

Assessment of genotoxic potential of herbomineral preparations – bhasmas

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The aim of this study was to generate and evaluate genotoxic data for herbomineral preparations (bhasmas), viz. *Abhrak* (mica) bhasma, *Mandura* (iron) bhasma, *Swas Kuthar ras* (mercury, sulphur) and *Smrit Sagar ras* (mixture of metals), using *in vivo* micronucleus (MN) assay and comet assay in Wistar rats of both sexes. No significant increase in MN frequency or DNA damage percentage was recorded in the bhasma-treated animals compared to the vehicle control groups in both sexes, indicating that the bhasmas tested were non-genotoxic under the experimental conditions and the test system employed.

Keywords: Bhasmas, comet assay, genotoxicity, herbal medicine, *in vivo* micronucleus assay.

AYURVEDA is a well-known traditional medicinal system practised in India for several centuries¹. Herbal medicines are being used by about 80% of the world's population, mainly in the developing countries, for primary health care². It prescribes the use of various plant parts such as leaves, roots, herbs, oils, common spices and other naturally occurring substances for curing a variety of ailments³⁻⁵. Several Western scholars have worked on the analysis of Indian plants and herbs for their therapeutic properties⁶. 'Bhasmas' (ash) commonly integrate heavy metals into primary herbal formulations, usually for their endorsed medicinal properties and to enhance potency, as recently defined by the World Health Organization (WHO)⁷. Several metals are found in ayurvedic remedies such as lead, copper, mercury, arsenic, iron, mercury, etc. which play a vital role in the biochemical process^{8,9}. Ayurvedically prepared bhasmas are considered to be more potent than any other healing preparations as they do not react with the tissues of the body¹⁰.

Information about the metal content of ayurvedic preparations is currently becoming significant. The Department of AYUSH, Ministry of Health and Welfare, Government of India has come out with a notification of permissible limits for four heavy metals (arsenic, lead, mercury and cadmium) and to regulate issues related to quality, safety, efficacy and practice of herbal medicines¹¹. With respect to safety, traditional remedies will definitely need to be assessed with the same parameters now used for modern pharmaceutical, nutraceutical and cosmeceutical products¹². WHO has accepted that tradi-

tional medicines may need less rigorous preclinical toxicological evaluations since their safety of use has been documented historically⁷. Although bhasmas have been used as effective drugs for centuries without any noticeable side effects, certain factors related to their preparations have not been explored. Though the materials have been well-established drugs, their current research is limited to the study based on an ayurvedic point of view.

Considering the lacuna in the safety evaluation of such preparations, we attempted to study genotoxic effects of a few preparations. Our previous studies with *Ras Manikya ras*, *Lauha* bhasma, *Tamra* bhasma and *Kajjali* bhasmas revealed no apparent genotoxic effects in Wistar rats using the micronucleus (MN) and comet assays¹³. The present study is an extension of the same with more bhasmas, viz. *Abhrak* bhasma, *Mandura* bhasma, *Swas Kuthar ras* and *Smrit Sagar ras*. The techniques involved mammalian erythrocyte micronucleus test¹⁴ and comet assay¹⁵. The current study would serve as a database of baseline information for genotoxicity of bhasmas, since there is apparently no literature on this aspect of herbomineral preparations.

MN assay was conducted according to the OECD guidelines for testing of chemicals (Section 4, No. 474, adopted 21 July 1997)¹⁶. The comet assay was performed following the procedure of Singh *et al.*¹⁵, with slight laboratory modifications.

Abhrak bhasma, *Mandura* bhasma, *Swas Kuthar ras*, and *Smrit Sagar ras* were obtained commercially. The other chemicals included cyclophosphamide (Sigma, USA). Foetal bovine serum (Gibco), DMSO (Sigma), normal melting agarose (Sigma), low melting agarose (Sigma), Tris base (Hi Media), Triton X 100 (Hi Media), ethidium bromide (Sigma) and corn oil.

The test system is described in Table 1.

The animals were identified uniquely and kept in cages for at least 5 days prior to the start of the study to allow for acclimatization to the laboratory conditions. The total number of animals was 60 (30 males + 30 females). The total number of groups was six and the number of animals/dose was 10 (5 males + 5 females) (Table 2).

The test facility temperature was maintained between 19°C and 25°C and relative humidity between 45% and 60%. The facility was provided with 12 h light and 12 h

Table 1. Test system

Species	: <i>Rattus norvegicus</i>
Strain	: Wistar
Source	: From the animal house facility at the International Institute of Biotechnology and Toxicology, Padappai
Body weight of the animal before start of experiment (at dosing)	: 120–140 g
Age of the animals	: 6–8 weeks

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Table 2. Treatment schedule

Group	Treatment	Schedule	No. of rats	Sacrifice time-point (h)
1	Vehicle control* corn oil (1 ml/100 g body wt)	Single	5M + 5F	24
2	Positive control cyclophosphamide (25 mg/kg body wt)**	Single	5M + 5F	24
3	<i>Abhrak</i> bhasma* (2000 mg/kg body wt)	Single	5M + 5F	24
4	<i>Mandura</i> bhasma* (2000 mg/kg body wt)	Single	5M + 5F	24
5	<i>Swas Kuthar ras</i> * (2000 mg/kg body wt)	Single	5M + 5F	24
6	<i>Smrit Sagar ras</i> * (2000 mg/kg body wt)	Single	5M + 5F	24

M, Male; F, Female. *Oral route; **Intraperitoneal route.

dark condition, controlled by an automatic timer. The temperature and relative humidity were recorded.

Standard polypropylene rat cages with stainless-steel top grill (supplied by M/s Vishnu Traders, Uttar Pradesh, India) were used to house the animals. The cages, grills and water bottles were autoclaved. Sieved and sterilized paddy husk was used as the bedding material. The animals were housed in groups according to their sex in cages, with each cage containing five animals.

Gamma-irradiated rodent pellet feed was supplied and reverse osmosis water was provided to the animals. Both drinking water and feed were provided *ad libitum*.

The bhasmas were prepared by vortexing thoroughly with appropriate volume of vehicle (corn oil).

The oral route of administration was used because it is recommended in the referenced guidelines and because human exposure to ayurvedic bhasmas occurs via this route.

A rat MN test was performed to detect genotoxic and cytotoxic potential of the above-mentioned bhasmas in *in vivo* mouse MN assay by assessing the induction of MN in polychromatic erythrocytes (PCE) in bone marrow cells. The data generated were considered adequate if at the initiation of the study, the weight variation of the animals did not exceed $\pm 20\%$ of the mean weight of each sex and the test system was sensitive to the known mutagens, as reviewed by the results in the concurrent positive control animals. After 24 h of dosing, the animals were sacrificed by cervical dislocation. Both the femurs from each animal were rapidly dissected out and cleaned to remove the adherent tissue. The epiphysis was removed to obtain access to the bone marrow canal. Bone marrow cells were flushed out with 3 ml foetal bovine serum and the recovered cells were centrifuged at 1600 rpm for 5 min. The bulk of the supernatant fluid was discarded and the cell pellet was resuspended in the remaining fluid. Smear was prepared by placing a single drop of the cell suspension on the slides and the slides were air-dried. The slides were then fixed in methanol, stained with Giemsa and mounted. With the oil-immersion objective, 2000 immature PCE were scored for the presence MN, which is defined as round, darkly staining nuclear fragment indicating chromosome damage. For evaluating any cytotoxicity induced by the bhasmas, the proportion of PCE to normo chromatic erythrocytes was determined in 200 cells for each animal in all treatment groups. The

data were subjected to statistical analysis by Mann-Whitney *U* test.

The alkaline comet assay is a short-term genotoxicity assay for rapid detection of DNA damage in almost any type of cell. In the present investigation, peripheral blood from the treated and control groups was collected and processed for comet assay. Briefly, 10 μ l of the sample was mixed with 100 μ l of low melting agarose (Sigma) and applied onto pre-coated microscopic slides. After solidification, the slides were treated with lysis buffer for 1 h, exposed to an alkaline solution (pH > 13) for 20 min and electrophoresed in the same buffer for 30 min. Following this, the slides were treated with neutralization buffer (Tris buffer, pH 7.2) and stained with ethidium bromide (2 μ g/ml). Hundred nucleoids per animal (50/slide) were analysed visually and categorized into one of the five degrees of damage (0 – undamaged, 1 – mild damage, 2 – moderate damage, 3 – severe damage, 4 – complete damage), according to Collins *et al.*¹⁷. The percentage of damaged nucleoids was calculated; an arbitrary unit (AU) was used to express the extent of DNA damage.

$$AU = \sum_{i=0}^4 n_i \times i,$$

where n_i is the number of cells with damage degree i (0, 1, 2, 3 or 4). Chi-square test was used to understand the statistical significance of the results obtained. *P* value was set at 0.05.

Table 3 represents the results of the MN assay. Corn oil was used as the vehicle control. Cyclophosphamide at 25 mg/kg body wt was used as the positive control. $P < 0.05$ represented statistically significant values as evaluated by Mann-Whitney *U* test.

Table 3 shows the ratio of PCE and the normo chromatic erythrocytes. No significant change in the treated versus the control groups (corn oil) was observed, indicating that the dose (2000 mg/kg body wt) was non-cytotoxic. Cyclophosphamide – the positive control chemical – is a covalent DNA-binding agent¹⁸. Its cytogenotoxicity has been reviewed and updated by Anderson¹⁹, and its use as a positive control chemical in genotoxicity tests has been recommended²⁰. In the present study cyclophosphamide (25 mg/kg body wt) indu-

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Table 3. Results of mammalian erythrocyte MN test in Wistar rats

Treatment group	Mean body weight (g ± SD)	Dose	Sex	% Mean MN PCE ± SD	% PCE
Vehicle control (corn oil)	129.2 ± 3.35	10 ml/kg body wt	M	0.10 ± 0.00	1.70
	131.2 ± 2.17		F	0.11 ± 0.02	1.94
<i>Abhrak bhasma</i>	131.6 ± 3.44	2000 mg/kg body wt	M	0.10 ± 0.04	1.76
	127.4 ± 7.16		F	0.11 ± 0.05	1.90
<i>Mandura bhasma</i>	134.2 ± 4.81	2000 mg/kg body wt	M	0.08 ± 0.03	1.36
	130.8 ± 4.43		F	0.11 ± 0.04	1.84
<i>Swas Kuthar ras</i>	128.6 ± 2.07	2000 mg/kg body wt	M	0.09 ± 0.07	1.68
	131.0 ± 2.24		F	0.11 ± 0.05	1.88
<i>Smrit Sagar ras</i>	133.6 ± 2.70	2000 mg/kg body wt	M	0.09 ± 0.04	1.61
	125.0 ± 5.43		F	0.09 ± 0.04	1.51
Cyclophosphamide	132.2 ± 3.27	25 mg/kg body wt	M	1.26 ± 0.55*	23.52*
	128.2 ± 5.26		F	1.38 ± 0.23*	24.22*

PCE, Polychromatic erythrocytes; % PCE, Quotient of micronucleated PCE to total PCE × 100.

* $P < 0.05$; MN, Micronucleus.

Table 4. Results of comet assay

Sample	Sex	Mean number of cells in each degree of damage					Damage (%)	DNA damage score*
		0	1	2	3	4		
Vehicle (corn oil)	Male	92.2 ± 1.92	5.8 ± 2.04	2 ± 1.58	0.0 ± 0.00	0.0 ± 0.00	7.8	9.8
	Female	91.2 ± 1.30	7.4 ± 0.89	1.4 ± 1.67	0.0 ± 0.00	0.0 ± 0.00	8.8	10.2
<i>Abhrak bhasma</i>	Male	95.6 ± 1.95	4.2 ± 1.64	0.2 ± 0.45	0.0 ± 0.00	0.0 ± 0.00	4.4	4.6
	Female	94.6 ± 2.61	4.4 ± 3.51	1.2 ± 1.64	0.8 ± 1.30	0.0 ± 0.00	6.4	9.2
<i>Mandura bhasma</i>	Male	93.4 ± 2.07	4.6 ± 1.34	1.8 ± 1.09	0.2 ± 0.44	0.0 ± 0.00	6.6	8.8
	Female	9 ± 2.45	4.2 ± 3.11	2.8 ± 0.84	0.0 ± 0.00	0.0 ± 0.00	7	9.8
<i>Swas Kuthar ras</i>	Male	96.0 ± 1.87	3.4 ± 1.14	0.6 ± 0.89	0.0 ± 0.00	0.0 ± 0.00	4	4.6
	Female	94.8 ± 1.79	4.0 ± 1.58	0.8 ± 0.84	0.4 ± 0.55	0.0 ± 0.00	5.2	6.8
<i>Smrit Sagar ras</i>	Male	93.6 ± 2.88	5.0 ± 1.22	1.0 ± 1.41	0.4 ± 0.89	0.0 ± 0.00	6.4	8.2
	Female	94.8 ± 2.28	4.4 ± 2.97	0.6 ± 0.89	0.2 ± 0.45	0.0 ± 0.00	5.2	6.2
Cyclophosphamide	Male	25.2 ± 1.79	3.2 ± 2.16	10.0 ± 1.22	27.4 ± 2.70	34.2 ± 3.27	74.8**	241.4
	Female	24.4 ± 2.51	2.0 ± 1.58	10.0 ± 1.41	26.2 ± 1.30	37.4 ± 1.82	75.6**	250.2

*Expressed in arbitrary units; ** $P < 0.05$ (chi-square test: over control).

ced significant increase in the number of micronucleated PCE. No statistically significant increase in MN frequency with respect to the control values was recorded in the vehicle control and treatment groups.

Table 4 shows the distribution of post-lysis nucleoids in the various degrees of damage in different groups, the percentage DNA damage and the DNA damage score, expressed in AU. The values represent mean of five animals scored (100 nucleoids/animal) and the standard deviation (SD) was recorded. The percentage DNA damage was calculated as follows: (Number of nucleoids in degree of damage 1 + 2 + 3 + 4)/100*100. AU was calculated as described previously and the parameters were statistically analysed using chi-square test. There was no statistically significant increase in the percentage of DNA damage or the DNA damage score (AU) in the treated groups over control, indicating that the Bhasmas tested do not induce DNA strand breaks in the alkaline comet assay.

Ayurveda was perhaps the first and still remains the only system of medicine, where metals have been mainly used as bhasmas, for the treatment of many chronic ailments since the 7th century BC. Most of the ayurvedic preparations are 'herbo-metallic' in nature, that is, they

contain minerals and metals as an integral part of their formulations (bhasmas). Use of metals in medicine is often associated with toxicity²¹, but they are made biocompatible in a particular chemical form by a detoxification process which removes the toxic potential from metals and imparts them with therapeutic efficacy of a high grade²². The use of bhasmas as potential drugs is facilitated mainly because of two reasons. First, because these materials are being routinely used as effective drugs for centuries and secondly, these drugs do not show any noticeable side effects with the recommended doses. In earlier times, the quality of the herbs and herbal preparations was not subjected to much review^{23,24}. Increased popularity of herbal medicines has also brought concerns and fears regarding the quality, efficacy and safety of the raw materials. In spite of the existence and use of traditional medicines over many centuries, the safety, efficacy and batch-to-batch consistency of herbal formulations are not up to the mark, to meet the criteria needed to support their use worldwide²⁵. Recently, a study conducted on ayurvedic medicines by the Harvard Medical School²⁶ also reported that some of these drugs had potentially harmful levels of lead and mercury. Therefore, herbal

Table 5. Bhasmas evaluated in the present study and their uses

Bhasma	Ingredient/description	Use
<i>Abhrak</i>	Mica	Hepatoprotective activity
<i>Mandura</i>	Iron	Nourishes blood and enhances vigour, and its astringency prevents blood from becoming too hot or too fluid, important role in oxygen transport
<i>Swas Kuthar ras</i>	Mercury, sulphur	Antispasmodic, diaphoretic, gastric stimulant indicated in case of asthma, fever, tonsillitis, painful neuralgic affections and as a restorative
<i>Smrit Sagar ras</i>	Mixtures of various bhasmas	Nervine stimulant, alternative and tonic. Epilepsy, Mania and weakness of memory

preparations and bhasmas need stringent quality control of the finished products, as many of them contain inorganic elements such as arsenic, mercury and lead, which are known to be highly toxic^{27,28}. Most herbal preparations and, in particular, bhasmas, are not subjected to scientific quality control and safety protocol. There are no well-established/documentated reports on the variation in the amount of major constituent elements. Therefore, strict quality control of the finished products is needed, as most of them contain inorganic elements in concentrated forms¹⁰. Several workers have pursued analysis of bhasmas for the characterization of various elements associated with the main constituent as well as other trace elements²⁹.

All the four bhasma preparations tested hold traces of lead, cadmium, mercury and arsenic according to their preparation protocols in Vedic literature. We did not quantitatively analyse the metal content of these preparations, since the objective was to assess if the mere presence of heavy metals, although in traces, which are calculatedly included to enhance the therapeutic efficiency, can be genotoxic in the mammalian system.

MN and comet assays were employed to fulfil this objective. Analysis of MN is a classic end-point for genotoxic exposure and effect¹⁵. It is expected to be a more sensitive technique than chromosomal aberration due to increased statistical power brought by increase in the number of cells analysed and scored. Accurate estimate of MN frequency can be obtained from the first post-mitotic interphase after exposure. The ability of MN assay lies in the detection of both clastogenic and aneugenic effects³⁰. The comet assay can detect DNA damage expressed as single-strand breaks and alkali-labile sites. The major advantages of the alkaline comet assay are its versatility and sensitivity. Hartmann *et al.*³¹ elucidated the predictive potential of the comet assay in comparison to other *in vivo* biological end-points of analysis, and recommended that this assay can be used for regulatory acceptance.

Our earlier reports on genotoxicity of bhasmas using MN and comet assays¹³ showed lack of MN induction and DNA damage. All the mean values of MN frequency were comparable with the corresponding negative control. In the present study, we have analysed four bhasmas which are mica-based (*Abhrak* bhasma), iron-based (*Mandura* bhasma), mercury and sulphur-based (*Swas Kuthar ras*) and several mixtures of metals (*Smrit Sagar*

ras) for their genotoxic effects. None of the bhasmas induced structural or numerical chromosomal damage in the immature erythrocytes of rat and are considered to be non-genotoxic in the mammalian erythrocyte MN test. Table 5 lists the names of the four bhasmas and their uses as described in the literature^{10,32,33}.

Abhrak bhasma is prepared by treating biotite (mica) with the juices of a number of reconstitute plants that make it a powerful cellular regenerator. It is a commonly used ayurvedic drug against many diseases, including hepatitis. It is also a nervine tonic and is widely used in respiratory-tract infections and anaemia. It contains iron, magnesium, potassium, calcium and aluminium in traces. *Abhrak* bhasma is an amorphous, powdery drug. Mica mainly contains iron sulphate, iron and copper oxide. *Abhrak* bhasma-treated animals that were exposed to CCl₄ showed marked improvement in enzyme profile³⁴. No biologically relevant increase in MN was found and also this bhasma did not illustrate any increase in DNA damage.

Mandura bhasma is an iron-based ayurvedic preparation containing iron, iron oxide and magnesium as active ingredients. Pandit *et al.*³² have evaluated the chemical and pharmacological action of different ayurvedic preparations containing iron. *Mandura* bhasma is prepared by purifying and calcinating iron rust. It is especially useful in anaemia, amenorrhoea, dysmenorrhoea, menorrhagia, chlorosis, and hepatic and splenic disorders. It is also used in diarrhoea, chronic bowel complaints, dyspepsia, intestinal worms, nervous system diseases, neuralgia, kidney diseases and albuminuria. It is a powerful hematinic and tonic, and is valuable in the treatment of hemolytic jaundice and microcytic anaemia. Iron nourishes the blood, and enhances vigour, and its astringency prevents blood from becoming too hot or too fluid³⁵. It is an essential element, that plays an important role in oxygen transport. Patil *et al.*³⁶ studied curative effects of *mandura* bhasma on liver and kidney of albino rats and noticed total recovery in two weeks. In our studies there was no incidence of MN or DNA damage induced by this bhasma.

Swas Kuthar ras is used as an antispasmodic, diaphoretic and gastric stimulant. It is also indicated in case of asthma, fever, tonsillitis painful neuralgic affections and as a restorative. As an herbal formulation containing mercury and sulphur, it was tested for genotoxic potential as mercury is primarily an environment contaminant and industrial hazard known for its toxicity, causing mina-

mata disease³⁷. It is said to be toxic in any form, the difference lying only in how it is absorbed, the clinical signs and symptoms, and the response to treatment modalities. It has been suggested that mercury can cure all diseases if it is properly prepared and used. In the present study, there was no incidence of genotoxicity induced by this bhasma in the MN or the comet assay.

Smriti Sagar ras contains *Kajjali*, *Harital*, *Swarna makshik* bhasma and *Tamra* bhasma. It is a nervine stimulant, alternative and tonic. It is used in epilepsy, mania and weakness of memory. *Smriti Sagar ras* did not induce MN formation or an increase in the percentage of DNA damage, showing its safety upon consumption.

The results of the current study point to the fact that the bhasmas tested are not genotoxic, as they do not exhibit clastogenic or aneugenic response in the *in vivo* bone marrow MN assay, nor did they induce strand breaks as evidenced by the alkaline comet assay. Our findings support the safety of the tested bhasmas from the genotoxic point of view. However, further studies on other parameters of toxicity are encouraged to support this observation.

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