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Full Length Research Paper

Protective effects of various fractions of *Launaea procumbens* on molecular markers in rat kidney

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Molecular markers; DNA, argyrophilic nucleolar organiser regions (AgNORs) and telomerase enzymes play important role in diagnosis of nephrotoxicity induced by potassium bromate ($KBrO_3$). In the present study, the effects of *Launaea procumbens* against $KBrO_3$ induced molecular changes in male rats are determined. 36 male albino rats (180 to 200 g) were divided into 6 groups. Group I was given saline (1 ml/kg body weight (b.w.), 0.85% NaCl) and DMSO (1 ml/kg b.w.); group II was treated with $KBrO_3$ (20 mg/kg b.w., i.p.); group III, IV, V and VI were administered with $KBrO_3$ and after 48 h with *L. procumbens* methanolic extract (200 mg/kg b.w. LPME), chloroform extract (200 mg/kg b.w. LPCE), ethyl acetate extract (200 mg/kg b.w. LPEE), and n-hexane extract (200 mg/kg b.w. LPHE), respectively. All the treatments were given twice a week for four weeks. The results revealed that $KBrO_3$ induced oxidative stress as evidenced by the significant alteration in DNA, AgNORs and telomeres enzymes of kidney which was restored with various fractions of *L. procumbens* might be due the presence of bioactive compounds.

Key words: Argyrophilic nucleolar organiser regions (AgNORs), *Launaea procumbens*, DNA, Telomerase enzymes.

INTRODUCTION

Phytotherapy means the use of bioactive compounds extracted from medicinal plants and their utilization as medicines or in the preparation of health-promoting drugs. Even though phytotherapy is usually known as "alternative medicine" in the Western countries, and is considered as an essential component of modern pharmaceutical drugs. All living organisms contain antioxidant metabolites and enzymes which ameliorate various free radical induced damages. Researchers have found a correlation between oxidative damage and the occurrence of diseases (Halliwell and Gutteridge, 1999). Many medicinal plants and their isolated compounds have been used for chemotherapy in living cells (Gilani et al., 1992). 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). *Launaea procumbens* (Roxb.) Amin. (Asteraceae) is used as a food and washing agent (Wazir et al., 2007) against rheumatism and is used as galactogogues in increasing milk production (Parikh and Chanda, 2006). It possesses salicylic acid, vanillic acid, synergic acid, 2-methyl-resercinol, gallic acid and is used against plant pathogenic fungi,

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nematocides and as allelopathic for inhibition of plant growth (Shaukat et al., 2003).

MATERIALS AND METHODS

Extraction and experimental design

Various fractions of *L. procumbens* were obtained as previously described by Khan et al. (2010a). To check the DNA, AgNORs and telomerase enzymes, 36 male albino rats were divided into 6 groups. Group 1 remain untreated, group II was given 20 mg/kg body weight (b.w.) potassium bromate ($KBrO_3$) in aqueous saline, group III, IV, V and VI was given 200 mg/kg extract of various fractions of *L. procumbens* (*L. procumbens* methanolic extract (LPME), *L. procumbens* chloroform extract (LPCE), *L. procumbens* ethyl acetate extract (LPEE) and *L. procumbens* n-hexane extract (LPHE)) for 4 weeks. After completion of the experiments, kidney was treated with liquid nitrogen for further analysis.

DNA fragmentation percentage assay

DNA fragmentation percentage 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 ethylenediaminetetraacetic acid (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 percentage fragmented DNA by the following formula;

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

AgNORs count

Silver staining technique was used according to the 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 decrease ethanol concentration (90, 70 and 50%) and were washed in distilled water for 10 min and were 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 nucleolar organelles (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 was 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 V. 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 ×g for 30 min at 4°C. Polymerase chain reaction (PCR) reaction mixture (total 48 µl) consisted of 36.6 µl diethylpyrocarbonate (DEPC) treated water, 2 µl (6 µg protein) extract, 5µl 10x TRAP reaction solution, 2µl (50 µM) each dNTP, 0.4µl (2 U) Taq DNA polymerase and 2 µl (0.1 µg) of transcribed spacer (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 90 s (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 was fixed in fixing solution (0.5% acetic acid and 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), it was then photographed.

Statistical analysis

To determine the treatment effects, one way analysis of variance was carried by computer software Statistical Package for Social Sciences (SPSS) 13.0. Level of significance among the various treatments was determined by least square difference (LSD) at 0.05% level of probability.

RESULTS

Effects of *L. procumbens* on AgNORs in rat kidney

Preventive efficacy of *L. procumbens* against $KBrO_3$ administration in rat on AgNORs count is as shown in Table 1. Administration of $KBrO_3$ significantly increased ($P < 0.01$) AgNORs count than control. Co-administration of various fraction of *L. Procumbens*, attenuated the $KBrO_3$ toxication and significantly ($P < 0.01$) reversed the level of AgNORs towards the control rats.

Effects of *L. procumbens* on kidney percentage DNA fragmentation in rat

Preventive efficacies of *L. procumbens* against $KBrO_3$ administration in rat on DNA fragmentation are as shown in Table 1. Administration of $KBrO_3$ significantly increased ($P < 0.01$) percentage DNA damages than control. Post-treatment of various fractions of *L. procumbens* erased the $KBrO_3$ toxication and significantly ($P < 0.01$) reversed

Table 1. Effect of different fractions of *L. procumbens* on AgNORs and percentage DNA fragmentation in rat kidney.

Group	Treatment	DNA fragmentation (%)	AgNORs/cell
I	Control	$6.833 \pm 0.422^{++}$	$2.617 \pm 0.317^{++}$
II	20 mg/kg $KBrO_3$	$37.67 \pm 3.24^{**}$	$12.50 \pm 1.48^{**}$
III	200 mg/kg LPME+ $KBrO_3$	$8.5 \pm 0.258^{++}$	$5.303 \pm 0.342^{**++}$
IV	200 mg/kg LPCE+ $KBrO_3$	$8.9 \pm 0.258^{++}$	$4.987 \pm 0.280^{**++}$
V	200 mg/kg LPEE+ $KBrO_3$	$8.8 \pm 0.258^{++}$	$4.853 \pm 0.205^{**++}$
VI	200 mg/kg LPHE+ $KBrO_3$	$14.0 \pm 0.258^{++}$	$5.9 \pm 0.307^{**++}$

Results are given as in Mean ± SE (n = 6 number). *,**, Significance from the control group at $P < 0.05$ and $P < 0.01$ probability level. $^{++}$, Significance from the $KBrO_3$ group at $P < 0.01$ probability level.

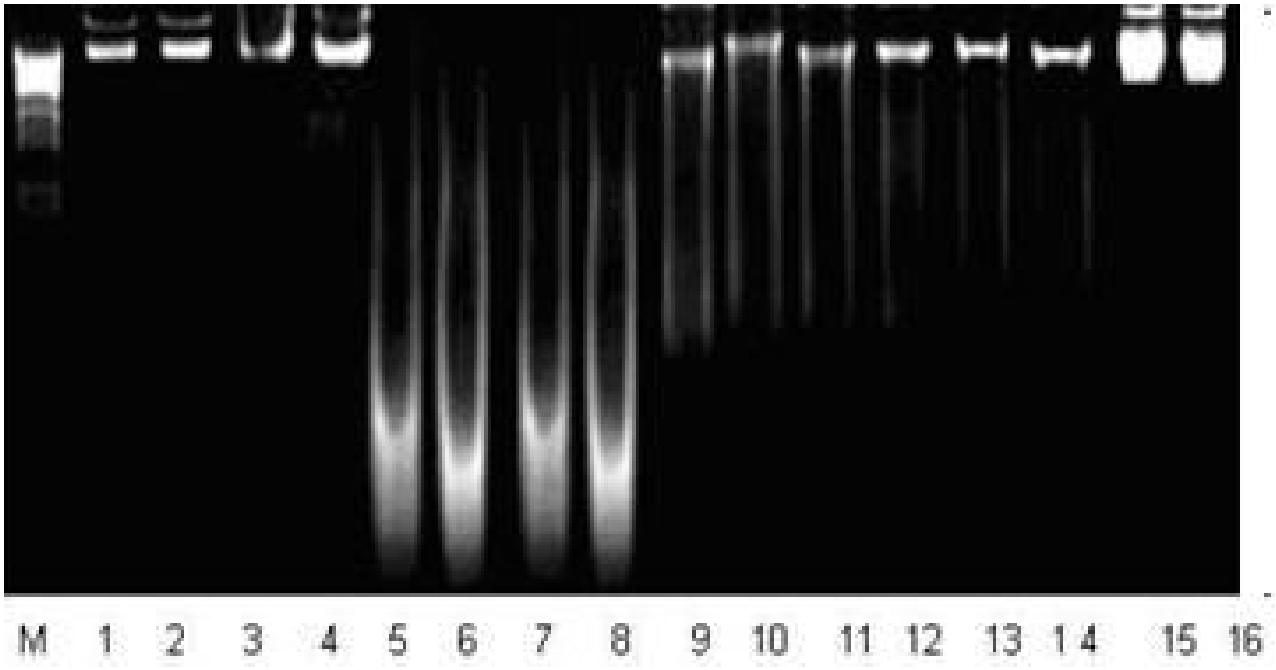

Figure 1. Agarose gel showing DNA damage by $KBrO_3$ and preventive effect of *L. procumbens* extracts in different groups. Lanes (from left) marker (M), Control (1 and 2), DMSO (3 and 4), $KBrO_3$ (5 to 8) and n-hexane, 200 mg/kg (9 and 10); ethyl acetate, 200 mg/kg (11 and 12); chloroform, 200 mg/kg (13 and 14); methanol, 200 mg/kg (15 and 16).

DNA damages towards the control rats.

Effect of *L. procumbens* 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 dimethyl sulfoxide (DMSO) treated group. However, $KBrO_3$ group showed severe DNA damages. Post-treatment of *L. procumbens* reduced the DNA damages as indicated by DNA band of *L. procumbens* comparatively to $KBrO_3$ group (Figure 1).

Effect of *L. procumbens* on reverse transcriptase-polymerase chain reaction (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 (Lane 7 to 8) showed telomeric repeat amplification protocol (TRAP) amplification product in group treated with $KBrO_3$. Figure 2 (Lane 1 to 4) shows that telomeric repeats bands were not present, indicating the protective effects of *L. procumbens*. These result suggested that *L. procumbens* possess potent anticancer as well as antitelomerase

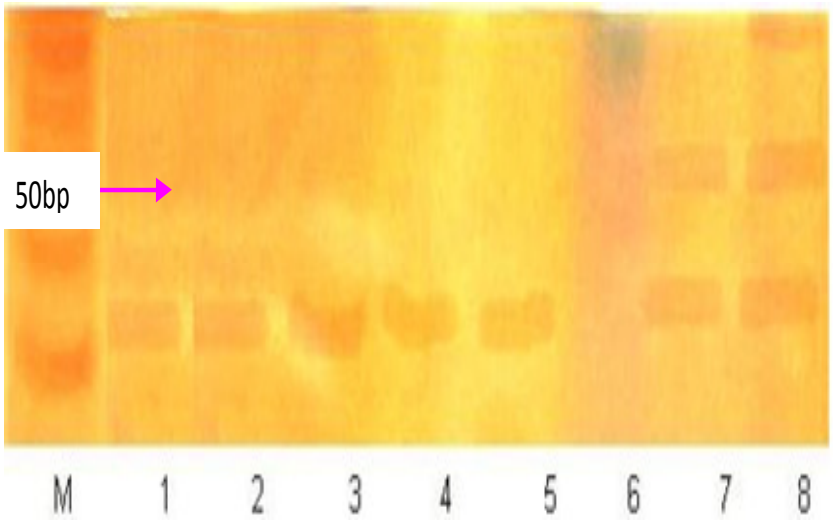

Figure 2. Polyacrylamide gel shows the telomerase enzyme activity in various groups of the study. Lane (1) LPME group, Lane (2) LPCE group, Lane (3) LPEE group, Lane (4) LPHE group, Lane (5 and 6) control group and Lane (7 and 8) $KBrO_3$.

activity might be the presence of bioactive anticancer compounds in the extract.

DISCUSSION

The data revealed that the treatment of KBrO3 causes significant oxidative DNA damage in various tissue of rats which are visualize on agarose gel by staining with ethidium bromide. Treatment with L. procumbens plants extracts significantly reduces these damages. Similar investigation was reported (Khan et al., 2009, 2010a, b) during the study of protective effects of *Digera muricata* against carbon tetrachloride induced nephrotoxicity in rats. Khan and Sultana (2005) reported that the induction of $KBrO_3$ cause oxidative DNA damages in rats which support our investigations. These results show that the extract of both plants contain bioactive compounds which play important role in DNA repair. Silver stained NORs per cell and chemical toxicity, 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 the number of AgNORs counts per cell and the prognosis of malignant tumor 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 is helpful in limitrophic lesions recognitation. In the 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. Rat treated with $KBrO_3$ showed amplification of telomeres which was completely devoid by administration with *L. procumbens*. Similar results were obtained by our lab researchers; however, free radicals induced telomeric activity was reversed by post-treatment with various fractions (Khan et al., 2009) in other studies (Ramachandran et al., 2002). This antitelomeric and anticancer effect of various fractions showed that this might be possible due to the presence of bioactive natural telomerase inhibitors compounds, which need to be further isolated and purified.

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