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Inhibition of potassium bromate-induced renal oxidative stress and hyperproliferative response by *Nymphaea alba* in Wistar rats

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Abstract

$KBrO_3$-mediated renal injury and hyperproliferative response in Wistar rats. In this communication, we report the efficacy of Nymphaea alba on $KBrO_3$ (125 mg/kg body weight, intraperitoneally) caused reduction in renal glutathione content, renal antioxidant enzymes and phase-II metabolising enzymes with enhancement in xanthine oxidase, lipid peroxidation, γ-glutamyl transpeptidase and hydrogen peroxide (H_2O_2). It also induced blood urea nitrogen, serum creatinine and tumor promotion markers, viz., ornithine decarboxylase (ODC) activity and DNA synthesis. Treatment of rats with *Nymphaea alba* (100 and 200 mg/kg body weight) one hour before $KBrO_3$ (125 mg/kg body weight, i.p.) resulted in significant decreases in xanthine oxidase ($P < 0.05$), lipid peroxidation, γ-glutamyl transpeptidase, H_2O_2 generation, blood urea nitrogen, serum creatinine, renal ODC activity and DNA synthesis ($P < 0.001$). Renal glutathione content, glutathione metabolizing enzymes and antioxidant enzymes were also recovered to significant levels ($P < 0.001$). These results show that *Nymphaea alba* acts as chemopreventive agent against $KBrO_3$-mediated renal injury and hyperproliferative response.

Keywords: *Chemoprevention, Nymphaea alba*, oxidative stress, potassium bromate, hyperproliferative response

Introduction

Over the last decade, considerable experimental evidence has supported the view that reactive oxygen species (ROS) play a key role in renal diseases [1]. Enzymatic and non-enzymatic systems preserve the oxidant/antioxidant status, but they are overwhelmed during oxidative stress, a metabolic derangement due to an imbalance caused by excessive generation of ROS or a diminished capacity of the antioxidant defense system. It has long been recognized that ROS are harmful for cells because they injure lipids, proteins and nucleic acids which leads to structural and functional impairments [2]. The resulting damage to cellular molecules has been involved in the development of various diseases including Alzheimer's disease, Parkinson's disease, diabetes mellitus, carcinogenesis, mutagenesis and ageing [3].

Potassium bromate ($KBrO_3$) is widely used as a food additive in the bread-making process for the maturation of flour because of its oxidizing properties. It is also used in the production of fish paste and fermented beverages. Consumers are exposed through the use of permanent-wave kits with potassium bromate neutralizer solutions. It is also found in drinking water as a by-product of ozone disinfection [4]. $KBrO_3$ has been reported to induce renal cell tumors in male and female rats after long term exposure [5] and induces renal carcinogenesis and acts as a tumor promoter in carcinogen-initiated animals. It also enhances *N*-ethyl-*N*-hydroxyethyl-nitrosamine-initiated renal tumors in rats [6]. It has been shown that there is enhancement in cellular proliferation in the kidney due to oxidative stress generated by $KBrO_3$. We have previously reported that $KBrO_3$ promotes *N*-diethylnitrosamine-initiated renal oxidative stress and also acts as a renal tumor promoter in Wistar rats [7–9]. It also causes DNA strand breaks and poly (ADPR) ribosylation in the kidney that is responsible for the proliferative response [10]. $KBrO_3$

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also induces 8-hydroxydeoxyguanosine (8-OH-dG), which is an oxidative DNA adduct, and this suggests that it indirectly induces DNA modification by way of oxygen radicals and that oxidative stress is involved in its carcinogenesis. It also induces chromosomal aberrations and micronucleus formation both *in vivo* and *in vitro* [5].

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasonings, beverages, cosmetics and medicines. We have shown the inhibitory effects of plants and natural products on chemically-induced oxidative stress and cancer [7–9,11].

Nymphaea alba L. (Family: Nymphaeaceae) is commonly known as water lily or water rose. It is widely used in Indigenous systems of medicine in India. It is commonly used as an antiseptic and astringent and has beneficial effects in diarrhoea, dysentery, leucorrhoea and gonorrhoea. The leaves and roots of the plant have been used in the form of a poultice to boils, scrofulous ulcers and inflamed skin. The pulp of the rhizome when applied externally acts as a rubefacient [12]. For the present study, we prepared a methanolic extract of the plant that contains ellagic acid, quercetin and apigenin as the major active constituents. The presence of these compounds in the methanolic extract was confirmed by Co-TLC with authentic samples under a UV lamp. Taking into account the previous findings, we elucidate the efficacy of the pretreatment of *Nymphaea alba* against $KBrO_3$-mediated renal oxidative stress and hyperproliferative response in Wistar rats.

Materials and methods

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, γ-glutamyl *p*-nitroanilide, glycylglycine, bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1,chloro-2, 4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD), glucose-6-phosphate, tween-20, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA) and potassium bromate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Diacetylmonoxime, urea, picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) and perchloric acid (PCA) were purchased from CDH, India. [^{14}C] ornithine (sp.act. 56 m Ci mmol) and [^{3}H] thymidine (sp.act. 82 ci mmol) were purchased from Amersham Corporation (U.K.). All other chemicals and reagents were of the highest purity commercially available.

Plant material

Nymphaea alba was collected from the herbal garden of Hamdard University, New Delhi, India. Professor Mohammad Iqbal, Head, Department of Environmental Botany, Hamdard University, verified the identity of the plant. The voucher specimen was deposited in the Department of Medical Elementology and Toxicology, Hamdard University. The plant material was dried in air and then milled to a fine powder of mesh size 1-mm as described by Antonio et al. [13].

Preparation of extract

The extraction procedure followed that described by Didry et al. [14]. Briefly, powdered plant material (400) was repeatedly extracted in a 3000 ml flask with 2000 ml solvents of increasing polarity commencing with petroleum ether, benzene, ethyl acetate, acetone, methanol and double distilled water. The reflux time for each solvent was 4 h. The extracts were cooled to room temperature, filtered and evaporated to dryness under reduced pressure in a rotatory evaporator (Buchi Rotavapor). The residues yielded from each solvent (14,10,16,20,31 and 24 g respectively) were stored at 4°C. The methanolic fraction was used for further study after preliminary *in vitro* tests viz., calf thymus DNA sugar damage, lipid peroxidation, xanthine-oxidase activity and cytochrome P_{450} level.

Animals

4–6 weeks old, female albino rats (130–150 g) of Wistar strain were obtained from the Central Animal House of Hamdard University, New Delhi, India. They were housed in polypropylene cages in groups of six rats per cage and were kept in a room maintained at 25 ± 2°C with a 12 h light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory feed. (Hindustan Lever Ltd., Bombay, India) and water *ad libitum.* The animals were sacrificed according to the guidelines of the current laws of CPCSEA (Ethical Committee for the purpose of control and supervision of experiments on animals), India.

Experimental protocol

Treatments

Different groups of animals were used for the various sets of biochemical studies. To study the effect of pretreatment with *Nymphaea alba* on $KBrO_3$-induced renal oxidative stress and ODC induction, thirty female Wistar rats were randomly allocated to 5 groups of 6 rats each. Group I received only saline injection intraperitoneally (0.85% NaCl) at a dose of 10 ml/kg body weight. Group II received only a single intra-peritoneal injection of $KBrO_3$ at a dose level of 125 mg/kg body weight. Group III received pretreatment with *Nymphaea alba* by gavage once daily for 5

days at a dose of 100 mg/kg body weight and Group IV and V received pretreatment with *Nymphaea alba* by gavage once daily for 5 days at a dose of 200 mg/kg body weight. After the last treatment with *Nymphaea alba*, the animals of groups II, III and IV received only a single intra-peritoneal injection of $KBrO_3$ at a dose level of 125 mg/kg body weight. After 24 h the animals were sacrificed by cervical dislocation and processed for sub-cellular fractionation, their kidneys were quickly removed and perfused in ice-cold saline. Just before they were killed, blood was collected in test tubes from retro-orbital sinus for the estimation of creatinine and blood-urea nitrogen.

To study the effect of pretreatment with *Nymphaea alba* on $KBrO_3$-mediated [^{3}H] thymidine incorporation into renal DNA, thirty female Wistar rats were randomly allocated to 5 groups of 6 rats each. The schedules for prophylaxis were as described above. After the last treatment with *Nymphaea alba*, the animals of groups II, III and IV received only a single intra-peritoneal injection of $KBrO_3$ at a dose level of 125 mg/kg body weight. 18 h after the treatment with $KBrO_3$, the rats were given [^{3}H] thymidine (30 μCi/animal) by i.p. injection and 1 h later they were sacrificed by cervical dislocation and their kidneys were quickly removed.

Post-mitochondrial supernatant (PMS) and microsome preparation

Kidneys were removed quickly, cleaned free of extraneous material and immediately perfused with ice-cold saline (0.85% sodium chloride). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%) using a Potter–Elvehjen homogenizer. The homogenate was filtered through muslin cloth, and was centrifuged at 3000 rpm for 10 min at 4°C using a Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4°C to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzymes. A portion of the PMS was centrifuged for 60 min by ultracentrifuge (Beckman L7-55) at 34,000 rpm at 4°C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%).

Biochemical determinations

Estimation of reduced glutathione

Reduced glutathione was determined by the method of Jollow et al.[15]. A 1.0 ml sample of PMS was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h and then centrifuged at 3000 rpm for 20 min at 4°C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (100 mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model- 21 D).

Estimation of lipid peroxidation

The assay for microsomal lipid peroxidation was done following the method of Wright et al. [16] as modified by Khan et al. [7]. The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsomes, 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM) was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by addition of 1.0 ml 10% trichloroacetic acid (TCA). Following addition of 1.0 ml 0.67% thiobarbituric acid (TBA), all the tubes were placed in a boiling water-bath for 20 min and then transfered to a crushed ice-bath before centrifuging at 2500 × g for 10 min. The amount of malondialdehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm using a spectrophotometer (Milton Roy 21 D) against a reagent blank. The results were expressed as nmol MDA formed $h^{-1}\,g^{-1}$ tissue at 37°C using a molar extinction coefficient of $1.56 \times 10^5\,M^{-1}\,cm^{-1}$.

Estimation of blood urea nitrogen

Estimation of blood urea nitrogen was carried out by the diacetyl monoxime method of Kanter [17]. A protein free filtrate was prepared. To 0.5 ml of protein free filtrate was added 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml sulphuric acid—phosphoric acid reagent (reagent was prepared by mixing 150 ml 85% phosphoric acid with 140 ml water and 50 ml of concentrated sulphuric acid). The reaction mixture was placed in a boiling water-bath for 30 min, cooled and the absorbance recorded at 480 nm.

Estimation of creatinine

Creatinine was estimated by the alkaline picrate method of Hare [18]. A protein free filtrate was prepared. To 1.0 ml serum was added, 1.0 ml sodium tungstate (5%), 1.0 ml sulfuric acid (0.6 N) and 1.0 ml distilled water. After mixing thoroughly, the mixture was centrifuged at 800 × g for 5 min. The supernatant was added to a mixture containing 1.0 ml picric acid (1.05%) and 1.0 ml sodium hydroxide (0.75 N) and the absorbance at 520 nm was recorded after 20 min.

Assay for hydrogen peroxide

Hydrogen peroxide (H_2O_2) was assayed by the H_2O_2—mediated horseradish peroxidase-dependent oxidation

of phenol red by the method of Pick and Keisari [19]. 2.0 ml of microsomes were suspended in 1.0 ml of solution containing phenol red (0.28 nmole), horse radish peroxidase (8.5 units), dextrose (5.5 nmole) and phosphate buffer (0.05 M, pH 7.0) and were incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.01 ml of NaOH (10 N) and then centrifuged at 800 × g for 5 min. The absorbance of the supernatant was recorded at 610 nm against a reagent blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2 gm tissue^{-1}h^{-1} based on the standard curve of H_2O_2-oxidized phenol red.

Assay for glutathione-S-transferase activity

Glutathione-S-transferase activity was assayed by the method of Habig et al. [20]. The reaction mixture consisted of 1.475 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM) and 0.3 ml PMS (10% w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg protein^{-1} using a molar extinction coefficient of $9.6 \times 10^3\,M^{-1}cm^{-1}$.

Assay for glutathione peroxidase activity

Glutathione peroxidase activity was assayed by the method of Mohandas et al. [21]. The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 int. units ml^{-1}), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg protein^{-1} using a molar extinction coefficient of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$.

Assay for glutathione reductase activity

Glutathione reductase activity was determined by the method of Carlberg and Mannervik [22]. The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized min^{-1} mg protein^{-1} using a molar extinction coefficient of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$.

Assay for γ-glutamyl transpeptidase activity

This was determined by the method of Orlowski and Meister [23] using γ-glutamyl *p*-nitroanilide as substrate. The reaction mixture in a total volume of 1.0 ml contained 0.2 ml 10% homogenate which was incubated with 0.8 ml substrate mixture (containing 4 mM γ-glutamyl *p*-nitroanilide, 40 mM glycylglycine and 11 mM $MgCl_2$ in 185 mM Tris–HCl buffer, pH 8.25) at 37°C. Ten minutes after initiation of the reaction, 1.0 ml 25%TCA was added and to terminate the reaction. The solution was centrifuged and the supernatant fraction read at 405 nm. Enzyme activity was calculated as nmol p-nitroaniline formed min^{-1} mg protein^{-1} using a molar extinction coefficient of $1.74 \times 10^3\,M^{-1}\,cm^{-1}$.

Assay for catalase activity

Catalase activity was assayed by the method of Claiborne [24]. The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed min^{-1} mg protein^{-1}.

Assay for glucose-6-phosphate dehydrogenase activity

The activity of glucose-6-phosphate dehydrogenase was determined by the method of Zaheer et al. [25]. The reaction mixture consisted of 0.3 ml Tris–HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose-6-phosphate (0.8 mM), 0.1 ml $MgCl_2$ (8 mM), 0.3 ml PMS (10%) and 2.1 ml distilled water in a total volume of 3 ml. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADP reduced min^{-1} mg protein^{-1} using a molar extinction coefficient of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$.

Assay for xanthine oxidase activity

The activity of xanthine oxidase was assayed by the method of Athar et al. [26]. The reaction mixture consisted of 0.2 ml PMS which was incubated for 5 min at 37°C with 0.8 ml phosphate buffer (0.1 M, pH 7.4). The reaction was commenced by adding 0.1 ml xanthine (9 mM) and kept at 37°C for 20 min. The reaction was terminated by the addition of 0.5 ml ice cold perchloric acid (10% v/v). After 10 min, 2.4 ml of distilled water was added and the mixture centrifuged at 4000 rpm for 10 min. and μ gm uric acid formed min^{-1} mg protein^{-1} was recorded at 290 nm.

Assay for quinone reductase activity

The activity of quinone reductase was determined by the method of Benson et al. [27]. The 3 ml reaction mixture consisted of 2.13 ml Tris–HCl buffer (25 mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM) and 50 μL(10%) PMS. The reduction of dichlorophenolindophenol (DCPIP) was recorded at 600 nm and enzyme activity was calculated as nmole of DCPIP

reduced min^{-1} mg protein^{-1} using a molar extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Assay for ornithine decarboxylase activity

ODC activity was determined by measuring the release of $^{14}CO_2$ from DL- [^{14}C] ornithine by the method of O'Brien et al. [28]. The kidneys were homogenized in Tris–HCl buffer (pH 7.5, 50 mM) containing EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and Tween 80 (0.1%) at 4°C. In brief, the reaction mixture contained 400 µl enzyme and 0.095 ml co-factor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brig 35 (0.02%) and [^{14}C] ornithine (0.05 µCi) in a total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and other test tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanolamine and methoxyethanol mixture in the central well and kept in a water bath at 37°C. After 1 h incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of the glass tubes. Finally, the central well was transferred to a vial containing 2 ml ethanol and 10 ml toluene based scintillation fluid was added. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as p mol $^{14}CO_2$ released h^{-1} mg protein^{-1}.

Assay for Renal DNA synthesis

The isolation of renal DNA and assessment of incorporation of [^{3}H] thymidine into DNA were carried out by the method of Smart et al. [29]. The rat kidneys were quickly removed and cleaned free of extraneous material and a homogenate (10% w/v) was prepared in ice-cold water. The precipitate thus obtained was washed with cold TCA (5%) and incubated with cold PCA (10%) at 4°C overnight. After this, incubation mixture was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%), incubated in a boiling water bath for 30 min and filtered through Whatman 50 paper. The filtrate was used for [^{3}H] counting in a liquid scintillation counter (LKB Wallace-1410) after adding scintillation fluid. The amount of DNA in the filtrate was estimated by the diphenylamine method of Giles and Myers [30]. The amount of [^{3}H] thymidine incorporated was expressed as dpm/µg DNA.

Estimation of protein

The protein concentration in all samples was determined by the method of Lowry et al. [31].

Statistical analysis

Differences between groups were analyzed using Dunnet's t-test followed by analysis of variance (ANOVA).

Results

The effect of pretreatment of animals with *Nymphaea alba* on $KBrO_3$-mediated renal glutathione content and on the activities of its metabolising enzymes, viz., glutathione-S-transferase and glutathione reductase is shown in Table I. Treatment with $KBrO_3$ alone resulted in the depletion of renal glutathione and reduction in the activities of glutathione-S-transferase and glutathione reductase to a level of 30, 42, and 48% respectively of that of the saline-treated control group. However, pretreatment of animals with *Nymphaea alba* at 100 mg/kg body weight and 200 mg/kg body weight partially recovered the levels to 20–25%, 23–32% and 19–34% respectively, as compared with the $KBrO_3$-treated control group.

The effect of prophylaxis with *Nymphaea alba* on $KBrO_3$-mediated reduction in the activities of renal

Table I. Effect of pretreatment in rats with *Nymphaea alba* on $KBrO_3$-mediated depletion of renal glutathione content and decrease in the activities of glutathione metabolizing enzymes, glutathione-S-transferase and glutathione reductase.

Treatment groups	Reduced glutathione (nmol GSH/g tissue)	Glutathione-S-transferase (nmole CDNB conjugate formed min^{-1} mg protein^{-1})	Glutathione reductase (nmole NADPH oxidized min^{-1} mg protein^{-1})
Saline (control)	0.516 ± 0.04	168.37 ± 11.2	258.37 ± 9.4
$KBrO_3$ (125 mg/kg body weight)	0.362 ± 0.02 ♣	97.45 ± 6.5 ♣♣	135.63 ± 6.5 ♣♣
Nymphaea alba (100 mg/kg body weight) alone + $KBrO_3$ (125 mg/kg body weight)	0.465 ± 0.03**	136.29 ± 5.7***	183.26 ± 5.5***
Nymphaea alba (200 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	0.492 ± 0.04*	151.32 ± 8.4***	223.56 ± 10.5***
Nymphaea alba (200 mg/kg body weight) alone	0.537 ± 0.05	188.54 ± 10.3	276.29 ± 4.7

Each value represents mean ± SE, n = 6.
♣$P < 0.01$ and ♣♣$P < 0.001$ compared with the corresponding value for saline-treated control.
*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with the corresponding value for treatment with $KBrO_3$.

Table II. Effect of pretreatment in rats with *Nymphaea alba* on $KBrO_3$-mediated depletion in the level of renal antioxidant enzymes.

Treatment groups	Catalase (nmol H_2O_2 consumed min^{-1} mg protein^{-1})	Glutathione peroxidase (nmol NADPH oxidized min^{-1} mg protein^{-1})	Glucose-6-phosphate dehydrogenase (nmol NADP reduced min^{-1} mg protein^{-1})
Saline (control)	158.73 ± 10.1	225.63 ± 8.9	42.67 ± 4.2
$KBrO_3$ (125 mg/kg body weight)	65.32 ± 6.3♣♣	132.29 ± 5.7♣♣	14.35 ± 2.3♣♣
Nymphaea alba (100 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	117.24 ± 5.4***	175.56 ± 6.4***	28.27 ± 3.1**
Nymphaea alba (200 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	134.65 ± 8.6***	198.51 ± 10.2***	37.56 ± 3.5***
Nymphaea alba (200 mg/kg body weight) alone	171.30 ± 7.6	241.73 ± 6.7	46.18 ± 1.9

Each value represents mean ± SE, n = 6.
♣P < 0.01 and ♣♣P < 0.001 compared with the corresponding value for saline-treated control.
*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the corresponding value for treatment with $KBrO_3$.

antioxidant enzymes is shown in Table II. $KBrO_3$ treatment caused a decrease in the activities of renal antioxidant enzymes such as catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase to levels 59, 41, and 66% respectively of that of the saline-treated control group. The recovery of the above enzymes ranged from 33–44%, 19–29% and 32–54% respectively, as compared with the $KBrO_3$-treated control group at lower (100 mg/kg body weight) and higher (200 mg/kg body weight) doses of *Nymphaea alba*.

$KBrO_3$ treatment enhances the susceptibility of renal microsomal membrane for iron-ascorbate induced lipid peroxidation to 46% and activity of xanthine oxidase to 87% while it causes a reduction in the activity of quinone reductase to 36% as compared with saline -treated controls (Table III). At 200 mg/kg body weight of *Nymphaea alba*, xanthine oxidase was reduced to 72% and lipid peroxidation to 31%, while the level of quinone reductase was increased to 26% as compared with the $KBrO_3$-treated control group.

The effect of pretreatment with *Nymphaea alba* on $KBrO_3$-mediated enhancement in the levels of blood urea nitrogen, serum creatinine, γ-glutamyl transpeptidase and H_2O_2 is shown in Table IV. $KBrO_3$ treatment leads to about 158%, 131%, 94% and 159% enhancement in the values of blood urea nitrogen, serum creatinine, γ-glutamyl transpeptidase and H_2O_2 respectively, as compared with saline -treated controls. *Nymphaea alba* pretreatment resulted in 69–132%, 40–101%, 41–69% and 57–86% reduction in these values, respectively as compared with $KBrO_3$-treated control group at both doses of *Nymphaea alba* (100 mg/kg body weight and 200 mg/kg body weight).

The effect of prophylactic treatment of rats with *Nymphaea alba* on $KBrO_3$-mediated induction of renal ODC activity is shown in Figure 1. Treatment with $KBrO_3$ resulted in 4.7-fold induction in ODC activity as compared with saline-treated controls. The pretreatment of rats with *Nymphaea alba* at a dose of 100 mg/kg body weight caused 278% and at a dose of 200 mg/kg body weight caused 374% inhibition in the elevation of ODC activity as compared with the $KBrO_3$-treated control group.

The effect of prophylaxis of rats with *Nymphaea alba* on $KBrO_3$-mediated enhancement in the incorporation of [^{3}H] thymidine into renal DNA is shown in Figure 2. Treatment with $KBrO_3$ alone caused a nearly 3-fold increase in the incorporation of [^{3}H] thymidine into renal DNA. At the lower dose of *Nymphaea alba* (100 mg/kg body weight) the enhancement was reduced

Table III. Effect of pretreatment in rats with *Nymphaea alba* on $KBrO_3$-mediated depletion of renal quinone reductase and enhancement of xanthine oxidase and renal microsomal lipid peroxidation.

Treatment groups	Quinone reductase (nmoles of dichloroindophenol reduced min^{-1} mg protein^{-1})	Xanthine oxidase (μgm of uric acid formed min^{-1} mg protein^{-1})	Lipid peroxidation (nmol MDA formed h^{-1} g tissue^{-1})
Saline (control)	179.56 ± 10.2	0.496 ± 0.04	3.86 ± 0.35
$KBrO_3$ (125 mg/kg body weight)	115.32 ± 5.0♣♣	0.463 ± 0.02♣♣	5.65 ± 0.23♣
Nymphaea alba (100 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	149.35 ± 4.5***	0.352 ± 0.05*	4.93 ± 0.17*
Nymphaea alba (200 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	161.29 ± 7.5***	0.306 ± 0.06*	4.46 ± 0.33*
Only *Nymphaea alba* (200 mg/kg body weight)	193.62 ± 6.8	0.242 ± 0.03	3.52 ± 0.27

Each value represents mean ± SE, n = 6.
♣P < 0.01 and ♣♣P < 0.001 compared with the corresponding value for saline-treated control.
*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the corresponding value for treatment with $KBrO_3$.

Table IV. Effect of pretreatment in rats with *Nymphaea alba* on $KBrO_3$-mediated enhancement of blood urea nitrogen, serum creatinine, γ-glutamyl transpeptidase and hydrogen peroxide.

Treatment groups	Blood-Urea Nitrogen (mg/100 ml) I.U./litre	Creatinine (mg/100 ml) I.U./litre	γ-glutamyl transpeptidase (nmoles *p*-nitroaniline formed^{-1} min mg protein^{-1})	H_2O_2 (nmoles H_2O_2 g tissue^{-1})
Saline (control)	21.87 ± 2.78	1.71 ± 0.22	425.37 ± 20.23	225.37 ± 10.84
$KBrO_3$ (125 mg/kg body weight)	56.35 ± 3.23♣♣	3.94 ± 0.15♣♣	826.51 ± 26.35♣♣	582.64 ± 8.63♣♣
Nymphaea alba (100 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	41.26 ± 2.21**	3.26 ± 0.23*	653.26 ± 15.72***	453.17 ± 6.52***
Nymphaea alba (200 mg/kg body weight) + $KBrO_3$ (125 mg/kg body weight)	27.44 ± 4.32***	2.23 ± 0.17***	532.63 ± 24.38***	387.28 ± 11.35***
Nymphaea alba (200 mg/kg body weight) alone	17.54 ± 2.19	1.62 ± 0.20	404.36 ± 19.37	192.62 ± 7.23

Each value represents mean ± S.E, n = 6.
♣P < 0.01 and ♣♣P < 0.001 compared with the corresponding value for saline-treated control.
*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the corresponding value for treatment with $KBrO_3$.

to 130% and at the higher dose (200 mg/kg body weight) the enhancement was reduced to 153% as compared with the $KBrO_3$-treated control group.

Discussion

Traditionally, plants have been used as folk medicine against various types of diseases. Country doctors and medical practitioners even today use crude extracts of medicinal plants to cure human ailments in several countries. Experimental work on several plants has been carried out to evaluate their efficacy against chemically-induced toxicity [32]. The present study demonstrates that *Nymphaea alba* diminishes $KBrO_3$-induced enzyme levels considered responsible for oxidative stress and tumor promotion response in Wistar rats. The medicinal uses and chemical composition of *Nymphaea alba* have been studied

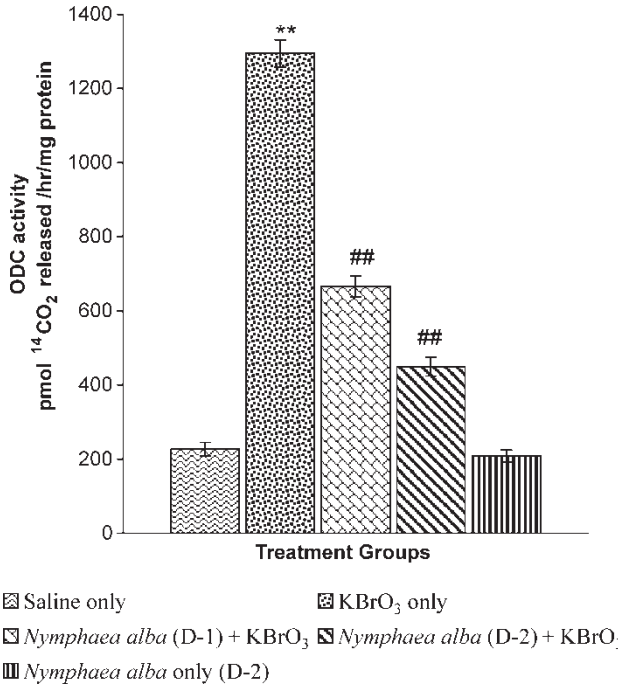

Figure 1. Effect of pretreatment of rats with *Nymphaea alba* on $KBrO_3$-induced enhancement of renal ornithine decarboxylase (ODC) activity. Each value represent mean ± SE of six animals. **Significant (P < 0.001) when compared with saline-treated control group. ## Significant (P < 0.001) when compared with $KBrO_3$-treated group. Doses (D1 and D2) represent 100 and 200 mg/kg body weight of *Nymphaea alba*.

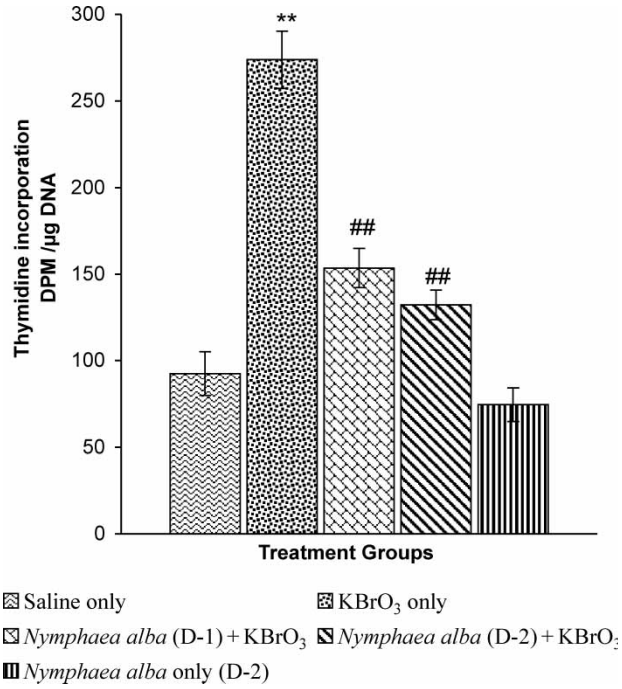

Figure 2. Effect of pretreatment of rats with *Nymphaea alba* on $KBrO_3$-induced enhancement of [^{3}H] thymidine incorporation into renal DNA. Each value represent mean ± SE of six animals. **Significant (P < 0.001) when compared with saline-treated control group. ## Significant (P < 0.001) when compared with $KBrO_3$ treated group. Doses (D1 and D2) represent 100 and 200 mg/kg body weight of *Nymphaea alba*.

widely. It has been reported to contain flavonoids like quercetin, kaempferol, isokaempferide, apigenin, tannins tocopheryl esters and phenolic acids [33]. Ellagic acid, gallic acid, their ethyl and methyl esters, as well as *p*-hydroxybenzoic, vanillic and ferulic acid have also been isolated from *Nymphaea alba* flowers [34]. The major active constituents of *Nymphaea alba* are ellagic acid, quercetin and apigenin. The rhizome of *Nymphaea alba* contains several tannic principles, viz., *tanno-nymphaein* ($C_{56}H_{52}O_{36}$), *nymphaeo-phlobaphene* ($C_{56}H_{48}O_{36}$), and *nymphaea-tannic acid* ($C_{56}H_{58}O_{38}$) [35]. Ellagic acid has anti-carcinogenic, anti-mutagenic, anti-bacterial and anti-viral properties and also inhibits esophagus, tongue, lung, colon, liver, and skin tumors [36]. Quercetin has also been shown to modulate several biochemical events associated with tumor promotion such as alteration in protein kinase C activity and interaction with calmodulin [37]. Apigenin is a constituent of many plants known to have a cancer-preventive effect. It has antiallergic, antibacterial, antihistaminic, anti-inflammatory, antioxidant and antimutagenic effects [38]. As the major constituents of *Nymphaea alba* have established efficacy in many studies, so the observed chemopreventive activity of *Nymphaea alba* in our study may be suggested as being due to the presence of these compounds.

ODC activity and [^{3}H] thymidine incorporation are characteristic changes of the proliferative response and are widely used as biochemical markers to evaluate the tumor promoting potential of an agent [39]. An enhancement in both renal ODC activity and [^{3}H] thymidine incorporation suggests a strong proliferative and tumor promoting potential of $KBrO_3$ in the kidney. As observed in the present study, *Nymphaea alba* dose-dependently inhibited the induction of ODC activity and [^{3}H] thymidine incorporation suggesting the antitumor potential of *Nymphaea alba*. A sharp decrease in the level of blood urea nitrogen and serum creatinine, marker parameters of nephrotoxicity in the *Nymphaea alba* pretreatment groups suggest that prophylaxis of the plant is effective in improving kidney function in the $KBrO_3$-treated group.

The inhibition of the $KBrO_3$-mediated activities of antioxidant enzymes, viz., glutathione peroxidase, glutathione reductase, catalase, glucose-6-phosphate dehydrogenase and phase-II metabolising enzymes such as glutathione-S-transferase and quinone reductase were ameliorated on prophylaxis with *Nymphaea alba*. As observed in the present study, the established antioxidant properties of the plant might have counteracted the oxidant effects of $KBrO_3$ by scavenging free radicals. Glutathione peroxidase catalyzes the detoxification of lipid hydroperoxides and other radicals with reduced glutathione, converting it to oxidized glutathione. Glutathione reductase with the aid of NADPH utilization reconverts oxidized glutathione to reduced glutathione maintaining the intracellular reduced glutathione content for the redox cycle. Glutathione-S-transferase has been shown to catalyze conjugation of xenobiotics with reduced glutathione to form glucuronyl-conjugates that are less toxic and easily excretable [40]. Similarly, quinone reductase catalyzes hydroquinone formation from quinones, thus escaping cytochrome P_{450}-dependent activation to toxic semiquinones and subsequent generation of reactive oxygen species. Inducible by a wide variety of xenobiotics, a strong correlation exists between its modulation and chemoprevention of cancer [41].

The enhancement in the activities of xanthine oxidase, γ-glutamyl transpeptidase, H_2O_2 and the depleted levels of renal glutathione content were also recovered on treatment with *Nymphaea alba*. The fast oxidation reaction between the glutathione and the oxidants generated by $KBrO_3$ might have resulted in the reduced levels of glutathione. Reduction of $KBrO_3$ by GSH results in the generation of short-lived intermediates that can react with DNA and cause single and double strand breaks. Bromine radicals ($Br^{\bullet}$) or oxides ($BrO^{\bullet}$, $BrO_2^{\bullet}$) are the species directly responsible for the observed cellular and cell free DNA damage. Pharmacokinetic studies have shown that $KBrO_3$ is degraded *in vivo* and *in vitro* to $BrO_3^{\bullet}$ and contact with renal tubular epithelium [42,43]. There was also a dose-dependent decrease in the $KBrO_3$-mediated susceptibility of the renal microsomal membrane for iron-ascorbate induced lipid peroxidation as shown by ameliorated malondialdehyde levels.

The major proposal for the action of *Nymphaea alba* seems to be its effectiveness to intercept free radicals and protect the cellular macromolecules from oxidative damage coupled with enhancement of the detoxification processes. Another possible mechanism may be its effectiveness in inhibiting ODC activity and DNA synthesis that are indicative of the hyperproliferative response and plays a major role in tumor promotion. Thus, it can be clearly deduced from our study that *Nymphaea alba* markedly diminished the severity of renal injury and it can be used as an effective chemopreventive agent against $KBrO_3$-mediated renal injury and hyperproliferative response in rats.

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