Effects of black seed oil on resolution of hepato-renal toxicity induced by bromobenzene in rats

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Abstract. – OBJECTIVE: Volatile halocarbon, bromobenzene (BB), is frequently encountered in table-ready foods as contaminants residues. The objective of this study was to investigate whether black seed oil could attenuate hepato-renal injury induced by BB exposure.

MATERIALS AND METHODS: The evaluation was done through measuring liver oxidative stress markers: reduced glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA). Hepatic succinate dehydrogenase (SDH), lactate dehydrogenases (LDH) and glucose-6-phosphatase (G-6-Pase) were estimated. Serum aspartate and alanine aminotransferases (AST, ALT) and alkaline phosphatase were also evaluated. Kidney function indices; blood urea nitrogen (BUN), creatinine, serum protein, nitric oxide (NO), Na-K-adenosine triphosphatase (Na+-K+-ATPase) and phospholipids were done. Liver and kidney histopathological analysis and collagen content were analyzed for results confirmation.

RESULTS: Treatment with black seed oil (BSO) alleviated the elevation of GSH, SDH, LDH, G-6-Pase, serum protein, NO, Na-K-ATPase, phospholipids levels and attenuated MDA, SOD, AST, ALT and ALP. Diminution of collagen content and improvement in liver and kidney architectures were observed.

CONCLUSIONS: BSO enhanced the hepato-renal protection mechanism, reduced disease complications and delayed its progression. Further studies are needed to identify the molecules responsible for its pharmacological effect.

Key Words: Hepatic toxicity, Kidney pathy, Nigella sativa, Bromobenzene, Biomarkers.

Introduction

Environmental pollution with bromobenzene (BB) may occur during its production as well as its use as a solvent in the chemical industry and chemical intermediates1. It has been detected at low frequencies and at low concentrations in samples of food, air, and water2,3. Bromobenzene is expected to have moderate to high mobility in soil1. Volatilization of bromobenzene from moist soil surfaces plays a significant role in toxicity of various organs4.

BB is subjected to biotransformation in the liver. The metabolites of BB are highly hepatotoxic while secondary metabolites are highly nephrotoxic5. In the liver, BB is hydrolyzed by cytochrome P450 monooxygenases which mediate epoxidation to yields the highly electrophilic compound; bromobenzene-3, 4-epoxide6. Phase II drug metabolizing enzyme; glutathione-S-transferases catalyzes the sequestration of the reactive epoxides through conjugation to glutathione. At high doses, conjugation to the metabolites depletes the hepatic GSH pool, where the intracellular protection against reactive oxygen species (ROS) and hazardous xenobiotics metabolites is lost6. This leads to a number of secondary events like elevation of lipid peroxidation, local inflammation, ATP depletion, mitochondrial dysfunction, energy imbalance, and intracellular calcium store lost7.

Today many botanicals natural products are used to treat different diseases10. Various therapeutic effects, such as antioxidant, anticancer11, antihistaminic12 and antibacterial13 have been described for Nigella sativa. Additionally, it has been shown that Nigella sativa has protective effect against ischemia reperfusion injury to various organs14. Oral administration of Nigella sativa seed oil (black seed oil; BSO) can decrease the disease scores in patients with bronchial asthma and atopic eczema15,16. Moreover, Nigella sativa has immunostimulatory and healing properties17.

Thymoquinone, the active constituent of Nigella sativa seeds, is a pharmacologically active quinone, which served as an analgesic and
Experimental Design

Thirty six rats were divided into six groups equally. Group 1: control rats received vehicle (0.5 ml corn oil, i.p.) as the administration regimens described above. Group 2: rats were orally received black seeds oil. Group 3: rats were intraperitoneally injected with bromobenzene. Group 4: rats were orally received black seeds oil thirty minutes before BB injection twice a week for three consecutive weeks. Group 5: rats received silymarin and BB; as in group 4. Group 6: rats were orally received silymarin only.

Sample Preparations

Serum sample: Blood collected from each animal by puncture the sub-lingual vein in a clean and dry test tube, left 10 min to clot and centrifuged at 3000 g for 10 min at 4°C for serum separation. The separated serum was stored at –80°C for further determinations of liver and kidney functions tests and serum protein.

Liver and kidney tissues were homogenized in normal physiological saline solution (0.9% NaCl) (1:9 w/v). The homogenate was centrifuged at 3000 g for 10 min at 4°C. The supernatant was used for estimation of hepatic antioxidant and marker enzymes as well as kidney disorder biochemical indices. Liver and kidney histopathological findings and collagen deposition were also evaluated.

Materials and Methods

Animals and Ethics

Male Wistar albino rats (100: 120 g) were selected for this study. They were obtained from the Animal House, National Research Center, Egypt. All animals were kept in controlled environment of air and temp with access of water and diet. Anesthetic procedures and handling of animals were in compliance with the ethical guidelines of Medical Ethical Committee of National Research Centre, Cairo, Egypt (Approval no: 10031).

Nigella Sativa Seed Oil

*Nigella sativa* L. (Ranunculaceae) seed oil was purchased from local market; Harraz Market for Medicinal Herbs, Cairo, Egypt. Harraz is a well known market for its products purity.

Doses and Route of Administration

The administration regimens was twice a week for three consecutive weeks. Black seed oil (BSO) was orally administered at a dose of 2 ml/kg body weight. Bromobenzene was intraperitoneally administered at a dose of 460 mg/kg body weight (1:9 w/v corn oil). Hepaticum as a reference herbal drug (active constituent; silymarin) was orally given at a dose 100 mg/kg body weight.

Experimental Oxidative Stress Markers

Malondialdehyde was determined by the method of Buege and Aust. Its concentration was calculated using the extinction coefficient value \(1.56 \times 10^5\) M\(^{-1}\) cm\(^{-1}\) and read at 535 nm.

Glutathione was assayed by the method of Moron et al using dithiobis-2-nitrobenzoic acid (DTNB) in phosphate buffer saline (PBS). The reaction colour was read at 412 nm (Novaspec II, LKB Pharmacia Technology, Uppsala, Sweden).

Superoxide dismutase was estimated by method of Nishikimi et al, where the increase of NADH oxidation was measured at 560 nm using its molar extinction coefficient 6.22 \(\times 10^3\) M\(^{-1}\) cm\(^{-1}\).

Hepatic Marker Enzymes

Succinate dehydrogenase (mitochondria marker) was estimated by the method of Rice and Shelton, where reduction of flavin adenine dinucleotide (FAD) is coupled with a reduction of tetrazolium salt as 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), the produced formazan of INT is measured colorimetrically at 490 nm. Lactate dehydrogenase (cytoplasm marker) was estimated by the method of Babson and Babson, where the reduction of
nucleoside derived amino acids (NAD) was coupled with the reduction of tetrazolium salt and phenazine methosulfate serving as an intermediate electron carrier; the produced formazan of INT was measured colorimetrically at 503 nm. G-6-Pase (microsome marker) was measured colorimetrically as inorganic phosphorus released at 660 nm²⁹.

**Serum Biomarkers of Liver Functions Tests**

Aspartate and alanine aminotransferases were measured by the method of Gella et al³⁰, where the transfer of the amino group from aspartate or alanine formed oxalacetate or pyruvate, respectively and the developed colour was measured at 520 nm. Alkaline phosphatase catalyze in alkaline medium the transfer of phosphate group from 4-nitrophosphatase to 2-amino-2-methyl-1-propanol (AMP) and the liberating 4-nitrophenol was measured at 510 nm³¹.

**Serum Biomarkers of Kidney Functions Tests**

Blood urea-nitrogen (BUN) was determined by the method of Tabacco et al³², where urea in the sample by urease enzyme gave a colored complex and the developed colour was measured at 600 nm.

Creatinine was measured by the method of Bartels et al³³, where creatinine in the sample reacted with picrates in alkaline medium and formed a colored complex at 500 nm.

Total protein, the Coomassie brilliant blue dye reacts with Bradford reagent to give a blue complex. The developed colour was read at 595 nm³⁴.

**Kidney Disorder Indices**

Nitric oxide, as vasodilatory chemokine was assayed by the method of Moshage et al³⁵, where Promega’s Griess Reagent System is based on the chemical reaction between sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic phosphoric acid condition to give colored azo-compound which can be measured colorimetrically at 520 nm.

Na⁺-K⁺-ATPase (cations transport marker) was determined by the method of Bonting et al³⁶, where the amount of Pi liberated were calibrated using Pi standard curve and Na⁺-K⁺-ATPase activity was calculated by subtracting total ATPase activity from Mg⁺² ATPase activity.

Phospholipid (membrane permeability marker) was measured by the method of Connerty et al³⁷. Phospholipids oxidized to phosphate with sulphuric and perchloric acid. The produced phosphate forms phospho molybdates complex reduced by stannous chloride to a blue color that measured colorimetrically at 620 nm.

**Histopathological Analysis**

Liver and kidney sections of all groups were stained with hematoxyline and eosin and Masson’s trichrome to detect changes in cells architecture and degree of fibrosis³⁸. Collagen content was determined in Masson’s trichrome sections and expressed as the volume of collagen in liver and kidney tissues, where % volume of collagen = number of points failing on 10 successive fields (1 cm² eye piece reticule) × 100/number of points in the reticule³⁹.

**Statistical Analysis and Calculations**

All data were expressed as mean ± SD of six rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Costat Software Computer Program.

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<th>% change</th>
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<td>% improvement</td>
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**Results**

**Potency of BSO on Oxidative Stress Markers**

Rats administrated with BSO or silymarin recorded insignificant changes in MDA, GSH and SOD (Figure 1). BB treated rats showed significant increase in MDA (56.33%) and SOD (52.49%), while significant decrease in GSH (39.02%) was recorded. Intoxicated rats treated with BSO recorded improvement in MDA, GSH and SOD by 48.59, 30.86 and 38.63%, respectively. Silymarin enhanced MDA and the antioxidant levels by 52.81, 34.75 and 42.51%, respectively.

**Effect of BSO on Hepatic Marker Enzymes**

Rats administrated with *Nigella sativa* seed oil or silymarin showed insignificant decrease in SDH, LDH and G-6-Pase (Figure 2). BB intoxicated group recorded significant decrease in SDH, LDH and G-6-Pase by 45.03, 25.99 and 37.35%, respectively. BB intoxicated group treat-
ed with BSO improved SDH, LDH and G-6-Pase by 29.52, 20.11 and 15.85%, respectively, while silymarin ameliorated the three enzymes by 31.88, 21.83 and 19.69%.

**Effect of BSO on Liver Function Enzymes**

Insignificant increase in serum AST, ALT and ALP levels after treatment of normal healthy rats with BSO or drug were observed (Figure 3). BB group showed significant increase in AST, ALT and ALP by 47.33, 28.55 and 57.29%, respectively. BSO treatment attenuated the enzymes by 15.89, 9.16 and 41.31%, while silymarin diminished the levels by 30.72, 17.29 and 49.01%, respectively.

**Effect of BSO on Kidney Function Parameters**

Blood urea nitrogen (BUN), creatinine and serum protein showed insignificant changes after treatment of normal rats with BSO or silymarin (Figure 4). BB group recorded significant increase in BUN and creatinine levels by 21.72, 47.70%, respectively, while serum protein
showed significant decrease by 10.66%. Treatment with BSO improved the kidney functions parameters by 33.80, 39.44 and 2.17%, respectively, while silymarin enhanced the levels by 18.20, 42.20 and 5.22%.

**Effect of BSO on Kidney Disorder Biomarkers**

Insignificant changes in NO, Na+-K+-ATPase and phospholipids in kidney tissue of normal rats treated with BSO or silymarin were observed (Figure 5). Intoxicated rats recorded significant decrease in NO (24.37%), Na+-K+-ATPase (24.94%) and phospholipids (22.61%). Intoxicated rats treated with BSO showed elevation in NO, Na+-K+-ATPase and phospholipids by 17.63, 15.93 and 11.01%, respectively, while treatment with silymarin elevated these biomarkers by 15.47, 16.51 and 6.18%, respectively.

**Histopathological Observations**

Liver histopathological features of control (Figure 6 a, d) and healthy rats treated with BSO (Figure 6 b, e) or silymarin (Figure 6 c, f)
showed normal hepatic lobular architecture. The hepatocytes are within normal limits and separated by narrow blood sinusoids. Portal tracts were extending with no lymphocytes infiltrations. No signs of fibrosis were detected.

Liver injured with BB showed portal loss of hepatic lobular architecture, ballooning of hepatocytes, deformed cord arrangement and disturbed sinusoids. The hepatocytes showed marked degree of hydropic changes, massive necrosis and marked number of chronic inflammatory cells (Figure 6 g).

Treatment of injured liver with BSO showed well formed nucleated hepatocytes, sinusoidal arrays and mild inflammatory lymphocyte infiltration (Figure 6 h). Treatment with silymarin showed well arranged cord of nucleated hepatocytes and sinusoids, while hepatocytes infiltrations were still present (Figure 6 i). Fibrous tissue was presented by 60% in BB intoxicated group (Figure 6 j), while mild fibrotic tissue (25%) was still present in BSO treated group (Figure 6 k). Silymarin treated group recorded collagen deposition by 21% (Figure 6 l).

Kidney histopathological features of control (Figure 7 a, d) and healthy rats treated with BSO (Figure 7 b, e) or silymarin (Figure 7 c, f) showed normal appearance of tubules, glomeruli and tubule.
lointerstitial cells. No signs of fibrosis were observed in the interstitial spaces of all control groups.

Kidney section of BB-treated rats showed glomerular basement membrane thickening, dilation of Bowman’s space, interstitial inflammation and focal patches of fibrosis in the interstitial epithelium (Figure 7 g). BB intoxicated rats treated with BSO showed normal architecture of the kidney, regeneration of glomeruli and tubules and absence of interstitial inflammatory cell infiltrations (Figure 7 h). Silymarin treated group still showed mild dilation of Bowman’s space (Figure 7 i). Collagen deposition reached to 52.2% in BB group (Figure 7 j). Mild collagen content was recorded in BSO treated group (15%) and silymarin treated one (20%) (Figure 7 k, l).

**Discussion**

Today, there is considerable interest in free radicals mediated damage to biological systems due to xenobiotics exposure. Some of these free radicals interact with various tissue components, resulting in dysfunction and injury to the liver and other organs. Lipid peroxidation has been suggest-
ed as one of the molecular mechanisms involved in toxicity. Free radical scavengers such as glutathione and superoxide dismutase may protect the biological systems from deleterious effects of free radicals induced by xenobiotics.

Liver has variety of redox systems, among which GSH is important. Therefore, it was worthwhile to investigate the GSH level in liver together with the activities of glutathione peroxidase, glutathione reductase, glutathione-S-transferase and superoxide dismutase since they may efficiently scavenge free radicals and be partly responsible for protection against lipid peroxidation. In parallel with these observations, our investigation recorded significant elevation in SOD and MAD, a marker of lipid peroxidation process, while glutathione level was depressed. Treatment with BSO or hepaticum attenuated the levels of MAD and SOD, while GSH was elevated. This gave an additional support of their role as free radical scavengers. In agreement with this observation, Ansari et al. and Hosseinzadeh et al. postulated the effective action of BSO as antioxidant to its rich with saponins and alkaloids.

Figure 5. Kidney disorder biomarkers in BB intoxicated rats. All data are mean ± SD of six rats in each group. Values are expressed as µmole/g tissue for NO, µPi/g tissue for Na+ K+-ATPase and mg lipid/g tissue for phospholipids. Unshared letters between groups are the significance values at \( p < 0.0001 \). Statistical analysis is carried out using one way analysis of variance (ANOVA), CoStat Computer Program using least significance difference (LSD) between groups at \( p < 0.05 \).
In addition, hepaticum as an antioxidant flavonoid complex derived from the herb milk thistle (*Silybum marianum*), has the ability to scavenge free radicals, chelate metal ions, inhibiting lipid peroxidation and preventing liver glutathione depletion. Hepaticum recorded more potent effect than any hepatoprotective drugs, where silymarin absorption is enhanced by micronization and inclusion in β-cyclodextrin as a complex.

Metabolites of BB are responsible for hepatotoxicity that harmfully affected various organelles. Mitochondrial injury has been proposed to play a key role in the initiating events that lead to cytotoxicities of several organelles by xenobiotics. Maellaro et al. examined the role of mitochondrial membrane potential alterations after BB cytotoxicity, and found that membrane potential was disrupted in the early stages of injury, before hepatic cell death and lipid peroxidation were evident. Wong et al. added that, mitochondrial glutathione was not depleted until three hours after BB administration. This delay in mitochondrial glutathione depletion may be attributed to the generation of reactive BB metabolites in the cytosol, resulting in localized decreases of the cytosolic glutathione pool, before being able to diffuse to the mitochondria and adversely affecting glutathione content within this organelle. Additionally, since mitochondria are incapable of de novo glutathione synthesis and are, therefore, dependent on glutathione import from the cytosol, so any stress on cytosolic glutathione levels would be expected to have a delayed effect in mitochondria. The present study showed that BB administration induced ROS and lipid peroxidation process that may affect the mitochondrial membrane integrity and lead to mitochondrial dysfunction. Depression of succinate dehydrogenase enzyme, which is localized in the inner mitochondrial membrane, gives an additional support of the harmful effect of BB intoxication on mitochondrial membrane integrity. Free rad-
icals involvement affected also plasma membrane permeability and lead to leakage of LDH enzyme into circulation\(^47,48\). Moreover, the decreased activity of G-6-Pase during toxicity confirms the microsomal membrane damage\(^49\). The functional integrity of the enzyme depends on the chemical composition and physical status of the lipid environment where it is embedded. The decrease in membrane phospholipids due to an increase in phospholipase A2 and C and increased lipid peroxidation could be the reason for the decreased enzyme activities\(^50\). Regulatory effect of BSO and hepaticum on hepatic marker enzymes was documented in our study. Their role as free radicals scavengers could in turn normalize microsomes, mitochondria and plasma membranes permeability and integrity which lead to restore the hepatic enzymes to normal levels.

It is clear that, serum activities of AST, ALT and ALP were significantly elevated in BB-treated rats compared to control group. This indicates injuries and impaired functions of liver as a result of BB intoxication\(^6\). Serum total proteins always measure the excretory and synthetic functions of liver\(^6\). In the present study, serum total proteins were significantly decreased in BB-treated rats as compared to untreated control group which indicates impaired excretory and synthetic functions of the liver. Treatment with BSO or hepaticum ameliorated the enzyme levels by variable degrees. Therefore, BSO acted as hepaticum in protecting hepatocytes plasma membrane and liver cells directly by stabilizing the membrane permeability and decrease leakage of the enzymes into the circulation\(^51\).

BB metabolites are formed in the liver and transported to the kidney, where they exerted the nephrotoxic action\(^8\). These metabolites; 2, 3-epoxybromobenzene, and 2-bromohydroquinone oxidized to 2-bromoquinone, which combined

\(\text{Figure 7. Hematoxylin \& eosin and Masson's trichrome stain kidney section (400}\times)\) of control rat (a, d), normal treated with BSO (b, e), normal treated with silymarin (c, f), BB intoxicated rats (g, j), BB intoxicated rats treated with BSO (h, k), BB intoxicated rats treated with drug (i, l). Arrows show normal glomeruli in normal and normal treated rats (a-f). Dilatation in Bowman capsules with massive infiltrations and fibrosis in interstitial space were observed in intoxicated rats (g, j). Normal glomeruli with minimal infiltrations and less fibrosis in interstitial space were seen in intoxicated rats treated with BSO and silymarin (h, i, k, l).

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with glutathione and gives various mono- and di-substituted derivatives\textsuperscript{52}. Glutathione conjugates with bromoquinone accumulate in the kidney and are nephrotoxic\textsuperscript{52}. Renal injuries may contribute to low level of serum protein that might have resulted from remarkable leakage into urine due to injuries in glomeruli and tubules\textsuperscript{53}. This was in accordance with our research through the recorded decrease in serum protein in BB intoxicated group. Impaired in renal function was also noticed by elevation of urea and creatinine levels. This was in agreement with Khan et al\textsuperscript{54} who reported that chronic renal injuries was associated with urea and creatinine elevation and consider as indicators of kidney injury, where the serum creatinine level does not rise until at least half of the kidney nephrons are destroyed.

Although NO was described initially as a vasodilatory chemokine\textsuperscript{55}, it plays a major role as antioxidant\textsuperscript{56}. The observed decrease of NO level in the kidney permitted vasoconstriction which contributed by diminution of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and phospholipids. The activity of renal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase varies in parallel with sustained changes in Na\textsuperscript{+} or K\textsuperscript{+} transport, indicating the participation of this enzyme in the chronic adaptation of the kidney to altered Na\textsuperscript{+} reabsorption or K\textsuperscript{+} secretory load\textsuperscript{47,57}. Not only these hemodynamic effects, but also alterations in membrane lipid composition that influence membrane fluidity, cation transport and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity can predispose renal tubular cells to injury\textsuperscript{58}. The observed improve in kidney function parameters gives an additional support that BSO mops up free radicals generated by BB and induces healthy state of renal cells, suggesting its role as renal protective agent.

The most commonly associated characteristic of liver and kidney fibrosis is the increased deposition of collagens\textsuperscript{59,60}. During liver fibrosis, altered collagen synthesis at both mRNA and protein levels is observed, with a dramatic increase in type I collagen along with smaller, but significant, increases in type III collagen\textsuperscript{59}. The excess deposition of extracellular matrix proteins disrupts the normal architecture of the liver which alters the normal function of the organ, ultimately leading to pathophysiological damage to the organ\textsuperscript{59}. The development of renal fibrosis involves the progressive appearance of glomerulosclerosis, tubulointerstitial fibrosis and changes in renal vasculature\textsuperscript{60}. The presence of kidney fibrosis seems mostly to be viewed as an endpoint or marker of tissue or organ failure and function-loss\textsuperscript{61}. In BSO and hepaticum treated groups, hepatocyte and glomeruli degeneration, fibrosis and infiltration of inflammatory cells were all apparently ameliorated and collagen deposition was also markedly reduced. Hepaticum as anti-fibrotic and anti inflammatory effects inhibits activation of hepatic stellate cells through the expression of transforming growth factor-beta1 and stabilization of mast cells\textsuperscript{62}. We compare the anti-fibrogenic effects of hepaticum with BSO and the results exhibited that BSO treatment had higher potency in inhibiting collagen deposition and fibrosis severity. The observed amelioration after BSO or hepaticum treatment was, at least in part, due to BB detoxification through inhibiting the cytochrome P450-dependent monooxygenase activities and enhancing the activity of epoxide hydrolase which detoxifies the toxic epoxide intermediate of bromobenzene produced upon oxidation by the cytochrome P450-mediated phase I metabolism\textsuperscript{63}.

**Conclusions**

BB impaired hepatic and renal functions, altered antioxidant levels, enhanced inflammation, induced fibrosis by highly collagen deposition and affected harmfully on the histological picture of the liver and kidney. The antioxidant effect of BSO may be contributed to the protection against bromobenzene toxicity. BSO can be considering as a nutraceutical agent or a complementary safe drug that may minimize hepato-renal damage, delay disease progression and reduce complications. Further studies are needed to identify the molecules responsible for its pharmacological activity and its clinical application.

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