2 dissociates from Keap1 and activates the release of antioxidant and detoxification enzymes [9]. Studies had reported that administration of Pb caused a clear downregulation for several antioxidant enzymes in the rats [10,11]. Therefore, Pb-induced oxidative damage was mainly attributed to the loss of the antioxidant enzymes rather than the generation of ROS [12].

Rosmarinic (RMA) and ascorbic acids (ASA) are among the phytochemicals that showed a documented antioxidant, anti-inflammatory and protective effects against heavy metals such as

cadmium [13,14], chromium [15] and Pb [7]. However, their comprehensive effects on the formed lipid hydroperoxides, antioxidant enzymes and Nrf2-Keap1 pathway had received little attention.

In sight of the previous facts, this study aimed at firstly confirming Pb-induced cytotoxicity and oxidative stress in HepG2 cells. Secondly, Pb-produced lipid hydroperoxides were quantitatively estimated using LC/MS. Thirdly, the effects of Pb on antioxidant enzymes were examined using quantitative real-time PCR (qPCR) and their dependent-activities. Fourthly, the protective effects of either RMA or ASA against Pb-induced adverse effects in HepG2 cells were investigated. Finally, the effects of either Pb or the tested phytochemicals on Nrf2-Keap1 reporter gene assay and protein concentrations were examined.

2. Materials and Methods

All experiments were conducted according to the guidelines of Hokkaido University, Japan.

2.1. Chemicals

Lead acetate, L-ascorbic acid, rosmarinic acid, oligonucleotides, TRI reagent, Dulbecco's modified Eagle's medium (DMEM), 2',7'-dichlorofluorescein diacetate (DCF-DA), 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reduced form (GSH), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ReverTrace qPCR master mix with gDNA remover was obtained from Toyobo, Osaka, Japan. Fast SYBR green master mix was purchased from Applied Biosystems, Gracuno, Vilnius, Lithuania. Chloroform, isopropanol, methanol, and water were of LC/MS grade and were purchased from Wako Pure Chemical (Osaka, Japan). Lipid hydroperoxide standards were synthesized in our laboratory based on previous reports [16-18], while other lipid standards were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Other chemicals and reagents of analytical grade were purchased from Kanto Chemical Industry (Tokyo, Japan) unless specified.

2.2. Cell culture conditions and treatment

Human liver hepatoma (HepG2) cell lines were from Cell Biolabs, Inc. (distributed by Funakoshi Co. Ltd., Tokyo, Japan). Cells were grown in DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin mixture, in a humidified incubator with 5% CO₂ at 37°C. Once cell confluency was reached, cells were exposed to either Pb (1.0 and 10 µM) for 24 h. In protection experiments, cells were co-exposed to Pb and either RMA or ASA at 1 or 10 µM for 24 h. The used concentrations of Pb and phytochemicals were relevant to their environmental concentrations, previous reports and based on a pre-experimental trials [7,19].

2.3. Cell viability assay

CCK-8 assay kit (Dojindo Molecular Technologies, Rockville, USA) was used to determine HepG2 cell viability, the manufacturer's instructions were followed (n = 6 per treatment).

2.4. Reactive Oxygen Species (ROS) measurement

Treated-HepG2 cells were stained with the fluorogenic probe DCF-DA [19] for measurement of ROS production. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using Wallac 1420 ARVO Mx plate reader, PerkinElmer, Tokyo, Japan (n = 6 per treatment).

2.5. Cell total lipids extraction

Total lipids were extracted from HepG2 cells according to the method of **Folch et al. [20]**. In short, cell lysates were extracted with 600 μ L of cold chloroform/methanol 2:1 (v/v, with 0.002% BHT and internal standards) twice, followed by dryness under vacuum. The dried lipids were dissolved in 200 μ L of methanol, centrifuged at 680 g at 4 °C for 15 min to remove any insoluble material, and thereafter stored at -80 °C until analysis. The entire sample pretreatment was finished within 1 h to avoid lipid degradation and auto-oxidation.

2.6. LC/MS conditions

Lipid extracts were separated on an Atlantic T3 C18 column (2.1 $\times$ 150 mm, 3 μ m, Waters, Milford, MA) connected to a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan). A flow rate of 200 μ L/min was used for the analysis, and the column and sample tray were held at 40 and 4 °C, respectively. LC gradient elution was performed with a mobile phase of 5 mM aqueous ammonium acetate, isopropanol, and methanol. MS analysis was carried out using an LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific Inc., San Jose, CA), with the

following parameters: The MS capillary voltage was set at 3.0 kV, the sheath gas (nitrogen) flow was set to 50 units, and the auxiliary gas (nitrogen) was set to 5 units. The MS¹ data were obtained in Fourier Transform mode with resolving power 60,000 and a 2 Hz scan speed, while the tandem MS data were acquired using collision-induced dissociation in ion-trap mode and data-dependent acquisition, which included scans on the most intense ions in MS² (collision energy of 35 V) and MS³ (collision energy of 45 V), respectively.

2.7. Data analysis

The extracted ion chromatograms (EICs) were drawn within the mass tolerance of 5.0 ppm by Xcalibur 2.2 (Thermo-Fisher Scientific Inc.). The LC/MS identification of lipid molecules were executed with the comparison of standards as well as the help of LipidBlast and in-house library [16,21,22]. The alignment, peak extraction, and EIC peak area integration from the raw data were utilized by the MS-label free differential analysis software package SIEVE 2.0 (Thermo-Fisher Scientific Inc.), of which the main parameters were set as follows: frames, 10 K; *m/z* window, 0.01 Da; retention time window, 1.00 min; intensity threshold, 10,000. The exported EIC peak area data were treated as the intensities of analytes and used for further statistical analysis.

2.8. RNA isolation and quantitative RT-PCR

Total RNA was isolated from HepG2 cells according to **Chomczynski et al. [23]**. In brief, cells were lysed in TRI reagent, followed by the addition of chloroform and centrifugation at 15000 rpm for 20 min at 4 °C for separation of the RNA. Precipitation of the RNA pellets was achieved using then using isopropanol combined with centrifugation under same conditions. The pellets were then washed with 70% ethanol and dissolved using RNase-free H₂O. RNA concentrations and qualities were determined using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford,

Conn., USA). For cDNA synthesis, ReverTraAce® qPCR RT Master Mix with gDNA remover was used as described in the manufacturer's instructions.

The mRNA expression levels of the antioxidant enzymes including glutathione-S-transferases (GST) O1, NAD(P)H dehydrogenase [quinone] 1 (NQO1), heme oxygenase (HO)1, superoxide dismutase (SOD) 1, 2, glutathione peroxidase 1 (GPx1), and catalase (CAT) were determined using qPCR. Reactions of qPCR were conducted in Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA). The PCR mixture contained 2 µL of cDNA (600 ng), 5 µL Fast SYBR® Master mix, 0.5 µL of each primer (5 µM), with RNase-free water added to a final volume of 10 µL. The reaction cycle comprised a holding stage for 20 s at 95°C, followed by 40 denaturation cycles of 3 s at 95°C and 30 s at 60°C and 15 s extension at 95°C. Single amplicon amplification was confirmed using melting curve analysis. The absence of primer dimers and genomic DNA amplification were confirmed by agarose gel electrophoresis. GAPDH was used for normalization by the comparative $\Delta\Delta C_t$ method (n = 6 plates per each treatment). Primer sets for the selected targets were designed using Primer3Plus software (<https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) and were displayed in **Table 1**. The efficiencies of the primers used in the present study ranged between 94-104%.

Table 1: Primer sequences of the target genes used in this study

Target	Sequence	Amplicon size	Accession number
GSTO1	F- 5'- AGGACGCGTCTAGTCCTGAA-3' R- 5'- TTCCCTGGGTATGCTTCATC-3'	191	NM_004832.2
NQO1	F- 5'- GGATTGGACCGAGCTGGAA-3' R- 5'- AATTGCAGTGAAGATGAAGGCAAC-3'	140	NM_001286137
SOD1	F- 5'- GCAGGTCCTCACTTTAATCCTCT-3' R- 5'- ATCGGCCACACCATCTTTGT-3'	111	CR541742
SOD2	F- 5'- GCCTGCACTGAAGTTCAATGG-3' R- 5'- TTCCAGCAACTCCCCTTTGG-3'	103	AY280719
HO1	F- 5'- ATGGCCTCCCTGTACCACATC-3'	55	NM_002133

GPx1	R- 5'- TGTTGCGCTCAATCTCCTCCT-3' F- 5'- GCAACCAGTTTGGGCATCAG-3' R- 5'- GTTCACCTCGCACTTCTCGA-3'	122	BC000742.2
CAT	F- 5'- TGAAGATGCGGCGAGACTTT-3' R- 5'- TGGATGTAAAAAGTCCAGGAGGG-3'	105	AY028632
Nrf2	F- 5'- CTTGGCCTCAGTGATTCTGAAGTG -3' R- 5'- CCTGAGATGGTGACAAGGGTTCTA-3'	124	NM_001313904
GAPDH	F- 5'- TCCAAAATCAAGTGGGGCGA-3' R- 5- TGATGACCCTTTTGGCTCCC-3'	115	DQ403057

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185 2.9. Preparation of the whole cell lysate from cells

186 Whole cell lysate from HepG2 cells exposed to Pb, RMA and ASA were prepared
187 according to the method described before [24]. The protein concentration in the whole cell lysates
188 was measured using Pierce™ BCA protein assay kit (Pierce Biotechnology, Thermo Scientific,
189 Rockford, USA), according to the manufacturer's guidelines.

190 2.10. Glutathione-S-transferases (GST) assay

191 Measurement of GST activity in HepG2 whole cell lysate was done utilizing CDNB as a
192 substrate, according to the method described by **Habig et al. [25]** with minor modifications. In
193 short, the reaction mixture contained 1 mg of cellular protein, 0.5 mM CDNB, and 0.5 mM GSH
194 in 0.1 M potassium phosphate buffer (pH 7.0). After preincubation at 37 °C for 2 min, the reaction
195 was started by the addition of GSH and lasted for 3 min, then terminated by the addition of 33%
196 trichloroacetic acid. The mixture was centrifuged at 1500g for 10 min. The absorbance of the
197 supernatant was determined at a wavelength of 340 nm.

198 2.11. Catalase assay

199 Measurement of catalase activity in the treated HepG2 cells was done following the
200 protocol of **Iwase et al. [26]** utilizing H₂O₂ as a substrate.

201 2.12. Nrf2 reporter gene assay

The assay for Nrf2 reporter gene assay was conducted based on the protocol described by **Joko et al. [27]**. In brief, HepG2 cells (4×10^5 cell/ml) were seeded into 96-well plates for 24h with DMEM supplemented with 10% FBS. We transfected pGL4.37 [luc2p/ARE/hygro] and pGL4.75[hRluc/CMV] (an internal control for transfection efficiency) vector at a 20:1 mass ratio using lipofectamine 3000 (Life Technologies, Tokyo) according to the manufacturer's protocol. After transfection, the transfection reagent/DNA mixture was removed, and samples solubilized in DMEM without FBS were separately applied to the transfected cells at various concentrations. Luciferase activity was assayed using dual luciferase system (Promega, Madison, WI, USA). Activity was measured with a Wallac 1420 ARVO Mx plate reader and corrected for transfection efficiency by normalizing to hRluc activity.

2.13. Estimation of Nrf2 and Keap1 protein concentrations

ELISA assays for quantitative estimation of both Nrf2 and Keap1 protein concentrations in the whole HepG2 cells lysates were conducted using human Nrf2 and Keap1 ELISA assay kits (MyBioSource, San Diego, California, United States). All assays were conducted according to the manufacturer's instructions. Absorbance was measured at 450 nm using a Wallac 1420 ARVO Mx plate reader.

2.14. Statistical analysis

Statistical significance was evaluated using Tukey-Kramer honestly HSD, Pairwise correlation analysis between Pb, cell viability and ROS production was conducted using JMP program, SAS Institute, Cary, NC, USA with $p < 0.05$ considered as significant.

3. Results and Discussion

3.1. *Pb-induced cytotoxicity, oxidative stress and production of lipid hydroperoxides in HepG2 cells*

Over the past decades, several studies had investigated the toxic effects of Pb and the mechanisms behind such effects in several organisms and cell models. There was almost an agreement that Pb-induced adverse effects are mainly associated with the induction of oxidative stress leading to lipid peroxidation and subsequently DNA damage, alterations in gene expressions, necrosis and apoptosis. However, there is no exact identification and quantification for the formed lipid hydroperoxides. The achieved results in this study firstly confirmed Pb-induced cytotoxicity and oxidative stress in HepG2 cells. Pb at 10 μ M caused a significant decrease in the cell viability (57.54%) compared with the control (**Fig 1A**). In parallel, Pb produced ROS in a concentration dependent manner (**Fig 1B**). The multivariate analysis showed positive correlations between Pb exposure, cell death and oxidative stress (**Fig 1C**). The ability of Pb to induce the generation of ROS and producing its cytotoxic effects on human cells had been reported in HepG2 cells [7], lymphoblastoid TK6 cells [28] and lung epithelial (A549) cells [29]. It was reported that the most important consequence of Pb-induced oxidative stress in cell is lipid peroxidation. Therefore, we quantitatively estimated Pb-produced hydroperoxides in HepG2 cells. To the best of our knowledge, the detailed identification of Pb-produced lipid hydroperoxides has not so far been explored. Interestingly, Pb produced a series of 20 lipid hydroperoxide species including 11 phosphatidylcholine hydroperoxides (PCOOH), 5 triacylglycerol hydroperoxides (TGOOH) and 4 cholesteryl ester hydroperoxides (CEOOH) (**Table 2; Fig 2**). Phosphatidylcholines are regarded as a structural lipids in the cellular membranes with other biological roles, particularly that related to cell signalling [30]. The dominant PCOOH

246 species formed after Pb exposure were PCOOH 34:2, PCOOH 34:3 and PCOOH 38:7 (**Fig 2A**).
247 PCOOH 34:2 was also formed during copper oxidation of low and high density lipoproteins [31].
248 Similarly, 5 TGOOH species were formed in HepG2 cells upon exposure to Pb, particularly
249 TGOOH 60:14, and TGOOH 60:15. Four CEOOH species were additionally formed in Pb-
250 exposed HepG2 cells, particularly, formed CEOOH 18:3 and CEOOH 20:4 (**Fig 2B, C**). In
251 agreement with these results, Pb exposure induced weight gain in adult rats with a significant
252 increase in the serum triglycerides and total cholesterol [32]. Furthermore, unfavourable total
253 cholesterol was highly formed in Chinese population exposed to arsenic and was positively
254 associated with oxidative DNA damage [33].

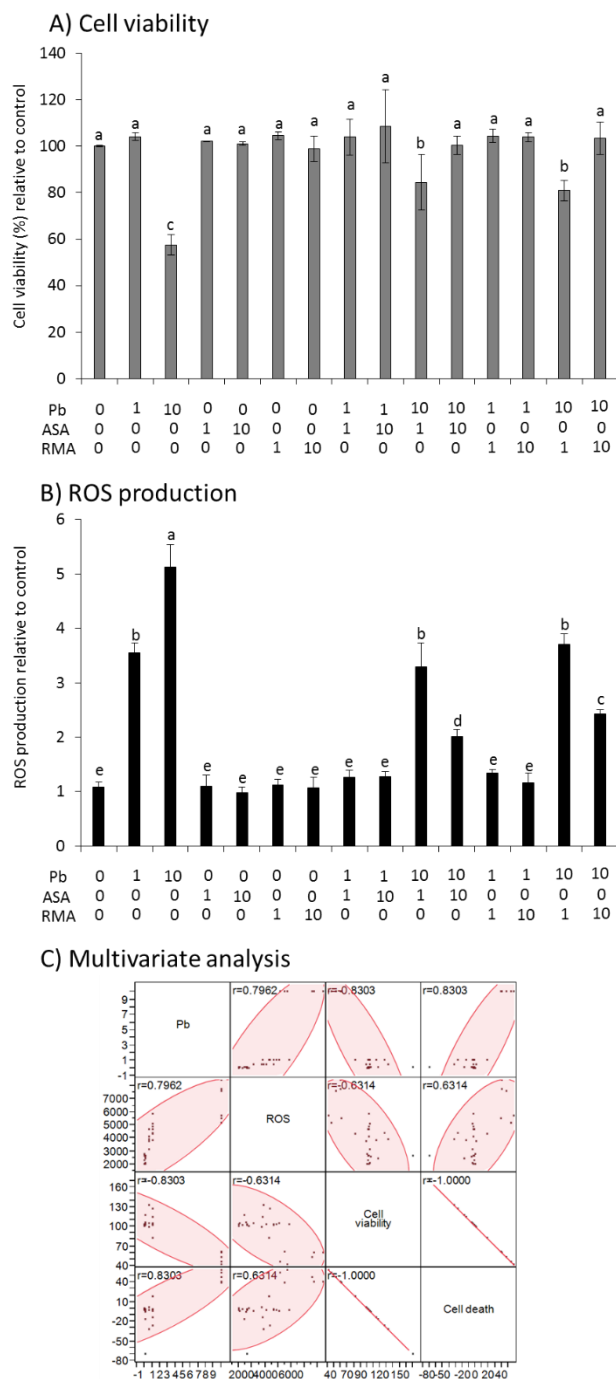


Fig. 1: Pb-induced cytotoxicity and oxidative stress in HepG2 cells and protection with RMA and ASA

Effects of co-exposure of HepG2 cells to Pb and/or RMA and ASA on **A)** cell viability as tested using CCK-8 assay. **B)** Oxidative stress as assayed using DCF-DA as a substrate. **C)** Multivariate analysis for Pb-induced cytotoxicity and oxidative stress in HepG2 cells. The data represent the average cell viabilities and levels of ROS $\pm$ SD relative to control (n = 6). Columns carrying different superscript letters are significantly different ($p < 0.05$).

264 **Table 2: LC/MS data of the identified lipids hydroperoxides**

No.	Lipid species	RT	Calculated m/z	Tested m/z	Δ ppm	Ion	Molecular formula
1	PCOOH34:2	6.54	790.5593	790.5594	0.13	$[M + H]^+$	C ₄₂ H ₈₀ O ₁₀ NP
2	PCOOH34:3	5.88	788.5436	788.5433	-0.38	$[M + H]^+$	C ₄₂ H ₇₈ O ₁₀ NP
3	PCOOH36:2	7.10	818.5906	818.5905	-0.12	$[M + H]^+$	C ₄₄ H ₈₄ O ₁₀ NP
4	PCOOH36:3	6.62	816.5749	816.5745	-0.49	$[M + H]^+$	C ₄₄ H ₈₂ O ₁₀ NP
5	PCOOH36:4	6.28	814.5593	814.5593	0	$[M + H]^+$	C ₄₄ H ₈₀ O ₁₀ NP
6	PCOOH36:5	6.08	812.5436	812.5439	0.37	$[M + H]^+$	C ₄₄ H ₇₈ O ₁₀ NP
7	PCOOH 38:4	6.83	842.5906	842.5903	-0.36	$[M + H]^+$	C ₄₆ H ₈₄ O ₁₀ NP
8	PCOOH38:5	6.54	840.5749	840.5753	0.48	$[M + H]^+$	C ₄₆ H ₈₂ O ₁₀ NP
9	PCOOH38:6	6.19	838.5593	838.5594	0.12	$[M + H]^+$	C ₄₆ H ₈₀ O ₁₀ NP
10	PCOOH38:7	5.90	836.5436	836.543	-0.72	$[M + H]^+$	C ₄₆ H ₇₈ O ₁₀ NP
11	PCOOH40:6	6.71	866.5906	866.5905	-0.12	$[M + H]^+$	C ₄₈ H ₈₄ O ₁₀ NP
12	TGOOH58:10	11.07	976.7600	976.7629	2.97	$[M + NH_4]^+$	C ₆₁ H ₉₈ O ₈
13	TGOOH58:11	10.82	974.7443	974.7489	4.72	$[M + NH_4]^+$	C ₆₁ H ₉₆ O ₈
14	TGOOH60:14	10.85	996.7287	996.7323	3.61	$[M + NH_4]^+$	C ₆₃ H ₉₄ O ₈
15	TGOOH60:15	10.17	994.7130	994.7166	3.62	$[M + NH_4]^+$	C ₆₃ H ₉₂ O ₈
16	TGOOH62:12	12.52	1028.7913	1028.7866	-4.57	$[M + NH_4]^+$	C ₆₅ H ₁₀₂ O ₈
17	CEOOH18:2	11.53	681.5816	681.581	-0.88	$[M + H]^+$	C ₄₅ H ₇₆ O ₄
18	CEOOH18:3	11.10	679.5660	679.5655	-0.74	$[M + H]^+$	C ₄₅ H ₇₄ O ₄
19	CEOOH20:4	8.32	705.5816	705.5809	-0.99	$[M + H]^+$	C ₄₇ H ₇₆ O ₄
20	CEOOH22:6	8.21	729.5816	729.5829	1.78	$[M + H]^+$	C ₄₉ H ₇₆ O ₄

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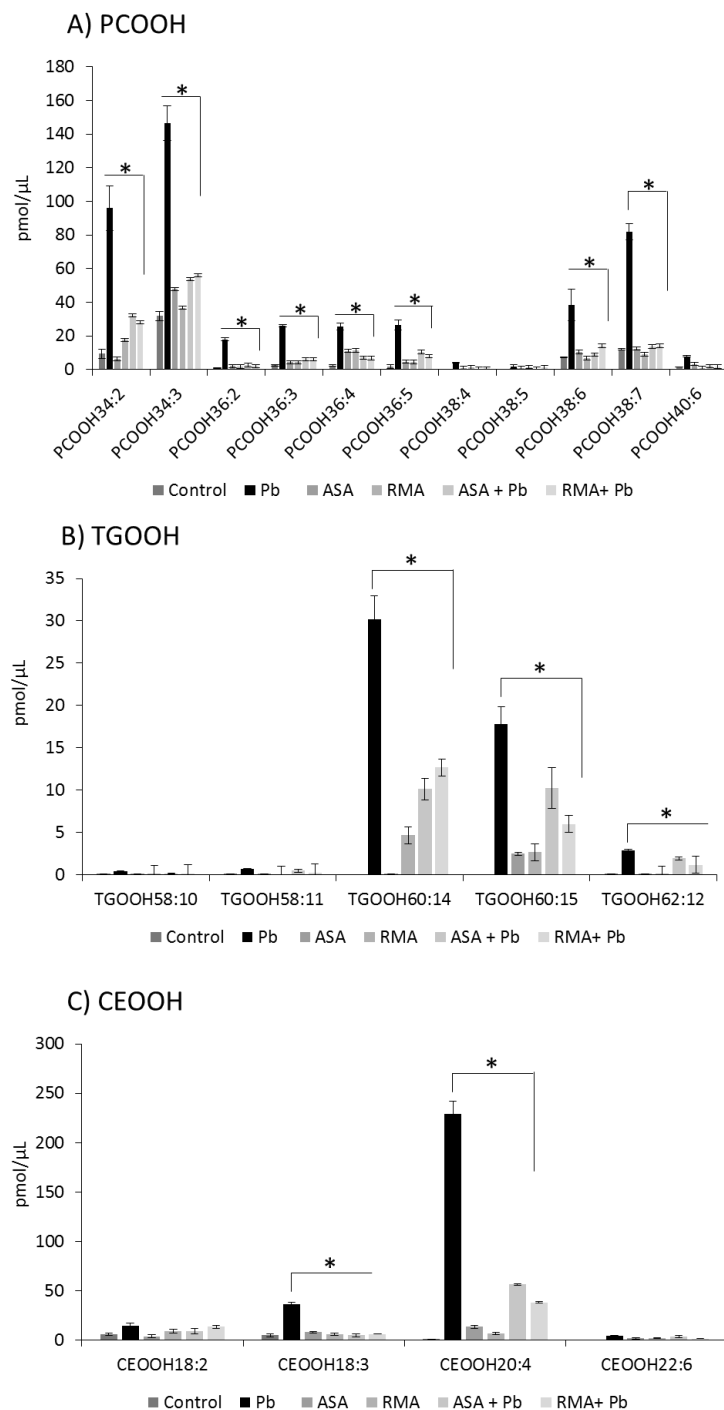


Fig. 2: Levels of lipid hydroperoxides produced in HepG2 cells after exposure to Pb, RMA and ASA

Levels of **A) PCOOH**, **B) TGOOH** and **C) CEOOH** produced in HepG2 cells after exposure to Pb and/or RMA and ASA as determined by LC/MS. Data are presented as the mean $\pm$ SD (n = 6). Column with star mark is significantly different ($p < 0.05$).

3.2. Effects of Pb on antioxidant enzymatic system in HepG2 cells

In order to investigate the possible reasons for the high level of lipid hydroperoxides formed after Pb exposure in HepG2 cells, mRNA expression levels of GSTO1, NQO1, SOD1, SOD2, HO1 and CAT were assayed using qPCR. It was clear from the achieved results that Pb at 10 μ M caused a significant downregulation in the mRNA expression of the assayed antioxidant enzymes (fold relative to control) as follows: GSTO1 (0.27 ± 0.03), NQO1 (0.55 ± 0.07), SOD1 (0.38 ± 0.12), SOD2 (0.29 ± 0.09), GPx1 (0.29 ± 0.09) and CAT (0.68 ± 0.03), respectively (**Fig. 3**). Unlikely, mRNA expression of HO1 was significantly upregulated (**Fig. 3E**). This might be attributed to the role of HO1 as an ideal biomarker for oxidative stress produced upon exposure to endotoxins, metals and inflammatory mediators as a kind of cytoprotection mechanism against oxidative stress [34]. The downregulation of the antioxidant enzymes was confirmed at the functional level as GSTO1 and CAT-dependent CDNB and H_2O_2 enzyme activities were significantly decreased (**Fig. 4**). This result indicates that the reduction of the antioxidant enzymes by Pb is through a mechanistic way. Similar downregulation of the antioxidant enzymes was reported in rats received Pb for 5 weeks in drinking water [35]. In addition, Pb caused significant depletion in the hepatic GST and CAT-dependent enzyme activities in rats chronically exposed to Pb in drinking water for 3 months [36]. Collectively, the obtained results possibly indicate that Pb-enhanced ROS levels are formed via the high production of lipid radicals and the downregulation of the antioxidant enzymes in human HepG2 cells.

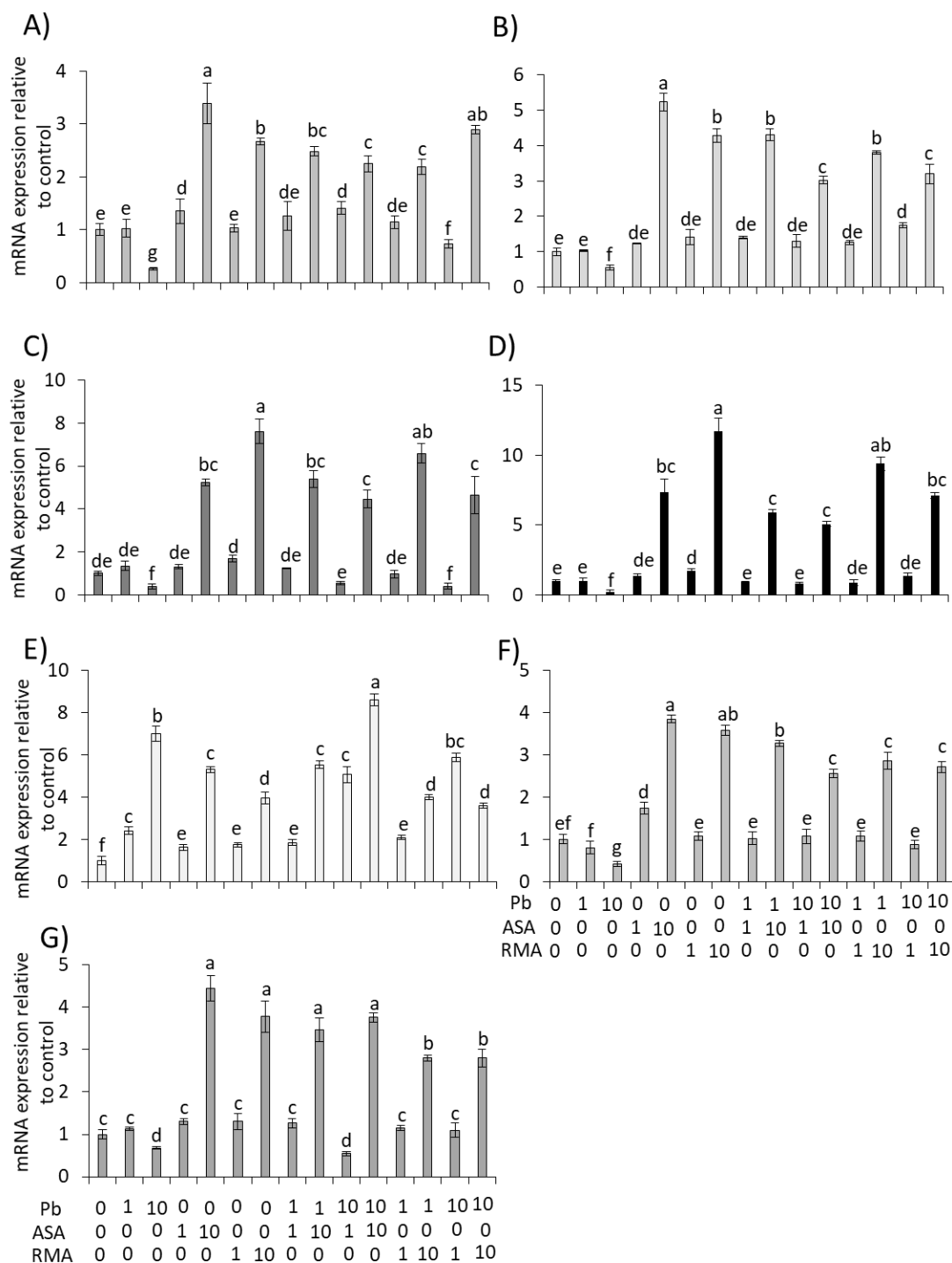


Fig. 3: Changes in mRNA expressions of antioxidant enzymes in HepG2 cells exposed to Pb and/or RMA and ASA

The effects of co-exposure of HepG2 cells to Pb and/or RMA and ASA on **A) GSTO1**, **B) NQO1**, **C) SOD1**, **D) SOD2**, **E) HO1**, **F) GPx1** and **G) Catalase** mRNA expressions as determined by qPCR. Data are presented as the mean $\pm$ SD (n = 6). Columns with different superscript letters are significantly different ($p < 0.05$).

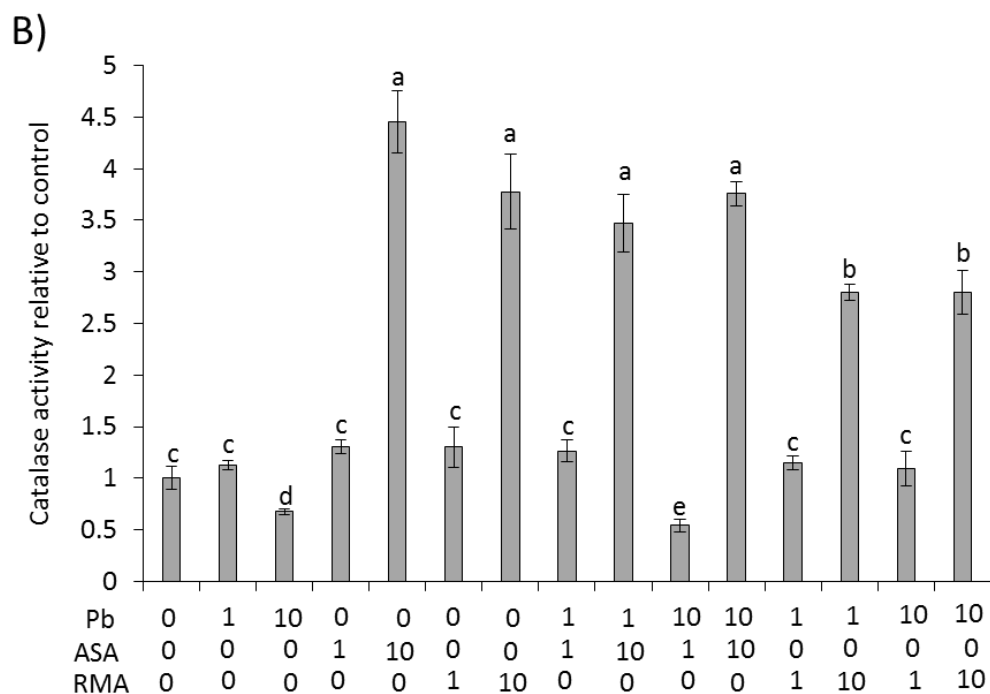
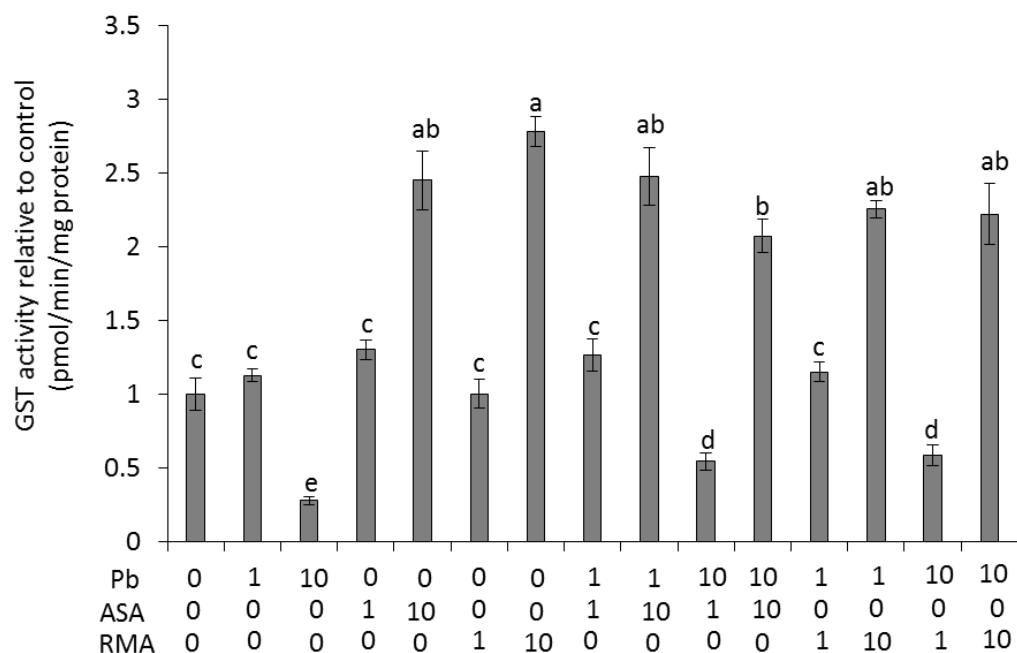


Fig. 4: Changes in GST- and catalase- dependent activities in HepG2 cells exposed to Pb and/or RMA and ASA

The effects of co-exposure of HepG2 cells to Pb and/or RMA and ASA on **A)** GST-, **B)** catalase-dependent activities using CDNB and H₂O₂ as substrates, respectively. Data are presented as the mean $\pm$ SD (n = 6). Columns with different superscript letters are significantly different ($p < 0.05$).

3.3. Protective effects of RMA and ASA against Pb-induced oxidative stress and cytotoxicity

In our recent report, RMA and ASA protected HepG2 cells against cadmium-induced cytotoxicity and oxidative stress [14]. It is known that metals behave differentially even in the same cell line [10,37]. Therefore, the protective effects of these phytochemicals were tested against Pb in HepG2 cells in parallel experiments. Furthermore, the mechanisms behind these effects were also investigated. The achieved results showed that coexposure of the hepatic cells to Pb and either RMA or ASA could reduce Pb-induced cytotoxicity and oxidative stress in a concentration dependent manner (Fig. 1). Both RMA and ASA significantly reduced the formed lipid hydroperoxides confirming their radical scavenging effects (Fig. 2). Furthermore, either RMA or ASA could upregulate mRNA expressions of the assayed antioxidant enzymes (GSTO1, NQO1, SOD1, SOD2, HO1, GPx1, and CAT) (Fig. 3) and the tested enzyme activities (GSTO1 and CAT) (Fig. 4). In particular, ASA had higher activities compared with RMA. The obtained results indicate that the protective effects of either RMA and ASA are via their radical scavenger effects and the induction and activation of the antioxidant enzymes. In agreement with the obtained results, Dashti et al. [15] reported the protective effects of RMA against chromium in the cultured cerebellar neuron cells. Furthermore, Kováčik et al. [38] confirmed the ameliorative effects of ASA (100 μ M) against Pb (100 μ M) and mercury (100 μ M) -induced toxicities in unicellular green alga *Coccomyxa subellipsoidea*. In addition, Darwish et al. [14] confirmed that ASA and RMA protected HepG2 cells against cadmium induced oxidative stress and cytotoxicity.

Nrf2 is a labile transcriptional factor that plays an important role in the release of antioxidant detoxification enzymes upon exposure to various xenobiotics. In the current study, the effects of RMA, ASA and Pb on the Nrf2 mRNA expression levels and its luciferase activity were investigated. Interestingly, ASA, RMA and Pb could upregulate Nrf2 at the transcriptional level.

Furthermore, the tested chemicals activated Nrf2 luciferase activity as follows: ASA (10.92 ± 0.64), RMA (5.53 ± 1.41) and Pb (2.94 ± 0.34), respectively (**Fig. 5A, B**). Nrf2 activation was confirmed at the protein level and it was associated with a significant reduction for Keap1 protein level, particularly at exposure to ASA or RMA (**Fig. 6A, B**). These results agree with **Vineetha et al. [39]** who reported that ASA attenuated arsenic-induced toxicity in H9c2 cardiomyocytes via activation of Nrf2. Furthermore RMA ameliorated H₂O₂ induced oxidative damage in L02 cells via activation of Nrf2 pathway **[40]**. Therefore, it is clear that Pb-, RMA- and ASA- modulation of antioxidant enzymes is possibly through a mechanistic way via activation of Nrf2-Keap1 pathway. Future approaches are still needed to compare the effects of Pb and such micronutrients in the human cancer and primary hepatic cells.

Conclusion

Pb had clear cytotoxic effects on human HepG2 cells. These toxic effects might be due to induction of oxidative stress. Pb-induced oxidative damage in the hepatic cells is probably due to production of lipid hydroperoxides such as PCOOH, TGOOH and CEOOH. Pb additionally downregulated antioxidant enzymes at both transcriptional and functional levels. RMA and ASA could significantly reduce the adverse effects of Pb on HepG2 cells. Such protective effects of RMA and ASA are possibly via activation of Nrf2-Keap1 pathway.

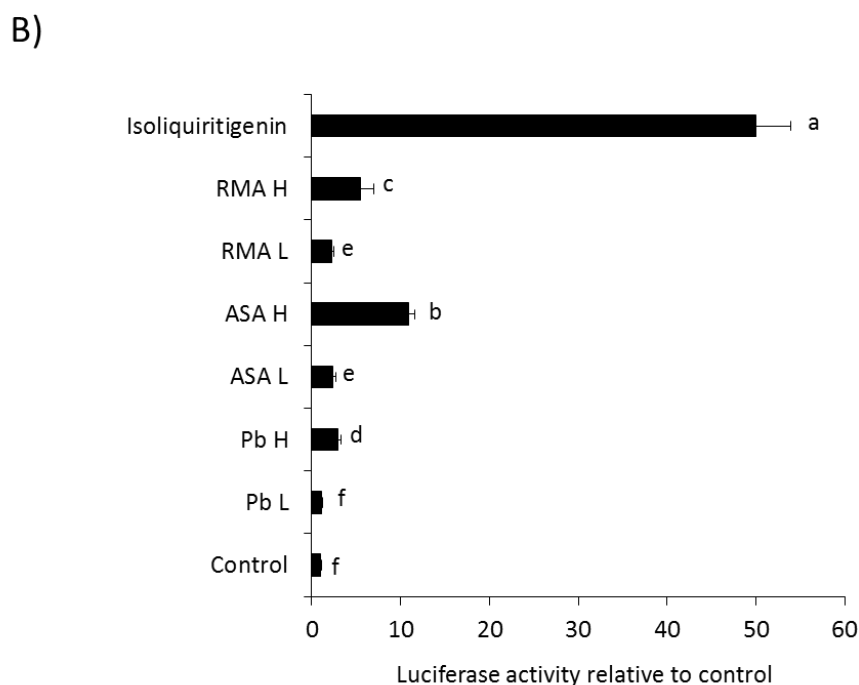
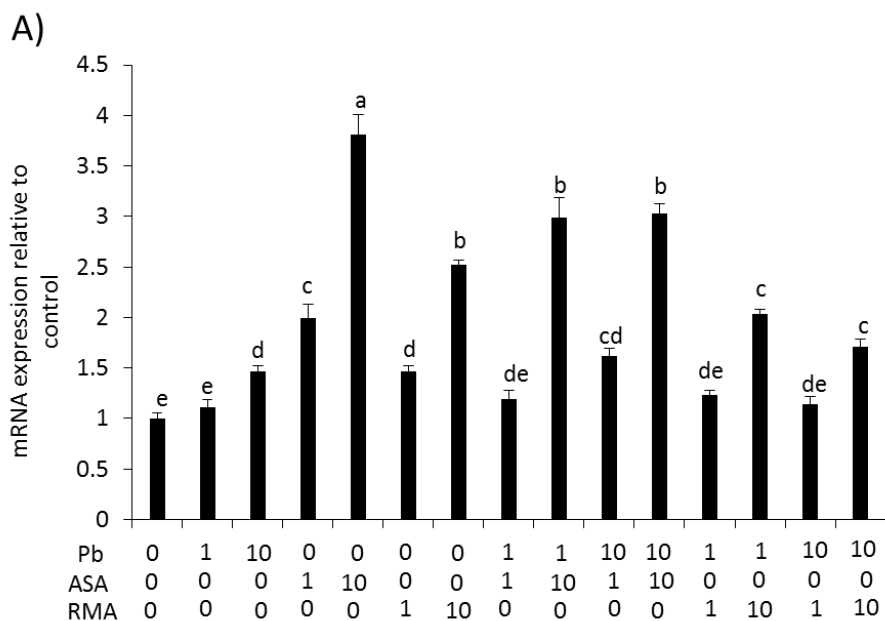


Fig. 5: Effects of Pb, RMA and ASA on Nrf2 mRNA expression and luciferase activity
 The effects of Pb, RMA and ASA on **A)** Nrf2 mRNA expression, **B)** Nrf2 luciferase activity. Isoliquiritigenin was used as a positive control when determining luciferase activity. Data are presented as the mean $\pm$ SD (n = 6). Columns with different superscript letters are significantly different ($p < 0.05$).

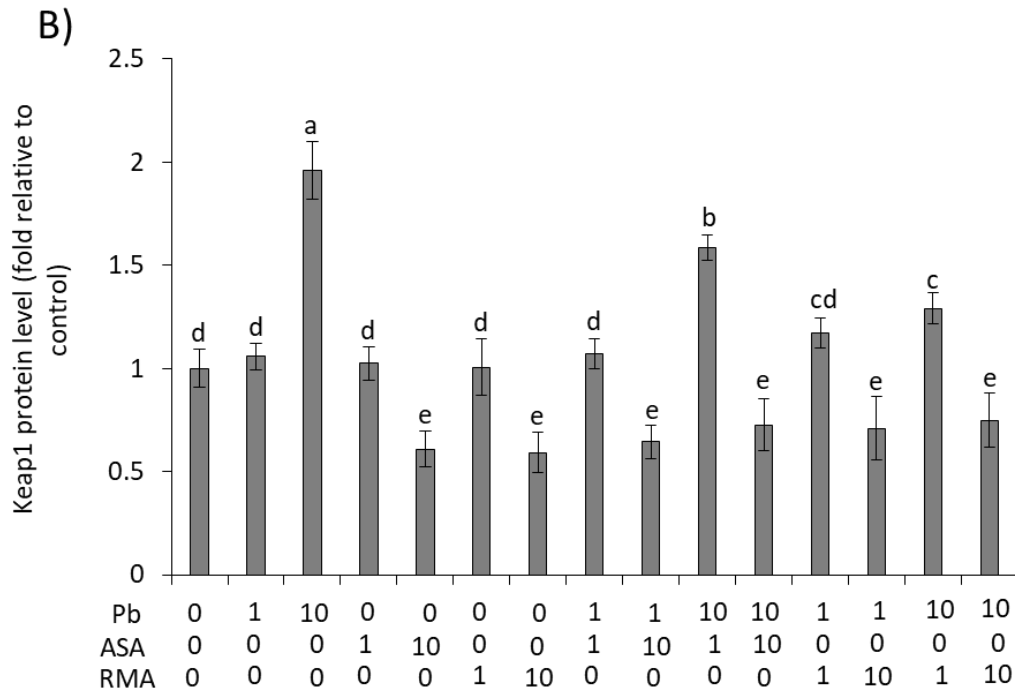
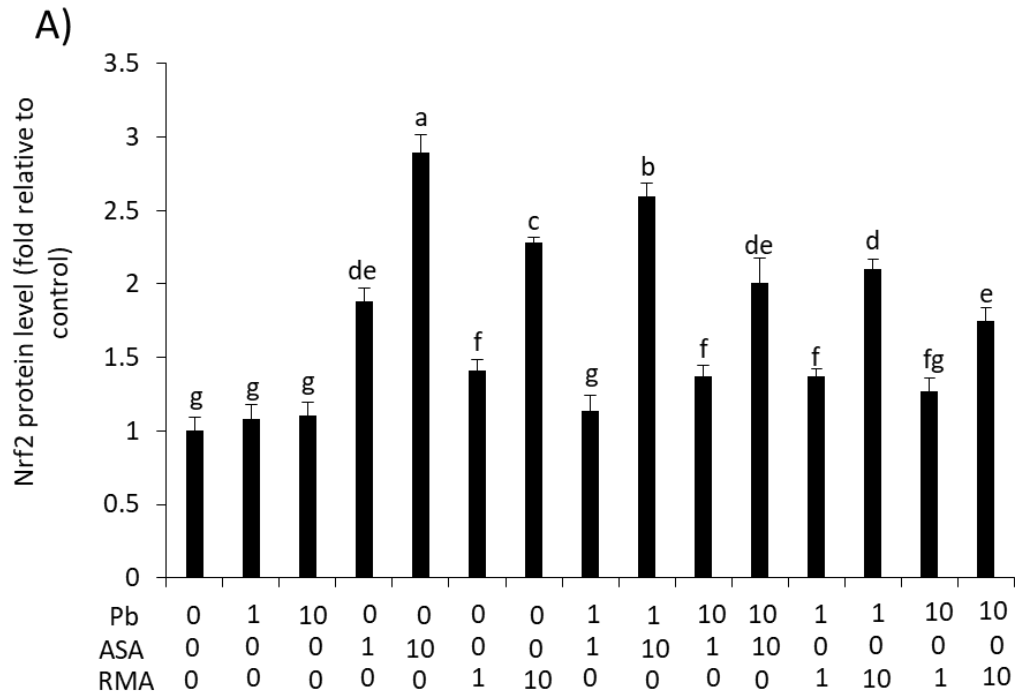


Fig. 6: Effects of Pb, RMA and ASA on the protein levels of Nrf2 and Keap1 in HepG2 cells
 The effects of Pb, RMA and ASA on the protein levels of **A)** Nrf2, **B)** Keap1 using ELISA kits. Data are presented as the mean $\pm$ SD (n = 6). Columns with different superscript letters are significantly different ($p < 0.05$).

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365 **Conflicts of interest**

366 The authors do not have any conflict of interest.
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