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Original article

In vivo assessment of genotoxic potential of brown shammah (smokeless tobacco) in bone marrow cells of mice

Pankaj Tripathi [a,*], Saeed Alshahrani [a], Hassan A. Alhazmi [b,c], Rina Tripathi [d], Abdul Hakeem Siddiqui [a], Waquar Ahsan [b], Mohammed Al Bratty [b]

[a] Department of Pharmacology, College of Pharmacy, Jazan University, P. Box No. 114, Jazan, Saudi Arabia
[b] Department of Pharmaceutical Chemistry, College of Pharmacy, Jazan University, P. Box No. 114, Jazan, Saudi Arabia
[c] Substance Abuse and Toxicology Research Centre, Jazan University, Jazan, P. Box No. 114, Jazan, Saudi Arabia
[d] Department of Clinical Pharmacy, College of Pharmacy, Jazan University, P. Box No. 114, Jazan, Saudi Arabia

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ABSTRACT

This study was aimed to assess the genotoxicity of brown shammah (BS), a local form of smokeless tobacco, popular in Middle East countries including Yemen, Saudi Arabia and Sudan. The genotoxicity was explored using *in vivo* chromosomal aberration (CA), micronucleus (MN) and sperm abnormality (SA) assays. In addition, oxidative stress was also determined using various hepatic markers. Swiss albino mice were selected for the study, which were divided in to 5 groups of six animals each. They include, negative control (NC, received only vehicle) as well as positive control group (PC, received vehicle for 2 weeks followed by administration of cyclophosphamide, CP). Depending upon their dose, three BS treated animal groups were BS-100, 300 and 900 mg/kg. Doses of BS were obtained by suspending BS in 0.5% CMC (carboxy methyl cellulose) and orally administered once a day for 2 weeks. Significant augmentation of the average percentage of aberrant metaphase (AM), CA per cells and suppressed mitotic activity was observed on post administration of BS. In addition, BS increased the occurrence of MNPCEs (micronucleated polychromatic erythrocytes) formation, induced cytotoxicity and increased percentage of abnormal sperms as compared to NC. Moreover, BS also induced oxidative stress as the activities of hepatic superoxide dismutase (SOD) and glutathione (GSH) were reduced and malondialdehyde (MDA) content were increased by BS. Cyclophosphamide was utilized as clastogen, showed anticipated positive results and confirmed the sensitivity of test system. Therefore, it may be deduced from the study that the BS possesses genotoxic effects on mice bone marrow and germ cells *in vivo*.

1. Introduction

Shammah is a variant of smokeless tobacco (ST), which is also marketed in form of chewing tobacco, oral and nasal snuff (Rogers et al., 2010; Alhazmi et al., 2018). Various ST products have collectively been categorized in Group I carcinogens (IARC, 2007). Roughly, 1.3 billion tobacco users are present globally, which account for ≥5 million deaths annually with 12 percent death in the age of 30 years or above (Malovichko et al., 2019). According to the surveys regarding use of current ST products, conducted by Parties since 2007, it was reported that the average global ST prevalence was 6.2% (8.1% of males and 4.4% of females) (WHO, 2018). Use of ST is predominantly prevalent in United States, various European countries, Asia (Yu et al., 2016), and Middle East countries including Saudi Arabia. In case of Saudi Arabia, the use of ST is found more prevalent in southern region, mainly in Jazan province, where it is locally known as shammah (Khalid et al., 2019). Shammah is a locally manufactured preparation of ST, where it may consist of powdered tobacco, black pepper, slaked lime, ash, oils, flavors and other substances (Abdulsallam, 2017). ST is generally used by placing tobacco into the oral cavity, mostly between the lips or cheeks and gums, and ingesting the saliva produced thereafter (Alsanosy, 2014; Brima, 2016). The ST usually consists of leaves of *Nicotiana tabacum*, *N. rustica*, *N. nepalensis* or *N. glauca* (Winter, 2000; Valdes et al., 2010).

* Corresponding author.
E-mail address: ptripathi@jazanu.edu.sa (P. Tripathi).
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Shammah is associated with a range of adverse health issues, including oral malignancies, cancer of esophagus, nasal cavities, lips and most importantly mouth cancer (Yu et al., 2016). Cytotoxicity investigations revealed that shammah promote the proliferation of cancer cells and killing the normal human cells; it could be the reason for many cancers including breast, ovary, and colon cancers (Khalid et al., 2019). According to the previous reports, shammah is one of the major factors in Arabian countries causing oral leukoplakia-like lesions, which over a period of time, increase the chances of cancers (Walsh and Epstein, 2000; Gupta et al., 2012; Al-Tayar et al., 2015). Shammah has also been identified as preventable cause of cardiovascular diseases such as hypertension, coronary heart disease, myocardial infarction and hypercholesterolemia (Alhazmi et al., 2019; Yu et al., 2016; Gupta et al., 2019). Besides, several adverse reproductive outcomes have also been reported including sperm abnormalities, reduction in total sperm count, premature birth and stillbirth (Das et al., 2016; Abdulsallam, 2017). Additionally, it has generally been found to induce micronuclei in oral mucosa (DeMarini, 2004). Recently, yellow shammah was reported to have significant genotoxic potential and found to produce chromosomal abnormalities, induce micronuclei formation in mice and augment various oxidative stress (Alshahrani et al., 2019).

Tobacco Harm Reduction Group suggested a strategic dialogue, that merging the results of preclinical cytotoxicity and genotoxicity, along with other testing such as, analysis of toxic substances, biomarkers of exposure and actual outcomes across various disease factors, is likely to establish the evidence base to reduce risk potential of tobacco products (Zeller and Hatsukami, 2009; Theophilusa et al., 2015). There are four varieties of shammah used in Saudi Arabia, generally categorized according to their color, which include white shammah, yellow shammah, red shammah, and brown shammah (Brima, 2016). Brown shammah is also known as shammah adani that comes in two variants, adani haar (hot) and adani baarid (cold). During analysis, our laboratory identified various toxic and carcinogenic components in several samples of shammah (Alhazmi et al., 2019; Khalid et al., 2019; Alhazmi et al., 2018). Earlier, we conducted the genotoxicity of yellow shammah (Alshahrani et al., 2019) and the interesting results prompted us to investigate the genotoxicity of brown shammah, which is another very popular variety among the users. Since, shammah is an admixture of tobacco with a variety of additives which are added to enhance its organoleptic and stimulant properties, these varieties of shammah differ with each other in various means. Their constituents are very much different and their effect would also be different. A number of harmful carcinogenic constituents are also added to shammah, therefore, we intended to perform the genotoxic studies on brown shammah to assess its carcinogenic potential. To the best of our knowledge, no such study has been performed previously.

2. Material and methods

2.1. Drugs and chemicals

Brown shammah powder was procured from an exclusive supplier of Jazan province, Saudi Arabia. The standard drug cyclophosphamide [Cytoxan; (CAS no.: 6055-19-2)], may-grunwald stain (CAS no. 68988-92-1), giemsa stain (CAS no.: 51811-82-6), colchicine (CAS no.: 64-86-8), bovine serum albumin (CAS no.: 9048-46-8), and eosin Y were purchased from Sigma Aldrich, USA. Other chemicals and solvents used during the experiments were purchased from commercial sources and were of laboratory reagent (LR) grade.

2.2. Animals

Swiss albino mice (6–8 weeks old) of either sex were procured from central animal facility of Jazan University. They were acclimatized in institutional animal house of college of pharmacy, Jazan University, for one week under standard conditions (temperature 22 ± 1 °C, humidity 50–55%, and a 12 h light/dark cycle). Food (commercial mouse pellets) and water were freely accessible to animals. Approval from the institutional animal ethics committee was obtained (approval no.: SARC/EC/79) prior to animal experimentation. All methods were conducted in accordance to the guidelines set by Organization for Economic Cooperation and Development (OECD) and International Conference on Harmonization (ICH) (OECD, 2013, 2015; ICH, 2008).

2.3. Experimental protocol

The experimental protocols used for CA, MN and SA assay were similar. Animals were divided into five groups of 6 animals each, which consisted of two controls and three BS-treated groups. Three doses 100, 300 and 900 mg/kg of BS were selected on the basis of LD_{50}. These dose levels were expected to cover a range from no or little toxicity to maximum tolerated dose (OECD, 2015). Suspension of BS was prepared in 0.5% CMC. Negative and positive control groups received 0.5% CMC once daily for two weeks. Treated animals received different doses of BS orally once a day for 2 weeks. Whereas, animals of positive control group were also administered with CP (40 mg/kg) intraperitonially (i.p.) 24 h before sacrifice.

2.4. Genotoxicity tests

2.4.1. In vivo CA assay

After 24 h of the last BS dose administered, tissue sampling was initiated. All the tested animals were injected with 0.05% colchicine (0.4 mL, i.p.), 90 min before sacrifice in order to arrest cell division in metaphase. Upon death, femurs of animals were dissected immediately, and the bone marrow was flushed out in 0.075 M KCl solution subsequently. The obtained cell suspension was then incubated at 37 °C for a period of 20 min followed by centrifugation (1000 rpm, 10 min) to collect the cells. They were then fixed with methanol/acetic acid (3:1) mixture thrice. The cell suspension thus obtained was burst over on previously coded clean and chilled glass slides, flame-dried and stained using 5% giesma solution to visualize chromosomes. Observations were made by microscope at a magnification of 100X using oil immersion lens (Tijio and Whang, 1962). One hundred cells with well-spread metaphases were scored per animal at random and average percentage of aberrant metaphases was calculated. All aberrations, such as chromatid and chromosomal gaps/ breaks, ring, dicentric ring, deletion, fragmentation, exchange, stickiness, and acentric fragments were considered equal, regardless of the number of breakages involved and reported as average aberrations per cell (CAs/cell) (Tice et al., 1987). Mitotic index (MI) was calculated from 1000 cells/animal and was expressed in percentage (Perez et al., 2008).

2.4.2. In vivo bone marrow MN assay

Bone marrow MN assay was carried out according to the previously reported standard technique (Adler, 1984). After 24 h of the last BS treatment, animals were sacrificed by cervical dislocation. Femurs were dissected and bone marrow was collected in centrifugation tubes using a syringe filled with 0.2 mL of 5% bovine serum albumin. Centrifugation was performed at 1000 rpm for a period of 5 min. Subsequently, supernatant was educed and cells were re-suspended in bovine serum. Smears were prepared on a clean microscopic slide using a small droplet. Slides were air dried and coded before fixing and staining using may-grunwald/giemsa

stains. Microscopic observations were performed at 100X magnification. Slides were scored randomly, and 1000 polychromatic erythrocytes (PCEs) were examined per animal for the presence of micronuclei. The polychromatic vs normochromatic erythrocytes (PCE/NCE) ratio was calculated by counting 1000 erythrocytes in each animal sample. Values were expressed as PCE/NCE ratio out of total erythrocyte counts in order to calculate the decrease in proliferation of erythroblasts (Schmid, 1975).

2.4.3. Sperm abnormality assay

Treated mice were sacrificed using cervical dislocation method followed by removal of cauda epididymis. Cauda were minced in 2 mL of phosphate-buffered physiological saline to prepare the sperm suspensions. To remove tissue fragments, the resulting suspension was pipetted and filtered through muslin cloth. Aqueous eosin Y (1%) was then mixed (10:1) with a fraction of each suspension and smears were made 30 min later. They were then allowed to dry in air and mounted under a coverslip using permount mounting medium. In each animal sample, one thousand sperm were observed for morphological abnormalities (Bruce et al., 1974), including hookless, folded, amorphous, banana shape as well as tail abnormality.

2.5. Determination of hepatic GSH, SOD activities and MDA content

Livers of treated animals were surgically removed and perfused in ice-cold normal saline (0.9% NaCl). The fresh tissue was used to prepare 10% homogenate in 0.1 M Tris HCl buffer (pH 7.4) and was used for the determination of protein content (Lowry et al., 1951). Concentration of MDA in tissue was determined according to the method previously described by Ohkawa et al. (1979). Glutathion concentration was measured according to previously described method by Teitz (1967), and the activities of SOD according to Misra and Fridovich (1972). Reaction products were measured spectrophotometrically.

2.6. Statistical analysis

Data generated for different endpoints of BS treated groups were presented as mean ± SD and compared with their respective NC data. Statistical analysis was performed by using Graphpad Prism software (Version 7.03) through one-way analysis of variance (ANOVA), followed by Tukeys multiple comparison test as a post-hoc test. Differences were examined at significance (*P* value) level of 0.05.

3. Results

3.1. Bone marrow CA assay

The results obtained for CA in dividing bone marrow cells of treated mice are presented in Table 1. Fig. 1a-j shows presence of these aberrations upon administration of BS. It was observed that incidences of CA/cell (0.85 ± 0.14) and average percent of aberrant metaphases (77.25 ± 7.58) significantly increased in case of CP treated positive control animals as compared to NC (0.047 ± 0.02 and 4.09 ± 2.09 respectively), confirming the sensitivity of test system. Interestingly, when given in high doses (900 mg/kg), BS also showed the high frequency of average aberrations per cell (0.41 ±

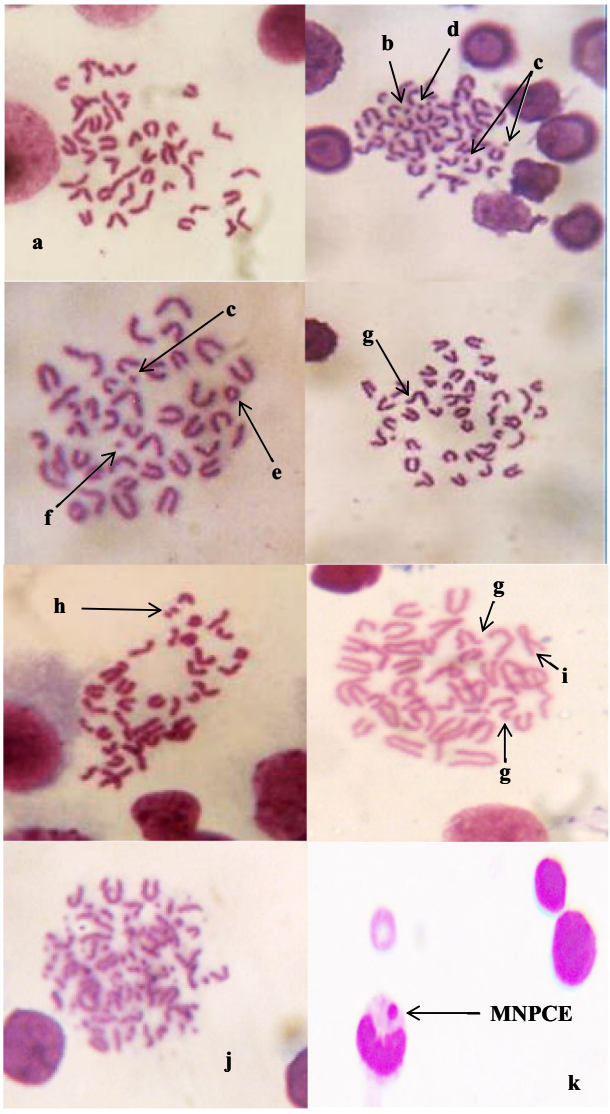

Fig. 1. (a–j) Abnormalities in chromosomes (a) Normal metaphase; (b) chromatid gap; (c) chromatid break; (d) chromosomal break; (e) ring chromosome; (f) deletion; (g) exchange; (h) acentric fragment; (i) dicentric ring; (j) fragmentation; (k) Micronucleated polychromatic erythrocyte.

Table 1
CA test in mice bone marrow cells treated with BS orally for 14 days.

Groups	Dose (mg/kg)	Total Metaphase	Total AM	Avg % of AM	gap	ctb	chb	R	DR	D	Ex	Frg	S	AF	Total aberrations excluding gap	Avg aberrations per cell	MI (%)
NC	–	598	24	4.09 ± 2.09	5	7	5	6	3	1	4	–	1	1	28	0.047 ± 0.02	8.1 ± 1.39
PC	40	602	465	77.25 ± 7.58d	113	127	52	86	69	77	25	21	27	29	513	0.85 ± 0.14d	1.22 ± 0.31[d]
BS-100	100	600	68	11.34 ± 4.97	9	27	14	17	8	13	4	3	4	7	97	0.16 ± 0.07	7.99 ± 1.24
BS-300	300	603	123	20.41 ± 7.31b	21	33	21	24	13	12	17	6	–	3	131	0.23 ± 0.06a	6.22 ± 1.03[a]
BS-900	900	602	217	36.06 ± 7.37d	39	89	27	31	21	22	24	9	13	11	247	0.41 ± 0.12d	3.97 ± 1.19[d]

Data are expressed as mean ± standard deviation (n = 6). [a]P < 0.05; [b]P < 0.01; [c]P < 0.001; [d]P < 0.0001, compared to NC. Abbreviation's: NC, negative control; PC, positive control; AM, aberrant metaphases ctb, chromatid break; chb, chromosomal break; R, ring; DR, dicentric ring; D, deletion; Ex, exchange; Frg, fragmentation; S, stickiness; AF, Acentric fragments; MI, mitotic index.

0.12) and aberrant metaphases (36.06 ± 7.37) as compared to NC. However, a marginal and statistically non-significant increase in average AM (11.34 ± 4.97) and CA/cell (0.16 ± 0.07) were seen at the lowest dose of BS (100 mg/kg), when compared to NC. Similarly, the middle dose of BS (300 mg/kg) resulted in 20.41 ± 7.31 as average percent of AM and 0.23 ± 0.06 average aberrations per cell, which was significantly higher as compared to NC.

Results of bone marrow cells toxicity (MI) are also summarized in Table 1. As evident from the data, in CP treated positive control animals, a highly significant ($P < 0.0001$) decrease in MI (1.22 ± 0.31) was recorded as compared to NC (8.1 ± 1.39). Among treatment groups, greater bone marrow suppression (3.97 ± 1.19) was observed at the highest dose of BS (900 mg/kg) which was significantly higher ($P < 0.0001$) than the NC. In addition, significant reduction ($P < 0.05$) in MI (6.22 ± 1.03) was also recorded in animals pretreated with middle BS dose (300 mg/kg), whereas, lower BS dose (100 mg/kg) did not show any significant reduction in mitotic activity as compared to NC.

3.2. Bone marrow MN assay

Results of bone marrow MN assay are depicted in Table 2. The average MNPCE percentage in NC group was 0.35 ± 0.29. In contrast, CP treated positive control showed 3.12 ± 0.87 MNPCEs which was highly significant ($P < 0.0001$) in comparison to NC. In BS treated groups, augmentation in percentage of MNPCEs were observed for all groups, however, it was non-significant at lower BS dose (100 mg/kg). On the other hand, higher dose of BS (900-mg/kg) showed multifold (2.21 ± 0.52) and highly significant ($P < 0.0001$) increased percent of MNPCEs as compared to NC. Similarly, the observed percentage of MNPCEs at middle dose of BS (300 mg/kg) was 1.22 ± 0.32, which was statistically significant ($P < 0.05$) as compared to NC. Fig. 1k shows the presence of MNPCEs upon treatment with high doses of BS.

Results of bone marrow MN assay also demonstrated the erythropoietic cell toxicity that was measured by determining the PCE/NCE ratio (Table 2). The positive control group showed the expected results of CP as highly significant ($P < 0.0001$) cytoxicity (0.54 ± 0.13) when compared to NC (1.25 ± 0.18). Meanwhile, the highest level of cytotoxicity was observed with BS-900 mg/kg group of animals, where the calculated PCE/NCE ratio was calculated to be 0.64 ± 0.16, which was highly significant ($P < 0.0001$) as compared to NC. Similarly, dose dependent significant ($P < 0.001$ and $P < 0.05$) decrease in PCE/NCE ratio was also noticed for BS-300 and 100 mg/kg, respectively.

3.3. Sperm abnormality assay

Sperm abnormality assay detects morphological changes, reflecting genetic damage in germ cells. Table 3 presents the results of sperm abnormality assay. CP treated positive control animals showed 20.42 ± 3.20 percent of abnormal sperms which was highly significant ($P < 0.0001$) as compared to NC 3.64 ± 0.72. On the other hand, different doses of BS (BS-100 to 900 mg/kg) reported a dose-dependent and statistically significant ($P < 0.05$ to $P < 0.0001$, respectively) increase in sperm abnormalities as compared to NC. The highest level of sperm abnormality (16.30 ± 1.82) was observed with BS-900 mg/kg group followed by BS-300 mg/kg (9.13 ± 1.68). Various sperm abnormalities upon administration of high doses of BS are shown in Fig. 2.

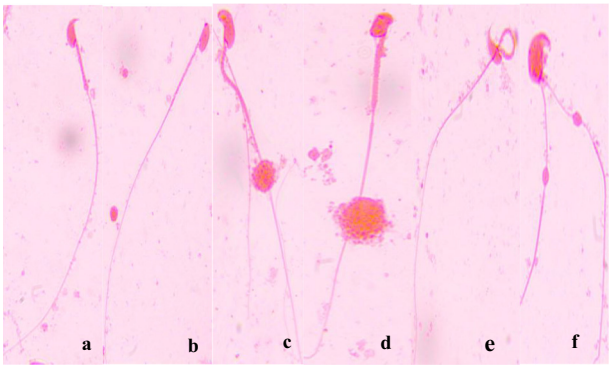

Fig. 2. Normal and abnormal sperms (a) normal; (b) hookless; (c) banana shaped; (d) amorphous; (e) folded; (f) two tailed.

Table 2
Average micronucleated PCEs in 1000 PCEs and the ratio between PCEs and NCEs in bone marrow cells after treatment with BS.

Groups	Dose mg/kg	Individual animal scores/1000 PCEs	% MNPCE (mean ± SD)	PCE/NCE (mean ± SD)
NC	–	1,7,5,0,6,2	0.35 ± 0.29	1.25 ± 0.18
PC	40	31,27,39,24,44,22	3.12 ± 0.87d	0.54 ± 0.13[d]
BS-100	100	7.8.3.7.11,6	0.72 ± 0.27	0.95 ± 0.16[a]
BS-300	300	13,10,9,12,11,18	1.22 ± 0.32a	0.79 ± 0.17[c]
BS-900	900	29,18,23,17,25,21	2.21 ± 0.52d	0.64 ± 0.16[d]

[a]P < 0.05; [b]P < 0.01; [c]P < 0.001; [d]P < 0.0001, compared to NC. Data are expressed as mean ± standard deviation (n = 6). Six animals per group (representing a total of 6000 PCEs) were analyzed for the presence of MNPCE and also for the ratio PCE/NCE.

Table 3
Average percent of abnormal sperms after treatment with BS.

groups	Dose mg/kg	Normal	total abnormal sperms	hookless	banana	amorphous	folded	two tailed	Average % of abnormal sperms
NC	–	5782	218	119	35	47	14	3	3.64 ± 0.72
PC	40	4775	1225	753	136	241	79	16	20.42 ± 3.20[d]
BS-100	100	5567	433	249	85	67	31	1	7.22 ± 2.13[a]
BS-300	300	5452	548	308	111	82	42	5	9.13 ± 1.68[c]
BS-900	900	5022	978	567	163	153	84	11	16.30 ± 1.82[d]

[a]P < 0.05; [b]P < 0.01; [c]P < 0.001; [d]P < 0.0001, compared to NC. Data are expressed as mean ± standard deviation (n = 6). Six animals per group (representing a total of 6000 sperm cells) were analyzed for the presence of abnormality.

Table 4
Effects of BS on hepatic GSH, SOD and MDA in mice.

Groups	Dose mg/kg	SOD (U/mg protein)	GSH (nM/mg protein)	MDA (nM/mg protein)
NC	–	8.15 ± 0.99	0.082 ± 0.015	3.09 ± 0.45
PC	40	3.44 ± 1.43d	0.031 ± 0.005d	7.45 ± 1.24[d]
BS-100	100	6.49 ± 1.09	0.062 ± 0.009a	4.21 ± 0.79
BS-300	300	5.97 ± 1.25b	0.057 ± 0.015b	4.94 ± 1.21[b]
BS-900	900	4.58 ± 1.12d	0.044 ± 0.014d	6.55 ± 0.83[d]

Data are expressed as mean ± standard deviation (n = 6). [a]P < 0.05; [b]P < 0.01; [c]P < 0.001; [d]P < 0.0001, compared to NC.

3.4. Determination of GSH, SOD activities and MDA content

The standard CP significantly reduced ($P < 0.05$ to $P < 0.0001$) the activities of GSH and SOD and increased the MDA content as observed in PC in comparison to NC (Table 4). BS administered groups (BS-300 and 900 mg/kg) also denoted an understandable decline in the activities of SOD and GSH ($P < 0.01$ to $P < 0.0001$). Nevertheless, in BS-100 mg/kg treated group, this reduction was non-significant for SOD but slightly significant for GSH ($P < 0.05$) activities. Also, the MDA contents elevated significantly in BS-300 and 900 mg/kg groups ($P < 0.01$ to $P < 0.0001$), but was non-significant with the lower dose (BS-100 mg/kg) when compared to NC.

4. Discussion

This study is first of its kind that investigated the genotoxic effects of BS. Five commonly used *in vivo* assays were select to detect the genotoxicity of BS on mice bone marrow and germ cells. The assessed end points in the present study clearly demonstrated the genotoxicity of BS in the murine test system. The highest toxicity was found with the BS-900 mg/kg dose whereas BS-100 mg/kg did not show any significant genetic toxicity in all test systems except in germ cells.

The results of CA assay clearly indicated the clastogenicity of BS, as evident from the subsequent increase of the frequencies of CAs and percentages of AM in 300 and 900-mg/kg treatment regimens. Highest dose of BS (900 mg/kg) demonstrated highest level of clastogenic activity, whereas, lowest dose did not produce statistically significant effect on bone marrow cells. Similarly, BS-300 and 900 mg/kg treated groups, the mitotic activity was significantly decreased, suggesting inhibition of the cellular proliferation in mice bone marrow cells (Tripathi et al., 2013a). In addition, increased percentage of MNPCEs at the similar dose levels of BS, indicating the breakage of chromatid and chromosomes forming acentric fragments due to 2 weeks BS treatment, which resulted in micronuclei formation (ICH, 2011). PCE/NCE ratio is a measure of cytotoxicity, which is calculated in micronuclei test. Brown shammah induced cytotoxicity as the PCE/NCE ratio decreased with increase in dose, which indicated that BS affected the healthy proliferation of bone marrow cells resulting in cytotoxicity (Tripathi et al., 2013a). This was in agreement with the previously reported cytotoxicity of BS by Khalid et al. (2019).

Genotoxic effects of any agent on the germinal cells give information about transmissible genetic damage from one generation to another (Au and Hsu, 1980; Tripathi et al., 2013b). Since, intensive use of ST affects normal sperm morphology more than sperm count and motility (Said et al., 2005), abnormal sperm morphology leads to infertility or causing irreversible epigenetic changes in future offspring (Sunanda et al., 2014). Data of the sperm abnormality test revealed that pretreatment with BS at different dose levels increased the morphological changes in sperm cells. This may be because of interference of BS or its constituents, with the genetically controlled differentiation process or the consequence of an abnormal chromosome complement (Tripathi et al., 2013b).

CP was used as positive reference standard in order to establish the efficacy of genetic toxicology test and to optimize the test system (OECD, 2015). In this study, the induction of significantly high percentages of CAs, aberrant metaphases, cytotoxicity, micronuclei formation, in mice bone marrow, and abnormal sperm by CP was in accordance with its previously reported clastogenicity (Alshahrani et al., 2019; Tripathi et al., 2013a, 2013b). CP is a bi functional alkylating agent which forms DNA adducts or cross-links DNA and proteins or cross-links two DNA bases either within one DNA strand (intra-strand cross-links) or on different DNA strands (inter-strand cross linkings). Repair of such damage, results in the indirect induction of DNA double-strand breaks (Kondo et al., 2010; Tripathi et al., 2019). In addition, pro-oxidant character of CP barges it into the tissue antioxidant defense system, producing highly reactive oxygen free radicals which lead to genotoxicity (Alshahrani et al., 2019; Kour et al., 2017; Tripathi et al., 2013a). The results obtained for CP in this study supported the above hypothesis as it suppressed GSH and SOD levels, as well as increased MDA content, leading to genotoxicity. Similarly, studies on antioxidant markers revealed pro-oxidant character of BS. The results obtained for BS were similar to CP as increased level of MDA, decreased levels of GSH and SOD were noticed which can be positively correlated to the expected toxicity mechanism of BS.

Tobacco has been reported to possess many hazardous chemicals which increase the risk of clastogenic cancer. Extensive research has been carried out in our laboratory on different varieties of shammah. Khalid et al. (2019) reported the presence of nicotine in different samples of BS ranging from 55.04% to 87.56%. Furthermore, Alhazmi et al. (2019), detected the phytocomponents of 21 different varieties of shammah using gas chromatography-mass spectroscopy (GC–MS) and reported the presence of nicotine in high concentration along with other components such as tridecane, pentadecane, 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-ris(trimethylsiloxy)tetrasiloxane and 13-docosenamide as toxic /carcinogenic components in BS (shammah baarid). Moreover, elemental profiling was also performed and presence of various elements were detected in order Sr > Mn > Zn > Ba > Li > Cu > Rb > Pb > U > Ti > Be > As > Cd as major elements in shammah baarid (BS) (Alhazmi et al., 2018), which are mainly present due to various additives in shammah.

Although, the exact mechanism behind genotoxicity of BS is yet to be discovered, role of nicotine and other elements cannot be ignored. Nicotine present in ST absorbs 3–4 times faster via buccal mucosa than smoking. The level of nicotine increase gradually with intensive practice of chewing and hence the residual chemicals continue to exist for extended period compare to smoking (Sunanda et al., 2014). Several studies proved the genotoxicity of nicotine and suggested strong DNA damage due to high concentration nicotine which is related to its pro-oxidant character. This pro-oxidant property of nicotine is due to increased activity of cytochrome P-450 2A6 system which generates free radicals during the metabolism of nicotine enantiomers within the cell and nico-

tine high doses. It has been reported that nicotine increases superoxide anions and peroxide production by disrupting the mitochondrial respiratory chain. This leads to decreased GSH, SOD levels and increase MDA content (Sobkowiak and Lesicki, 2009; Sassen et al., 2005; Hukkanen et al., 2005). It can be correlated to our study where we took the high dose of BS in order to detect the genotoxicity if any, and found that BS also decreased GSH, SOD levels and increased MDA content in the similar manner. In addition, presence of heavy metals, such as Sr (Çelik et al., 2011), As (Faita et al., 2013; Yedjou and Tchounwou, 2006), Cd (Mouchet et al., 2007; Tchounwou et al., 2001), Cr (De Flora et al., 1990; Patlolla et al., 2009), and Pb (García-Lestón et al., 2010; Yedjou and Tchounwou, 2007; Tchounwou et al., 2004), may also be the reason for carcinogenic effects due to their capability to produce ROS in biological systems, might be contributing to the genotoxic effects of BS.

5. Conclusion

In conclusion, BS was found capable of inducing genotoxicity in bone marrow and germ cells of mice at higher doses. This genotoxic effect of BS was based on its ability to generate ROS that may lead to enhance lipid peroxidation and other cell-damaging effects including membrane and DNA damage. Since BS is a complex mixture containing many hazardous constituents such as nicotine and heavy metals ions, which may play vital role in induction of genotoxicity. Nevertheless, further investigations are required to have more insight.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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