ORIGINAL ARTICLE

Caesalpinia bonduc Linn Extracts Exhibit Hepatoprotective Effect on HepG2 Cells against Paracetamol by Up-regulating Glutathione Related Genes

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Abstract:
Background: Caesalpinia bonduc (CB) is said to own various pharmacological and therapeutic application against a number of diseases. It is used widely in folk medicine to treat liver diseases. In the present study, we have made a sincere effort to evaluate the hepatoprotective activity of CB leaf extracts against Paracetamol (PCM) induced toxicity and their mechanism of hepatoprotection in the human Hepatocarcinoma cells (HepG2 cells) thereby providing scientific evidence for the same. *Material and Methods:* The hepatoprotective activity of CB was assessed *in-vitro* by the estimation of glutathione (GSH) and Malondialdehyde (MDA), anti-apoptotic assay/Annexin V and the expressions of genes such as Glutathione Reductase (GS-R) and Glutamate-Cysteine Ligase, Catalytic (GCLC). *Results:* The obtained results suggest that the aqueous extract of CB possess significant hepatoprotective activity. This activity may be due to the possible antioxidant property and the free radical scavenging ability of the extracts, which might clear the toxic metabolites of PCM. *Conclusion:* The present study suggests that the aqueous extract of CB have potential hepatoprotective activity, which may prevent the lipid peroxidation of the cell membrane by its antioxidant properties.

Keywords: *Caesalpinia bonduc,* Hepatoprotection, Hepatocarcinoma cells, Paracetamol

Introduction:

Liver is one of the major digestive glands of the human body; it has a major role in elimination and biotransformation of toxic substances. It is well known for its complexity and diverse functions like metabolism, bile acid synthesis and detoxification of various drugs and xenobiotic. It is this prime function of detoxification, which makes the liver susceptible to liver damage, liver toxicity and liver failure [1]. Liver damage and liver failure may be caused by various chemicals. A handful among them is Carbon Tetrachloride (CCl_4), Paracetamol (PCM), alcohol, *tert*-butyl hydroperoxide (t-BH), Galactosamine (GalN), acetaminophen, and Isoniazid (IZN). Cell damage by oxidative stress and lipid peroxidation is the most common cause for hepatotoxicity [2-5]. PCM is a most common drug which is frequently used worldwide, when taken in over dose, leading to hepatic centrilobular necrosis. Thus it is also considered as reliable model for drug induced hepatotoxicity [6, 7].

Although there are a number of disorders pertaining to the liver, no effective treatment or drug is found to seize the progression of the ongoing disease or its complications. Most of the existing remedies, however, support or promote the healing or regeneration of the liver. The lack of proper medication for the liver ailment, the focus of the treatment regimen has now shifted to alternative medicine. Even though many herbal medicines or formulations are available, utmost importance needs to be given to justify its medicinal value scientifically [8].

Caesalpinia bonduc from the Caesalpiniaceae family is one among such wonder drugs. It is found in the tropical and subtropical regions worldwide and has been used substantially in traditional medicine [9-16]. Hepatoprotective activity of the plant although mentioned in Ayurveda is not discussed well with scientific evidence. Further, there is a dearth in the literature for studies related to the hepatoprotective activity of CB on PCM induced liver toxicity.

The present study was therefore designed to evaluate the hepatoprotective activity of *Caesalpinia bonduc* on PCM induced toxicity in human Hepatocellular carcinoma (HepG2) cell line.

Material and Methods:

Reagents and chemicals:

HepG2 cell line was obtained from National Center for Cell Science Pune, India. All the chemicals were obtained from SD Fine Chemicals Mumbai i.e., 3-(4, 5 Dimethyl thiazol -2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dexamethasone, silymarin, insulin, Dulbecco's Modified Eagle Medium (DMEM), antibiotics and PCM were procured from Sigma. Enzyme kits-ALT Kits were purchased from Aspen Laboratories, India.

Plant material and extraction:

Leaves of CB were collected in the month of February from Soans farms Moodabidri, Karnataka, India, and were authenticated by the botanist. A voucher specimen was deposited in the Pharmacognosy Department of Manipal Academy of Higher education, Manipal. Aqueous extract of CB was prepared by hot maceration method- Leaves of CB were shade dried and coarsely powdered. A decoction of the CB leaf powder was then prepared at 75-80^{0}C by dissolving the leaf powder (200 g) in 1500 ml of distilled water, which was then cooled, filtered and evaporated to dryness using lyophilizer.

Preparation of drug solution:

The concentrated aqueous extract CB was dissolved in Phosphate Buffer Solution (PBS) to prepare the stock solution and was syringe filtered before exposing it to the cell culture. The stock solution of silymarin was prepared by dissolving it in the universal solvent Dimethyl Sulfoxide (DMSO). Care was taken to note final concentration of the DMSO in the solvent (>0.2%). Further serial dilutions were made with the media to get various concentrations of extracts and used for *in-vitro* experiment.

Cell culture:

HepG2 cells were cultured in DMEM, supplemented with streptomycin (1 mg/mL), penicillin (0.134 mg/mL), 10% Foetal Bovine Serum (FBS). Cells were incubated in a humidified incubator containing 95% air and 5% CO_2 at 37°C and when cells reached confluence they sub cultured by trypsinisation and treated as mentioned below.

Treatment plan/ Experimental protocol:

HepG2 cells were seeded in a 96-well plate (5×10^3cells/well) and incubated for 24h in a humidified incubator containing 5% CO_2 and 95% air, at 37°C. The cells were then treated with various concentrations of test materials along with 5mM and 10 mM concentration of the PCM, and incubated for 24 /48h. Silymarin (50 µg/ml) was used as a reference standard [17].

MTT assay:

Following treatment, cells were then incubated with 0.5 mg/mL MTT for an additional 4 h. Subsequently, after discarding the culture medium the purple formazan crystals formed were dissolved with DMSO (100 µL/well). By using a microplate reader (Bio-Rad Laboratories, Orlando, FL, USA, model 3550) absorbance was monitored at 540-630 nm. The result was then compared to the control, and expressed as a percentage of viable cells.

Evaluation of total intracellular Glutathione (GSH):

HepG2 cells were seeded in six-well dishes (2×10^5 cells/well), after attachment, the medium was replaced and cells were incubated in fresh medium containing the CB extract for 24 h at 37°C. The non-treated cells served as the control. At the end of incubation, the cells were washed, trypsinized and centrifuged in PBS attain cell palate. To extract cellular GSH, 500 µl of cell lysate was added to the cell palate and centrifuged at 7000 rpm. To the 250 µl resultant supernatant 5 µl of 5% Trichloroacetic Acid (TCA) were added to form the stock solution. This mixture was then centrifuged at 7000 rpm for 10 min at 4ºC. To 100 µl of stock solution 600 µl of PBS and 100 µl of 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) solution (Ellman's reagent) was added vortexed, and incubated for 10 minutes. Finally Optical Density (OD) was read at 412 nm against a reagent blank. Absorbance values were compared with a standard curve generated from known GSH.

Malondialdehyde (MDA) Determination of Thiobarbituric Acid Reactive Substances (TBARS):

Lipid peroxidation is estimated by colorimetry by measuring TBARS [18]. At the end of treatment to 100 µl of supernatant 500 µl of trichloroacetic acid, thiobarbituric acid and butylated hydroxytoluene mixture was added. This mixture was then heated for 10min at 90ºC in a water bath. After incubation, the tubes were allowed to set to room temperature. Supernatant organic layer of the mixture obtained by centrifugation (2000 rpm for 5 min) was taken and its OD was read at 525 nm against an appropriate blank. The lipid peroxides were expressed as millimoles of TBARS/100gram of liver tissue using an extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Annexin V-Fluroscein Isothiocyanate (FITC) /Propidium Iodide (PI) apoptosis assay:

Cells were seeded (1.0×10^6) in a six-well culture plate, and treated with CB extracts for 48 h. After incubation, the cells were trypsinzed, washed with PBS. Normal, necrotic and apoptotic cells were identified by using an Annexin V-FITC/ PI assay kit (Beyotime Bioengineering Institute, China). 5µl of Annexin V-FITC and 5µl PI was then added to the samples, incubation for 15 minutes at room temperature in the dark. Post incubation cells were re-suspended in 400µl of binding buffer and the flow cytometry analysis was performed within 1 h.

Ribonucleic Acid (RNA) isolation and Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR):

Cells were cultured on 6 well plates and treated with different concentrations of aqueous extract of CB (1, 2.5 and 5 µg/ml) extract, PCM (10 and 5mM), PCM + CB and silymarin (50µg/ml) was taken as the standard control. Total RNA was obtained from different groups using Pure Link RNA isolation kit (Invitrogen, USA) and complementary Deoxyribonucleic Acid (cDNA) was prepared using Superscript III First strand synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. Semi quantitative RT-PCR was done using primers specifically designed for mRNA templates which could distinguish between genomic DNA and cDNA. Only cDNA template was amplified in all primers sets. PCR was carried out in 25µl reaction mixture having 1X PCR buffer (Thermo scientific, USA), 0.1µg of forward and reverse primers (Bioserve, India), 0.25mM deoxynucleotide (dNTP) mix (Applied Biosystems, USA) and 2 units of DNA polymerase (Thermo scientific, USA) in Peq STAR Thermal cycler (Peq Lab, Germany).

PCR was performed by using PEQLAB PCR machine. Samples were initially denatured at 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30s and DNA extension/elongation at 72°C for 45s. The final DNA extension was done at 72°C for 10 min.

The PCR products were then run through electrophoresis on a 1% agarose gel stained with 5 µg/ml ethidium bromides. The separated DNA bands were then visualized using a UV trans illuminator, and the images were captured using a SYNGENE gel documentation camera. The intensity of the bands was analyzed using IMAGE J software (NCBI). Scanned values were expressed in arbitrary unit, and the expression of the selected genes relative to that of a housekeeping gene Glyceraldehyde 3-phosphate Dehydrogenase (GADPH) was then determined The intensity of the PCR products of selected genes were divided by intensity of the PCR products of housekeeping gene GAPDH to normalize the data. The value of control well was taken as 1 and other test samples were calculated to have fold change in gene expression compared to control.

Statistical analysis:

The obtained data in each experimental group was computed and expressed as Mean ± Standard Deviation (SD). All the results were analyzed by GraphPad Prism software (Microsoft, San Diego, CA, USA). Groups were compared using one way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test for comparison of the treatment groups with control. $p<0.05$ was considered statistically significant.

Results:

Cytotoxicity of the extracts and PCM:

The non-cytotoxic concentrations of the aqueous extracts of CB were determined on the basis of the MTT assay. IC_{50} value of CB for 48h was 26.08µg/Ml. Therefore, 5µg/mL, 2.5µg/mL and 1 µg/ml of concentration of CB were used for further studies. Similarly the IC_{50} value of PCM for 48h was 9.80 mM. Based on the % viability of the cells for 48h, 5 and 10mMconcentration of the PCM was selected for further studies.

Effect CB on intracellular GSH and MDA levels:

The results showed that there was a significant ($p<0.0001$) decrease in the GSH levels in the groups treated with PCM. In groups where the HepG2 cells were co-treated with CB and PCM these antioxidant levels were found to be significantly ($p<0.0001$) increased (Fig.1).

The results showed a significant ($p<0.0001$) increased in the malondialdehyde (MDA) levels in PCM exposed group. When HepG2 cells were co-treated with PCM and CB, relatively less activity of MDA was observed compared to PCM. The restoration of the GSH levels and fall in the MDA levels glorifies the antioxidant nature of aqueous CB extract and its ability to ameliorate the damage by free radicals (Fig. 2).

Evaluation of the anti-apoptotic effect CB extract by flow cytometry:

When the HepG2 cells exposed to PCM, the percentages of apoptotic cells were increased to 18.7 and 17.36 respectively. HepG2 cells when exposed to PCM with different concentration of CB, there was a significant dose dependent reduction in the percentages of apoptotic cells observed i.e. 11.1, 10.16, 3.86 and 6.63, 4.36, 3.43 (Fig. 3 and Fig. 4). This justifies the fact that the extract of CB is effective in combating the toxicity by promoting cell proliferation and preventing cell death.

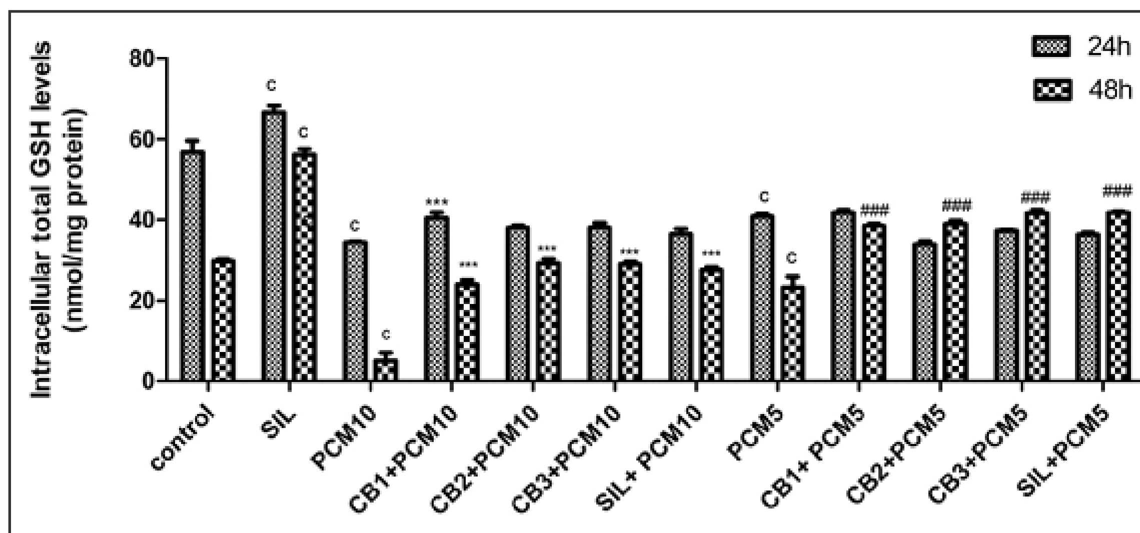

Fig. 1: Effect of Exposure of Aqueous Extract of CB and PCM on Total Glutathione Levels in HepG2 Cells

The values are expressed in mean ± SD where: a = $p<0.05$; b= $p<0.01$; c =$p<0.001$ compared to control. *$p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to 10mM PCM. #$p<0.05$; ## $p<0.01$; ### $p<0.001$ compared to 5mM PCM. PCM10-Paracetamol 10mM concentration; PCM5-Paracetamol 5mM concentration; SIL-Silymarin 50µg/ mL; AQ-aqueous leaf extracts of CB expressed in µg/mL. CB1, 2, 3:1, 2.5,5 µg/ mL

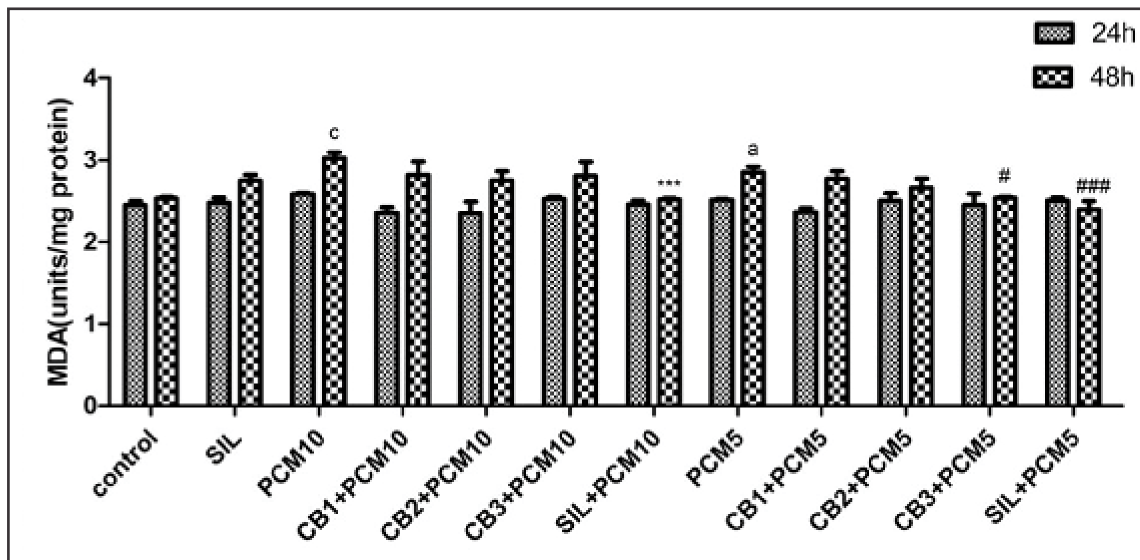

Fig. 2: Effect of Aqueous Extract of CB and PCM on the Levels on MDA in HepG2 Cells

The values are expressed in mean ± SD where: a = $p<0.05$; b= $p<0.01$; c =$p<0.001$ compared to control. *$p<0.05$ compared to PCM; ** $p<0.01$ compared to PCM; *** $p<0.001$ compared to PCM. #$p<0.05$ compared to PCM; ## $p<0.01$ compared to PCM; ### $p<0.001$ compared to PCM. PCM-Paracetamol 5mM concentration; SIL-silymarin 50µg/ mL; AQ-aqueous leaf extracts of CB expressed in µg/ mL. CB1, 2,3:1,2.5 and-5 µg/ mL

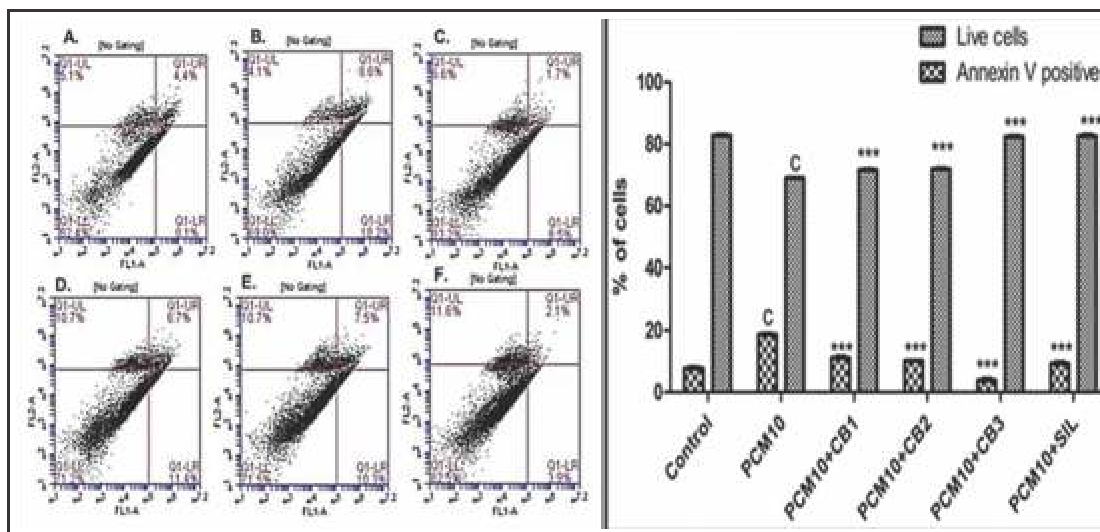

Fig. 3: Graphical Representation of Inhibition of 10mM PCM Induced HepG2 Cells Apoptosis by CB Extract

A= Control; B=PCM; C=PCM +SIL; D=1 µg/ ml of CB+ PCM; E=2.5 µg/ ml of CB+PCM; F=5 µg/ ml of CB+PCM. CB-aqueous extract of CB, PCM10-10mM Paracetamol, CB1,2,3=1, 2.5 and-5 µg/ml of CB; SIL- 50 µg/ml silymarin; c =$p<0.001$ compared with control. ***$p<0.001$ compared with PCM.

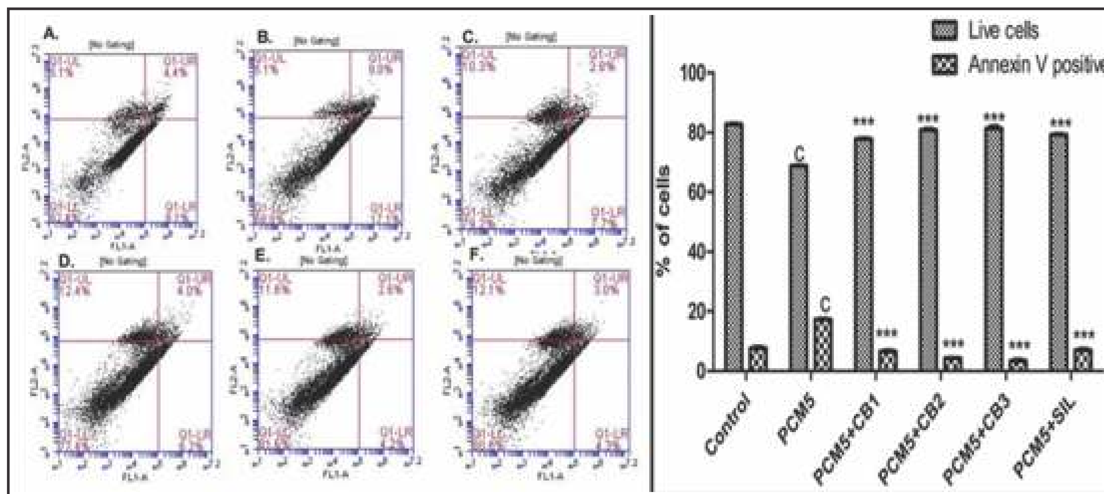

Fig. 4: Graphical Representation of Inhibition of 5mM PCM Induced HepG2 Cells Apoptosis by CB Extract

A=Control; B=5mM PCM; C=PCM +SIL; D=1 µg/mL of CB+PCM; E=2.5 µg/ mL of CB+PCM; F=5 µg/mL of CB+PCM. CB-aqueous extract of CB, PCM5-5mM Paracetamol, CB1-1 µg/ml of CB; CB2-2.5 µg/ml of CB; CB3-5µg/ml of CB; SIL-50µg/ml silymarin; c =$p<0.001$ compared with control. ***$p<0.001$ in comparison to PCM

Effect of aqueous CB extract on the expression of Glutathione Reductase (GS-R) and Glutamate-Cysteine Ligase, Catalytic (GCLC) genes:

In this study, the two essential genes for glutathione synthesis i.e., GS-R and GCLC were examined by RT-PCR. These genes are rate-limiting enzymes of the GSH biosynthesis. As shown in Fig. 6, HepG2 cells exposed to PCM showed significant reduction in the expression of GS-R(0.3, 0.25 fold) and GCLC(0.22 ,0.21 fold) was observed. This might be due to the action of N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite of PCM on GSH. When HepG2 cells are exposed to CB (1 , 2.5 and 5µg/ml) along with 5mM PCM for 48h, a significant increase in the expression of GCLC (0.31 and 0.48 fold) was observed in groups treated with 1 and 2.5µg/mL of CB. The findings were however insignificant in the expression levels of GCLC in other treated groups of CB. Further, it was noted that the groups treated with 2.5µg/ml of CB exhibited better expression of GCLC than the standard silymarin. The groups co-treated with 1 and 2.5µg/ml of CB with 10Mm PCM showed 0.16 and 0.15 fold increase respectively in the expression of GSR gene when compared to PCM. When HepG2 cells were treated with CB (1, 2.5 and 5µg/ml) at 5mM PCM toxicity, 0.16, 0.35 and 0.07 fold increase expression of GSR genes were observed. The expression of GSR was maximum (0.35 fold) in the group treated with 2.5 µg/ml of CB, than the cells treated with the standard silymarin (0.19fold) at 5mM PCM toxicity. This experiment demonstrated that in PCM intoxication, there is a down regulation of GCLC and GS-R genes. The use CB helps in the up regulation of these genes, which are a key enzyme for the synthesis of the antioxidant, Glutathione. This suggests that CB may be effective in restoring the GSH content depleted by the PCM toxicity (Fig. 5).

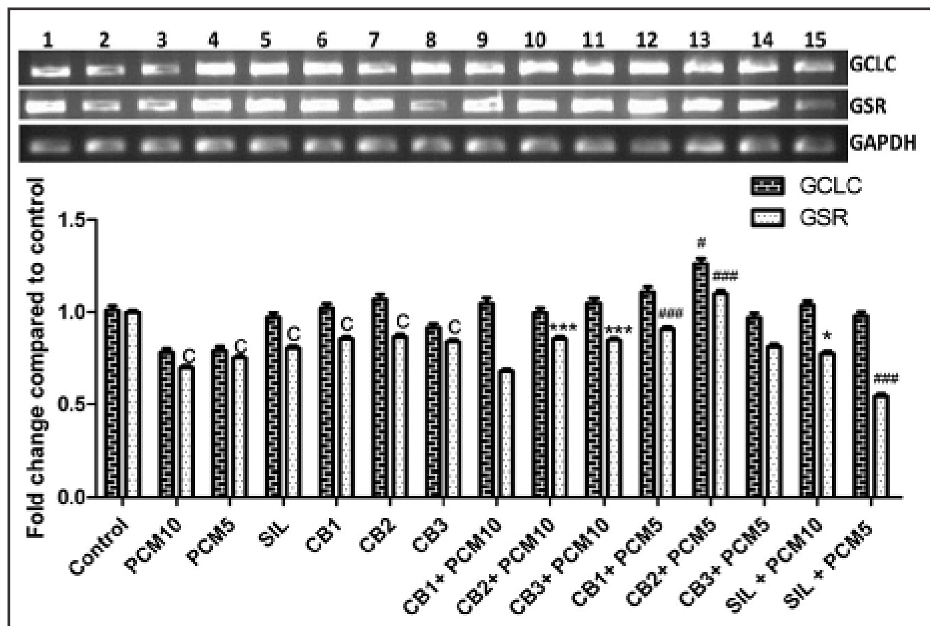

Fig. 5: mRNA Level of GCLC and GS-R Genes in HepG2 Treated with CB and PCM at 48h

1- Control;2- PCM 10mM; 3-5 PCM mM; 4- SIL ; 5- CB 1; 6- CB2; 7- CB 3; 8- CB 1+ PCM 10mM; 9- CB 2+ PCM10mM; 10 - CB 3+ PCM10mM; 10 -CB 1+ PCM 5mM; 11-CB 2+ PCM 5mM; 12 -CB 3+ PCM 5mM; 13- SIL + Para 10mM; 14 - SIL + PCM5mM. Where, PCM- Paracetamol; CB- aqueous leaf extract of CB; CB1- 1 µg/ml; CB2- 2.5 µg/ml and CB3- 5 µg/ml of CB extract respectively. SIL-silymarin 50µg/ml. The values are expressed in mean ± SD where c =$p<0.001$ when compared with control.*$p<0.05$; *** $p<0.001$ when compared with PCM10; # $p<0.05$; ### $p<0.001$ when compared with PCM5.

Discussion:

In the present study, amelioration of toxic effect of PCM was studied by CB extracts on HepG2 cell line. This cell line is extensively used for biochemical and drug toxicity studies. It is considered to be most reliable model to estimate the hepatoprotective activity, since it possess many morphological and biochemical features of normal hepatocytes [19-21]. PCM is most commonly and widely used analgesic and antipyretic drug. It is safe in therapeutic doses, but produces fatal hepatic necrosis at higher concentrations. About 80% of the ingested PCM is excreted out of the body as sulfate and glucuronide conjugates and only 5% is converted into NAPQI when PCM is consumed in lower doses. When PCM is administered in toxic dose the sulfation and glucoronidation routes become saturated, thus more and more PCM molecules are oxidized by enzyme cytochrome-450 into highly reactive NAPQI molecules. The free radicals released will cause lipid peroxidation of the cell membrane leading to tissue damage [22]. The present study focused on alteration of these changes induced by PCM on HepG2 cell line using extract of CB, which are known for its hepatoprotective effects. Water extracts derived from many natural products possess hepato-protective effects [23, 24]. Previously authors have studied its effects in in-vitro models [25] and they found aqueous and methanol extract of CB

was effective in CCl_4 and PCM toxic model respectively. In the present study, the hepato-protective activity of CB was evaluated by the MTT assay; further, the hepatoprotection was confirmed by other biomarkers.

High levels of PCM reported to induce damage and decrease viability of the cells by up to 80% at 24 h [26]. In the present study, cells when exposed to PCM exhibited similar findings, and the cell death was increased with increase in exposure time. In similar studies, where HepG2 cells were treated with serial concentrations of PCM for 24 and 72h, it was observed that dose at or above 10mM lead rapid to increase in the level of MDA [27]. Contrary to the previous study, in the present study, the excessive MDA release was only evident at 48h. It is well established that HepG2 cells are killed by PCM [28], but the mechanism of action responsible for cell death differs between HepG2 cells and cells that form a reactive metabolite. For this reason, GSH depletion does not occur in HepG2 cells at 12h, [29] and our finding that GSH started to decrease only after 12h i.e, 24 or 48h after exposure to PCM is consistent with this observation. Experimental results using various mediators of oxidative stress confirm the involvement of free radicals acting through lipid peroxidation, as reported for this toxic agent [30-32].

Glutathione (GSH/GSSG) is regarded as the principal redox buffer in cells. Glutathione plays an important role in the elimination of ROS and protects the thiols in bio macromolecules [33]. Depletion of glutathione results due to enhanced toxicity to chemicals, including PCM [34]. The results of our present study showed that the cells intoxicated with PCM showed low glutathione level than the control. However, co-treatment of CB and PCM increased the level of total glutathione significantly. These results suggested that CB could exert its hepatoprotective and radical-scavenging activities by preventing the formation of free radicals originating from PCM metabolism. These findings are further supported by our previously published reports that have shown to enhance the antioxidant defense system, which is well exhibited and proved by the DPPH activity of the CB [24].

MDA is the product of lipid peroxidation, and it is considered as a biomarker of lipid peroxidation for several decades. It is one of the means for assessing the lipid peroxidation in various biological materials [35-37]. PCM treatment led to a significant increase in the level of MDA in HepG2 cells. The changes induced by PCM were alleviated gradually with the increasing concentration of CB. Therefore suggested that the possible underlying mechanism for the hepatoprotective effect of CB *in vitro* was because of its ability to increase antioxidase activity, inhibit lipid peroxidation and maintenance of glutathione in reduced state.

To further confirm the controlling levels of GSH by the CB, expression profile for GSH was evaluated after 48h with co-treatment of PCM. The maintenance of the levels of the cellular GSH was attained by the rate limiting enzymes like GS-R and GCLC genes [38]. The expression profiles of GCLC and GS-R genes encoding the antioxidant enzymes GSH were investigated by RT-PCR in the present study to provide a holistic insight into the mechanisms governing the protective activities of CB. When HepG2 cells

were exposed to PCM for the48hsignificant fall in the expression levels of GCLC and GS-R genes were observed this is due to the rapid depletion of GSH levels by the reactive metabolites of PCM, In PCM toxicity, the rate of depletion of the GSH exceeds its replenishment [39]. A significant rise in the expression of GCLC was seen in the groups treated with a low concentration of the extract, which might be due to the assessable changes at a lower concentration. The expression of GS-R (the other rate limiting enzyme for GSH synthesis) showed a significant increase in the levels of expression in most of the treatment groups. The combinations of higher concentration of PCM with a lower concentration of extract and lower concentration of PCM with a higher concentration of the extract did not produce any significant increase in GSR expression. This might be due to the inability of the combinations to prevent the toxic effect of PCM.

From the expression studies, it can be stated that administration of CB alone or in combination to PCM induces increased expression of GCLC and GS-R genes, compared to PCM. Further, the activity of CB was better pronounced than the standard silymarin. This may be because the CB is effective in supplementing and boosting the antioxidant enzyme activities of the liver by up regulating the gene expressions. Encoding the antioxidant thus provides strong protection against the cytotoxicity.

Annexin V staining assay was further performed to confirm the anti-apoptotic activity of CB in HepG2 cells. The Annexin V staining is a method, which allows studying the mechanism of apoptosis by staining the phospholipids and phosphatidyl serine of the cell membrane. Annexin V particularly/ precisely stains the PS and thereby it helps in identifying the cells undergoing apoptosis. The necrotic or dead cells with disruption of the internal and external membranes has been stained by the vital dye PI [40]. In the present study, we found a significant decrease in the number of apoptotic cells when co-treated with high dose of CB along with PCM for 48h, in comparison with PCM intoxicated cells. Thus, the result of Annexin V staining assay confirmed the anti-apoptotic activity of CB on HepG2 cells by reducing apoptosis by six fold.

Conclusion:

Based on the results of the present study, it can be concluded that the aqueous extracts of CB have proven itself as a significant hepatoprotective agent and a considerably good antioxidant against PCM intoxication in the liver. Therefore the usage of this extract in the treatment of liver toxicity may provide promising results.

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