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Amelioration of bromobenzene hepatotoxicity by *Withania somnifera* pretreatment: Role of mitochondrial oxidative stress

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ABSTRACT

The present study investigated the possible protective role of *Withania somnifera* (Linn.) Dunal (Solanaceae) root powder against bromobenzene-induced oxidative damage in rat liver mitochondria. Administration of bromobenzene (10 mmol/kg body weight) to rats resulted in increased levels of liver marker enzymes, lipid peroxidation, TNF-α, IL-1β and VEGF. There was also marked depletion in the levels of mitochondrial enzymes and antioxidant activity. Pre-treatment with *W. somnifera* significantly decreased the levels of liver marker enzymes, TNF-α, IL-1β, VEGF and ameliorated histopathological manifestations in bromobenzene-treated rats. The molecular docking analysis predicted that the pro-inflammatory mediator NF-κB showed significant interaction with selected various active components of *W. somnifera* (withaferin A, withanolide D and withanolide E). This study demonstrates a good protective effect of *W. somnifera* against bromobenzene-induced oxidative stress.

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1. Introduction

Bromobenzene (C_6H_5Br) is a well-known organic solvent and has profound use in the manufacture of various drugs and chemicals. It is metabolized in the liver by primary cytochrome enzymes to form various oxides of bromobenzene, of which bromobenzene-3, 4-oxide is highly reactive. Bromobenzene-3, 4-oxide is sequestered by binding with reduced glutathione (GSH) and subsequently depleting hepatic glutathione levels. This results in reduced protection against intracellular reactive oxygen species (ROS) leading to secondary events such as mitochondrial dysfunction, changes in membrane permeability and oxidative stress [1]. Mitochondria is a vital site for energy metabolism and ATP production. Hence, its malfunction leads to cellular damage and contributes to a wide range of diseases [2]. It can be suggested that mitochondrial dysfunction would be diminished by enriching the mitochondria with antioxidants, thereby reducing the oxidative stress.

Recent studies have focussed on the potential of various natural compounds against liver pathological conditions. Silymarin and *N*-acetyl-L-cysteine are being already used

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Abbreviations: BB, Bromobenzene; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-s-transferase; WS, *Withania somnifera*; ROS, reactive oxygen species.

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in the clinical treatment of liver injury and they exhibit a potent hepatoprotective activity, but with certain limitations such as gastric irritation, allergies and limited efficacy [3,4]. This indicates that there is still the need of finding highly effective and reliable drugs with minimal side effects for the prevention of acute liver failure.

Withania somnifera (Linn.) Dunal (Solanaceae) is a well-known Ayurvedic medicine known to possess pharmacological properties such as antistress, antioxidant, immunomodulating and anti-arthritic activities [5,6]. These properties may be due to the presence of various biologically active chemical constituents such as alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanoloides with a glucose at carbon 27 (sitonidoside XI and X) [7,8]. The roots of *W. somnifera* are the most pharmacologically active part of the plant and are known to possess free radical scavenging and antioxidant activity [9]. Therefore, an attempt was made to evaluate the hepatoprotective effect of *W. somnifera* root powder against bromobenzene-induced acute liver necrosis which has not been reported to the best of our knowledge. Silymarin has been used as the standard reference drug in the present study. Also, the effect of selected active components of *W. somnifera* on NF-κB was analyzed using molecular docking.

2. Materials and methods

2.1. Animals

Wistar albino rats of either sex, weighing 120–150 g were obtained from animal house, VIT University, Vellore. They were fed with commercial pelleted feed from Hindustan Lever Ltd. (Mumbai, India) and water ad libitum. The animals were maintained according to the guidelines recommended by the Committee for the Purpose of Supervision and Control of Experiments on Animals (CPSCEA), Government of India, Chennai, Tamil Nadu. Experimental procedure for the present study has been approved by the ethical committee (VIT/IAEC/VIIth/17) of VIT University, Vellore, India.

2.2. Drugs and chemicals

Commercially available *W. somnifera* root powder was obtained from Indian Medical Practitioners Co-operative Stores and Society (IMCOPS, Chennai, India). Silymarin, a standard hepatoprotective drug, was obtained from Micro Labs Ltd (Goa, India). All other reagents used were standard laboratory reagents of analytical grade and were purchased locally. The effective dosage of bromobenzene [10] and *W. somnifera* [11] were based on the basis of previous studies. Aqueous suspension of silymarin (100 mg/kg body weight) and *W. somnifera* (250 and 500 mg/kg body weight) were made in double distilled water for administration to rats.

3. Experimental procedure

Animals were allocated randomly in six groups of six animals each. In this study, all group of rats except group I and group VI received bromobenzene dosage (intragastric tube) only once. The animals were treated as shown in Table 1:

Table 1
Experimental animal design for the study.

Groups	Treatment
Group I	Normal control (received single dose of 0.1 ml coconut oil through intragastric intubation once and sacrificed after 19 h)
Group II	Bromobenzene (10 mmol in 0.1 ml coconut oil by intragastric intubation) once and sacrificed after 19 h
Group III	*Withania somnifera* (250 mg/kg, orally) for 8 days and a single dose of bromobenzene (10 mmol/kg in 0.1 ml coconut oil, intragastric intubation) on the 8th day and sacrificed after 19 h
Group IV	*Withania somnifera* (500 mg/kg, orally) for 8 days and a single dose of bromobenzene (10 mmol/kg in 0.1 ml coconut oil, intragastric intubation) on the 8th day and sacrificed after 19 h
Group V	silymarin (100 mg/kg, orally) for 8 days and a single dose of bromobenzene (10 mmol/kg in 0.1 ml coconut oil intragastric intubation) on the 8th day and sacrificed after 19 h
Group VI	*Withania somnifera* (500 mg/kg, orally) for 8 days and sacrificed after 19 h

All six groups were fasted for 24 h before and 19 h after the administration of silymarin/*W. somnifera*/bromobenzene/coconut oil. After the collection of blood, samples from liver tissues (approximately 0.05–0.1 g) were homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate [12]. This homogenate was centrifuged at 3000 g and 4 °C for 10 min; the supernatant was stored at −20 °C until analysis.

3.1. Serum biochemical parameters, antioxidant status, plasma ceruloplasmin and total sulfhydryl group determination

The activities of aspartate aminotransferase (AST), alkaline aminotransferase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin and direct bilirubin were determined according to the manufacturer's protocol using kits (Autospan diagnostics, Bangalore, India) in the serum of control and experimental rats.

Antioxidant status was determined in the plasma and liver tissue of control as well as experimental rats. Lipid peroxidation was determined by the procedure of Ohkawa et al. [13] and malondialdehyde (MDA), which forms as end product of lipid peroxidation, was measured. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and reduced glutathione were also evaluated [14–18]. Total protein was estimated according to the method of Lowry et al. [19] using bovine serum albumin as standard. Furthermore, plasma ceruloplasmin [20] and total sulfhydryl groups [21] were measured in plasma and liver respectively in the control and experimental rats.

3.2. Isolation of mitochondria

For the isolation of liver mitochondria, the method of Johnson and Lardy [22] was followed and the protein content was measured according to manufacturer's protocol using kit obtained from Autospan diagnostics, Bangalore, India.

3.3. Evaluation of mitochondrial enzymes

Isocitrate dehydrogenase activity was measured using a calibration curve with α-ketoglutarate as standard and was expressed as nmol of α-ketoglutarate formed/h/mg protein [23]. The activity of α-ketoglutarate dehydrogenase was assayed by the method of Reed and Mukherjee [24] and the reaction was terminated by the addition of tricarboxylic acid (TCA). The colour developed was measured at 540 nm. A standard containing potassium ferrocyanide was assayed simultaneously. The activity of succinate dehydrogenase was assayed according to the method of Slater and Bonner [25] using sodium succinate and potassium ferricyanide, and the change in optical density (OD) was recorded at 15 s intervals for 5 min at 420 nm. Succinate dehydrogenase activity was expressed as nmol of succinate oxidized/min/mg protein. The activity of malate dehydrogenase was assayed by the method of Mehler et al. [26]. Reaction mixture containing phosphate buffer, NADH, oxaloacetate and mitochondrial suspension was prepared. The change in OD at 340 nm was measured for 2 min at intervals of 15 s in a UV spectrophotometer. The activity of the enzyme was expressed as nmol of NADH oxidized/min/mg protein. The activity of cytochrome c oxidase was assayed according to the method of Pearl et al. [27] and the change in absorbance was recorded at 550 nm for 5 min at intervals of 15 s. The enzyme activity was expressed as change in absorbance/min/mg protein. The enzyme activity of NADH dehydrogenase was assayed by the method of Minakami et al. [28] and was expressed as nmol of NADH oxidized/min/mg protein.

3.4. Measurement of serum TNF-α, IL1β and VEGF

The serum concentrations of TNF-α, IL1β and VEGF were measured by ELISA according to manufacturer's protocol using commercial kits (Krishgen BioSystems, Bangalore, India) and were expressed in nanograms per millilitre.

3.5. Docking study of active components of *W. somnifera* with NF-κB

A three dimensional structure of the binding domain of NF-κB (PDB ID: 3U21) was downloaded using the protein databank database (http://www.rcsb.org/pdb/home.do) (Fig. 4). The sequences of selected active components of *W. somnifera* such as withaferin A, withanolide D and withanolide E were obtained from pubchem (http://www.ncbi.mlm.nih.gov.pccompound) and submitted in SMILES format on Corina (http://www.molecular-networks.com/node/84) to generate 3-Dimensional structures of the ligands (Fig. 4). Docking was done using PatchDock server and the scoring in PatchDock is based on both geometric fit and atomic desolvation energies.

3.6. Histopathological studies

Immediately after sacrifice, a portion of the liver was fixed in 10% formalin, then washed, dehydrated in descending grades of isopropanol and finally rinsed with xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 mm thickness, stained with haematoxylin and eosin and were observed microscopically for histopathological changes (400× magnification).

3.7. Statistical analysis

All results were expressed as mean ± SD compared to normal control rats. The intergroup variation between various groups was measured by one way analysis of variance (ANOVA) and the comparisons between the groups were conducted by Student Newman–Keul's test using the software Graph Pad InStat version 3.10. Results were considered statistically significant when $^*p < 0.05$. Comparisons were made as follows: a-group I vs groups II–VI; b-group II vs group III–VI.

4. Results

4.1. Effects of *W. somnifera* on liver weight and serum biochemical parameters

The results observed in groups which were pre-treated with *W. somnifera* and silymarin with respect to the induction of hepatotoxicity using bromobenzene are given in Table 2 and 3. Rats which were treated with bromobenzene developed liver damage as evidenced by increase in the activities of liver functional markers; ALT, AST, ALP, total and direct bilirubin (Table 3). Moreover, there was an increase in the liver weight and decrease in the albumin levels in bromobenzene treated group as compared to the control group of rats (Table 2). *W. somnifera* (250

Table 2

Effect of the administration of bromobenzene on liver weight with or without prior administration of *Withania somnifera* in control and experimental rats.

Groups	Liver weight (g)	Body weight initial (g)	Body weight before sacrifice (g)
Group I (Control)	5.19 ± 0.55	118.32 ± 13.20	122.17 ± 14.30
Group II (Bromobenzene-10 mmol/kg)	6.27 ± 0.27a*	117.17 ± 15.31	120.50 ± 12.07
Group III (*Withania somnifera*-250 mg/kg +Bromobenzene-10 mmol/kg)	5.38 ± 0.18b*	119.05 ± 8.15	125.16 ± 11.09
Group IV (*Withania somnifera*-500 mg/kg + Bromobenzene-10 mmol/kg)	5.16 ± 0.13b*	118.00 ± 15.33	129.50 ± 13.41
Group V (Silymarin-100 mg/kg + Bromobenzene-10 mmol/kg)	4.86 ± 0.26b*	116.33 ± 14.70	119.36 ± 12.80
Group VI (*Withania somnifera* -500 mg/kg)	5.47 ± 0.36b*	120.75 ± 10.90	129.87 ± 14.97

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V, VI; b– group II vs group III, IV, V, VI. Statistical analysis was calculated by one way analysis of variance (ANOVA) followed by the Student Newman–Keul's test.

* The symbols represent statistical significance at $p < 0.05$.

Table 3
Effect of the administration of bromobenzene (BB) on levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, direct and total bilirubin with or without prior administration of *Withania somnifera* (WS) in serum of control and experimental rats.

Parameters	Group I (Control)	Group II (BB – 10 mmol/kg)	Group III (WS - 250 mg/kg + BB)	Group IV (WS – 500 mg/kg + BB)	Group V (Silymarin – 100 mg/kg + BB)	Group VI (WS – 500 mg/kg)
AST (U/L)	114.04 ± 0.73	236.07 ± 1.15a*	145.1 ± 0.52a*b*	134.45 ± 3.58a*b*	136.26 ± 3.01a*b*	128.14 ± 1.63b*
ALT(U/L)	48.44 ± 3.12	113.09 ± 1.65 a*	57.13 ± 1.67 b*	55.25 ± 2.51 a*b*	57.68 ± 2.32 b*	54.41 ± 1.77 b*
ALP(U/L)	87.04 ± 3.08	167.88 ± 9.95 a*	113.04 ± 3.16 a*b*	106.02 ± 6.70 a*b*	135.01 ± 2.91 a*b*	103.60 ± 7.61 b*
Total Bilirubin (mg/dl)	0.88 ± 0.07	3.29 ± 0.48a*	2.40 ± 0.23a*b*	1.28 ± 0.08b*	1.51 ± 0.09a*b*	1.01 ± 0.03b*
Direct Bilirubin (mg/dl)	0.03 ± 0.01	0.36 ± 0.05 a*	0.19 ± 0.01 a*b*	0.12 ± 0.01 a*b*	0.15 ± 0.02 a*b*	0.04 ± 0.01 b*
Albumin (g/l)	42.52 ± 1.44	35.38 ± 1.62a*	41.36 ± 1.38	44.80 ± 1.60b*	48.15 ± 0.54b*	42.18 ± 0.21b*

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V, VI; b-group II vs group III, IV, V, VI. Statistical analysis was calculated by one way analysis of variance (ANOVA) followed by the Student Newman–Keul's test.

[*] The symbols represent statistical significance at $p < 0.05$.

and 500 mg/kg) pre-treatment restored the levels of ALT, AST, ALP, total bilirubin, direct bilirubin and albumin in bromobenzene treated group of rats.

4.2. Effect of W. somnifera on antioxidant levels

Malondialdehyde (MDA) is the main oxidative degradation product during lipid peroxidation and functions as a marker of oxidative injury to cellular membranes. Antioxidant status was found to be depleted (Table 4) accompanied with an increase in the levels of MDA in both plasma and hepatic tissue of bromobenzene-treated group as compared to the control group. Levels of CAT, SOD and glutathione were significantly enhanced, whereas levels of MDA were reduced in *W. somnifera* and silymarin treated groups. The highest dose (500 mg/kg) of *W. somnifera*

Table 4
Effect of the administration of bromobenzene (BB) on antioxidant status, lipid peroxidation, plasma ceruloplasmin and total sulfhydryl groups with or without prior administration of *Withania somnifera* (WS) in plasma and liver tissue homogenate of control and experimental rats.

Parameters	Group I (Control)	Group II (BB – 10 mmol/kg)	Group III (WS – 250 mg/kg + BB)	Group IV (WS – 500 mg/kg + BB)	Group V (Silymarin – 100 mg/kg + BB)	Group VI (WS – 500 mg/kg)
Liver						
Catalase (Units/min/mg protein)	64.05 ± 0.93	21.57 ± 0.30a*	31.94 ± 1.52b*	60.18 ± 7.09b*	59.4 ± 8.47a*b*	61.84 ± 2.08b*
SOD (U/mg protein)	68.85 ± 7.06	23.85 ± 2.07a*	43.90 ± 4.82a*b*	66.9 ± 7.84b*	63.5 ± 7.12b*	66.42 ± 7.83b*
Lipid Peroxidation (nmol/mg protein)	1.44 ± 0.03	3.25 ± 0.09a*	1.53 ± 0.03b*	1.50 ± 0.06b*	1.51 ± 0.04b*	1.56 ± 0.02b*
GST (nmol/minmg protein)	19.97 ± 0.10	7.65 ± 0.14a*	15.94 ± 0.15a*b*	19.43 ± 0.31b*	19.12 ± 0.16b*	19.57 ± 0.17b*
Reduced Glutathione (mmol/mg protein)	7.95 ± 0.21	3.33. ± 0.67a*	8.27 ± 0.12b*	7.93 ± 0.47	8.25 ± 0.49	7.68 ± 0.12
Glutathione Peroxidase (µg of GSH utilized/min/mg protein)	26.42 ± 3.85	16.5 ± 1.93a*	24.40 ± 3.02b*	25.70 ± 2.20b*	25.08 ± 1.16b*	25.76 ± 4.03b*
Total Sulfhydryl groups (nmol/mg protein)	18.5 ± 3.08	7.08 ± 0.79a*	12.23 ± 1.52a*b*	15.16 ± 1.60b*	13.50 ± 2.88a*b*	17.83 ± 2.31
Plasma						
Catalase (Units/min/mg protein)	62.99 ± 6.85	43.55 ± 1.45a*	55.32 ± 3.59	59.68 ± 1.75b*	56.13 ± 2.83	60.8 ± 3.02a*
SOD (U/mg protein)	52.60 ± 7.77	24.50 ± 2.73a*	42.4 ± 4.49b*	50.21 ± 4.12a*	42.35 ± 10.46b*	51.28 ± 5.47
Lipid Peroxidation (nmol/mg protein)	3.04 ± 0.12	6.18 ± 0.82a*	4.16 ± 0.17a*	3.60 ± 0.31b*	3.72 ± 0.71b*	3.83 ± 0.26
GST (nmol/min mg protein)	16.63 ± 2.36	8.41 ± 2.92a*	14.78 ± 0.17	15.27 ± 0.18a*	15.22 ± 0.11b*	16.32 ± 0.14b*
Reduced Glutathione (mmol/mg protein)	15.86 ± 0.38	7.65 ± 0.67a*	12.89 ± 1.94a*	13.26 ± 2.01	10.43 ± 1.45a*	14.69 ± 0.27a*
Glutathione Peroxidase (µg of GSH utilized/min/mg protein)	29.16 ± 3.17	18.58 ± 2.92a*	27.12 ± 2.24	23.42 ± 1.94b*	28.94 ± 2.11	28.32 ± 1.23
Plasma ceruloplasmin (g/l)	0.75 ± 0.08	1.89 ± 0.62a*	0.94 ± 0.07	0.77 ± 0.12	0.89 ± 0.10	0.78 ± 0.44

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V, VI; b– group II vs group III, IV, V, VI. Statistical analysis was calculated by one way analysis of variance (ANOVA) followed by the Student Newman–Keul's test.

[*] The symbols represent statistical significance at $p < 0.05$.

Table 5

Effect of the administration of bromobenzene with or without the prior administration of *Withania somnifera* or silymarin on mitochondrial enzymes in liver of control and experimental rats.

Parameters	Group I (Control)	Group II (BB – 10 mmol/kg)	Group III (WS – 250 mg/kg + BB)	Group IV (WS – 500 mg/kg + BB)	Group V (Silymarin – 100 mg/kg + BB)	Group VI (WS – 500 mg/kg)
Isocitrate dehydrogenase (U_A/mg protein)	404.11 ± 9.60	293.14 ± 11.01a*	348.13 ± 10.78a*b*	391.93 ± 10.34b*	374.62 ± 10.17a*b*	397.07 ± 11.82b*
α-Ketoglutarate dehydrogenase (U_B/mg protein)	99.93 ± 8.71	59.12 ± 6.92a*	89.53 ± 4.31a*b*	94.10 ± 10.52a*b*	85.08 ± 10.13a*b*	98.94 ± 1.95b*
Succinate dehydrogenase (U_C/mg protein)	46.12 ± 2.52	18.21 ± 1.78a*	34.12 ± 2.53a*b*	39.94 ± 2.56b*	31.45 ± 1.93a*b*	45.11 ± 1.46 b*
Malate dehydrogenase (U_D/mg protein)	283.52 ± 16.53	174.22 ± 19.24a*	220.83 ± 18.53a*b*	263.12 ± 10.41a*b*	253.14 ± 7.28a*b*	278.14 ± 9.53 b*
NADH dehydrogenase (U_E/mg protein)	46.24 ± 4.62	19.93 ± 3.46a*	31.56 ± 2.93a*b*	38.43 ± 3.48b*	31.57 ± 8.34a*b*	42.14 ± 3.23b*
Cytochrome-c-oxidase (U_F/mg protein)	12.43 ± 1.47	3.53 ± 1.45a*	5.31 ± 1.97a*b*	9.25 ± 1.32b*	8.57 ± 1.20 a*b*	11.92 ± 2.04b*

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a– group I vs groups II, III, IV, V, VI; b– group II vs group III, IV, V, VI. UA, nmol of a-ketoglutarate formed/h; UB, nmol of ferrocyanide formed/h; UC, nmol of succinate oxidized/min; UD, nmol of NADH oxidized/min; UE, nmol of NADH oxidized/min; UF, change in OD 9 10–2 min^{-1}. Statistical analysis was calculated by one way analysis of variance (ANOVA) followed by the Student Newman–Keul's test.

[*] The symbols represent statistical significance at $p < 0.05$.

showed maximum recovery of antioxidant activities in the experimental animals.

4.3. *Effects of W. somnifera on total sulfhydryl groups and plasma ceruloplasmin content*

Table 4 shows increased plasma ceruloplasmin levels while there was depletion in the total sulfhydryl levels in bromobenzene treated group in comparison to the control group of rats. Pre-administration of *W. somnifera* restored the levels of plasma ceruloplasmin and total sulfhydryl groups to near normal levels.

4.4. *Effect of W. somnifera on mitochondrial enzymes*

The activities of TCA cycle enzymes (Isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase) and respiratory enzymes (NADH dehydrogenase and cytochrome c oxidase) were found to be significantly reduced ($p < 0.05$)

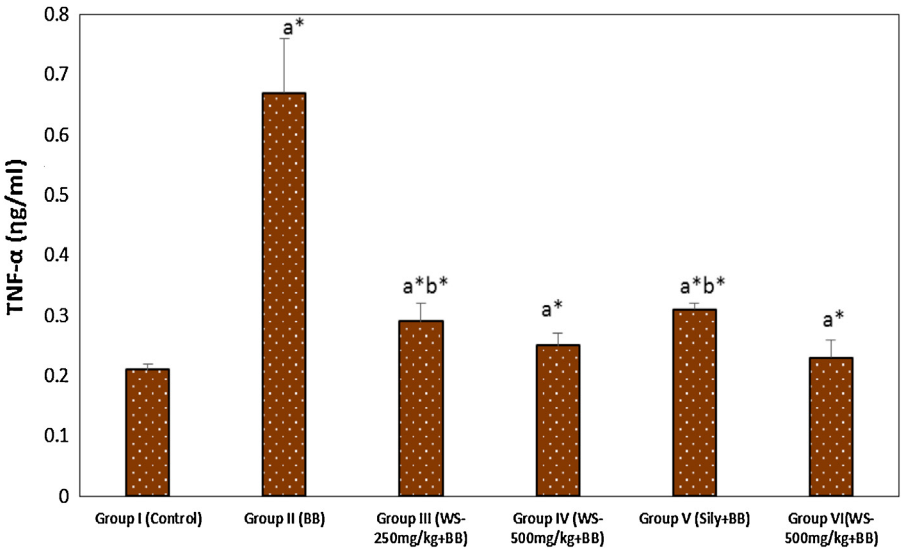

Fig. 1. Effect of the administration of bromobenzene on serum levels of TNF-α with or without prior administration of *Withania somnifera* in control and experimental rats. Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V, VI; b-group II vs group III, IV, V, VI. The symbols represent statistical significance at $^{*}p < 0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

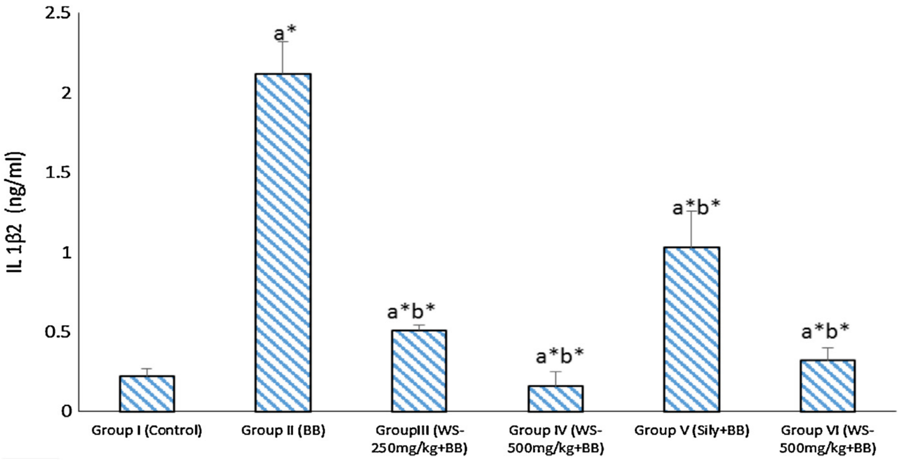

Fig. 2. Effect of the administration of bromobenzene on serum levels of IL1β with or without prior administration of *Withania somnifera* in control and experimental rats. Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V, VI; b- group II vs group III, IV, V, VI. The symbols represent statistical significance at $^*p < 0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

due to the administration of bromobenzene to rats. *W. somnifera* (250 and 500 mg/kg) or silymarin pre-treatment restored the activities of mitochondrial enzymes and brought them to near normal levels (Table 5).

4.5. Measurement of serum TNF-α, IL1β and VEGF

It is already known that pro-inflammatory cytokines (TNF-α and IL1β) and signal protein (VEGF) promote oxidative stress, therefore, their concentration in serum was found to be significantly enhanced in the bromobenzene alone treated group (Figs. 1–3). Prior administration of *W. somnifera* (250 and 500 mg/kg) and silymarin prevented the increase in serum concentrations of TNF-α, IL1β and VEGF.

4.6. Docking analysis

The molecular docking analyses showed that there was a strong interaction between selected active components of *W. somnifera* i.e., Withanolide D, Withanolide E and Withaferin A with NF-κB. Due to these interactions, the selected active components of *W. somnifera* block normal pathway of NF-κB for release of inflammatory mediators (Fig. 4 and Table 6).

4.7. Histopathological examination of liver damage

The light microscopy examination of the representative sections of control rat liver clearly shows complete hepatic lobules with distinct hepatic cells. Hepatic cells were arranged in cord like fashion, which were separated by sinusoids and central vein was clearly visible (Fig. 5a) and the liver sections of bromobenzene treated rats showed massive fatty changes (Fig. 5b). Pre-treatment with *W. somnifera* (250 and 500 mg/kg) was effective in restoring the bromobenzene-induced histopathological lesions, however highest dose was found to be more effective (Fig. 5c and d). The histological architecture of liver

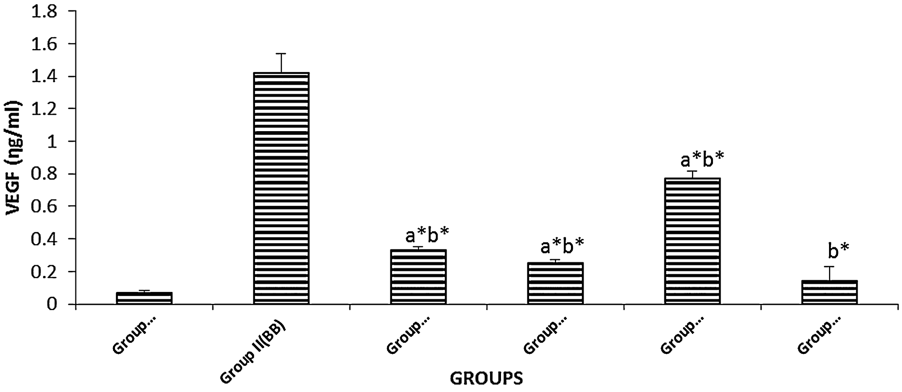

Fig. 3. Effect of the administration of bromobenzene on serum levels of VEGF with or without prior administration of *Withania somnifera* in control and experimental rats. Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V, VI; b-group II vs group III, IV, V, VI. The symbols represent statistical significance at $^*p < 0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

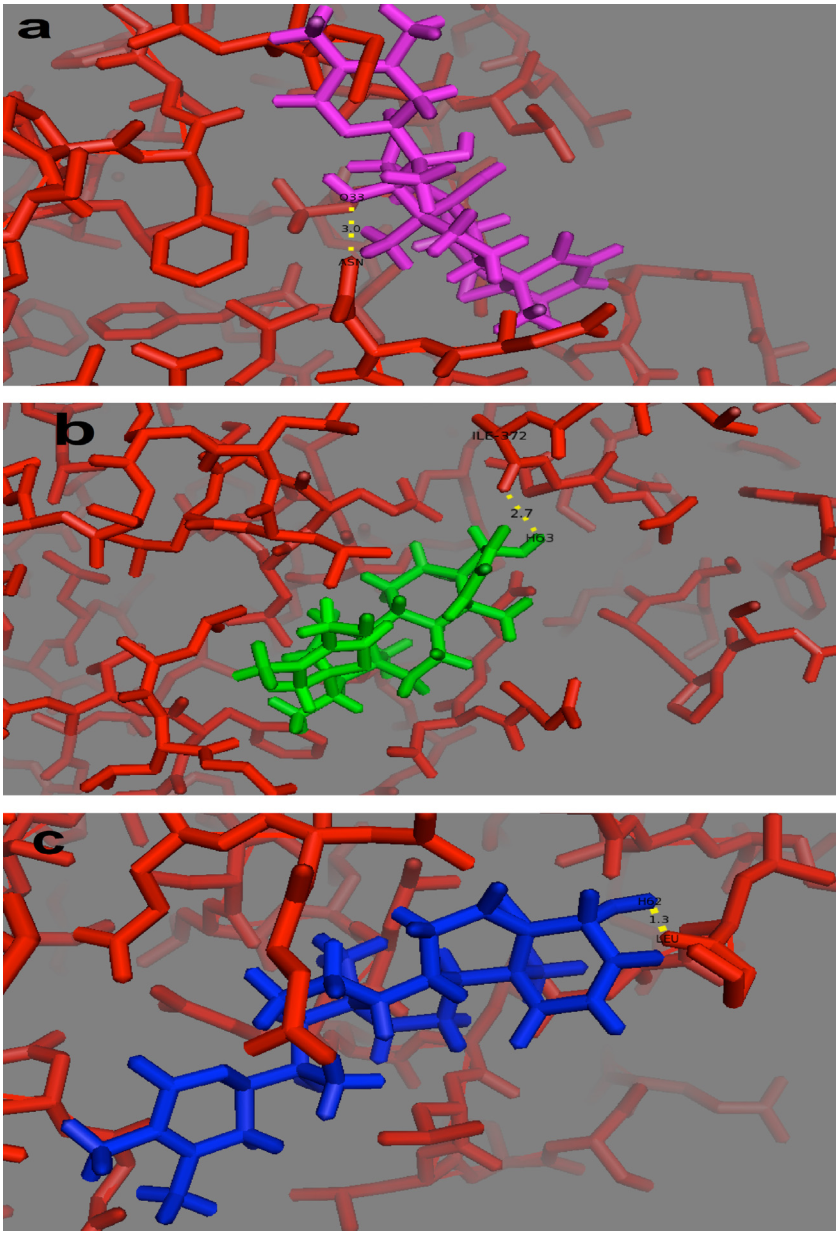

Fig. 4. Molecular docking of Withanolide E (purple) (a), Withaferin A (green) (b) and Withanolide D (blue) (c) with NF-kB (Red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sections of rats that received silymarin showed absence of cell necrosis, but with minimal inflammatory conditions (Fig. 5e). In case of administration of *W. somnifera* at 500 mg/kg dose, the micrographs exhibited an almost normal architecture (Fig. 1f).

5. Discussion

Bromobenzene is subjected to biotransformation in the liver by cytochrome P450 enzymes to form highly hepatotoxic metabolites [29]. It is known to cause functional and

Table 6
Docking studies of active components of *Withania somnifera* (Withaferin A, Withanolide D and Withanolide E) with pro-inflammatory mediator NF-kB.

Active component	Atomic contact energy (ACE)	Docking score	No. of hydrogen (H) bonds	Residues involved	Length of the H bond
Withaferin A	–234.51	5120	1	Leu-H62	1.3 A°
Withanolide D	–232.17	5520	1	Ile-H63	2.7 A°
Withanolide E	–205.70	5378	1	Asn-O33	3.0 A°

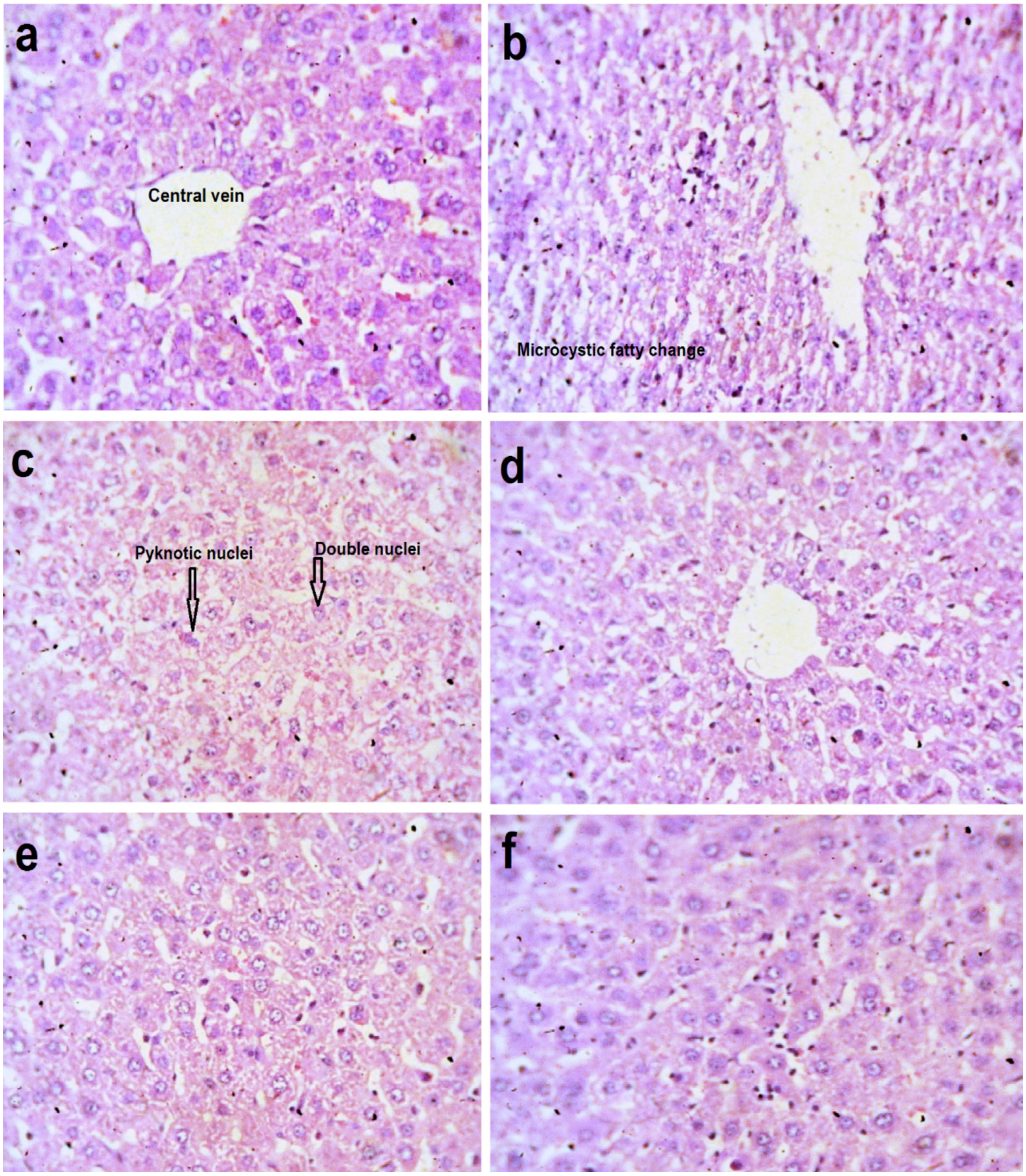

Fig. 5. Histopathological monograph of the liver. (a) Group I (Control), (b) Group II (bromobenzene), (c) Group III (*Withania somnifera* – 250 mg/kg + bromobenzene), (d) Group IV (*Withania somnifera* – 500 mg/kg + bromobenzene), (e) Group V (Silymarin 100 mg/kg+ bromobenzene), and (e) Group VI (*Withania somnifera* – 500 mg/kg), all the pictures are taken under 400× magnification.

morphological alterations in the liver cell membrane, as evidenced by increase in AST, ALT and ALP, which were in accordance with the previous studies [30]. Pre-treatment with *W. somnifera* depicted its protective action against cellular injury and normalized the levels of AST, ALT and ALP, which may be due to its membrane stabilizing action (Table 3). A high concentration of bilirubin in serum is an indication for increased erythrocyte degeneration rate. *W. somnifera* helped in decreasing the significantly ($p < 0.05$) altered levels of total and direct bilirubin, thus bringing liver to normal functionality (Table 3).

It is known that BB-3,4 oxide causes glutathione depletion, therefore, a decrease of the general cellular red/ox balance will automatically increase the ROS level (there are other sources to ROS than a damaged mitochondrion). Furthermore, BB metabolites such as bromobenzene phenols can be oxidized to hydroquinones, which also may form ROS. Intragastric intubation of bromobenzene at 10 mmol/kg in 19 h elicited a significant alteration of some antioxidant mechanisms and drug metabolizing enzymes in the liver [2,31]. Pre-treatment with *W. somnifera* at both doses caused marked elevation in the levels of these antioxidants which may be associated with its free radical scavenging and antioxidant enhancing abilities as reported in previous studies. Furthermore, reduction in total sulfhydryl groups possibly means that these might have been used to combat the action of toxic by-products formed from bromobenzene metabolism [32]. Plasma ceruloplasmin levels were also increased due to acute phase response [33] in bromobenzene treated rats. The active components of *W. somnifera* probably react directly with and detoxify the reactive electrophilic bromobenzene (BB) metabolite BB-3,4-oxide, as well as possible ROS molecules formed. Furthermore, the ROS molecules can be secondary to damage mitochondrion as suggested, but the mitochondrial damage may not be the only source for ROS. Also, there was a decrease in the activities of liver mitochondrial enzymes due to the administration of bromobenzene to rats and *W. somnifera* pre-treatment prevented the decrease in their levels, which is in concordance with the previous studies [34].

Reactive oxygen species promote inflammation by enhancing the activation of transcription factor NF-κB, which controls the formation of cytokines, chemokines, and adhesion molecules [35,36]. In the present study, through docking analysis it was shown that some selected active components of *W. somnifera* show significant interactions with NF-κB. Also, in rats which were treated with bromobenzene only, levels of TNF-α, IL1β and VEGF were found to be elevated as a result of inflammation (Figs. 1–3). Prior administration of *W. somnifera* was able to reduce their levels in serum in a dose dependent manner. Thus, it can be suggested that *W. somnifera* is able to counteract bromobenzene-induced hepatotoxicity and oxidative stress due to the presence of its active components by interacting with the inflammatory mediator, NF-κB (Fig. 4).

The hepatoprotective effect was supported by the liver histological changes produced by *W. somnifera* as compared to the control and bromobenzene treated rats. *W. somnifera* pre-treatment markedly attenuated the bromobenzene-induced liver cell necrosis in a dose dependent manner and restored more or less the same histopathological picture as observed in the control group (Fig. 5c and d).

6. Conclusion

It can be concluded from this study that the roots of *W. somnifera* offer significant dose-dependent protection against bromobenzene-induced liver injury to rats and the hepatoprotective effect was comparable to silymarin, which is the standard drug of reference for hepatoprotection. Moreover, results obtained in this study support the antioxidant properties of *W. somnifera* [34]. However, further pharmacological evidence supporting the role of *W. somnifera* against bromobenzene-induced liver injury is needed and more studies are required to explore the mechanisms for the same.

Conflict of interests

The authors declare that there is no conflict of interest between them.

Transparency document

The Transparency document associated with this article can be found in the online version.

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