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Anti-oxidative and hepatoprotective effects of methanolic extracts of seeds of *Brassica nigra* L., against CCL_4 induced hepatic damage in $HEPG_2$ cell lines

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Abstract
Accumulation of an excess amount of reactive oxygen species can cause hepatotoxicity that may result in liver damage. So development of antioxidant is needed for reducing liver toxicity. This study investigated the antioxidative and hepatoprotective activity of methanolic extract of seeds of *Brassica nigra L.*, on carbon tetrachloride induced acute liver damage *in vitro.* The results of MTT assay indicated that methanolic extract of seeds of *Brassica nigra L.*, was good antioxidant. The CCl_4 exposed $HepG_2$ cell line exhibited decreased cell viability, and elevated LPO, and decreased SOD, GSH and also increased the enzymes ALT, AST and LDH levels. Methanolic extract of seeds of *Brassica nigra* increased the cell viability and restoration of enzymes AST, ALT, LDH, and increased SOD, GSH levels and decreased the LPO and also restored the morphological changes of CCl_4insulted $HepG_2$celllines.

Keywords: *Brassica nigra* L.,, carbon tetrachloride, $HepG_2$cell lines, Liver enzyme markers

Introduction
Carbon tetrachloride is a industrial solvent that is used to induce chemical hepatitis and liver injuries in experimental animals. Carbon tetrachloride induced liver injuries are the most common experimental model for monitoring the hepatoprotective activity of certain drugs. A single exposure to CCl_4 as being a strong hepatotoxic xenobiotic directly leads to severe liver necrosis and steatosis[1,2]. Mechanistic studies offer evidence that metabolism of CCl_4 via CYP2E1 to strongly reactive free radical metabolites plays a crucial role in the proposed mode of action. The major metabolites trichloromethyl (CCl_3^-) and trichloromethyl peroxy (($CCl_3O_2^-$) free radicals are extremely reactive and are capable of covalent bind to cellular macromolecules, preferring fatty acids of the membrane phospholipids. The free radicals induce cell membrane lipid peroxidation via disrupting polyunsaturated fatty acids within these membranes, initiating a sequential free radical chain reaction[1].

Materials and Methods
Cell culture[3]
HepG2 human liver hepatoma cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown in standard conditions: supplemented DMEMA at 37°C in a humidified 5% carbon dioxide atmosphere. When the cells reached at 30,000 cells per well in a 96-well plates. $1x10^6$ cells per well in six plates, or $5x10^7$ cells per well in a single dish, depending on the determination. The cells were used after attachment.

CCL_4 induced toxicity in HepG2 cells
HepG2 cells were incubated in medium or treated with toxic agent (30 mM CCL_4 in 0.05% DMSO). The evaluation assays were performed using standard methods.

Effect of Methanolic extract of *Brassica nigra (L).*, seeds on HepG2 cells
The hepatoprotective effects of Methanolic extract of *Brassica nigra (L).*, seeds on HepG2 cells were measured as follows; Normal control cells were incubated with DMEMA in DMSO (0.05%v/v) for 12 h. For toxic treatment, cells were incubated with DMEMA in DMSO (0.05%v/v) for 12 h and then treated with DMEMA with 30mM CCL4 for 1.5 h. For met-BN-S treatment cells were incubated with DMEMA with met-S.BN at 150 µg/ml for 12 h and then treated with 30mM CCL4 for 1.5 h

Cell viability assay
Cell viability was assessed using the MTT reduction assay with slight modifications. This colorimetric assay involves the conversion of MTT to purple formazan derivative by mitochondrial succinate dehydragenase, which

is present only in viable cells. The cells were treated with met-BN-S and or the toxic agent. The medium was then removed and the cells were then incubated with MTT (0.5 mg/ml) for 2 h after which the formazan crystals were dissolved with 200 µl/well of DMSO. Absorbance was measured at 570 nm. Viability was defined as the ration of the absorbance of treated cells to that of untreated control cells and is expressed as a percentage.

Measurement of AST, ALT and LDH activities

AST, ALT, and LDH activities were measured using an instrumentation Laboratory assay kits. HepG2 cells were treated with met-BN-S and or the toxic agent. The supernatant was removed from the wells. And the enzyme activities were measured immediately. The results are expressed as IU/L.

Measurement of GSH level

GSH level was quantified using a Glutathione assay kit. The assay kit is based on the enzymatic 5, 5'-dithiobis-2-(nitrobenzoic acid), disulfide dimer-oxidized GSH-reductase recycling method (DTNB). After treatment the medium was removed from the wells, and the adherent cells were scraped off and suspended in 0.5 ml of 50mM phosphate, pH 6.5, containing 1mM ethylene diamine tetra acetic acid sonicated and placed on ice. The supernatant of lysed cells was used to measure GSH level. Absorbance of the yellow product in the well was measured at a wavelength of 405 nm on a microplate reader at 5 min intervals for 30 min. the total GSH activity was measured using the kinetic method from a standard curve of GSH. The results are expressed as micromoles of GSH per litre.

Measurement of SOD activity

SOD activity was measured using a Superoxide Dismutase Assay kit, which uses a colorimetric assay to measure the concentration of formazan crystals. This assay uses a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. After treatment, the medium was removed from the wells, the adherent cells were scraped off and suspended in 20mM HEPES buffer, pH 7.2 containing 1mM EGTA, 210 mM mannitol, and 70mM sucrose, sonicated and placed on ice. To measure SOD activity the diluted radical detector and the supernatant of lysed cells or standard were added to each well of a 96 well plate, and xanthine oxidase was added. Absorbance in the well was measured at a wavelength of 460 nm after 20 min on a microplate reader. The results are expressed as IU/ml.

Measurement of Lipid peroxidation

The concentration of MDA the end product of lipid peroxidation was measured using a thiobarbituric acid reactive substance (TBARS) assay kit. After treatment the medium was removed from the wells, adherent cells were scraped off, suspended in cold PBS, sonicated and placed on ice. The supernatant from lysed cells or standard, sodium dodecyl sulphate, and the color reagent were added to each vial. The vial was heated at 100 C for 1 h and then immediately cooled in an ice bath and centrifuged; the content of each vial was transferred to a well in a microplate. The absorbance of the product was measured at a wavelength of 540 nm on a microplate reader. The extent of lipid peroxidation was quantified by estimating the MDA concentration. The results are expressed as micromoles of MDA equivalents formed per litre.

Measurement of IL-1β & TNF-α (Pro-inflammatory cytokines) by ELISA

The levels of IL-1β and TNF-α were assessed in cell lysates by using respective ELISA kits (Invitrogen, R&D systems & Alpha diagnostics, USA) as per the manufacturer' sprotocol. Briefly, 150µl distilled water was added to the standard an db lank wells for standard calibration and 100µl distilled water and 50µl of each supernatant were added in duplicate in to the wells. After incubation for 3 hour s at room temperature, the wells were emptied and washed three times with 150µlof wash buffer. TMB substrate (100µl) was added to each well and incubated for 15 min at room temperature followed by addition of 100µl stop– solution to all wells including blank wells. The absorbance was determined at 410nm using above mentioned ELISA reader (Roeske-Nielsen *et al*. 2004).

Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA) Tukey's multiple comparison tests using Graph pad prism Version 6.0. Differences between means were considered significant at $P< 0.05$.

Results

Cell viability assessment – MTT assay [in presence of CCl_4 (30mM)]

Table 1: Effect of methanolic extract of seeds of *Brassica nigra* on cell viability level in CCl_4 intoxicated HepG2 cell lines.

S. No	Groups	% viability of cells
1	Control	99.13 ± 0.210
2	CCl_4 (30mM)	$09.61 \pm 1.271^{***}$
3	Met-BN-S	$61.14 \pm 3.287^{###}$

Statistical significance was determined by one way ANOVA followed by Tukey's multiple comparison tests using Graph pad prism Version 6.0. Values are represented as Mean ± SEM, Superscript *** denotes p<0.001 vs control, ### denotes p<0.001; vs CCl_4 groups respectively, (CCl_4: Carbon tetrachloride).

Incubation of CCl_4 in HepG2cell lines have shown significant (p<0.001) reduction in cell viability in comparison to control cells indicate the intensity of hepatotoxicity exhibited by CCl_4. Post-treatment with met-BN-S (p< 0.001) have shown notable increase in cell viability of $HepG_2$ cell lines evidence the protective effect exerted by extract in CCl_4 intoxicated condition.

Morphology

The control HepG2 cells are tiny, highly light retractile, fibroblast-like and good morphological features and growing densely. CCl_4 (30mM) intoxication caused morphological changes with condensed cell density, swollen, decreased proliferation and differentiation of cells in comparison to the control cells. Post-treatment with Met-BN-S have significantly protected the cells from the CCl_4 toxicity which is evidenced by increased cell density and proliferation.

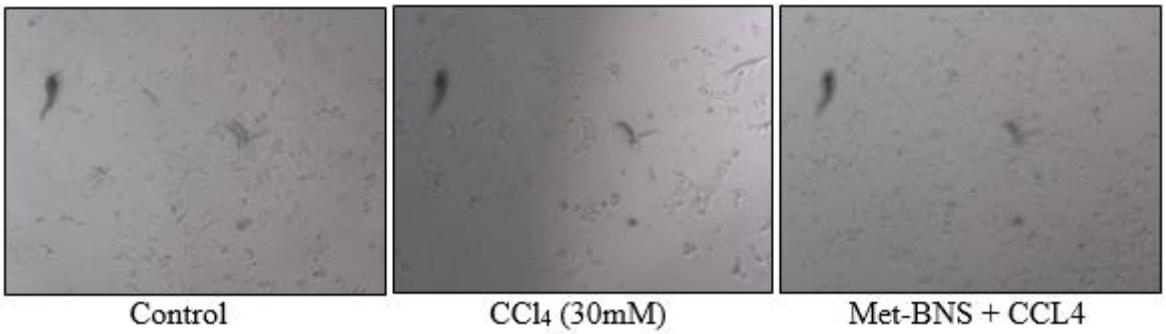

Fig 1

Table 2: Effect of methanolic extract of seeds of *Brassica nigra* (met-BN-S) on cellular LPO, GSH and SOD level in CCl_4 intoxicated $HepG_2$ cell lines.

S. No	Groups	LPO (n/mol)	GSH (n/mol)	SOD (n/mol)
1	Control	09.14 ± 0.57	91.27 ± 2.57	12.81 ± 0.72
2	CCl_4 (30mM)	76.89 ± 1.27***	19.78 ± 1.79***	1.27± 0.96***
3	Met-BNS + CCl_4	41.29 ± 1.57###	65.67 ± 3.57###	8.71 ± 0.73##

Statistical significance was determined by one way ANOVA followed by Tukey's multiple comparison tests using Graph pad prism Version 6.0. Values are represented as Mean ± SEM, Superscript *** denotes p<0.001 vs control, ### denotes p<0.001; vs CCl_4 groups respectively, (CCl_4: Carbon tetrachloride).

The SOD and GSH level (p<0.001) was significantly reduced in CCl_4 intoxicated $HepG_2$ cell lines in comparison to control cells. Post-treatment with met-BN-S (p<0.001) have remarkably elevated the SOD level in comparison to CCl_4 group indicate the free-radicals scavenging capacity of above extract in hepatotoxicity conditions. While the LPO level was significantly (p<0.001) increased in CCl_4 intoxicated cell lines in comparison to control cells indicate the induction of oxidative stress. Post-treatment with met-BN-S (p<0.001) have remarkably decreased the LPO level when compared to CCl4 treated cells represent the reduced lipid peroxidation level in CCl_4 treated $HepG_2$ cell lines while treated with met-BN -S.

Table 3: Effect of methanolic extract of seeds of *Brassica nigra* (met-BN-S) on AST, ALT, LDH level in CCl_4 intoxicated $HepG_2$ cell lines.

Group	ALT(U/L)	AST(U/L)	LDH(U/L)
Control	8.53±2.55	12.51±0.45	290.25±0.62
CCl_4 (30mM)	27.51±1.68***	34.55±1.10***	790.18±0.54***
Met-BN-S + CCl_4	15.45±3.61###	24.35±1.31###	600.20±0.61###

Statistical significance was determined by one way ANOVA followed by Tukey's multiple comparison tests using Graph pad prism Version 6.0. Values are represented as Mean ± SEM, Superscript *** denotes p<0.001 vs control, ### denotes p<0.001; vs CCl_4 groups respectively, (CCl_4: Carbon tetrachloride).

The LDH, ALT, AST level was significantly (p<0.001) increased in CCl_4 intoxicated cell lines in comparison to control cells indicate the damage of hepatocytes that release LDH into culture medium. Post-treatment with met-BN-S (p<0.001) have remarkably decreased the LDH, ALT, AST level when compared to CCl_4treated cells represent the hepatoprotective effect of the met-BN-S on $HepG_2$ cell line

Anti-inflammatory assessments – ELISA

Table 4: Effect of methanolic extract of seeds of *Brassica nigra* (met-BN-S) on IL-1β and TNF-α level in CCl_4 intoxicated $HepG_2$ cell lines.

S.No	Groups	IL-1β (pg/ml)	TNF-α (pg/ml)
1	Control	105.23±1.22	133.71±5.37
2	CCl_4 (30mM)	889.87±17.89***	977.84±36.52***
5	Met-BN-S	623.87±32.65###	675.24±18.14###

Statistical significance was determined by one way ANOVA followed by Tukey's multiple comparison tests using Graph pad prism Version 6.0. Values are represented as Mean ± SEM, Superscript *** denotes $p<0.001$ vs control, ### denotes $p<0.001$; vs CCl_4 groups respectively, (CCl_4: Carbon tetrachloride).

The IL-1β, TNF-α level was significantly ($p<0.001$) increased in CCl_4 intoxicated $HepG_2$ cell lines in comparison to control cells indicate the inflammation of hepatocytes. Post-treatment with Met-BN-S have decreased the level of IL-1β, and TNF-α level when compared to CCl_4 treated cells represent the reduced inflammatory response by above extract in CCl_4 treated $HepG_2$ cell lines.

Discussion

This research work deals with antioxidant and hepatoprotective activities of methanol extract of seeds of *Brassica nigra*. Liver is the second largest organ of our body involved in vital functions like detoxification of drugs and toxins through metabolism. CCl_4 is the toxin which produce trichloromethyl radical (CCl_3) which activated by cytochrome P450. This radical associated with CCl_4 induced hepatic damage. It reacts covalently with sulfhydryl-containing proteins in cells which leading to membrane lipid peroxidation and cell necrosis.

HepG2 cell line was used to evaluate the hepatoprotective activity of methanolic extract of seeds of *Brassica nigra (L).*, against liver damage induced by CCL_4. Cells exposed to this toxic agents loss cell viability, release liver enzymes into the culture medium, do not metabolize the tetrazolium salt, and exhibit significantly changed levels of MDA, SOD and GSH[79-80]. We found that, compared with control cells, HepG2 cells exposed to CCl_4 showed significantly reduced viability, reduced GSH and SOD levels and increased AST, ALT, LDH and MDA activities. Oxidative stress plays major role in CCl_4 toxicity. Oxidative stress occurs when the generation of reactive oxygen species overwhelms the ability to detoxify the reactive intermediates or exceeds the capacity to repair the resulting damage[4].It has been reported that CCl_4cause injury and reduce viability, increased AST, ALT, LDH. Enzyme levels are significant markers to determine the severity of damage to particular tissue. CCl_4 administration cause significant increase in enzyme levels like AST, ALT, LDH, which indicate the liver damage. Restoration of abnormal enzyme levels to normal indicating the protection of Methanolic extract of seeds of *Brassica nigra* against the liver injury by CCl_4.

It is important to mention that one way of indirectly assessing the damage to HepG2 cells caused by free radicals is by measuring the activities of intracellular enzymes GSH and SOD and the viability of cultured cells by MTT assay. These measurements are useful for assessing the *in vitro* antioxidative actions of the hepatoprotective plant extracts [5-9]. By-products of LPO cause hepatic damage in hydrophobic core of bio-membranes. Significant decline in LPO was observed in $HepG_2$ cell lines treated with methanolic extracts of seeds of *Brassica nigra*. We further studied non-enzymatic antioxidants like GSH and endogenous enzymes like SOD which involves in neutralizing free radicals. Suppression of these enzyme levels is an indicator for hepatic damage. CCl_4 administration cause decline in all the hepatic antioxidant levels in $HepG_2$ cell lines. Increased SOD and GSH levels was observed in $HepG_2$ cell lines treated with Met-BN-S indicates the antioxidant potential of the above extract which is a positive sign of liver protection. CCl_4 metabolism stimulated the Kupffer cells which activate TNF-α and IL-1β is a strong inflammatory cytokines which involves in the production of prostaglandins [10], macrophage activation and neutrophil infilteration. Activation of inflammatory cytokine cascade produces significant damage to the cells especially to hepatocytes. $HepG_2$ cell lines pre-treatment with Met-BN-S protects the hepatocytes from CCl_4 induced toxicity. Decreased TNF-α and IL-1β in $HepG_2$ cell lines indicates the anti-inflammatory potential of the Met-BN-S.

Conclusion

We concluded that the methanolic extract of seeds of *Brassica nigra* offered significant hepatoprotection against the oxidative damage induced by CCl_4 in $HepG_2$ cells, as revealed by reduction of ALT, AST and LDH levels in the culture medium and remarkable decrease in intracellular LPO content, together with elevation of SOD activity and GSH concentration. Our recent study supported the *In vivo* study for the hepatoprotective effect of methanolic extract of seeds of *Brassica nigra*, thus further demonstrating that Met-BN-S could serve as a potent hepatoprotective agent in the food supplement, pharmaceutical and medical industries.

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