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Ph.D. Thesis Summary

Some aspects of molecular mechanisms of xenobiotics' hepatotoxicity and hepatoprotection

Modulatory roles of natural polyphenols

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Information regarding PhD thesis will be provided at the Dean's Office of First Faculty of Medicine, Charles University

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ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Casp3	caspase 3
CAT	catalase
CD	conjugated dienes
COX-2	cycloxygenase 2
D-GalN	D-galactosamine
GPx	glutathione peroxidase
GSH	reduced glutathione
INF-γ	interferon gamma
LPS	lipopolysaccharide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor kappa B
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS-2	nitric oxide synthase-2
TBARS	thiobarbituric acid reactive substances
TBOOH	*tert*-butyl hydroperoxide
TNF-α	tumor necrosis factor-alpha

SUMMARY

Background & Aims: Oxidative stress and apoptosis are proposed mechanisms of cellular injury in studies of xenobiotic hepatotoxicity. The aim of this work is to find early signal markers of drug-induced injury of the liver by focusing on select antioxidant/oxidant and apoptotic genes. As well, to address the relationship between conventional liver dysfunction markers and the measured mRNA and protein expressions in the D-galactosamine/lipopolysaccharide and tert-butylhydroperoxide hepatotoxicity models. Furthermore, potential hepatoprotective capabilities of antioxidant polyphenols quercetin and curcumin were evaluated in relation to its modulation of the oxidative stress and apoptotic parameters in the given xenobiotic hepatotoxicity models.

Methods: Biochemical markers testing the hepatic function included aminotransferases (ALT, AST) and bilirubin. Measurements of TBARS and conjugated dienes were used to assess lipoperoxidation. Plasma levels of catalase and reduced glutathione were used as indicators of the oxidative status of the cell. Real time PCR was used to analyse the mRNA expressions of the inducible nitric oxide synthase (NOS-2), heme oxygenase-1 (HO-1), superoxide dismutase (SOD-1), glutathione peroxidase (Gpx-1), caspase 3 (Casp3), BH3 interacting domain death agonist (Bid) and Bcl-2 -associated X protein (Bax), and tumor necrosis factor α (TNF- α)mRNAs. Additionally, the protein expressions of HO-1 and NOS-2 were assessed with the use of Western blot method. Morphometric evaluation of hepatocytes at the light microscopical level was done on

semithin epon sections stained by toluidine blue using Leica IM 500 program for digital recording and measurements. Statistical analysis was performed using ANOVA and post hoc Boneffori, Tukey- Kramer comparison test or unpaired T-test with Welch correction.

Results: Overall, the results of this study have revealed the early activation of oxidative stress and apoptosis in the given hepatotoxic models as seen by the relevant changes in the tested parameters. Hepatoprotective effects of curcumin and quercetin were demonstrated, where the induction of the antioxidant enzyme HO-1 and its products played the most important cytoprotective role. In case with curcumin, this effect was paralleled with the concomitant reduction of NOS-2 and TNF-α expressions.

Conclusion: Understanding the mutual regulatory mechanisms of the tested parameters in hepatocyte injury should provide important clues to the diagnosis and treatment of liver damage. The research data from the present study paves a way for those interested in further research of these dietary polyphenols, curcumin and quercetin as it gives an overview of the potential cytoprotective mechanisms and effective doses in the given models.

SOUHRN

Zdůvodnění a cíle: Oxidační stres a apoptóza jsou navrhovanými mechanismy buněčného poškození v studiích hepatotoxicity vyvolané cizorodými látkami. Cílem této práce bylo nalézt časné signální markery jaterního poškození cizorodými látkami soustředěním na vybrané antioxidační/oxidační a apoptotické geny. Cílem mé práce dále bylo oslovit vztah mezi obvyklými markery jaterní dysfunkce a mRNA a proteinovou expresí v modelech hepatotoxicity s D-galaktozamin/lipopolysacharidem a tert-butylhydroperoxidem. Kromě toho, byly vyhodnocené hepatoprotekční možnosti antioxidačních polyfenolů quercetinu a kurkuminu v souvislosti s jejich modulacemi oxidačního stresu a apoptotických parametrů v stanovených modelech hapatoxicity způsobené cizorodými látkami.

Metody: Biochemické markery zkoumající jaterní funkce zahrnují aminotransferázy (ALT, AST) a bilirubin. Meření TBARS a konjugovaných dienů bylo použito k stanovení peroxidace lipidů. Hladiny katalázy a redukovaného glutathionu byly použity jako indikátory oxidačního stavu v buňce. Pomocí ‘real-time PCR’ byly analyzovány mRNA exprese inducibilní syntázy oxidu dusnatého (NOS-2), hemoxygenázy-1 (HO-1), superoxiddismutázy (SOD-1), glutathionperoxidázy (Gpx-1), kaspázy 3 (Casp3), BH3 interacting domain death agonist (Bid) and Bcl-2 -associated X protein (Bax), a tumor necrosis factor α (TNF- α)mRNAs. Proteinové exprese HO-1 a NOS-2 byly stanoveny pomocí metody Western blot. Morfometrické hodnocení hepatocytů použitím světelného mikroskopu bylo uděláno na polotenkých segmentech, které byly obarveny toluidinovou modří. Pro tento účel byl použit Leica IM 500 program pro digitální záznamy a měření. Statistická analýza byla udělána pomocí ANOVA a post hoc Boneffori, Tukey- Kramer porovnávácího testu nebo nepárového T-testu s Welch korekcí.

Výsledky: Výsledky této studie nám odhalují časnou aktivaci oxidačního stresu a apoptózy v daných modelech hepatotoxicity, což je vidět z příslušných změn měřených parametrů. Hepatoprotekční účinky kurkuminu a quercetinu byly prokázany, přičemž indukce antioxidačního enzymu HO-1 a jejich produkty hrály nejdůležitější cytoprotekční

roli. V případě kurkuminu, paralelně s tímto účinkem, jsme pozorovali i průvodní redukci NOS-2 a TNF-α expressí.

Závěr: Porozumění vzájemných regulačních mechanismů testovaných parametrů při poškození hapatocytů by mělo poskytnout důležité stopy k diagnóze a léčbě jaterní nemoci. Data vyplývající z této studie připravují cestu pro ty, kteří mají zájem o další výzkum těchto dietních polyfenolů, kurkuminu a quercetinu, neboť dávají přehled potenciálních cytoprotekčních mechanismů a účinných dávek v daných modelech.

1 INTRODUCTION

Xenobiotic induced hepatotoxicity, such as those caused by medications, has been an ongrowing problem largely attributable to the increasing amounts of new drugs entering the market (Jaeschke *et al*. 2012, Rangnekar and Fontana 2011). Additionally, the growing number of xenobiotics found in our environment increases the chance of exposure of liver to these toxic substances. Growing scientific evidence attributes oxidative stress and increased reactive oxygen species production as the main mediators of pathology of both acute and chronic liver injury caused by xenobiotics. Oxidative stress can be induced by toxins as it causes accumulation of reactive oxygen/nitrogen species, further causing a misbalance in pro-oxidant/antioxidant steady state. Protein crossslinking, lipid peroxidation, mitochondrial dysfunction and induction of cell death pathways are some of the proposed mechanisms of cellular damage due to this misbalance (Jaeschke 2011).

Activation of nuclear transcription factors by hepatotoxins may be an important mechanism in liver cell defence and survival in toxic injury. Activation of transcription factor, such as antioxidant response element, leads to increased transcription of antioxidant enzymes including heme oxygenase-1, superoxide dismutase-1 and glutathione peroxidise . These antioxidant enzymes are important for maintaining a homeostatic redox state and their deficiency or over-expression may cause irreversible damage to the tissues of the body. One such example of cellular damage is a resultant increase in cytokine TNF-α level. This cytokine has been shown to induce apoptosis mediated by executive caspases and the members of Bcl-2 family proteins, Bid and Bax (Morgan *et al*. 2010, Van Herreweghe *et al*. 2010). Interestingly, enzymes such as nitric oxide synthase-2 exhibit both pro-oxidant and antioxidant characteristics, depending on the current cell conditions and may exhibit relationship with other antioxidant enzymes. Elucidation of the steps involved in this complex interaction between the oxidative stress and apoptotic mechanisms has yet to be determined. For this reason, studies on the oxidative stress mechanisms as pertaining to liver damage and clarification of exact mechanisms involved in different settings are of crucial importance. In addition, complete understanding of the role of antioxidant system pathways as well as their inter-relationship and their modulation will be of great benefit.

Fortunately, in the recent years scientific field has seen a growth in research studies done on a genomic scale, which help further our understanding of the toxicity mechanisms. Specifically, the field of toxicogenomics involves identification of genomic biomarkers of toxicity through analysis of mRNA profiling. The information obtained from these molecular analyses discloses gene changes that have important effects on cell function, without necessarily exhibiting changes in the morphology of the cell. As well, there exists a possibility of induction of target genes by xenobiotics without involving

any changes in the DNA sequence. These epigenetic effects include chromatin modifications such as alteration in histones, as well as post-transcriptional changes (Heijne *et al.* 2005). In the future, the combination of application of epigenetics, genomics and proteomics will lead to more accurate assessments of the risks associated with xenobiotic exposure.

Substances isolated from natural sources have been used traditionally in the pharmacotherapy of various diseases, as they are relatively inexpensive and widely available. Silymarin and reservatrol are some of many examples of natural substances that have shown a strong hepatoprotective potential due to their antioxidant, anti-inflammatory and liver regeneration capabilities (Pradhan and Girish 2006, Glauert *et al.* 2010). On the other hand, substances such as curcumin and quercetin have shown to exhibit antioxidant and cytoprotective properties, but their use as hepatoprotectants has not been extensively investigated (Zhou *et al.* 2011, Chirumbolo 2010). The need for seeking other novel potential hepatoprotective substances is important in regards to the aim to reduce the occurrence of xenobiotic mediated liver injury. Conversely, the hepatotoxic potential of natural supplements should also be taken into account.

This research work will try to encompass all of these before mentioned concepts with the overall goal to evaluate mechanisms of xenobiotic induced hepatotoxicity as well as to seek and assess novel potential hepatoprotective substances.

2 RESEARCH AIM & HYPOTHESIS

Understanding the exact mechanism of xenobiotic hepatotoxicity is one of the major challenges hepatologists are faced with today. Several mechanisms have been shown to play the major role in liver toxicity, including oxidative stress and apoptosis. It can be hypothesized that during the events of oxidative stress, there will be an increase in gene expression of both antioxidant (eg. HO-1, SOD-1, GPx, Cat) and pro-oxidant (eg.NOS-2) enzymes. Extensive oxidative stress will lead to activation of cell death mediators (eg.Bid, Bax, Casp3) and inflammatory cytokines (eg.TNF-alpha).

Furthermore, agents which are able to alter expression of anti-/pro-oxidant enzymes and apoptotic mediators have a potential in modulation of hepatotoxicity.

Thus, the aim of this research work is:

- To elucidate some underlying molecular mechanisms of xenobitic hepatotoxicity in the given *in vitro* Tert –butyl hydroxyperoxide (TBOOH) and *in vivo* D-Galactosamine and lipopolysaccharide (D-GalN/LPS) models, in regards to the involvement of oxidative stress and apoptotic cell death pathways.
- To evaluate the activities of specific antioxidant enzymes (HO-1, SOD-1, GPx, CAT), pro-oxidant enzyme (NOS-2), the inflammatory cytokine TNF-α and apoptotic mediators (Bid, Bax, Casp3) involved in the given hepatotoxicity models and to see whether there exists a potential mutual relationship between the given parameters.
- To evaluate the potential hepatoprotective properties of the natural substances, curcumin and quercetin, as pertaining to their abilities to ameliorate oxidative stress and apoptosis.

3 MATERIALS & METHODS

3.1 Animals and Experimental Design

This study was performed on male Wistar rats (200–300g) that were fed granulated diet ad libitum under standard conditions; light (i.e. 12 h light and 12 h dark); temperature (22 ± 2ºC); relative humidity (50 ± 10%). All rats received humane care according to the general guidelines and approval of the Ethical Committee of the First Faculty of Medicine, Charles University in Prague.

For the purposes of *in vivo* experiments rats were injected i.p. with a dose of specific substance of interest (eg. D-GalN, curcumin). The rats were sacrificed at 24 hours following injection. For the purposes of liver extraction, rats were injected with heparin into the vena cava, followed by liver perfusion with specific buffer for several minutes. Subsequently, hepatocytes were isolated using the standard two-phase colagenase perfusion method that allows for disruption of intercellular connection in liver tissue. Purified hepatocyte suspension was obtained by centrifugation, and suspensions having a cell count greater than 85% were used in the subsequent steps (Moldeus *et al.* 1978).

The polystyrene Nunclon™ culture plates covered with collagen suspension diluted in acetic acid and washed with Williams E medium were used for cell culture. Incubation of plates for the first 24 hours was carried out in complete medium containing Willams E medium, penicilin (1%), streptomycin (1%), glutamine (1%), insulin (0.06%) and fetal bovine serum (5%). In the following 24 hours plates were incubated with a mix of complete medium and/or appropriate testing substances. All cell cultures were incubated in an incubator with a high relative humidity of ± 90% in atmosphere containing 5% CO_2 at 37°C.

3.2 Biochemical and Lipoperoxidation Parameters Measurements

Determinations of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin plasma levels were carried out using Fluitest® GPT ALT, Fluitest® GOT AST and Fluitest® BIL-Total kits respectively. Concentration of urea in the medium was directly measured using the diagnostic Fluitest® Urea Kit manufacturer‘s instructions. Nitric oxide production was determined spectrophotometrically (540 nm) by measurement of one of its stable oxidation products, NO_2^-, in plasma using Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine, 2.5% H_3PO_4). The medium nitrite levels were calculated by comparison with a sodium nitrite standard curve. The measurement of catalase in plasma was performed according to the reaction between H_2O_2 and molybdenium ammonium as previously reported (Goth 1991). Assessment of reduced gluathione in homogenate was based on the method that depends on a reaction between thiol group with 5,5-dithio-2-nitrobenzoic acid which can be measured spectrophotometrically (Sedlak and Lindsay 1968). The measurement of catalase in plasma was performed according to the reaction between H_2O_2 and molybdenium ammonium as previously reported (Goth 1991). The lipid peroxidation of the rat liver was assayed by the thiobarbituric acid (TBARS) method, and the spectrofluorometric assay for conjugated dienes (CD) was carried out as described earlier (Yagi 1976, Ward *et al.* 1985). The results were expressed in nmol/mg of total protein.

3.3 Real-time PCR Gene Expression Measurements

Total RNA isolation was carried out according to the manufacturers' instructions of the Qiagen® RNeasy plus kit. Subsequently, the reverse transcription from total RNA to cDNA was carried out with universal GeneAmp® RNA PCR kit, using a murine leukemia virus (MuLv) as reverse trancriptase. The obtained total mRNA was reverse transcribed to cDNA with the help of ABI PRISM 7900, and TagMan® Gene Expression master mix. Expressions of genes of interest were evaluated using real-time polymerase chain reaction (RT-PCR). The TagMan® Gene Expression Assays Kit was used. NOS-2, HO-1, TNF-α, CAT, SOD-1, Bid, Bax and Casp 3 were used as genes of interest (target genes) and beta-actin gene as a control (housekeeping) gene, using the FAM colored primes and probes. Housekeeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. The obtained Ct values were used in relative quantification of gene expression measurements relative to the endogenous gene control Ct measurements, and the relative gene expression was calculated using the ΔΔCt method (Arocho *et al.* 2006).

3.4 Western Blot Analysis of Proteins

Isolated liver samples were lysed with lysis buffer (1 MTris HCl) and homogenized with an electric homogenizer. The cell lysates were mixed (1:1) with sample buffer (b-mercaptoethanol, 10% SDS, glycerol, bromophenol blue, 0.5 M Tris HCl pH 6.8) and then heated for 5 min at 95 °C. Protein from the tissue samples (30 μg protein measured by Bradford method) were separated on 10% SDS-acrylamide gel and transferred to a nitrocellulose membrane by electrophoresis overnight at 4 °C. Membranes were blocked for 1 h with 5% non-fat milk in Tris-buffered solution, at room temperature. Membranes were then washed in a washing buffer (NaCl, KCl, Na2HPO4, KH2 PO4, Tween, H2O). They were then incubated with rabbit primary antibody against NOS-2 (1:1000), mouse HO-1 primary antibody (1:500) or mouse beta actin primary antibody (1:5000). After eluting the unconjugated primary antibody with the wash buffer, the membrane was then incubated with corresponding secondary antibody anti-rabbit IgG HRP conjugate (1:20,000) or anti-mouse IgG HRP conjugate (1:100,000). This step was followed by chemiluminescence labeling with Super SignalWest Pico Chemiluminescent Substrate for 2 min. Bands were detected with the use Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software. Optical densities of NOS-2 and HO-1 bands were normalized by the corresponding loading control (ACTB) and then to the mean of the corresponding control group.

3.5 Measurements of Apoptotic/Necrotic Parameters and Morphological Evaluation

Caspase 3 activity was measured according to the instructions of Sigma-Aldrich fluorometric caspase 3 assay kit. The results were expressed as percentage of caspase 3 activity in the treated goup relative to the control. Cell viability was measured using the MTT test method (Mosmann 1983), in which a yellow compound, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, is reduced to the purple formazan in the living cells by the action of the mitochondrial reductases. The absorbance of the measured spectrophotometrically at the wavelength of 540 nm.

Morphological evaluation of hepatocytes at the light microscopical level was done on semithin epon sections (1-2 μm thick) stained by toluidine blue using Leica IM 500 program for digital recording and measurements.

3.6 Statistical Evaluation

All of the executed experiments were performed using a minimum of five rats per tested group. The collected data was adjusted using the Dixon test that excluded the outliers. The data was then processed using the GraphPad statistical program. All of the data passed the normality test. Thus the statistical significance of the mean scores was performed using the One-way Analysis of Variance with the use of appropriate post tests (post hoc Boneffori, Tukey- Kramer comparison test, unpaired T-test with Welch correction) to evaluate the plasma, tissue, protein and/or genetic alterations between the treated vs. untreated animals (negative control). In each case, there was just one independent measurement of each parameter from each animal. The p-values less than 0.05 were considered significant.

4 RESULTS

4.1 *In vitro* tert-butylhydroperoxide hepatotoxicity model: The effects of quercetin

Treatment with TBOOH resulted in significantly higher levels of ALT (5-fold) and AST (3-fold) production that was ameliorated by the addition of quercetin (TBOOH + Q). However, when given alone, quercetin did not have any influence on AST and ALT production compared to control group. The results of MTT test have shown that the application of to the cell culture, has significantly decreased viability of hepatocytes by 3-fold in 0.25 mM, and 5.5-fold in 0.50 mM concentrations. Quercetin significantly improved cell viability in TBOOH treated cells, which is shown by significant increases in all quercetin treated hepatocytes (Fig. 1).

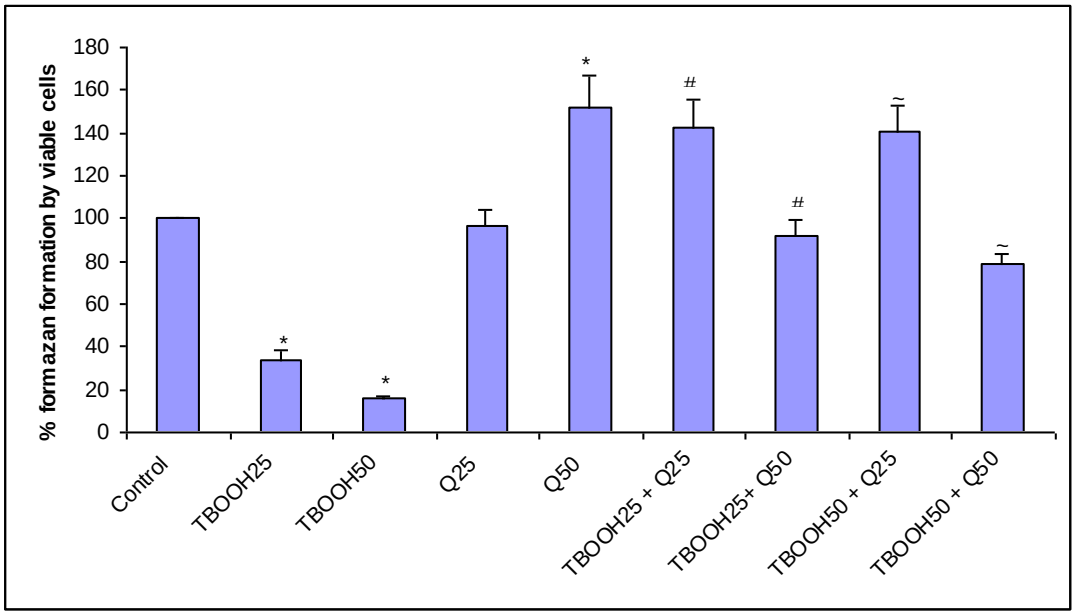


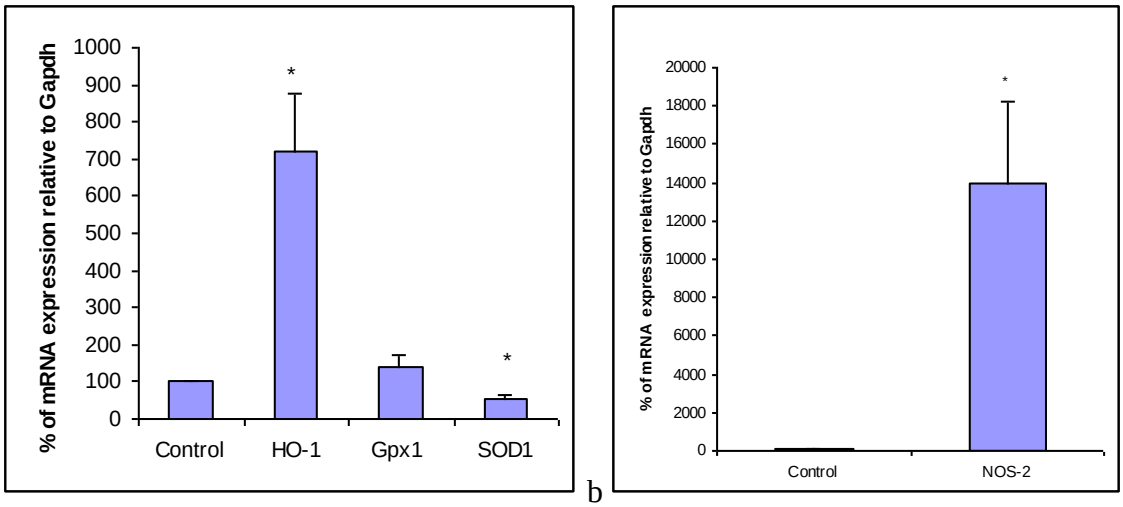
Fig. 1. *The effects of tert-butylhydroperoxide and quercetin treatment on mitochondrial dehydrogenase activity (MTT test), expressed as a percentage of formazan formation by viable hepatocytes.*

Administration of TBOOH had no effect on HO-1 mRNA expressions, whereas quercetin treatment (without TBOOH) of hepatocytes increased HO-1 mRNA expressions significantly. Interestingly, treatment with 250 μM quercetin significantly increased HO-1 mRNA expressions in both of the TBOOH groups, but 500 μM had no effect. The administration of TBOOH to hepatocytes increased mRNA expressions of NOS-2 significantly. Mostly, quercetin administration had no effect on NOS-2 mRNA expressions, with the exception of 500 μM concentration given alone, which significantly increased NOS-2 mRNA expression. The inflammatory cytokine TNF-α mRNA gene expressions were increased in TBOOH 0.5 mM group, but not in 0.25 mM TBOOH group. Furthermore, quercetin alone, and combination of TBOOH 0.25 mM and quercetin 500 μM had no effect on TNF-α mRNA gene expressions. Addition of quercetin in other combination groups (Q+TBOOH) significantly reduced TNF-α mRNA gene expressions.

4.2 *In vivo* xenobiotic hepatotoxicity model (D-Galactosamine and Lipopolysaccharide): Involvement of oxidative stress and apoptosis

The combination of D-GalN (i.p., 400 mg/kg) and LPS (i.p., 50 μg/kg) treatment in rats has produced hepatic failure as it significantly increased the levels of aminotransferases in plasma. There was no significant effect of D-GalN/LPS on the lipid peroxidation as there were no differences in the levels of TBARS and CD between the two groups . Additionaly, there were observed increases in plasma levels of antioxidant enzyme catalase in D-GalN/LPS treated rats.

As for the antioxidant enzymes of interest, D-GalN/LPS treatment has caused a highly significant increase in the HO-1 gene expression, a decrease of SOD1 gene expression, while it had no significant effect on the Gpx1 expressions, in comparison to the untreated control group, using Gapdh as endogenous control (Fig. 2a). In comparison to the untreated control animals, D-GalN/LPS treatment induced simultaneous statistically significant increase in both plasma NO_2^- levels and NOS-2 gene expression (Fig. 2b).



a b

Fig. 2. *The effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) HO-1,Gpx1, SOD-1 (a) and NOS-2 (b) gene expressions relative to Gapdh as the endogenous control.*

The evaluation of gene expressions of the selected apoptotic parameters including Casp3, Bid and Bax show significant increases in all of the three parameters as a result of D-GalN/LPS treatment (Fig.3).

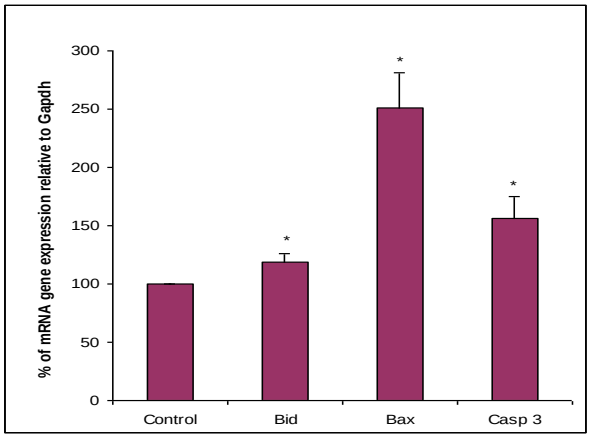


Fig. 3. *The effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) Bid, Bax and Casp3 gene expressions relative to Gapdh as the endogenous control.*

The morphological evaluation has shown the well preserved cytological features of the control liver tissues, which was affected as a result of D-GalN/LPS insult (Fig. 4a). At the lower magnification (Fig. 4b) striking necrotic lesions can be observed in the peripheral and intermediate regions of the central vein lobule. Furthermore, aggregation of heterochromatin near the nuclear envelope confirms the apoptosis in some hepatocytes and the presence of pycnotic nuclei is clearly visible (Fig. 4 c & d).

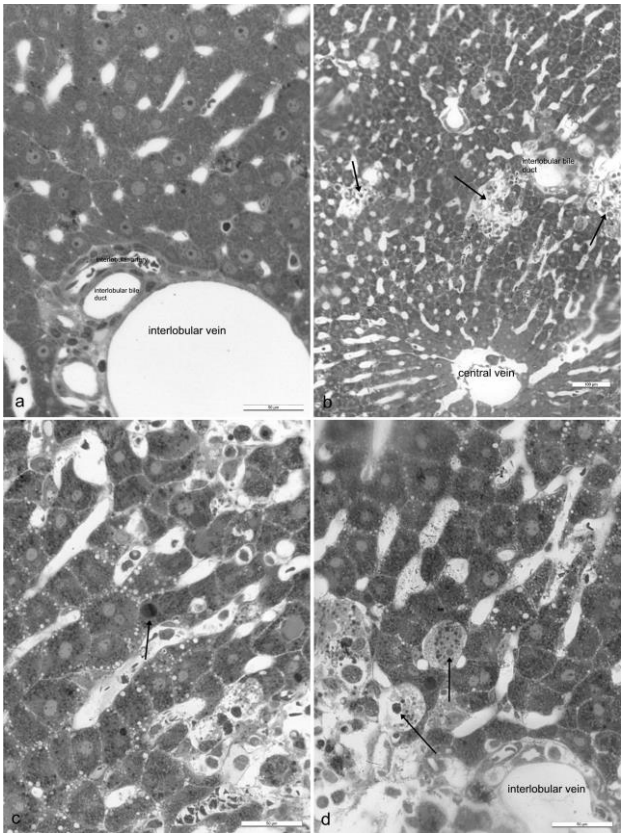


Fig. 4. *Light microscopy morphological findings of rat liver of control and D-GalN/LPS treated samples: a) control hepatocyte; the effect of D-GalN/LPS treatment b) low magnification, bar 100 μm; c) higher magnification, bar 50 μm; d) presence of pycnotic nuclei (arrows), bar 50 μm;*

4.3 The effects of curcumin treatment on *in vivo* D-GalN/LPS xenobiotic hepatotoxicity model

In this study, the influence of curcumin treatment (*i.p*., 100 mg/kg) on D-GalN (*i.p*., 400 mg/kg) and LPS (*i.p*., 10 μg/kg) model of hepatotoxicity was tested. The combination of D-GalN/LPS developed acute hepatotoxicity within 24 hours of the insult as seen in the observed several-fold increase in plasma levels of transaminases (AST and ALT). Pretreatment with curcumin in D-GalN/LPS rats significantly decreased the levels of both ALT and AST. In the groups treated with D-GalN/LPS, we observed significant 5.2-fold increase in bilirubin and 2.4-fold increase in catalase plasma levels, which was further increased by the curcumin pre-treatment in D-GalN/LPS treated rats. However, curcumin had no effect when given alone. D-GalN/LPS treatment resulted in 3.5-fold significantly higher levels of CD compared to that of control, indicating presence of lipoperoxiation, which was ameliorated by the curcumin pre-treatment.

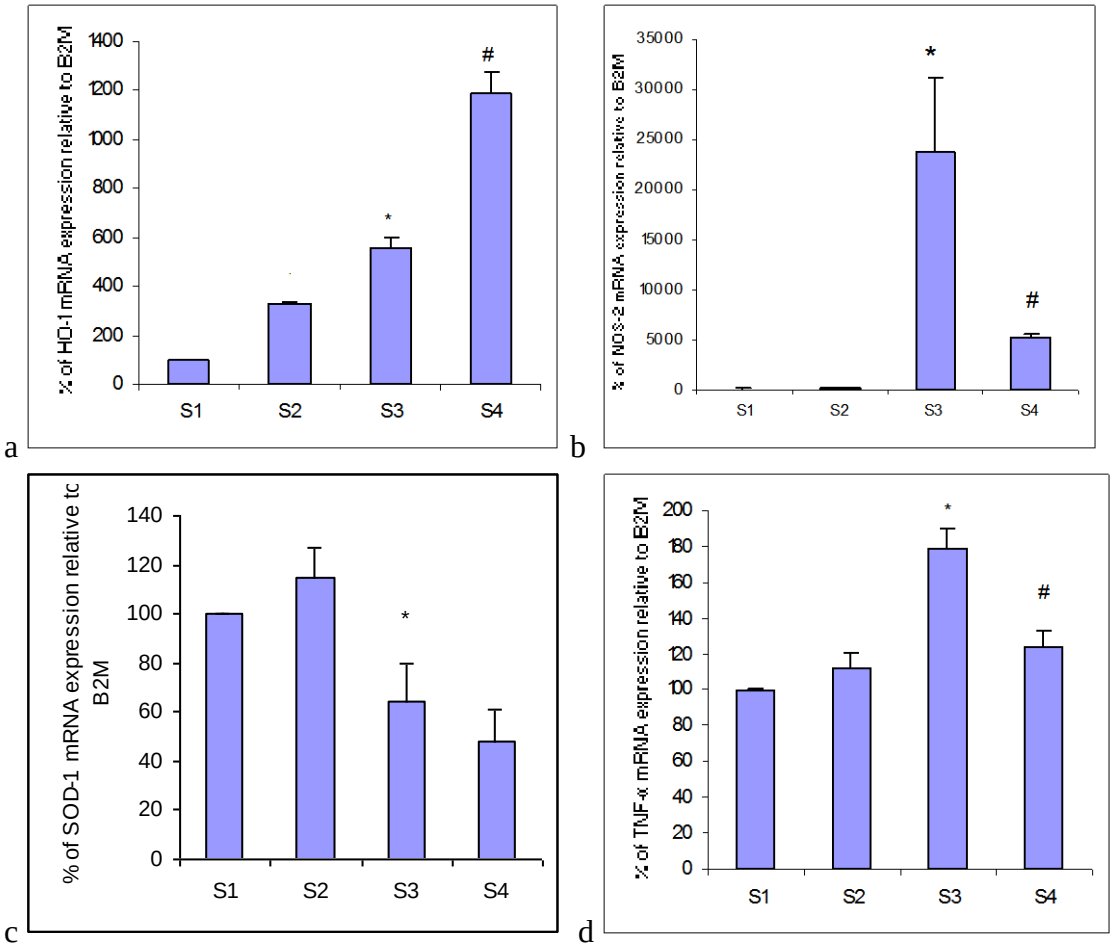


Fig. 5. *The effects of curcumin pre-treatment on HO-1 (a), NOS-2 (b) SOD-1 (c) and TNF-α mRNA expressions in D-GalN/LPS hepatotoxicity model. S1-vehicle only; S2: curcumin 100 mg/kg; S3: D-GalN 400 mg/kg + LPS 10 µg/kg; S4: D-GalN/LPS+curcumin.* Indicates significant values (p≤0.05) compared to the control group (vehicle only); # Indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS).*

The administration of D-GalN/LPS has resulted in significant 5-fold increase in the HO-1 and a 240-fold increase in NOS-2 mRNAs expressions (Fig. 5 a&b). Conversely, D-GalN/LPS administration resulted in a 1.6-fold decrease in SOD1 mRNA expression (Fig.5c). Pre-treatment with curcumin of D-GalN/LPS rats resulted in further increase in HO-1 mRNA expression (2.2-fold); a decrease in NOS-2 mRNA expression (4.7-fold); while it had no significant effect on SOD1 mRNA expression. Administration of D-GalN/LPS significantly increased the expression of proinflammatory cytokine TNF-α, whereas curcumin pre-treatment significantly decreased its mRNA level, indicating the potential anti-inflammatory effect (Fig. 5c).The mRNA expressions measurements of Bid, Bax and Casp3 were used as determinants of potential apoptotic process (Fig. 6). As might be expected, the administration of D-GalN/LPS has resulted in significant increases in all three parameters; Bid (1.5-fold); Bax (2-fold); and Casp3 (1.4-fold). Pre-treatment with curcumin reduced the apoptotic process, as can be seen by statistically

significant decreases of both Bid and Bax mRNA expressions in D-GalN/LPS treated rats. There was no statistically significant difference in Casp3 mRNA expression.

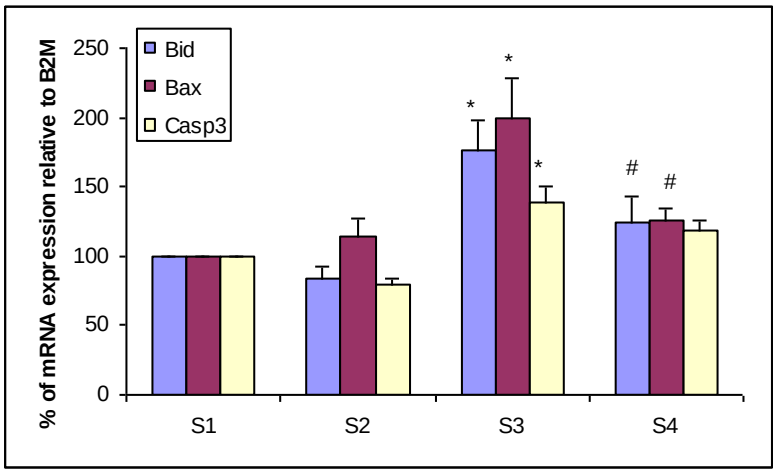


Fig. 6. *The effects of curcumin pre-treatment on Bid, Bax and Casp3 mRNA expressions in D-GalN/LPS hepatotoxicity model. S1-vehicle only; S2: curcumin 100 mg/kg; S3: D-GalN 400 mg/kg + LPS 10 μg/kg; S4: D-GalN/LPS+curcumin.* Indicates significant values (p≤0.05) compared to the control group (vehicle only); # Indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS).*

4.4 The effects of quercetin treatment on *in vivo* D-GalN/LPS xenobiotic hepatotoxicity model

In this study, the influence of quercetin treatment (*i.p.*, 50 mg/kg) on D-GalN (*i.p.*, 400 mg/kg) and LPS (*i.p.*, 10 μg/kg) model of hepatotoxicity was tested. The results show that rats treated with the combination of D-GalN/LPS developed acute hepatotoxicity, which was confirmed by a several-fold increase in plasma levels of transaminases (AST and ALT) that are indicative of failing liver function. Treatment with quercetin (Q) in D-GalN/LPS rats resulted in significant lowering of the ALT (5.6-fold) and AST (5.9-fold) plasma levels, compared to the group receiving the hepatotoxic D-GalN/LPS combination. Furthermore, in the groups treated with D-GalN/LPS, we observed significant 6.4-fold increase in bilirubin and 5.5-fold increase in catalase plasma levels. However, quercetin treatment had no effect on the levels of these two antioxidants.

The second question to be addressed in this study was to determine whether, and how, quercetin influences the activity of the potent antioxidant enzyme HO-1, as well as that of the enzyme NOS-2 in D-GalN/LPS model of hepatotoxicity. As postulated, both HO-1 mRNA and protein expressions were significantly increased (30.6-fold and 2-fold respectively, $p \leq 0.05$) in D-GalN/LPS group compared to control. Conversely, quercetin alone did not exert any significant changes in HO-1 mRNA nor protein expressions.

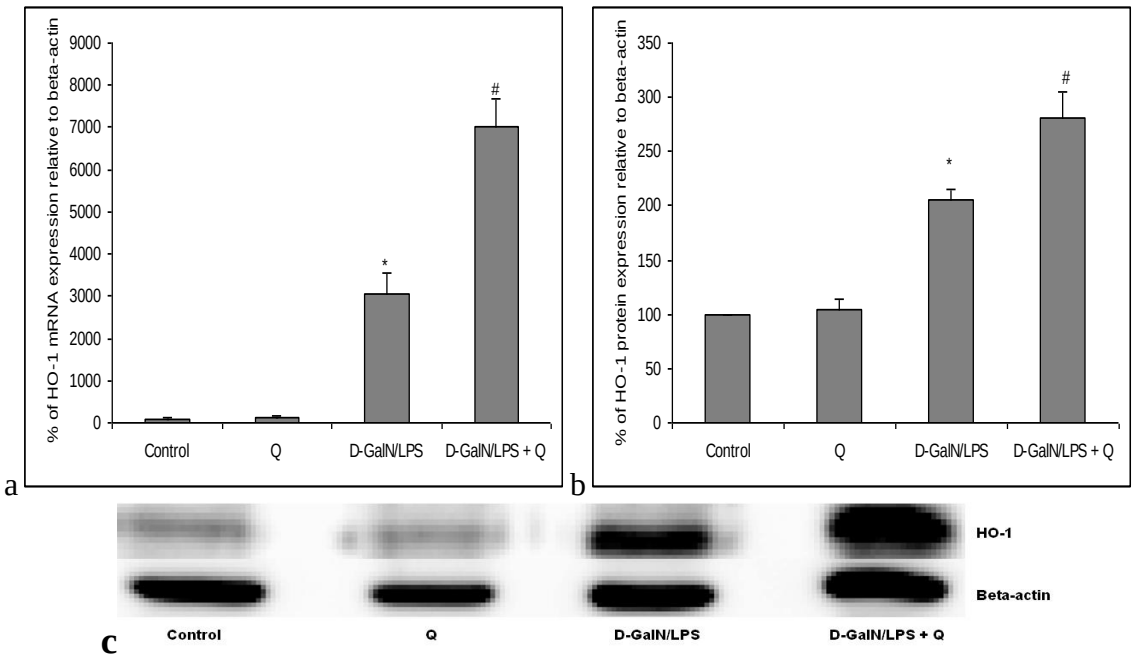


Fig. 7. *Increase in HO-1 gene and protein expressions by quercetin treatment in D-GalN/LPS hepatotoxicity model. Gene expression of HO-1 (a)***,** *optical density (b) of HO-1 protein expression and protein bands (c). * Indicates significant values (p≤0.05) compared to the control group); # Indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS).*

However, when given to D-GalN/LPS treated rats, quercetin significantly (p≤0.05) increased the levels of HO-1 mRNA expression (by 2.3-fold) and protein expression (by 1.2-fold). These results clearly indicate the induction of HO-1 under hepatotoxic conditions, where quercetin further increased its activity under hepatotoxic, but not under physiological, conditions (Fig. 7).

Furthermore, D-GalN/LPS challenge resulted in a significant 12.3-fold increase in NOS-2 mRNA and 2.4-fold increase in NOS-2 protein expressions in comparison to the control group (p≤0.05) (Fig. 8). Interestingly, however, there was no significant difference in NOS-2 mRNA and protein expressions between the D-GalN/LPS and D-GalN/LPS+Q groups, despite the notice of slight decrease in these levels upon addition of quercetin. Moreover, quercetin did not show to have any influence on NOS-2 mRNA and protein expressions when given under normal physiological conditions (Fig. 8).

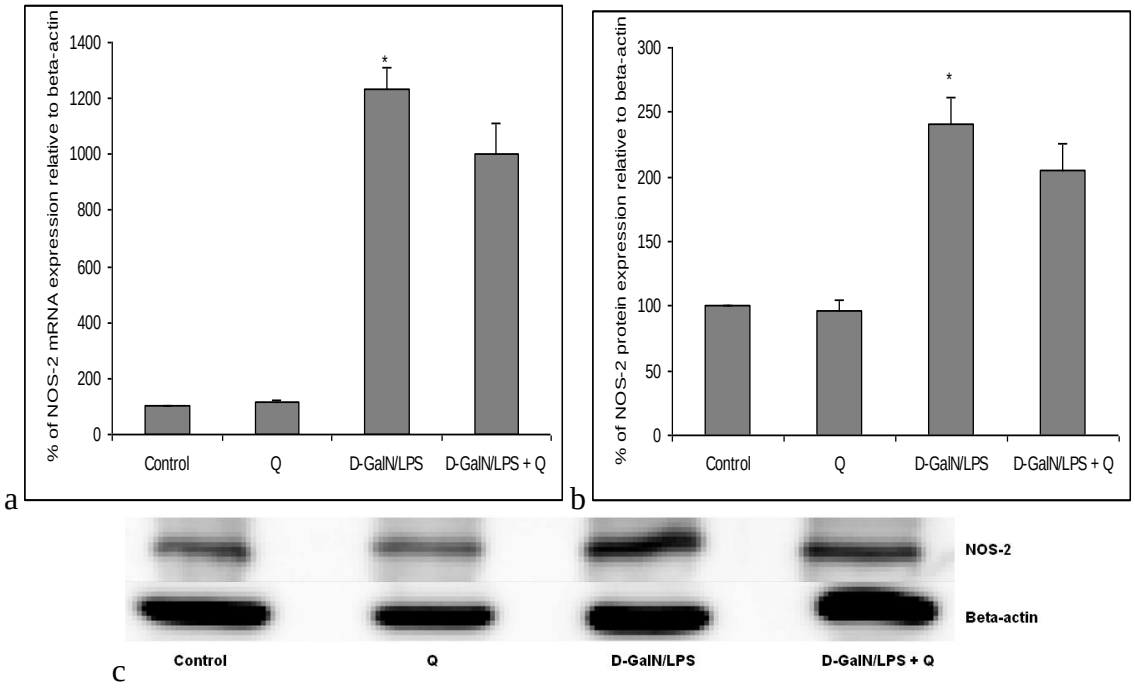


Fig. 8. The effects of quercetin treatment on NOS-2 on gene and protein expressions in D-GalN/LPS hepatotoxicity model. Gene expression of NOS-2 (a)**,** optical density (b) of HO-1 protein expression and protein bands (c). * Indicates significant values ($p \leq 0.05$) compared to the control group); # Indicates significant values ($p \leq 0.05$) compared to the respective positive control group (D-GalN/LPS).

5 DISCUSSION

To test the first aim, the elucidation of the underlying molecular mechanism of xenobiotic hepatotoxicity in regards to the involvement of oxidative stress and apoptotic cell death pathways, two models of hepatotoxicity were explored, one *in vitro* (TBOOH) and the other *in vivo* (D-GalN/LPS).

In short, TBOOH is able to decrease cellular antioxidant status, increase intracellular ROS production and lipoperoxidation, activate caspase 3, reduce mitochondrial membrane potential and increase cytochrome C release resulting in initiation of cell death pathways (Ghosh *et al*. 2011, Vidyashankar *et al.* 2010). In the *in vitro* study, 24 hours following the treatment of hepatocytes with TBOOH resulted in severe cytotoxicity. Additionally, there were significant parallel increases in the mRNA expressions of the NOS-2 and pro-inflammatory cytokine TNF-α, but there were no significant changes in the HO-1 mRNA expressions as a result of TBOOH insult. The increased expression of NOS-2 may trigger an augmented production of nitric oxide, and consequently result in increase in RNS formation. It can be concluded that the noted decrease in cell viability of TBOOH treated cells is due to the accumulation of RNS and pro-inflammatory cytokine TNF-α, all of which contribute to the initiation of the cell death pathways. Despite the noted increases in the cytoprotective HO-1 mRNA expressions, there were no significant results that would indicate a potential relationship in this model of hepatotoxicity between the cytoprotective HO-1 and the proinflammatory NOS-2 and TNF- α.

The combination of D-GalN/LPS is a useful *in vivo* model of hepatotoxicity, as it resembles hepatitis of various etiologies. In short, D-galactosamine exerts its damaging actions on the cell through inhibition of protein synthesis by depleting uridine triphosphate pool, resulting in early generation of reactive oxygen species and apoptosis (Choi *et al.* 2011, Gonzalez *et al.* 2009). On the other hand, lipopolysaccharide increases the release of pro-inflammatory cytokines (mainly TNF-α), induces the release of reactive nitrogen species and inflammatory prostaglandins due to the activation of NOS-1 and cycloxygenase 2 (COX-2) respectively (Devaux *et al*. 2001, Nolan 2010).

In this study, 24h following the D-GalN/LPS failing liver function was noted as the levels of aminotransferases were raised. On the other hand, the extent of lipid peroxidation was of no significance in this model.

One of the important physiological antioxidants is the reduced glutathione (GSH), which protects cells from oxidative injury by scavenging reactive oxygen/nitrogen species and a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins (Ballatori *et al*. 2009). In addition to the antioxidant action of GSH, the antioxidant enzymes SOD1, GPx1 and catalase work together to counteract the oxidation of proteins, lipids and DNA, by removing reactive oxygen species from the cell (Yuan and Kaplowitz 2008). Specifically, SOD reduces the harmful superoxide into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase (Valdivia *et al.* 2009). It might be expected that under the present experimental conditions, the responses of these three parameters would be increased in parallel, however, under D-GalN/LPS toxicity the expected mutual relationship of these antioxidant enzymes was not seen. Differential response of these enzymes may be dependent on the dictating cellular needs in fight against increased levels of reactive oxygen species in induced oxidative stress states (Djordjevic *et al*. 2010).

One of the ways lipopolysaccharide exerts its cellular harm is by the stimulation of production of pro-inflammatory cytokine TNF-α by the Kupffer cells (Lichtman *et al.* 1994). Also, the high levels of TNF-α increase the expression of NOS-2 enzyme, whose product NO can in turn stimulate additional production of TNF-α and amplify the inflammatory damage (Sass *et al.* 2001). The activity of NO as reflected in the measurement of nitrites in plasma was significantly increased upon the D-GalN/LPS treatment which was paralleled with the gene expression of NOS-2 enzyme. As well, there was a noted parallel increase in both NOS-2 and TNF-α mRNA expressions due to D-GalN/LPS administration as noted in the second part of this study dealing with hepatoprotective effects of curcumin.

The products heme degradation pathway, biliverdin/bilirubin and CO, are the key mediators of HO-1 mediated cytoprotection for the reason that they help restore intracellular homeostatic balance and reduce the release of pro-inflammatory cytokines (Gomes *et al.* 2010, Paine *et al.* 2010). For instance, it has been shown that the cooperative action of biliverdin/bilirubin and CO is responsible for the prolonged survival of mice due to TNF-α reduction in D-GalN/LPS toxicity model (Sass *et al*. 2004). Additionally, the induced enzyme heme oxygenase-1 has the ability to exert its cytoprotective effect through the inhibition of inflammatory NOS-2 induction and a decrease in Caspase 3 activity as is noted in some studies (Sass *et al.*, 2003, Wen *et al.* 2007). This is in accordance to the present findings of this analysis, in so far as the relationship between HO-1 and billirubin from one hand and NOS-2 and TNF- α gene

expression on the other. Thus the observed increase in HO-1 expression in this study is a result of the cellular adaptive response in times of oxidative stress.

The data from the past and current literature indicates that D-GalN/LPS cell death involves TLR-mediated inflammatory pathway that results in increased levels of TNF-α, NF-κB, IFN-β, c-Jun N-terminal kinases, as well the activation of executioner caspases, which further commits the hepatocytes to cell death (Kang *et al*. 2013). Similarly in this study, we have observed the measured increases in the gene expressions of Bid, Bax and Casp 3 that D-GalN/LPS treated rats. This signifies initiation of the apoptotic pathways. This was further confirmed by the presence of pycnotic nuclei as seen in the morphological evaluation of the liver tissue of D-GalN/LPS treated rats

The second part of this study focused on evaluating dietary substances quercetin and curcumin as potential hepatoprotective agents in regards to their ability to ameliorate hepatotoxicity. Quercetin exhibited a clear cytoprotective effect in both *in vitro* and *in vivo* models as it decreased the levels of aminotransferases and increased the cell viability. Additionally quercetin had an HO-1 inducing effect; it effectively lowered TNF-α mRNA expressions; whereas it did not have any effect on NOS-2 mRNA expressions in TBOOH treated cells. Similarly, quercetin treatment increased the HO-1 gene and protein activities in D-GalN/LPS+Q rats, which is in agreement to our findings as well as to other studies demonstrating the cytoprotective effect of quercetin by induction of HO-1 (Yao et al. 2007).

Some studies have reported that quercetin mediates its cytoprotective actions by lowering NO production (Zhang *et al.* 2011). However, in this study quercetin did not have a significant reducing effect on NOS-2 activity or nitrate levels in both hepatotoxicity models. Thus, it could be postulated that there seems to be an alternative mechanism of HO-1 induction protection that is not related directly to NOS-2 activity reduction. One possible explanation is that the products of HO-1 pathway, bilirubin and CO are mainly responsible for the quercetin mediated cytoprotection given their aforementioned cytoprotective actions. It could also be postulated that the mechanism of HO-1 cytoprotection is due to its effect on the upregulation of anti-inflammatory mediators such as IL-10, and inhibition of production of pro-inflammatory cytokines such as TNF-α (Park *et al.* 2010). The discussed possible mechanisms of the quercetin cytoprotection are summarized in the figure 9.

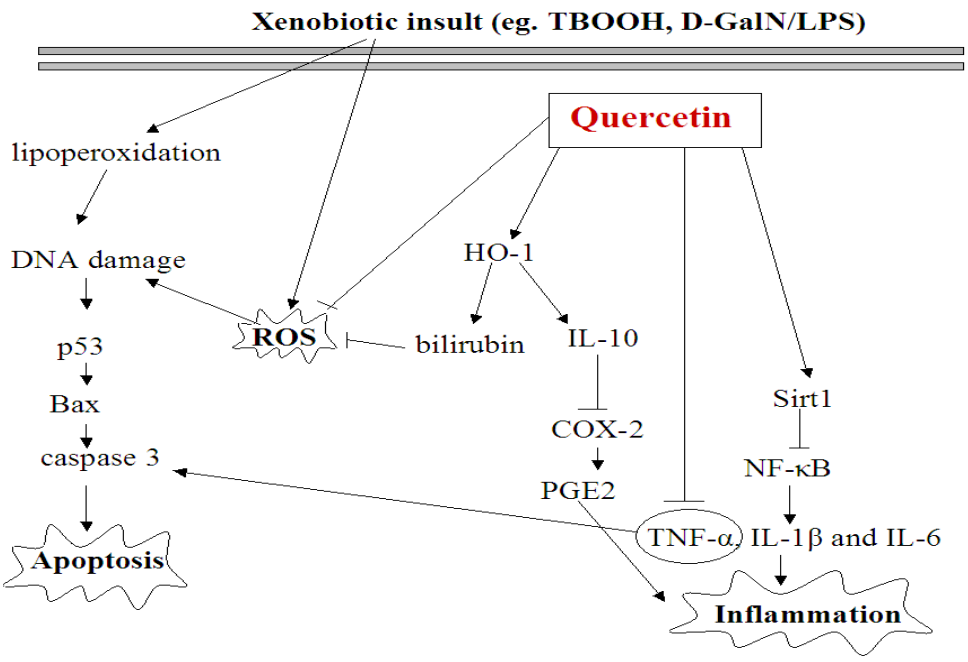


Fig. 9. Molecular mechanisms of potential cytoprotective effects of quercetin.

Activation of important antioxidative enzymes such as HO-1, induction of sirtuin activity, inhibition of pro-inflammatory cytokines such as TNF-α are just some of the many proposed beneficial properties of curcumin (Sikora *et al.* 2010). As might have been anticipated, curcumin pretreatment notably improved liver function and decreased lipoperoxidation. Furthermore, it increased the oxidative status of the cell as it as it elevated the plasma levels of catalase and bilirubin, and increased the HO-1 mRNA expression in D-GalN/LPS rats.

Moreover, the potential relationship between mRNA expressions of HO-1, NOS-2 and TNF-α was also explored in this model. As previously discussed, HO-1 and its products exert much of their cytoprotective effect by decreasing the production of the pro-inflammatory cytokines (Sass *et al*. 2004), by inhibiting inflammatory NOS-2 induction and decreasing caspase 3 activity (Sass *et al.,* 2003), which was in agreement with the results of this study.

The oxidative stress induced by lipopolysaccharide leads to TNF- α mediated apoptosis that involves activation of the executive caspases as well as proteins Bid and Bax (Morgan *et al.* 2010). Our results have shown that curcumin pre-treatment resulted in a significant decrease in both Bid and Bax, but it had no significant effect on the Casp3 mRNA expressions in the D-GalN/LPS rats. It could be postulated that the those cells that have committed themselves to apoptosis due to the activation of execution caspases were not affected by quercetin; Whereas curcumin had an inhibitory effect only on the early activators of apoptosis, Bid and Bax, thus preventing the potential activation of the downstream executioner caspases and the resulting cell death. However, it must be noted that when given alone curcumin had no significant effect on these parameters, suggesting that curcumin's effect is different in physiological versus pathological conditions. This molecule therefore pre-conditions the cell to increase its own antioxidant systems in

combat of further oxidant challenge. This is relevant to the observed increase in HO-1 mRNA expressions, and concominant reduction in NOS-2, Bid and Bax mRNA expressions, demonstrating a potential relationship between these tested parameters. The following figure nicely summarizes the discussed cytoprotective molecular mechanisms of curcumin (Fig. 10).

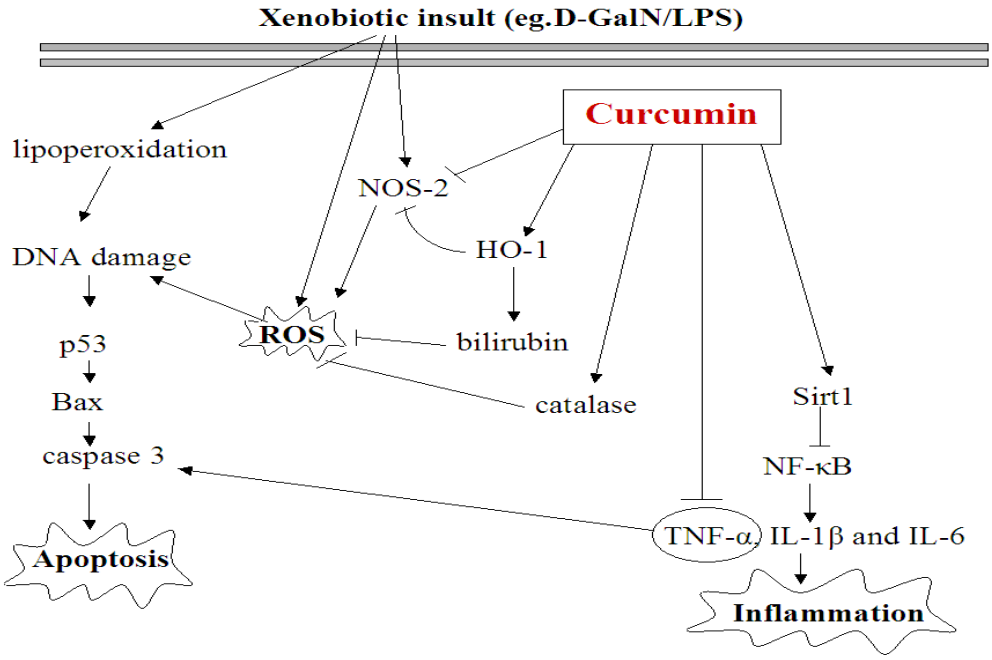


Fig. 10. Molecular mechanisms of potential cytoprotective effects of curcumin.

6 CONCLUSION

In summary, both xenobiotic hepatotoxicity models resulted in differential oxidative stress response as reflected by the alterations in expressions of certain oxidant/antioxidant genes, while the expression of others remained unchanged. Even though our findings were not able to confirm a direct relationship between the oxidative and apoptotic parameters that were tested, a parallel relationship between selected enzymes' gene expressions and their respective biochemical markers was seen. The enzyme HO-1 has been shown to be the main regulator of cytoprotection in all tested models.

Moreover, the results of this study have shown that both quercetin and curcumin exhibit cytoprotective effects through modulation of HO-1, NOS-2 and/or TNF-α systems and inhibition of early initation of cell death. Both of these polyphenols induce HO-1, however curcumin relies on concomitant NOS-2 activity reduction, while this is not the requirement with quercetin. Thus quercetin's significant induction of HO-1 alone, without concomitant NOS-2 activity reduction, might be sufficient in combating cellular damage.

The research data from the present study paves a way for those interested in further research of these dietary polyphenols as it gives an overview of the potential cytoprotective mechanisms and effective doses in the given models.

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