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Research Article

Antigenotoxic studies of *Salvadora persica* (Miswak) root extract in mice

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ABSTRACT

The present study was aimed to investigate *in-vivo* antigenotoxic effect of repeated exposure of hydro-alcoholic extract of *Salvadora persica* (SP). Two doses of root extract of SP (200 and 400 mg/kg) were administered to mice twice daily via gavage followed by induction of genotoxicity by Cyclophosphamide (CP-40 mg/kg), 24 hours before sacrifice. Animals were sacrificed, bone marrow & liver extracted for various estimations. Studies such as bone marrow chromosomal aberration assay; micronucleus test, and hepatic antioxidant enzymes were conducted for standard and treated mice. Results were statistically analyzed by one way ANOVA followed by Tukys test ($P<0.05$). Results showed that CP produced a significant increase in average percentage of aberrant metaphases, chromosomal aberrations (CAs), formation of micronuclei (MN) in polychromatic erythrocytes (PCE) and confirmed genotoxicity of CP in mouse bone marrow cells. CP also markedly inhibited the activities of glutathione (GSH) and increased malondialdehyde (MDA). Pretreatments with SP significantly inhibited the frequencies of aberrant metaphases, MN formation, CAs and reduced genotoxicity in mouse bone marrow cells induced by CP. SP also improved CP-induced GSH activities and reduced MDA content in the liver. Our studies revealed that SP has protective effect against genotoxicity and oxidative stress induced by CP.

Keywords: *Salvadora persica*, Cyclophosphamide, Chromosomal aberration, Micronucleus, Antioxidant enzymes.

INTRODUCTION:

Around the world, 182 species of plants have been used as chewing sticks for oral hygiene, the most important being ***Salvadora persica*** (SP) - belong to family- *Salvadoraceae,* commonly known as Miswak. It is considered to be 'the first toothbrush of mankind and has been documented by Babylonians, Greeks, Romans, Jews, and Egyptians[1,2]. In addition, the Islamic tradition (Sunnah) also recommends using Miswak for oral hygeine. It is still used as aid for the oral hygiene around the Islamic world and beyond[3].

The main constituents of SP are trimethylamine alkaloid salvadorine; chloride; sulphur; terpenes; large amounts of fluoride; silica; tannins; steroids; saponins; vitamin C; glycosides; phenolics, and flavonoids (rutin and quercetin)[4,5,6]. Benzylisothiocynate was also isolated from root[7].

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The root-bark and young twigs of SP have been used in folk medicine of India for a very long time for the treatment of a variety of conditions such as cough, asthma, scurvy, leprosy, gonorrhea, piles, rheumatism, headaches and hepatic and other diseases[8]. The leaves are used for scurvy, rheumatism, cough, asthma and as an antiinflammatory and antisyphilitic[9]. In addition, pharmacological data indicate antibiotic, anti-inflammatory and hypoglycemic activity[10].

Several clinical studies have reported that the miswak has a positive effect on gingivitis and removal of plaque[11,12]. It was reported to exhibit antiulcer, anticonvulsant, analgesic[13], antibacterial[14], and antihyperlipidemic activity[7]. According to Galati et al., (1998)[15] the decoction of SP demonstrated a significant protective action against stress-induced and ethanol induced ulcers in rats. Moreover, the decoction of stem of SP showed hypoglycemic effects in normal rats, an increase in plasma immunoreactive insulin (IRI), and increased oral-glucose tolerance[16]. Flavonoid

and phenolic compounds from the hydroalcoholic root extract of SP were found to possess antioxidant property[17]. As there were no studies on its protective action against genotoxicity, but yet possess an excellent antioxidant property, we aimed our current project to investigate the usefulness of SP root extract as an antigenotoxic agent.

METHODS

Drugs and chemicals

Cyclophosphamide, Colchicine, bovine albumin Fraction V, Geimsa's stain, and May-Grunwald's stain (all purchased from Sigma Aldrich, Germany) were used for the study. All other chemicals (analytical grade) were purchased from the commercial sources.

Preparation of plant extract

Roots of *Salvadora persica* were purchased from local areas of Jazan in the months of October /November. It was authenticated by Dr Maksood Ali, department of Pharmacognosy, College of Pharmacy, Jazan University, Jazan. Roots were shade dried initially followed by dried in vacuum oven at 60°C and powdered. The powder was passed through 60 # sieve and extracted with petroleum ether to remove the fatty materials. The residue was then extracted with ethanol:water (70:30) using Soxhlet apparatus. The yield of the extract was 10.5 % w/w (on dry weight basis of the crude material).

Experimental design

Swiss albino mice (8–12 weeks old) of either sex were procured from the institutional animal house of the College of Pharmacy, Jazan University, Jazan KSA. Mice were acclimatized for 7 days under standard husbandry conditions (i.e., room temperature maintained at 25 ± 5°C, relative humidity of around 45–55%, and a 12-hour light-dark photoperiod), with water *ad libitum*. For animal experimentation, approval from the local institutional animal ethical committee was taken before the experiments.

Group distribution

Healthy mice [approximately 25 g body weight each] were selected and divided randomly into six groups (n = 5) for each study. The group distribution for each study was as follows: Group 1, negative control (distilled water); Group 2, positive control (CP 40 mg/kg); Group 3 animals treated with, SP (200 mg/kg); Group 4 animals treated with, SP (400 mg/kg); Group 5 animals treated with, SP (200 mg/kg+CP) and Group 6 animals treated with SP (400 mg/kg+CP). The SP extract was diluted in distilled water in the ratio of 1:1, administered orally, twice daily for 7 days. Genotoxicity was induced by administering CP (40 mg/kg, intraperitoneally; i.p.) 24 hours before tissue sampling.

Bone marrow chromosomal aberration assay

Colchicine (0.4 ml of 0.05%) was administered intraperitoneally to the animals 90 minutes before sacrifice in order to arrest the mitotic process in metaphase. After sacrifice, both femurs were immediately dissected out and bone marrow was extracted in 0.075M of KCl and the cell suspension was incubated for 20 min at 37°C. Cells were collected by centrifugation at 1000 rpm for 10 min and were fixed three times with a solution of methanol/acetic acid (3:1). Chromosome slides were prepared by cell suspension being dropped onto clean chilled slides, which were flame dried, coded and stained in dilute Giemsa solution. The microscopic observations were performed with a magnification of 100X oil immersion[18]. Hundred well spread metaphase were scored per animal (around 500 metaphase per treatment group) at random. The types of aberration were scored and recorded with strict accordance of the method of Tice et al (1987)[19]. All aberrations (chromatid gaps, chromosomal gaps, deletion, chromatid and chromosomal breaks, ring and fragmentation) were considered equal regardless of the number of breakages involved. Percentage of aberrant metaphases (inclusive of metaphases with gaps) and aberrations (excluding gaps) per cell in them were calculated. From the same slides, 500 cells from each animal were taken into consideration for mitotic index (MI) study[20].

Bone marrow micronucleus assay

Mice were sacrificed and 5% w/v of bovine serum albumin was used to flush the bone-marrow cells. The obtained cell suspension was centrifuged (1000 rpm, 5 min), the supernatant was removed and the pellet re-suspended in bovine serum albumin. A drop of the suspension was smeared onto a clean slide, air-dried, fixed in methanol and stained with May-Grunwald and Giemsa as

originally described by Schmid (1975)[21]. About 1000 PCE were scored for the presence of micronuclei for each animal. For evaluation of bone marrow toxicity, the ratio of polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated by counting 1000 erythrocytes[22].

Determination of hepatic GSH activities and MDA content

Firstly, the livers were excised and then perfused with ice cold saline (0.9% sodium chloride). A 10% liver homogenate was made with fresh tissue in 0.1 M TrisHCl buffer at pH of 7.4. The tissue homogenate was used for the estimation of protein content[23], MDA[24] and GSH[25]. The reaction products were determined by spectrophotometry.

STATISTICAL ANALYSIS

The average data generated at different end points of the treated groups of mice was calculated and compared with the respective data of negative and positive control groups. For statistical analysis, the one-way ANOVA was first applied, followed by tuky's test for multiple pair-wise comparisons using GraphPad Prism 6 software. The statistical significance was examined at the P-value of 0.05.

RESULTS

Bone marrow CA assay

Mice of the negative control group showed 4.69±2.66% aberrant metaphases with 5.36±2.90 aberrations (excluding gaps) per hundred metaphases (Table 1). The positive control group mice showed 75.18±10.80% aberrant metaphases with 84.11±11.77 aberrations (excluding gaps) per hundred metaphases. The percentages of aberrant metaphases and CAs in mice of the positive control group were statistically significant ($P < 0.001$), compared to that of the respective negative control groups of mice (Table 1).

The average percentage of aberrant metaphases in SP-200 and 400 alone treated animals were 3.51±1.92 and 3.53±1.08 respectively, and aberrations (excluding gaps) per hundred metaphases were 6.38±3.58 and 4.31±2.52, respectively. SP-200 and 400-induced average percentages of aberrant metaphases in mice were 49.76±10.06 and 26.38±7.28, respectively, after induction of clastogenicity by CP. The average aberrations (excluding gaps) per hundred metaphases in the same mice were 58.12±8.42 and 38.09±7.96, for SP-200 and SP-400 respectively. The percentages of aberrant metaphases and aberrations per hundred metaphases induced by both tested doses of SP in mice were significantly ($P < 0.001$) lower than the positive control group of mice (Table 1).

Mice of the negative control group showed 8.12±0.60 percentage of MI, whereas their counterparts in the positive control group have shown of 3.42±0.49%, with a significant difference ($P < 0.001$) from that of the negative control mice (Table 2). In the group of mice that received SP-200 and 400 alone, the average percentages of dividing cells (MI) were 7.96±0.62, 8.28±0.39 and were found to be non significant compared to negative control. Percentage of dividing cells in the SP 200 and 400 mg/kg-treated groups combined with CP were increased to 5.78±1.19 and 7.30±0.75 respectively from that of the positive control mice and were statistically significant ($P < 0.001$, $P < 0.01$).

Bone marrow micronucleus assay

Average micro-nucleated polychromatic erythrocytes (MNPCs) per thousand PCEs of the negative control group of mice were 4.20±1.92; whereas the positive control group showed 27.80±7.33, which was significantly ($P < 0.001$) higher than that of the negative control group. SP 200 and 400 mg/kg in combination with CP induced 18.50±3.06 and 13.40±4.16 average MNPCEs respectively. The decrease in MN in both SP-treated groups of mice were found to be statistically significant from that of the respective control group ($P<0.001$ and $P<0.05$); however, both doses of SP (200 and 400 mg/kg) when treated alone did not produce the significant increase in MNPCs (Table 2).

In the current study, erythropoietic cell toxicity was calculated by the PCE/NCE ratio. CP-induced clastogenicity (0.59±0.10) in the positive control animals; whereas, in the animals pretreated with the different doses of SP (200 and 400 mg/kg+CP), significant restoration (0.89±0.16 or 1.02±0.19, $P<0.05$) in PCE/NCE ratio were observed (Table 3).

Table 1: Chromosomal aberration test in mice bone marrow cells pretreated with hydroalcoholic extract of *Salvadora persica* roots (1 weeks continuous treatments)

Groups	No. of metaphase analyzed	AM	Chromatid		Chromosomal		del	rg	dc	Ex	frg	Total no. of aberrations (excluding gap)	Avg. percentage of AM	Avg. aberrations
			gp	br	gp	br								
NC	505	23	7	4	5	2	7	6	4	4	-	27	4.69±2.66	5.36±2.90
PC (CP)	517	388	67	71	79	68	84	98	55	57	12	445	75.18±10.80[a]	84.11±11.77[a]
SP-200	497	27	2	3	5	3	10	8	3	5	-	32	3.51±1.92[c]	6.38±3.58[c]
SP-400	508	18	2	2	3	4	7	5	-	3	1	22	3.53±1.08[c]	4.31±2.52[c]
SP-200+CP	510	252	37	54	38	39	71	62	18	38	10	292	49.76±10.06[a,c]	58.12±8.42[a,c]
SP-400+CP	519	138	24	21	29	33	41	28	13	34	2	172	26.38±7.28[b,c]	38.09±7.96[a,c]

Data are expressed as mean±SD (n = 5). Abbreviations: NC, negative control; PC, positive control; AM, aberrant metaphases; gp, gap; br, break; rg, ring; del, deletion; dc, di centric; Ex, exchange; frg, fragmentation; SP, *Salvadora persica* extract; CP, Cyclophosphamide (40 mg/kg). ap< 0.001; bp< 0.01significant when compared with the negative control. cp< 0.001 significant when compared to positive control group (CP).

Table 2: Mitotic index of bone marrow cells pretreated with *Salvadora persica*.

Groups	No. of cell analyzed	% Mitotic index
NC	1000	8.12±0.60
PC (CP)	1000	3.42±0.49[a]
SP-200	1000	7.96±0.62[c]
SP-400	1000	8.28±0.39[c]
SP-200+CP	1000	5.78±1.19[b,d]
SP-400+CP	1000	7.30±0.75[c]

Data are expressed as mean±SD (n = 5).ap < 0.001; b p < 0.01; significant when compared with the negative control. cp< 0.001; dp< 0.01; significant when compared to positive control group (CP).

Table 3: Percentage of MNPCE in 1000 PCE and the ratio between PCE and NCE) in treatment with *Salvadora persica*.

Groups	MNPCE	PCE/NCE
NC	4.20±1.92	1.18±0.15
PC (CP)	27.80±7.33[a]	0.59±0.10[a]
SP-200	4.40±2.07[d]	0.94±0.18[c,f]
SP-400	3.80±1.92[d]	1.01±0.12[c,f]
SP-200+CP	18.50±3.06[a,f]	0.89±0.16[f]
SP-400+CP	13.40±4.16[c,d]	1.02±0.19[f]

Data are expressed as mean±SD (n = 5). Abbreviations: MNPCE, micronucleated polychromatic erythrocytes; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; ap< 0.001; bp< 0.01; cp< 0.05, significant when compared with the negative control. dp < 0.001; e p < 0.01; f p < 0.05; significant when compared to positive control group(CP). Five animals per group (representing a total of 5000 PCE) were analyzed for the presence of MNPCE and for the ratio PCE/NCE.

Determination of SOD, CAT, GSH activities and MDA content

As shown in Table 4, the activities of GSH were substantially reduced and MDA content was significantly increased in the CP-treated group compared to control group (P < 0.001). In the groups treated with SP (200 and 400 mg/kg) in combination with CP, the activities of GSH were significantly increased (P < 0.05 and P < 0.001) and MDA content was significantly reduced (P < 0.001) compared to positive control group (P < 0.05 to P < 0.01). Further, there were no significant changes in the activities of GSH and MDA content found in mice given SP at 200 and 400 mg/kg, alone, compared to negative control.

Table 4: Effects of *Salvadora persica* extract on hepatic GSH and MDA in mice.

Groups	GSH n mol / mg protein	MDA n mols / mg protein
NC	0.626±0.114	2.75±0.99
PC (CP)	0.249±0.111[a]	6.58±1.62[a]
SP-200	0.599±0.142[c]	2.65±0.78[b]
SP-400	0.645±0.122[c]	2.88±0.69[c]
SP-200+CP	0.539±0.134[d]	4.17±0.76[d]
SP-400+CP	0.609±0.148[b]	2.97±1.14[c]

Data are expressed as mean±SD (n = 5).ap < 0.001; significant when compared with the negative control. bp < 0.001; c p < 0.01; d p < 0.05; significant when compared to positive control group (CP).

DISCUSSION

The antigenotoxic potential of the hydro-alcoholic extract of roots of SP was investigated in the present study using mice bone marrow chromosomal aberration assay and micronucleus assay. Genotoxicity was induced by Cyclophosphamide (CP) since it is recommended as the standard genotoxicant as per OECD guidelines.

Chromosomal analysis of bone marrow cells *in vivo* from mammals is a standard method for testing potential mutagenic effects of viruses, radiation, drugs and chemicals pollutants. The assay is based on the ability of a test agent to induce structural or numerical alterations in the chromosome that can be visualized microscopically. The target tissue for the chromosomal aberration assay is the bone marrow as it is a rapidly dividing, well-vascularized tissue. The DNA is organized in chromosomes in higher organisms, and the induced aberrations of chromosomes can lead to cellular lethality and mutation[26].

In the present study, positive control CP is shown to induce significant increase (P<0.001) in number of aberrant metaphases as compared to the negative control group. It was also seen that administration of both the doses of SP (SP-200 and SP-400) did not increased the number of metaphases as opposed to negative control. On the contrary, in groups SP-200+CP and SP-400+CP, it was observed that SP significantly reduced the number of aberrant metaphases even in presence of CP (P<0.001). Similarly, the average aberrations excluding gaps were found significantly higher (P<0.001) in the CP treated group when compared to the negative control, while SP in presence of CP, reduced the frequencies of aberrations significantly (P<0.001) in a dose dependent manner (Table 1).

The relationship between cell cycle progression and inhibition of cell proliferation was examined by determining the mitotic index (MI) which is also called growth index[26]. MI is used as indicator of adequate cell proliferation for genotoxicity studies along with cytostatic and cytotoxic action of various environmental hazards or therapeutic agents[27]. For mitotic index study, it was found that CP treated positive control group demonstrated a significant decrease in mitotic activity, which was the indicator of bone marrow suppression. Treatment of the SP-200 and SP-400 alone did not show any significant changes in the mitotic activity while at the same time, it was found that both the doses of SP (200 and 400 mg/kg) countered the mitotic suppressant effect of CP significantly (P<0.01 and 0.001) (Table 2).

The mouse bone marrow micronucleus assay *in vivo* allows a sensitive indication of both chromosomal damage and chromosome loss caused by chemical substances. The presence of micronuclei in cells is considered as a biomarker of damage to the DNA. This assay is based on an increase in the frequency of micronucleated polychromatic erythrocytes found in bone marrow from animals treated with the test substance compared to that of negative control animals[21].

The data of the present study demonstrated that the root extract of SP (dose levels of 200 and 400 mg/kg) alone did not increase the percentage of micronucleated PCE compared to the negative control group. In the positive control group (CP), it was found that CP significantly (P<0.001) increased the percentage of MNPCE, which is the reflection of the result of CP treated group in the chromosomal aberration assay. Moreover, in Group 5 and 6, SP at the doses 200 and 400 mg/kg, significantly (P<0.05& 0.001) reduced the percentage of MNPCEs against CP (Table: 3).

The micronuclei test used in this study detects the cytotoxic effects by the PCE/NCE relationship. The PCE/NCE ratio may decrease when normal proliferation of bone marrow cells is affected by a toxic substance[28] and therefore, it is regarded as an indicator for toxicity affecting the cellular integrity of the bone marrow[29].

Both doses of SP (200 and 400 mg/kg) fail to show the cytotoxicity as no significant difference in PCE/NCE ratio was observed when compared to the negative control animals. Moreover, CP treated animals showed a high level of cytoxicity as the PCE/NCE ratio drastically decreased as compared to the negative control group which was highly significant (P<0.001). However, when the same observation was done in the animals pretreated with SP (200 and 400 mg/kg +CP) it was found that SP significantly reduced the cytotoxicity of CP (P<0.05 & P<0.01) (Table: 3).

The standard genotoxicant in the present study is CP which binds covalently to the DNA[30]. According to Sladek, (1971, 1988)[31,32], for the therapeutic and the toxic effects of CP, it is required that CP metabolically activated by the hepatic microsomal cytochrome P450 system. Acrolein and Phosphoramide mustard are the two active metabolites of CP, where the antineoplastic effects are related with the phosphoramide mustard, while the acrolein produces toxic side effects[33]. It is stated that Acrolein interferes with the tissue antioxidant defense system[34], and thereby produces highly reactive oxygen free radicals[35] and suppresses the antioxidant activities[36] which is genotoxic to mammalian cells[37]. The induction of significantly ($P<0.001$) high percentage of aberrant metaphases, CAs (excluding gaps), and MN per thousand PCEs in mouse bone marrow, by CP (40 mg/kg b.w. of mice) in the present study are in complete agreement with its earlier reported clastogenicity.

Accordingly, CP has a pro-oxidant character and administration of CP produces oxidative stress in liver, lungs and serum of mice and rats, which results in reduction of activities of anti-oxidant enzymes and increases lipid peroxidation in these tissues[38] as shown in table 4. Among all the markers of oxidative stress, it is believed that GSH binds covalently to CP metabolites thereby produces cytotoxicity. As discussed earlier, after hepatic metabolism, acrolein released intracellularly by CP reduces the GSH content resulting in DNA single strand breaks[39]. Our results also show that CP significantly increases the MDA content, which is consistent with the results of GSH ($P<0.001$), may also be involved to induce genotoxicity[37].

Pretreatment with SP root extract significantly increased the hepatic GSH content ($P<0.01$ & $P<0.05$), and decreased the MDA level against CP in mice ($P<0.05$ & $P<0.01$). This implicates that the chemoprotective mechanism of SP root extract against CP-induced genotoxicity can be linked, at least partially, to its elevated GSH content and decreased MDA level. This can also be ascribed to its phenolic and flavonoid content. Since, phenolic content is attributed to the multidimensional effect of the phenolics including scavenging of free radicals and alteration of gene expression, notably the antioxidant response elements (ARE) which involve the phase II detoxifying enzymes[40,41].

Plant phenols also modify the DNA repair pathway after DNA damage and inhibit the formation of DNA adducts or methylation[42,43]. Moreover, it is also believed that antigenotoxic activity of plant phenols is due to the interaction of the compound with the target tissue DNA which, in turn blocks the site(s) of DNA to the electrophilic attack by reactive carcinogenic moieties and therefore associated with low incidence of various cancers[44,45].

On the other hand, flavonoids present in the SP like quercetin, have been reported to scavenge free radicals and inhibit lipid peroxidation[46,47]. These compounds reportedly suppress the cytotoxicity of superoxide ion[48,49], increasing the expression of DNA polymerase beta gene which is an enzyme responsible for the error-free DNA repair[50] that helps to increase the cell survival.

According to Ezmirly (1981)[51], the roots of SP contain 90% benzylisothiocyanate which is classified as one of the chemo-preventive agents thought to prevent carcinogenic and other genotoxic compounds from reaching or reacting with the target sites on the treated tissue. Hence, the protective effect of the SP against the CP induced genotoxicity seems to be due to its antagonizing effect against oxidative stress which may probably be due to the synergistic effect of the multiple phytochemicals and also protects the DNA by multiple mechanisms.

CONCLUSION

The present investigation provides evidence that SP inhibits *in vivo* genotoxicity of CP. Moreover, the present results also support the notion that the protective effect of SP against genotoxicity and oxidative stress is due to the synergistic & combined effects of a complex mixture of phytochemicals, the total activity of which may result in considerable health benefits.

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