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Full Length Research Paper

Potassium bromate ($KBrO_3$) induced nephrotoxicity: Protective effects of n-hexane extract of *Sonchus asper*

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Potassium bromate ($KBrO_3$) is an oxidizing agent used in industries for the formation of hair solution, cosmetics and as a by product during ozonation of water, causes infections in kidney and has been classified as 2B group toxic chemical a probable human carcinogen. In the present study, *Sonchus asper* non polar n-hexane extract (SAHE) is used against $KBrO_3$ induced nephrotoxicity in rats. During this study 32 male albino rats were randomly divided into 4 groups and experiment was proceeded for 6 weeks. Results revealed that induction of $KBrO_3$ in rats significantly reduced activities of antioxidant enzymes *(p<0.01)* while enhanced NORs/cell and telomerase enzyme activity and lipid peroxidation which were markedly improved by co-treatment of SAHE. $KBrO_3$ also considerably distorted *(p<0.01)* DNA fragmentation in kidney comparatively to control. These changes are noticeably *(p<0.01)* reimbursed with treatment of SAHE. These data proved that SAHE extract has ability of DNA fragmentation repairing, reduces rgyrophilic nucleolar organiser regions (AgNORS), restoring enzyme activity and telomerase enzymes, which might be due to the presence of plant bioactive compounds.

Key words: *Sonchus asper*; DNA fragmentation, argyrophilic nucleolar organiser regions (AgNORs), telomerase enzyme inhibition, antioxidant enzymes.

INTRODUCTION

Medicinal plants play important role in improving human health. These are composed of some bioactive phytochemical substances, which regulate various physiological and molecular action in living organisms (Hill, 1952). Today many natural products extracted from medicinal plants are being tested for the presence of new drugs with new modes of pharmacological action. Special features of higher plants are their capacity to produce a large number of secondary metabolites (Castello et al., 2002). Recent studies are involved in the identification and isolation of new therapeutic compounds of medicinal importance from higher plants for specific diseases (Khan et al., 2009; Khan et al., 2010a, b; Sahreen et al., 2010). Some bioactive compounds derived from plants include tannins, alkaloids, cardiac glycosides, flavonoids, sterols, triterpenes and anthraquinones which play main role in nutrition, physiology and control of various diseases (Sahreen et al., 2011; Khan et al., 2011a, b) and in many biological activities including; spasmolytic activity of smooth muscles and as antioxidant in protecting the body against oxidative stress (Tona et al., 2000). *Sonchus asper* (L.) Hill. have spiny leaves and yellow flowers. The leaves are simple, bluish-green in color, lanceolate, with wavy and lobed margins.

The stem and leaves emit a milky sap on cutting. Their flowers grow in clusters. This plant is native to Pakistan, but also found as a common weed in North America in open fields and road sides. According to binomial nomenclature *S. asper* belongs to Kingdom Plantae, Order Asterales, Family Asteraceae, Tribe Cichorieae, Genus *Sonchus*, and Species *asper*. *S. asper* locally named as Mahtari used in the treatment of liver injuries and cardiac dysfunction (Khan et al., 2011) and kidney inflammation (Khan et al., 2010b).

Therefore, the study is arranged to investigate protective effects of SAHE against $KBrO_3$ induced oxidative depression of antioxidant enzymes, DNA

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fragmentations, enhanced AgNORs counts and telomerase enzyme.

MATERIALS AND METHODS

Extraction and experimental design

Crude methanolic extract of *S. asper* was obtained as previously described by Khan et al. (2010b) and was further fractionated with n-hexane to get non polar fraction, stored at 4°C for *in vivo* investigation in rats. To check the activities of antioxidant enzymes, lipid peroxidation, DNA damages, AgNORs count and telomerase enzyme activity 32 male albino rats were purchased from National Institute of Health (NIH), Islamabad, Pakistan, acclimatized for 7 days, then randomly divided into 4 groups. Group 1 was remain untreated, group II was given 20 mg/kg bw $KBrO_3$ in aqueous saline, group III and group IV was given 100 and 200 mg/kg SAHE respectively for 6 weeks. Study protocol was approved by ethical committee of Quaid-i-Azam, University, Islamabad, Pakistan. After completion of experiments kidney was treated with liquid nitrogen for further analysis.

Effect of SAHE on antioxidant status

For determination of antioxidant status, 10% solution of tissues was made in phosphate buffer (100 mmol) and EDTA (1 mmol), centrifuged at 12,000 × g for 30 min at 4°C to collect the supernatant. Activities of antioxidant enzymes; catalase assay (CAT) (Chance and Maehly, 1955), superoxide dismutase (SOD) (Kakkar et al., 1984), glutathione-S-transferase (GST) (Habig et al., 1974), glutathione reductase (GSR), (Carlberg and Mannervik, 1975), glutathione peroxidase (GSH-Px) (Mohandas et al., 1984) and contents of reduced glutathione (GSH) were estimated with protocol of Jollow et al. (1974) while lipid peroxidation (TBARS) was estimated with Iqbal et al. (1996).

DNA fragmentation% assay

DNA fragmentation % assay was conducted using the procedure of Wu et al. (2005) with some modifications. The tissue (50 mg) was homogenized in 10 volumes of a TE solution pH 8.0 (5 mmol Tris-Hcl, 20 mmol EDTA) and 0.2% triton X-100. 1.0 ml aliquot of each sample was centrifuged at 27,000 × g for 20 min to separate the intact chromatin (pellet, B) from the fragmented DNA (supernatant, T). The pellet and supernatant fractions were assayed for DNA content using a freshly prepared Diphenylamine (DPA) solution for reaction. Optical density was read at 620 nm with (SmartSpecTM Plus Spectrophotometer catalog # 170-2525) spectrophotometer. The results were expressed as amount of % fragmented DNA by the following formula:

$$\% \text{ Fragmented DNA} = T \times 100/T+B$$

AgNORs count

Silver staining technique was used according to Trere et al. (1996). The AgNORs technique was performed on dried slides as follows; unstained fixed slides were dewaxed by dipping for 3 min in xylene. After complete removal of wax the slides were hydrated in descending order of ethanol concentration (90, 70 and 50%) and washed in distilled water for 10 min and dried in an oven. After drying slides were treated with one drop of colloidal solution (2% gelatin and 1% formic acid) and two drops of 50% $AgNO_3$ solution onto the slide and incubated at 35°C for about 8 to 12 min. The progressive staining was followed under microscope to get golden colored nuclei and brown/black NORs. Then, the slide was washed in distilled water, treated for 1 min with 1% sodium thiosulphate at room temperature to stop the reaction, and washed in tap water. The cells were examined under light microscope at 100 × magnification and number of AgNORs was counted per cell.

DNA ladder assay

DNA was isolated by using the methods of Wu et al. (2005) to estimate DNA damages. 5 µg of rat DNA was separately loaded in 1.5% agarose gel containing 1.0 µg/ml ethidium bromide including DNA standards (0.5 µg per well). Electrophoresis was performed for 45 min at 100 Volt. After electrophoresis gel was studied under gel doc system and was photographed through digital camera.

RT-PCR analysis (TRAP assay)

Telomerase activity was determined by the protocol of Wen et al. (1998) with some modifications.100 mg kidney was washed in ice-cold wash buffer (10 mM Hepes-KOH pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 20 µl RNAs inhibitors), and homogenised in 200 µl ice cold lysis buffer. The homogenate was incubated on ice for 30 min and then centrifuged at 10,000 xg for 30 min at 4°C.

PCR reaction mixture (total 48 µl) consisted of 36.6 µl DEPC treated water, 2 µl (6 µg protein) extract, 5 µl 10xTRAP reaction solution, 2 µl (50 µM) each dNTP, 0.4 µl (2 U) Taq DNA polymerase, and 2 µl (0.1 µg) of TS primer sequence (5'-AATCCGTCGAGCAGAGTT-3'). The PCR reaction mixture was incubated at 25°C in water bath for 30 min for extension of TS primer. CX primer sequence (5'-CCCTTACCCTTACCCTTACCCTAA-3') 2 µl (0.1 µg)) was added. The reaction mixture (total 50 µl) was subjected to PCR cycles (25) at 94°C for 30 s, 55°C for 30 s, and 72°C for 90s (then 10 min for the final step). After amplification 5 µl of loading dye (0.25% bromophenol blue, 0.25% xylenocyanol and 50% glycerol) was mixed to each PCR product and 25 µl of each sample were loaded onto a 12.5% non-denaturing polyacrylamide gel. After complete running of gel it was fixed in fixing solution (0.5% acetic acid,10% ethanol) and stained with 0.2% $AgNO_3$ for 10 min, followed by 15 min incubation in developing solution (0.1% formaldehyde and 3% NaOH) and then photographed.

Statistical analysis

To determine the treatment effects one way analysis of variance was carried by computer software SPSS 13.0. Level of significance among the various treatments was determined by LSD at 0.05% level of probability.

RESULTS

Effect of SAHE on antioxidant status

Protective effects of SAHE against $KBrO_3$-induced toxicity are shown in Table 1. SAHE significantly increased *($p<0.01$)* the activities of antioxidant enzymes; CAT, SOD, GST, GSH-px and GSR as well as GSH contents while reduced contents of TBARS in kidney as was altered by induction of $KBrO_3$.

Table 1. Effect of SAHE on antioxidant status in rat.

Treatment	CAT (U/min)	SOD (U/min)	GST (nM /min/mg protein)	GSH-Px (nM /min/mg protein)	GSR (nM /min/mg protein)	GSH (M/g tissue)	TBARS (nM /min/mg protein)
Control	12.233±0.250++	45.33±5.65++	223.3±10.3 ++	173.8±15.3++	59.61±2.5++	1.7±0.57++	5.5±0.075++
20 mg/kg $KBrO_3$	5.535±0.185**	25.35±2.08 **	134.5±24.7**	113.8±26.1**	33.99±0.9**	0.76±0.59**	11± 0.68**
100 mg/kg SHE+ $KBrO_3$	9.893±0.0992++	32.87±2.68**+	169.3±9.92**++	151.2±32.8++	46.13±1.3**++	1.3±0.06++	8.0±0.49++
200 mg/kg SHE+ $KBrO_3$	11.251±0.171++	42.65±3.88++	205.17±17.1++	167.50±7.78++	58.46±3.6++	1.7±0.09++	6.0±0.27++

Mean ± SE (n = 8 number). ** indicate significance from the control group at $p<0.01$ probability level. ++ indicate significance from the $KBrO_3$ group at $p<0.01$ probability level.

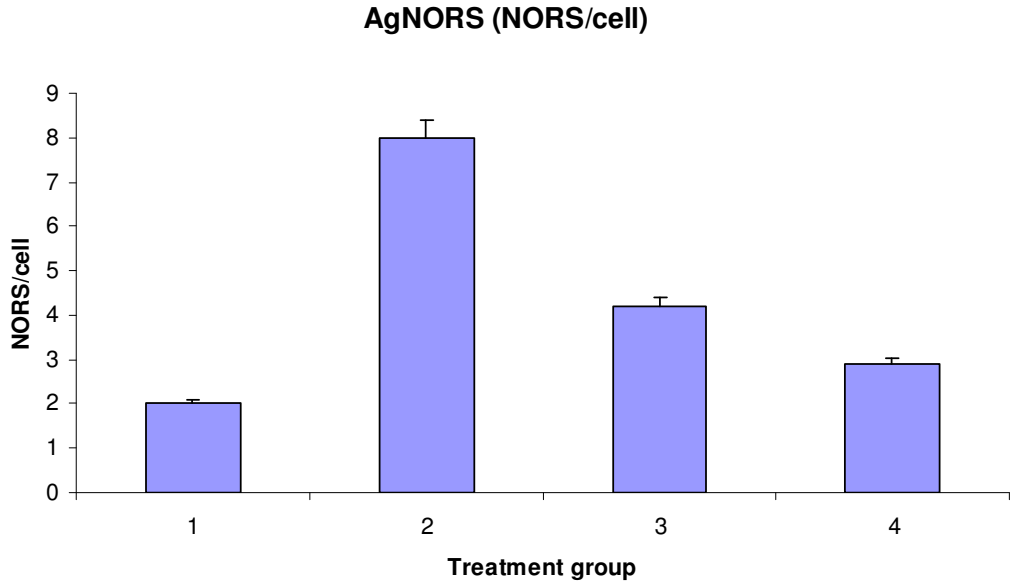

Figure 1. Effects of SAHE on AgNORs counts.

Effects of SAHE on kidney AgNORs in rat

Preventive efficacy of SAHE against $KBrO_3$ administration in rat on AgNORS count as shown in Figure 1.

Administration of $KBrO_3$ significantly increased *(p<0.01)* AgNORS count than control. Post-treatment with SAHE erased the $KBrO_3$ toxication and significantly *(p<0.01)* reversed the level of AgNORs towards the control rats.

Effects of SAHE on kidney % DNA fragmentation in rat

Preventive efficacies of SAHE against KBrO3 administration in rat on DNA fragmentation are

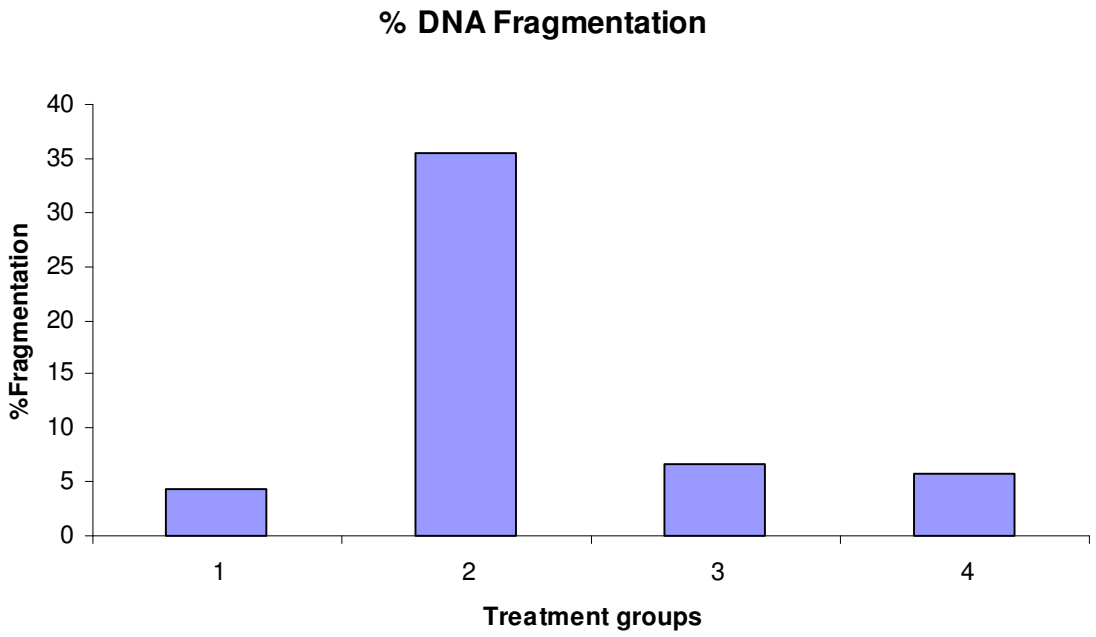

Figure 2. Effects of SAHE on % DNA fragmentation.

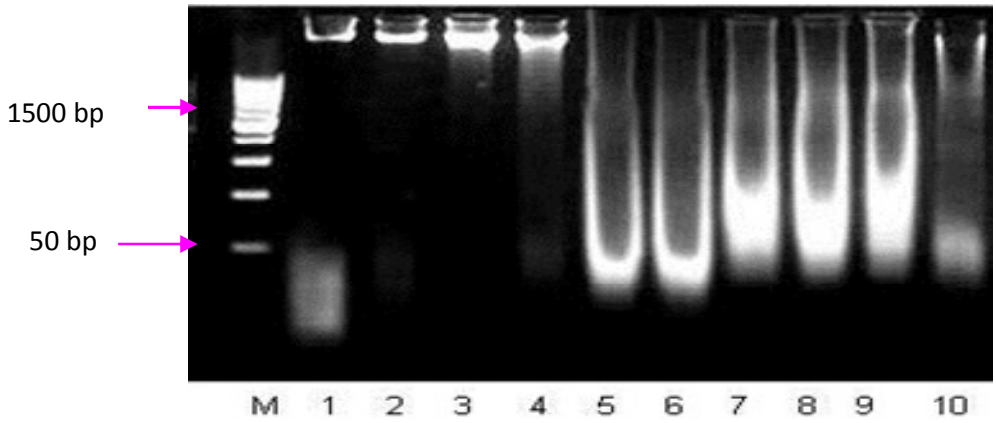

Figure 3. Agarose gel showing DNA damage by $KBrO_3$ and preventive effect of SAHE, Lanes (from left) DNA marker (M), Control (1 to 4), $KBrO_3$ (5 to 8), 100, 200 mg/kg b.w., SAHE (9, 10).

shown in Figure 2. Administration of $KBrO_3$ significantly increased *(p<0.01)* % DNA damages than control. Post-treatment of SAHE erased the $KBrO_3$ toxication and significantly *(p<0.01)* reversed DNA damages towards the control rats.

Effect of SAHE on kidney DNA damages (DNA ladder assay)

$KBrO_3$ forming DNA-free radical adduct, induces DNA damages in the kidney tissues of rats. DNA ladder assay showed that DNA damage was present in control as well as DMSO treated group. However, $KBrO_3$ group showed severe DNA damages. Post-treatment of SAHE reduced the DNA damages as indicated by DNA band of SAHE comparatively to $KBrO_3$ group (Figure 3).

Effect of SAHE on RT-PCR analysis

Telomerase enzyme play important role in oxidative stress and cancer. The results for telomeric repeat amplification protocol assay showed a single band in control group (Lane 5 to 6) which revealed the absence of telomerase enzyme activity while (Lanes 7 to 8) showed

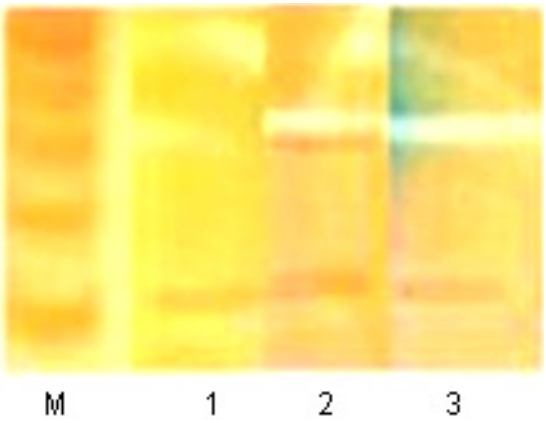

Figure 4. Polyacrylamide gel shows the telomerase enzyme activity in various groups of the study. From left to right marker Lane (M), control group Lane (1), $KBrO_3$ group Lane (2) and SAHE Lane (3).

TRAP amplification product in group treated with $KBrO_3$. Figure 4 (Lanes 1 to 4) shows that telomeric repeats bands were not present, indicated the protective effects of *S. asper*. These result suggested that SAHE possess potent anticancer as well as anti telomerase activity might be the presence of bioactive anticancer compounds in the extract.

DISCUSSION

The data revealed that the treatment of $KBrO_3$ causes significant alteration in antioxidant enzymes and oxidative DNA damage in kidneys of rats which are visualize on agarose gel by staining with ethidium bromide. Treatment with SAHE significantly improved the activities of CAT, SOD, GSH-px, GST, GSR and reduced DNA damages. Similar investigation was reported by Khan et al., (2009, 2010a, b) during study of protective effects of *Digera muricata* against carbon tetrachloride induced nephrotoxicity in rats. Khan and Sultana (2005) reported that the induction of $KBrO_3$ caused oxidative DNA damages in rats, which support our investigations. These results show that the SAHE contain bioactive compounds which play important role in DNA repair. Silver stained nucleolar organelles (NORs) per cell and chemical toxicity are directly correlated each other. Various studies reported that the quantity of protein AgNORs/cell is directly related with cell proliferation. It has also been reported from various investigation that number of AgNORs counts per cell and the prognosis of malignant tumour are directly related to one another. According to Irazusta et al. (1998), the quantification of AgNORs proteins per cell has been useful in diagnostic pathology especially in the differentiation of benign from malignant tumors and helpful in limitrophic lesions recognitation. In present study, statistically significant difference indicated the presence of invasive neoplasia (Wilkinson , 1990). The results inferred from the current data revealed that $KBrO_3$ induce telomerase activity in rats. The highly sensitive TRAP assay was used to detect the telomerase activity. The rat treated with $KBrO_3$ showed amplification of telomeres which was completely devoid by administration with SAHE. Similar results were obtained by our laboratory group of researcher. Those free radicals induced telomeric activity was reversed by post-treatment with *Sonchus asper* (Khan et al., 2009) and in other studies (Ramachandran et al., 2002). This antitelomeric and anticancer effect of various fractions showed that this might be possible due the presence of bioactive natural telomerase inhibitors compounds, which needs further isolation and purification.

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