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ORIGINAL ARTICLE

Inhibitory Effect of *Salvadora persica* (Miswak) against Cigarette Smoke-induced Mutagenicity and Sperm Abnormalities in Rats

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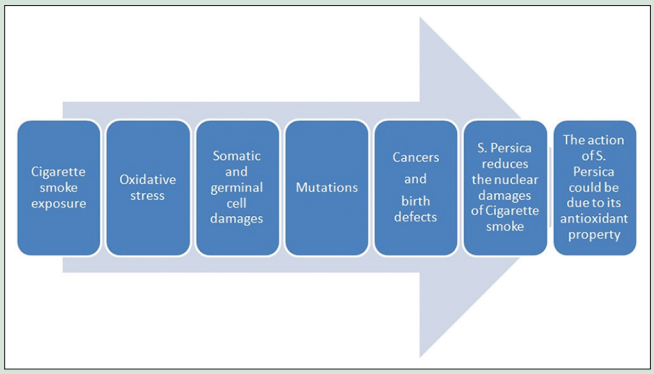
ABSTRACT

Objective: The objective of the study is to evaluate the inhibitory effect of *Salvadora persica* against the cigarette smoke-induced mutagenicity and sperm damages in rats. **Materials and Methods:** Young male Wistar rats of weight 120–140 g were exposed to the cigarette smoke for 8 weeks. The lyophilized decoction of *S. persica* was administered daily for 4 weeks by oral route at the doses 50, 100, and 200 mg/kg. The somatic and germinal cells damages were determined by peripheral blood micronucleus and sperm abnormalities tests, respectively. *In vitro* antioxidant activity of the decoction was determined by hydrogen peroxide scavenging assay. Ginseng was used as a standard herbal agent. The results were analyzed statistically by one-way ANOVA followed by Tukey test. P <0.05 was considered to indicate the significance of results. **Results:** The data from the present study indicated that cigarette smoke exposure significantly (P < 0.01) increased the population of micronucleated erythrocytes and sperm shape abnormalities and, reduced the polychromatic: normochromatic (P/N) ratio, and total sperm count compared to control. The administration of *S. persica* produced a dose-dependent inhibition on somatic and male germinal cell damages induced by cigarette smoke and the significant (P < 0.05) activity was observed at 200 mg/kg. However, none of the tested doses enhanced significantly the P/N ratio. Ginseng at 100 mg/kg significantly (P < 0.01) prevented the cigarette smoke-mediated damages. The *in-vitro* antioxidant activity indicated both *S. persica* and ginseng possess scavenging activity against the hydrogen peroxide free radicals. **Conclusion:** The observation suggests that the decoction of *S. persica* can prevent the somatic and germinal cell nuclear damages induced by cigarette smoke exposure. These actions could be related to its antioxidant property.

Key words: Antioxidant, cigarette smoke, micronuclei, *Salvadora persica*, sperm abnormalities

SUMMARY

- Cigarette smoke is known to produce noxious effects on almost all the systems of the body
- The actions of cigarette smoke on the somatic and germinal cells were studied using peripheral blood micronucleus test and sperm abnormalities test
- Exposure of cigarette smoke for 8 weeks increased the population of micronucleus cells, sperm shape abnormality and reduced the polychromatic: normochromatic ratio and total sperm count
- *Salvadora persica* commonly known as Miswak was tested for the nuclear damage preventive activity against the cigarette smoke
- The results indicated that *S. persica* at a higher tested dose (200 mg/kg) inhibited the changes induced by cigarette smoke on micronucleated erythrocytes population and sperm abnormalities
- *S. persica* exhibited marked scavenging activity in the *in vitro* hydrogen peroxide assay
- Ginseng used as standard also reduced the somatic and germinal cells damages induced by Cigarette smoke besides exhibiting the antioxidant potential
- The findings suggest that *S. persica* inhibited the cigarette smoke-induced micronucleated and sperm defects and the action could be related to its antioxidant potential.



Abbreviations Used: *S. persica*: *Salvadora persica*; P/N: Polychromatic: Normochromatic erythrocytes; g/gm: Gram; mL: Milliliter; µg: Microgram

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INTRODUCTION

Tobacco smoking is common among the population of all races. There are more than 8000 chemicals of smoke constituents identified in tobacco smoke, and among them, 100 have been reported to cause potential diseases such as lung cancer, cardiovascular disease, and emphysema.[1] Some of the major toxic substances reported are arsenic, benzene, benzo (a) pyrene, carbon monoxide, heavy metals (e.g. lead and cadmium), hydrogen cyanide, and tobacco-specific nitrosamines.[2]

Cigarette smoke is known to contain various chemicals that have a tendency to produce mutations. Improper repair of the genetic damages is the leading cause for several somatic and germinal cell disorders. The somatic damages can be manifested as disease condition in the present generation such as cancer, cardiovascular, and respiratory diseases, while the germinal cell damages have the tendency to produce abnormalities in the progeny.[3]

Batteries of tests are available to detect the somatic and germinal cell mutations in the experimental setup. The micronucleus test in the

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peripheral blood is popular for determining the somatic mutations wherein the frequency of micronucleated erythrocytes was evaluated after respective treatments. Micronuclei are the fragment of main nucleus that left-behind in the cytoplasm after nuclear damage when the main nucleus is expelled during erythropoiesis.[4] The ratio of two types of erythrocytes, i.e. polychromatic and normochromic can be used to indicate the influence of treatment on the proliferation of cells.[5] Abnormalities of sperm shape and sperm count were routinely employed to determine the qualitative and quantitative germinal cells damages, respectively. These tests are reported to be used for evaluating both clastogenic and anticlastogenic potential of agents.[6]

Plants from ancient times are the major sources of medicine. Plants-derived medicines have been a part of our traditional health-care system and are reported to be more effective and less harmful, as *per se* they are devoid of major side effects.[7] *Salvadora persica* L. (Miswak) belonging to the family *Salvadoraceae* is an evergreen shrub, 4–6 m tall with a short trunk, white bark, and smooth green leaves. The roots of the plant are referred by different names in different cultures such as Chewing sticks, toothbrush tree, Siwak, and Arak.[8]

S. persica L. is most commonly used medicinal plants for oral hygiene and is popular among millions of people in Africa, South America, the Middle East, and Asia.[9] A variety of chemical components have been identified in *S. persica* extracts. The important constituents reported are salvadourea, salvadorine, trimethylamine, lignans, oleic, alkaloids, and linoleic acids and have been suggested to contribute in various pharmacological activities such as antidiabetic, antioxidant, antiulcerogenic, and lipid-lowering effects.[7-9]

However, the potential benefit of *S. persica* juice in the tobacco smoke-induced mutagenic complications is not well documented in the literature.

MATERIALS AND METHODS

Plant material

Fresh roots of the plant *S. persica* grown in the Makkah region of Saudi Arabia were purchased. The plant material was authenticated by Dr. Hamdoon, Pharmacognosist in the Department of Phytochemistry and Medicinal Chemistry, College of Pharmacy, Qassim University and a voucher specimen was deposited in the herbarium.

Preparation of decoction

The decoction was prepared as per the procedure described by Galati *et al*. 1999.[10] 100 g of dried powdered roots of *S. persica* was boiled with 1000 ml distilled water for 30 min. After filtration, the decoction was lyophilized. The quantity of lyophilized powder obtained from 100 g of the drug was 12.6 g. The lyophilized powder was administered, in the morning, by oral gavage, at doses of 50, 100, and 200 mg/kg, dissolved in aqueous vehicle, in a volume of 0.5 ml/l00 g of body weight.

Animals

Young adult rats of Wistar strain (120–140 g) were used for the present study. The experimentation was conducted after obtaining the permission from the Institutional Animal Ethics Committee (Approval ID # 2019-CP-4). Animals were housed in the central animal house facility maintained under standard laboratory conditions. Animals were provided pelleted food and water *ad* labitum under 12 h dark and light environment.

Experimental grouping

The experimental animals were divided into six groups as follows: Group 1 is control (Saline – 0.5 ml/100 g, body weight per day), Group 2 is positive control where animals were exposed to cigarette smoke daily for 6 days in a week for 8 weeks, Group 3–5 were treated with *S. persica* to the cigarette smoke-exposed animals at three doses namely 50, 100, and 200 mg/kg, per oral for 4 weeks[11] and Group-5 was standard treatment group (Ginseng – 100 mg/kg, per oral, 4 weeks).[12]

Experimental design

Each group comprised of 8 rats. The experimental animals were exposed to the cigarette smoke in the chamber, a glass box in a cube shape (aquarium shape) with the size of 30 cm × 40 cm × 80 cm for keeping the rats and a hood over the aquarium-shaped box to evacuate the extra smoke from the environment as described by Ypsilantis *et al*., 2012.[13] Animals were daily exposed to cigarette smoke (2–3 cigarettes) for a total duration of 30 min (with intermittent exposure to fresh air for 2 min after every 10 min of smoke exposure). After initial 4-week exposure, tests were done to find the influence of cigarette smoke on micronucleus formation. For this, a drop of blood from the tail vein was collected under mild ether anesthesia, and the percentage of micronuclei was determined. The changes in the body weight, general health condition, water, and food consumption were also monitored weekly. Once a significant level of clastogenicity was observed in the animals (8th week), Groups 3–6 were subjected to respective drug treatments for 4 weeks, simultaneously exposing the animals to cigarette smoke.

Mutagenicity studies

Peripheral blood micronucleus test

A drop of blood collected on a clean glass slide was smeared immediately. The micronuclei test described by Hayashi *et al.*, was used to study the frequency of micronuclei in two types of RBCs namely polychromatic (P) and normochromatic (N) erythrocytes.[14] The dried, smeared slides were sequentially stained in Wrights' and Giemsa stains. The presence of micronuclei was identified using light microscope under oil immersion objective.[15] A total of 1000 cells were counted and the ratio of polychromatic to normochromatic (P/N) was used to study the effect of treatment on erythropoiesis.

Sperm shape abnormality test

The suspension of the sperms isolated from the caudal epididymis was filtered to remove the tissue debris. A 3–4 drops of 1% aqueous Eosin-Y stain was added, and the smeared is prepared in the clean glass slide. After air drying, the smears were observed under light microscope. Six types of sperm abnormalities such as hookless, tailless, banana-shaped, double hooked, and double tailed[15] as per the procedure described by Wyrobek *et al*.[16]

Total sperm count

The analysis of total sperm count was done as per the method described by D'Souza.[17] The total sperm count was done using the Neubauer's chamber. The stained solution was taken in the WBC pipette up to 0.5" mark and immediately diluted with phosphate buffer up to 11" mark. The solution was mixed for 1–2 min and then charged into the chamber. The sperm count was done after allowing the sperm to settle in the chamber. The total number of sperms present in the four chambers were taken and represented as cubic millimeter after multiplying with dilution factor (50,000).

In vitro antioxidant activity

The *in vitro* antioxidant assay was done by the procedure of Ruch *et al.*[18] In this method, a 40 mM solution of the hydrogen peroxide was prepared in phosphate buffer (50 mM, pH 7.4). The different concentration of *S. persica* and ginseng (10–160 μg/mL) was added to the hydrogen peroxide solution; the absorbance was recorded at 230 nm after allowing

the mixture to stand for 15 min. The percentage scavenging activity was calculated from the formula:

$$\text{Percentage scavenging activity} = \frac{(\text{Abs of control} - \text{Abs of test})}{\text{Abs of control}} \times 100$$

Statistics analysis

The data obtained from the study were statistically evaluated by one-way ANOVA, followed by *post hoc* analysis by Tukey. $P < 0.05$ was considered to indicate the significance of the result.

RESULTS

Effect of *Salvadora persica* decoction on the percentage micronuclei in polychromatic erythrocytes (P) and normochromatic erythrocytes (N) in cigarette smoke-exposed animals

The data from Table 1 indicated that the exposure of cigarette smoke to animals significantly increased the percentage of micronuclei in polychromatic ($P < 0.01$) and normochromatic erythrocytes ($P < 0.001$) compared to the control animals. The administration of *S. persica* at 50 mg/kg did not produce significant difference in the micronuclei percentage. However, *S. persica* at 100 mg/kg showed a significant ($P < 0.05$) reduction in the population of micronucleated polychromatic erythrocytes compared to CSE group. Further, when *S. persica* was tested at 200 mg/kg, a significant ($P < 0.01$) reduction in micronuclei was observed in normochromatic erythrocytes in addition to polychromatic erythrocytes. The treatment of standard drug, i.e. ginseng showed a significant reduction on micronuclei in both polychromatic ($P < 0.01$) and normochromatic ($P < 0.001$) compared to cigarette smoked rats.

Effect of *Salvadora persica* decoction on the percentage sperm shape abnormality and total sperm count in cigarette smoke-exposed animals

The present studies conducted to determine the qualitative and quantitative defects in the male germinal cells suggested that the exposure of cigarette smoke significantly enhanced ($P < 0.001$) the percentage sperm shape abnormalities and reduced ($P < 0.05$) the total sperm count compared to the normal group. The treatment of *S. persica* produced a dose-dependent variation in the sperm anomalies. At 50 mg/kg, *S. persica* showed nonsignificant variation, however, when the dose was increased to 100 mg/kg, a significant ($P < 0.05$) reduction in the sperm shape abnormalities was observed when compared to the challenge group. The administration of *S. persica* at 200 mg/kg further reduced the sperm shape defects ($P < 0.01$) and also enhanced the total sperm count ($P < 0.05$) compared to the cigarette smoked animals. On the other hand, ginseng exhibited a significant reduction in the sperm shape abnormalities ($P < 0.001$) and increased the sperm count ($P < 0.05$) when compared with a positive control group [Table 2].

Effect of *Salvadora persica* on the polychromatic: normochromatic ratio in cigarette smoke exposed animals

The observations from the present study conducted to determine the P/N ratio indicated that cigarette smoke exposure reduced the ratio significantly ($P < 0.01$) compared to the control group. The administration of *S. persica* in three doses produced a nonsignificant elevation in the P/N ratio compared to the challenge group. However, when ginseng was tested at 100 mg/kg, a significant ($P < 0.05$) elevation in the P/N ratio was observed when compared to the cigarette smoked animals [Graph 1].

Table 1: Effect of *Salvadora persica* decoction on the percentage micronuclei in polychromatic erythrocytes (P) and normochromatic erythrocytes (N) in cigarette smoke-exposed animals

Treatment	Percentage micronuclei in	
	Polychromatic erythrocytes	Normochromatic erythrocytes
Control	0.54±0.02	0.61±0.03
CSE	0.68±0.08**	0.93±0.11***
CSE + *S. persica* (50 mg/kg)	0.71±0.09 NS	0.90±0.12 NS
CSE + *S. persica* (100 mg/kg)	0.61±0.04[a]	0.86±0.09 NS
CSE + *S. persica* (200 mg/kg)	0.59±0.06[a]	0.75±0.06[b]
CSE + ginseng (100 mg/kg)	0.56±0.06[b]	0.71±0.07[c]

Values are represented as Mean±SEM, n=8, NS, Statistics: One-way ANOVA followed by posttest Tukey. **P<0.01, ***P<0.001 compared with control, [a]P<0.05, [b]P<0.01, [c]P<0.001 compared with CSE group. NS: Not significant; SEM: Standard error of mean; CSE: Cigarette smoke-exposed; *S. persica*: *Salvadora persica*

Table 2: The effect of *Salvadora persica* decoction on the percentage sperm shape abnormality and total sperm count in cigarette smoke-exposed animals

Treatment	Percentage sperm shape abnormality	Total sperm count (10^6 per cu mm)
Control	10.62±1.24	32.07±8.19
CSE	19.88±3.39***	21.77±7.68*
CSE + *S. persica* (50 mg/kg)	19.03±2.97 NS	24.96±9.17 NS
CSE + *S. persica* (100 mg/kg)	15.65±2.71[a]	26.04±8.58 NS
CSE + *S. persica* (200 mg/kg)	14.93±2.18[b]	29.68±6.89[a]
CSE + ginseng (100 mg/kg)	13.06±2.13[c]	30.92±6.41[a]

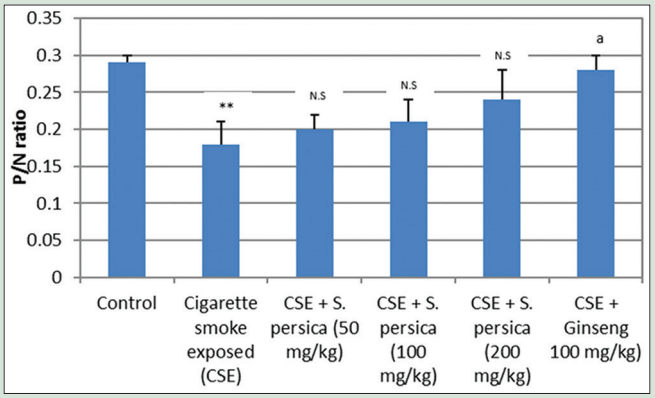
Values are represented as mean±SEM, n=8, NS, Statistics: One-way ANOVA followed by posttest Tukey. *P<0.05, ***P<0.001 compared with control, [a]P<0.05, [b]P<0.01, [c]P<0.001 compared with CSE group. NS: Not significant; SEM: Standard error of mean; CSE: Cigarette smoke-exposed; *S. persica*: *Salvadora persica*

In vitro percentage hydrogen peroxide scavenging activity

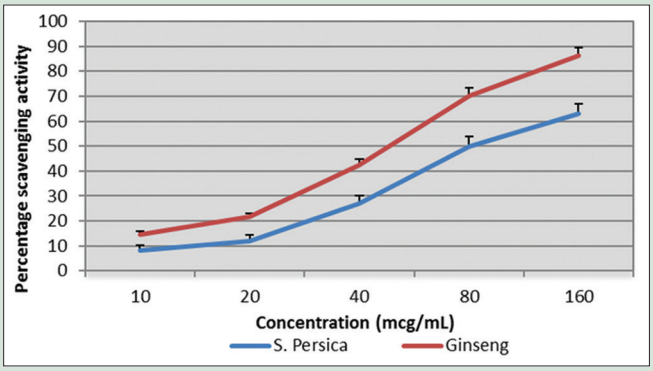
The percentage hydrogen peroxide scavenging activity was tested in five concentrations namely 10, 20, 40, 80, and 160 µg/mL. At the lowest tested dose (10 µg/mL), *S. persica* produced 8.11% scavenging activity and at the highest dose (160 µg/mL) 62.83%. On the other hand, ginseng at lowest tested dose (10 µg/mL) showed 14.82% and at highest dose (160 µg/mL) 86.11% scavenging activity. The 50% scavenging effect for *S. persica* was observed at 81.9 µg and for ginseng the effect was found at 52.2 µg concentrations [Graph 2].

DISCUSSION

Cigarette smoking is one of the leading causes of mortality and morbidity in many countries worldwide. The gender distribution among smoker indicated that around 26.5% are male and 9% are habituated to tobacco smoking.[1] Epidemiological data suggested that various chemicals present in cigarette can directly affect the reproductive health of both active and passive smokers. Cigarette smoke contains a large number of recognized carcinogens and mutagens. Genetic damages are known to contribute in several disease conditions when the host defense mechanisms fails to repair the mutations.[2] If the genetic damage occurs to somatic cells, then it will contribute in diseases such as cancer, heart ailments, and central nervous system defects, and if it happens in germinal cells, then the defects will



Graph 1: The effect of *Salvadora persica* on the P/N ratio in cigarette smoke exposed animals. Values are represented as mean ± standard error of mean, $n = 8$, NS = Not significant. Statistics: One-way ANOVA followed by post-test Tukey. $^{**}P < 0.01$ compared with control. $^{a}P < 0.05$ compared with CSE group



Graph 2: *In vitro* percentage hydrogen peroxide scavenging activity. Values are represented as mean ± standard error of mean, $n = 4$

be transferred to the progeny besides reducing the ability to reproduce.[3]

The observations from this study indicated chronic exposure of animals to cigarette smoke increased the frequency of micronuclei in both polychromatic and normochromatic erythrocytes, besides reducing the rate of erythropoiesis [Table 1 and Graph 1]. Exposure to cigarette smoke also induced significant qualitative and quantitative defects in the male germinal cells [Table 2]. The results suggest that cigarette smoke exposure induced both mutational and the male germinal cell damages in the experimental animals. As per the previous research, the carcinogens present in the cigarette smoke can cause several structural damages to the nuclear component of the cells. Besides, the over-production of free radicals due to cigarette smoke has the ability to induce these changes directly as well to augment the actions of carcinogens in the host cells.[19] Proto-oncogenes and tumor suppressor genes are considered to be the critical targets of the cigarette smoked mutagens.[2]

One more observation of the study is that cigarette smoke reduced the polychromatic/normochromatic erythrocytes ratio (P/N ratio) significantly compared to control animals [Graph 1]. The ratio is determined to find the influence of treatment on the rate of proliferation of cells. The findings suggested that the complex components present in the cigarette smoke has the ability to interfere in the proliferation of erythrocytes.[5] Treatment with ginseng enhanced significantly the P/N ratio in the cigarette smoked animals. However, the administration of *S. persica* in the tested doses produced a non-significant improvement in the P/N ratio. The observation suggests that the tested doses and duration of *S. persica* treatment is not sufficient to reverse the changes in diminished P/N ratio induced by cigarette smoke.

As reported, one of the best methods of reducing the complications of environmental pollutants is to enhance the supplementation of protective agents. Plant-based products are traditionally used for both treatment and prevention of various diseases.[20] Our observations indicated that the administration of *S. persica* reduced the cigarette smoke-induced somatic nuclear damages and germinal cell defects in rats. Ginseng used as a standard also showed a significant reduction in micronuclei frequency and sperm morphology and sperm count defects [Tables 1 and 2].

Several studies in the past suggested that antioxidant activity of a compound has the potential to prevent the mutagenic complications of environmental pollutants. Ginseng is traditionally used for various medicinal purposes and is known to contain one of the potent antioxidant such as ginsenosides.[21] *S. persica* is also reported to possess the antioxidant activity and is linked to the presence of phytochemicals such as flavonoids and phenolic compounds.[22] The *in vitro* antioxidant study also indicated that both *S. persica* and ginseng has the ability to produce the scavenging activity of free radicals such as hydrogen peroxide [Graph 2]. Therefore, it can be suggested that the antioxidant potential of *S. persica* and ginseng could be responsible for reducing the cigarette smoke-induced somatic and germinal damages in rats. Hence, chewing Miswak not only provide oral hygienicity and may also reduce the mutagenic-complications of environmental pollutants such as cigarette smoke.

CONCLUSION

The present study indicated that cigarette smoke exposure increased the frequency of micronuclei, sperm shape abnormality, and reduced the P/N ratio and total sperm count. The administration of *S. persica* showed dose-dependent prevention in the somatic and germinal cells defects without producing significance change in P/N ratio, and the maximum activity was observed at 200 mg/kg. These activities can be related to the antioxidant potential of *S. persica*.

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Conflicts of interest

There are no conflicts of interest.

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