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Genotoxic and Mutagenic Activity of Suvarna Bhasma

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Abstract

Genetic toxicity and mutagenicity of Suvarna Bhasma (SB), an Ayurvedic drug, was determined using a battery of tests. The results of In-vivo Micronucleus assay and COMET assay did not reveal any significant increase in % Micronucleus frequency(MN) in bone marrow cells of mice and DNA damage in blood lymphocytes respectively after the oral administration of SB at various concentrations (3,-30 mg/kg bw) in treated animals as compared to vehicle control in either sex. The in-vitro chromosome aberration (CA) assay carried out with and without metabolic activation at different concentrations of SB in human lymphocyte culture did not cause any effect on structural or numerical chromosome aberrations. Suvarna Bhasma did not induce any mutagenic activity in presence and absence of S9 fractions in Ames assay employing three strains of salmonella typhimuriumTA98, TA100 and TA102.These results demonstrated that Suvarna Bhasma preparation evaluated in this study is not genotoxic and mutagenic at the concentrations tested under the experimental conditions.

Keywords: Suvarna Bhasma, Genotoxicity, Mutagenicity, Micronucleus Assay, COMET Assay, Chromosome Aberration Assay, Ames Assay

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1. Introduction

SuvarnaBhasma is an eminent ayurvedic product with well documented therapeutic benefits in traditional Indian literature [1].It is comprised of ash of purified (*shodhit*) gold. It is one of the supreme adaptogenics and therefore is used singly as well as in combination of other ingredients of herbal, mineral and animal origin.Suvarna Bhasma possesses aphrodisiac, spermatogenic, anti-aging, adaptogenic, cardiotonic properties[1] and also used in clinical conditions like bronchial asthma, rheumatoid arthritis, diabetes mellitus, and nervous system diseases[5-9].

In view of increased interest in the traditional medicines, the regulatory authorities have renewed their strategies for herbal drugs development and discovery[2]. World Health Organization (WHO) is keen regarding usage of traditional medicine and has been active in creating strategies, guidelines and standards of botanical medicines [2-3]

The efficacy of Suvarna Bhasma in various therapeutic ailments has made it a point of attraction for the researchers. The study on the rodents has revealed the safety of Suvarna Bhasma when administered orally for 8 weeks[4]

It is now well established that genetic perturbations, both gene mutations and chromosomal anomalies, contribute to reproductive failure, genetic disorders, onset of diseases at later age and polygenic conditions. Hence, evaluating the effects of Suvarna Bhasma on genetic machinery is essential as a part of the safety assessment process. Currently, no data is available on genetic toxicity of Suvarna Bhasma. Hence the present study was carried out to determine genetic toxicity and mutagenicity of Suvarna Bhasma, if any.

Genetic toxicity[15] of test substance can be evaluated using an in-vitro and in-vivo tests designed to detect compounds that induce genetic damage by various mechanisms. These tests dealwith the identification of

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DNA damage by revealing the form of gene mutations[11] large scale chromosomal damage/aberrations, or recombination. A single test system cannot give the information for universal detection of all the relevant genotoxic effects of Suvarna Bhasma, hence a battery of tests were selected as per Schedule Y and tests were performed as per OECD guidelines[12-13].

2. Materials and Methods

Chemicals: Dimethyl Sulphoxide, 2-aminoanthracene,4 nitro-o-phenylenediamine (4NOPD), sodium azide, ethanediol (ED), mitomycin C (MMC), ethidium bromideand cyclophosphamide were obtained from Sigma Aldrich, Germany. RPMI-1640 media and fetalbovine Serum,S9 mix, low-melting point (LMP) and normal melting point (NMP) agarose were obtained from Gibco BRL _MD, USA.Giemsa stain, May–Grunewald stain, colchicine powder from HiMedia (Mumbai). The Salmonella typhimurium strains used for Ames assay were procured from American Type Culture Collection (ATCC). All other chemicals used were purchased from Merck.

Test Material (Suvarna Bhasma): The test substance "SuvarnaBhasma (SB)" (batch no: .P110600209) was obtained from renowned manufacturer of ayurvedic products Shree Dhootapapeshwar Limited, Panvel - 410 206. SB was a light brown colored powder, stable at room temperature and stored in amber colored glass bottle.

Animal handling and care: Healthy Swiss adult male and female mice (8 weeks of age, bodyweight≈25g) that were randomly bred at the institutional animal house wereused for the study. The animals were kept in cages with autoclaved paddy husk forbedding and maintained under standard laboratory conditions (14 h: 10 h dark/ light cycle, a temperature of (22±2 °C), and 50–70% humidity). The animals were fed adiet of soy-free, in-house-prepared pellets (consisting of crude protein, fiber and nitrogen free extract) and water (purified by UV and reverse osmosis) ad libitumthroughout the study. The quality of food and water provided was routinely monitored by qualitative and quantitative analysis. The ethical clearance for the use of animals in the study was obtained from the institutional animal ethics committee. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA). Study approval number was NIRRH/IAEC/ 38-11.

Dose selection: For i*n-vivo* micronucleus/COMET assay SB doses were selected based on human therapeutic dose (TD). Low dose corresponds to human therapeutic dose (3mg/kg BW), mid dose (15mg/kg BW i.e. five time of TD and high does30mg/kg BW ie.10time of TD). We have also carried out sub-chronic systemic toxicity study of the same preparation using same doses (communicated for publication). For *in-vivo* Micronucleus assay and COMET assay SB was suspended in 2% CMC whereas, for *in-vitro* chromosomal aberration assay, Suvarna Bhasma was dissolved in Dimethyl Sulphoxide (DMSO) and then it was further diluted with phosphate buffer for serial dilutions, whereas for Ames assay Suvarna Bhasma was suspended in Ethanediol (ED).

Human Blood Collection: Human peripheral blood samples were obtained from the healthy volunteers (age ≤ 30 years), from blood bank with permission. Basically after collection of blood from donor, bag was sealed tube was cut and remaining blood in the tube was collected aseptically in the heparinized glass tube. Immediately blood was transferred to the laboratory.

***In-vivo* Micronucleus test (MN Test):** Suvarna Bhasma was administered to swiss mice with oral gavage once. After 24hr of test drug administration, animals were sacrificed by CO_2 asphyxiation. Femoral bones were dissected out and Bone marrow cells were aspirated using syringe and needle with 3.0 ml of fetal bovine serum (FBS) and centrifuged at 800 x g for 10 min. The supernatant was discarded; pellet was suspended in 100 µl of fresh FBS, smeared on clean glass slide fixed in methanol for 5 min. The fixed smear was stained with undiluted May Grunewald stain for 5 min followed by diluted May Grunewald stain (1:1 v/v in distilled water) for 3 min. The slides were washed with distilled water, stained with Geimsa (10% v/v in Sorenson buffer) for 10 min and observed under light microscope (Carl, Zeiss, Berlin, Germany). A total number of 2,000 polychromatic erythrocytes (PCEs) and normochromic erythrocytes (NCEs) were examined per animal. PCEs were examined

for presence of micronucleus. In addition, the PCE/NCE ratio per animal was recorded to evaluate bone marrow toxicity[10].

***In-vivo* Single Cell Gel Electrophoresis (Comet Assay):** The assay was carried out according to the description of Singh et al [10]with some modifications. Suvarna Bhasma was administered to swiss mice with oral gavage once. Blood samples were collected24hr after of test drug administration, from each animal through retro-orbital sinus in heparinized tubes for comet assay.

20µl of blood sample were mixed with 130µl of prewarmed 0.8 %w/v (0.1 M) phosphate buffered saline. 1% Normal Point agarose slides were prepared and blood was mixed with LMP agarose were added on 1% NPA. A coverslip was placed immediately on each slideand agarose was allowed to solidify at 4°C for 10 min. The coverslip was then removed from each slide and incubated in cold freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 1%TritonX100, 10% DMSO, pH 10 for 1 hr. at 4°C. The slides were gently removed from lysis solution and placed in electrophoresis tank filled with electrophoresis buffer (300 mM NaOH, 1mM EDTA, pH >13) for 20 min to allow DNA unwinding. The slides were then subjected to electrophoresis for 30 min at 25 V and 300 mA, drained and neutralized with three change of neutralization buffer (0.4M Tris-HCL, pH 7.5) for 5 min. All steps were carried out under dark to prevent additional DNA damage. The slides were stained with 75 µl of 1Xethidium bromides (20 µg/ml) for 5 min and viewed under fluorescence microscope (Carl, Zeiss Germany) using a 40X objective, with 490 nm excitation and 515 mm emission filters. Total, 50 cells were randomly selected and images were captured for each animal using a camera and analyzed using comet scan software (Metasystem, Germany) to determine tail length (TL) of comet, tail moment(TM), tail moment olive (TMO, the product of TL and percent of DNA in the tail) and percent DNA damage.

Ames assay: Preincubation Ames assay was carried out for Salmonella typhimuriumTA98,TA100 and TA102 strain according to the method described by Maron and Ames (1983)[11,14]. Metabolic activation (with S9 mix) Ames assay involves exposing the tester strains (0.1ml) for 20-30 min to SuvarnaBhasmawith buffer or S9 mix (10%) (0.1ml) prior to addition on plates of minimum glucose agar (MGA).The mixture consisting of bacteria and SuvarnaBhasmawas plated in soft agar on minimum glucose agar plates. These plates were then incubated at 37^{0}C for 48hr and revertant colonies were counted. Experiments were performed in duplicate using three plates perconcentration.DMSOblank (for measuring spontaneous reversion), vehicle controls (i.e. DW) (for measuring toxic or inhibitory effects if any) and positive controls without metaboloic activation 4-nitro-o-phenylenediamine(for TA98) Sodium Azide(For TA 100) Mitomycin-C(For TA 102)2-Aminoanthracenefor with metabolic activation for all the strains were included to compare response of known mutagens in each assay. The test concentrations were selected as per the dose range finding study, SuvarnaBhasma powder was suspended in Ethanediol and accordingly final concentrations were prepared by serial dilutions.

***In-vitro* Chromosomal Aberration Assay:** The human peripheral blood cells were used as a test system. Whole blood (0.5ml) from healthy donors was added to 4.5ml of complete medium (1:4 fetal bovine serum and RPMI 1640 media with glutamine). 100µl of human phytoagglutenine (PHA) was added to the medium and cultures were incubated at 37^{0}C for period of 48 hours in CO_2 incubator (5% CO_2). After completion of 48hrs, cells were treated with Suvarna Bhasma without metabolic activation(-S9) and with metabolic activation (by incubating with 4% S9 mix for 3hrs prior completion of 48hrs i.e. +S9 mix). Negative Control (phosphate buffer) and positive control (Mitomycin-CandCyclophosphamide) were used to check the sensitivity of the assay. To arrest the cells at metaphase stage colchicines 0.10µg/ml was added to the culture before 1 hour of harvesting. For harvesting culture was centrifuged at1200 rpm for 15 min, and treated with hypotonic solution (0.56% KCl) and incubated at 37 ^{0}C for 30 min.The pellet was fixed in chilled methanol: glacial acetic acid (3:1). This step was repeated three times, slides were prepared by adding a drop of cell suspension on chilled glass slide from a height of about 2-2.5 feet and slides were air dried. The slides were stained with freshly prepared Giemsa stain(10%, v/v in Sorenson buffer) for 10 min followed by washing with distill water,scanned under 10× objective using metaphase analyzer (Carl Zeiss, Germany). Thegalleries of images generated with metaphases were selected and captured using63× objective under oil immersion. These images were subsequently analyzed manuallyfor chromosome aberrations. A total

number of 100 metaphases per donor were evaluated for chromosomal aberrations.

3. Statistical analysis

The statistical evaluation was performed using one-way ANOVA with Tukey multiple comparison tests. All values reported (Mean±S.D).

The significance level was set at $P < 0.05$.

4. Results

***In-vivo* Micronucleus test (MN Test):** Micronucleated polychromatic and normochromatic erythrocytes (MN-PCEs) present in the bone marrow of Male and Female mice are shown in table 1, table 2 and fig.1 given bellow. Administration of Suvarna Bhasma at the dose levels of 3, 15 and 30 mg/kg bw showed no effect on frequency of MN-PCEs and the PCE/NCE ratio in mice bone-marrow

Table 1. Micronucleated polychromatic erythrocyte (MN-PCEs) in the bone marrow cells of male mice exposed to various doses of Suvarna Bhasma

Groups	Treatment	Dose (mg /kg bw)	MN/ 10000cells	% MNfrequency	PCEs/NCEs ratio
I	Vehicle Control	0	8	0.08±0.044	0.84±0.05
II	SB[a]	3	8	0.08±0.027	0.81±0.10
III	SB [a]	15	8	0.08±0.057	0.81±0.09
IV	SB [a]	30	10	0.10±0.035	0.81±0.06
V	Cyclophosphamide[b]	40	82	0.82±0.103**	0.80±0.08

Abbreviations: **p<0.01, against control group, a-oral administration, b- intra peritoneal administration. MN- Micronucleus, PCE-Polychromatic Erythrocyte, NCE-Normochromatic Erythrocyte, SB- Suvarna Bhasma. n=5 male mice/group.

Table 2. Micronucleated polychromatic erythrocyte (MN-PCEs) in the bone marrow cells of Female mice exposed to various doses of Suvarna Bhasma

Groups	Treatment	Dose (mg /kg bw)	MN/ 10000cells	% MNfrequency	PCEs/NCEs ratio
I	Vehicle	0	8	0.08±0.447	0.85±0.05
II	SB [a]	3	9	0.09±0.041	0.81±0.08
III	SB [a]	15	9	0.09±0.0418	0.80±0.07
IV	SB [a]	30	11	0.11±0.065	0.84±0.08
V	Cyclophosphamide[b]	40	82	0.82±0.067**	0.82±0.09

Abbreviations: **p<0.01, against control group, a-oral administration, b- intra peritoneal administration. MN- Micronucleus PCE-Polychromatic Erythrocyte, NCE-Normochromatic Erythrocyte, SB- Suvarna Bhasma.. n=5 female mice/group.

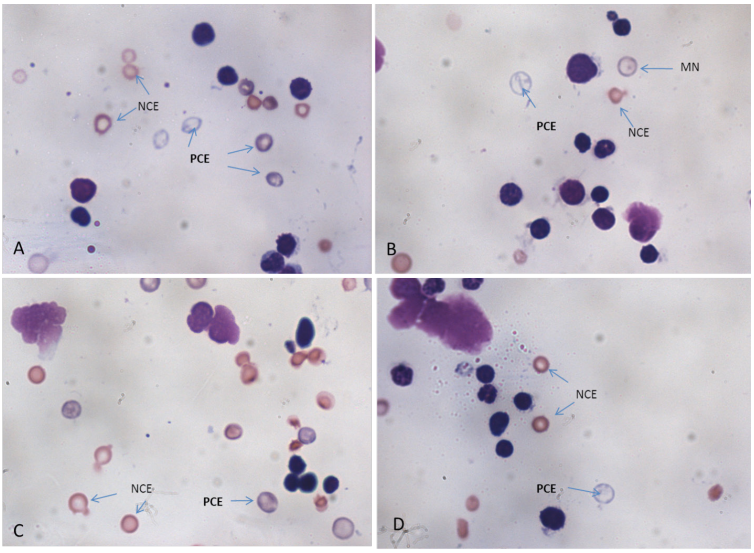


Figure 1. Representative bone marrow cellsstained with May Grunewald-Giemsa and exhibiting the presence of micronuclei, (A)-Vehicle Control, (B)-Cyclophosphamide showing micronucleus, (C)- Suvarna Bhasma 3mg/kg body weight and (D)- Suvarna Bhasma 30mg/kg body weight, NCE-Normochromic Erythrocytes, PCE-Polychromic Erythrocytes, All images are at 40× magnifications.

cells. There was no significant increase in number of MN-PCEs in both male and female mice treated with Suvarna Bhasma at various doses. There was no significant difference in % MN frequency in treatment groups as compared to vehicle control in either sex. However, an increase in frequency of MN observed in positive control group demonstrates the sensitivity of the test system.

***In-vivo* Single Cell Gel Electrophoresis (Comet Assay):** Administration of Suvarna Bhasma at the dose levels of 3, 15 and 30 mg/kg bw showed no significant difference in the tail movement olive, tail movement, tail length and percentage DNA damage in blood lymphocytes of both male and female mice when compared with the control group animals (Fig. 2 and Fig 3).

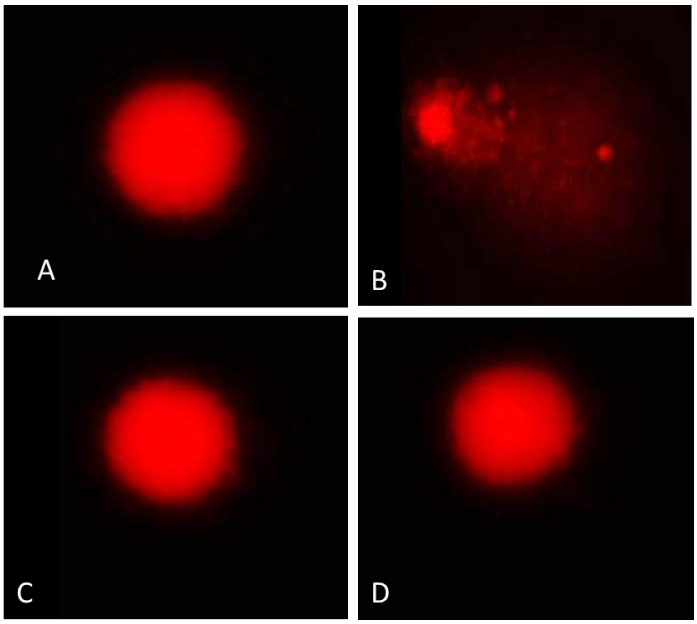


Figure 2. Extent of DNA damage assessed by comet assay in blood lymphocytes obtained from mice administered with different concentrations of Suvarna Bhasma. (A) Vehicle control; (B) cyclophosphamide exposed positive control and (C) Suvarna Bhasma treated at 3mg/kgbw; (D) 30mg/kgbw; All images are at 40× magnifications.

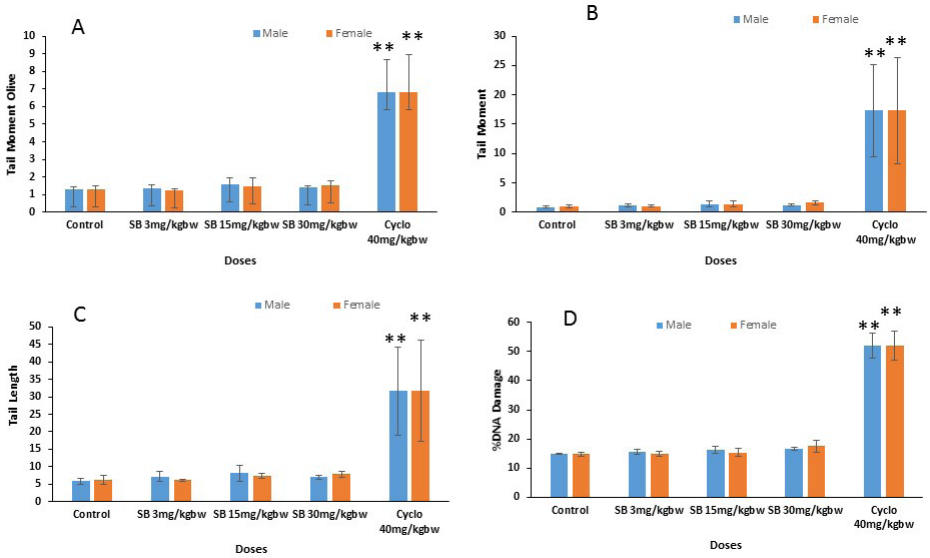


Figure 3. Bar diagrams showing the quantitative analysis comet with the treatment of Suvarna Bhasma. (A) Olive tail moment, (B), tail moment, (C) tail Length and (D) % tail DNA damage. Data are shown as Mean±S.D. One-way ANOVA was used for statistical analysis with Tukey multiple comparison tests (**P < 0.01). The significance level was set at P < 0.05.n= 5 mice per group.

AMES ASSAY: The results of Ames assay carried out on different strains of Salmonella typhimurium (TA 98, TA 100, and TA 102) exposed to Suvarna Bhasma are summarized in table no. 3 and table no.4.

The mutagenic response was not observed in any of the tester strains at the various concentrations of Suvarna Bhasma in presence and absence of S9 fractions.

***In-vitro* Chromosome Aberration Assay:** The results of the in vitro chromosome aberration test did not show any significant difference between vehicle control and treatment groups and supports the results obtained in the in vivo micronucleus test (Table No. 5, Table No. 6).

Table 3. Mutagenicity of Suvarna Bhasma by using Ames assay with S. typhimurium tester strain TA 98, TA 100, TA 102 with metabolic activation

Groups	Treatment	Dose/Plate	TA 98	TA 100	TA 102
I	VC (Ethanediol)	100 µl	36.50±4.42	89.00±7.01	36.50±4.42
II	Positive Control (2AA)	10 µg	660.67±8.48**	162.00±28.01**	660.67±8.48**
III	SB	0.62 mg	32.17±4.17	90.00±6.91	32.17±4.17
IV	SB	1.25 mg	29.50±4.76	85.00±8.45	29.50±4.76
V	SB	2.5 mg	31.83±4.07	98.00±12.67	31.83±4.07
VI	SB	5 mg	33.17±3.76	88.00±9.14	33.17±3.76
VII	SB	10 mg	31.00±5.10	97.00±7.21	31.00±5.10
VIII	VC (DMSO for 2AA)	100 µl	37.67±3.50	91.00±17.81	37.67±3.50

Abbreviations: **p<0.01, against control group, 2AA- 2-Aminoanthracene, DMSO-Dimethylsulphoxide, VC- Vehicle Control, SB-Suvarna Bhasma.. n=6 plates/dose

Table 4. Mutagenicity of Suvarna Bhasmaby using Ames assay with S. typhimurium tester strain TA 98, TA 100, TA 102 without metabolic activation

Groups	Treatment	Dose/Plate	TA 98	TA 100	TA 102
I	VC Ethanediol	100 µl	45.83±4.71	120 ± 10.82	420.67±30.96
II	4-nitro-o-phenylenediamine	20 µg	356.17±13.50**	--	--
	Sodium Azide	20 µg	-	2178 ± 147. 75**	--
	Mitomycin-C	0.5 µg	--	--	3307.17±17.90**
III	SB	0.625mg	44.00±3.85	119 ± 25.44	429.50±34.36
IV	SB	1.25mg	47.67±3.93	117 ± 22.64	395.50±22.42
V	SB	2.5mg	43.83±6.54	109 ± 18. 68	407.83±18.07
VI	SB	5mg	46.17±3.92	108 ± 12.06	416.67±24.51
VII	SB	10mg	41.50±3.45	115 ± 15.63	411.50±33.01
VIII	VC DW	100 µl	44.83±3.31	130 ± 11.51	424.50±27.06

Abbreviations: **p<0.01, against control group, DW- Distilled Water, VC- Vehicle Control, SB- Suvarna Bhasma. n=6plates/dose

Table 5. *In Vitro* Structural chromosomal aberration in Human blood without metabolic activation exposed to Suvarna Bhasma

Type of Aberration	Total	Gap	Break	Frag	Pulverization	Complex exchange	Total-Gap
Phosphate Buffer	12	0	12	0	0	0	12
SB (0.5µg/ml)	13	5	7	0	1	0	8
SB(5µg/ml)	18	8	9	0	1	0	10
SB (50µg/ml)	30	10	18	1	1	0	20
Mitomycin C (0.5µg/ml)	114	9	87	8	8	2	105**

Abbreviations: Frag-Fragment, **p<0.01, against control group, (Gap not included in final analysis), SB- Suvarna Bhasma. n=4donor/group.

Table 6. *In Vitro* Structural chromosomal aberration in Human blood with metabolic activation exposed to Suvarna Bhasma

Type of Aberration	Total	Gap	Break	Frag	Tri/Quadriradial	Pulverization	Complex exchange	Total (ExceptGap)
Phosphate Buffer	17	10	6	0	1	0	0	7
SB (0.5µg/ml)	20	7	9	4	0	0	0	13
SB(5µg/ml)	28	8	19	0	0	0	0	20
SB (50µg/ml)	36	11	24	1	0	0	0	25
Cyclophosphamide (40µg/ml)	269	21	168	31	25	8	16	248**

Abbreviations: Frag-Fragment, **p<0.01, against control group, (Gap not included in final analysis), SB- Suvarna Bhasma n=4donor/group.

5. Discussion

Genotoxicity and mutagenicity testing are important part of the hazard assessment of chemicals for regulatory purposes. To assess Genotoxicity and mutagenicity, different end points must be taken into considerations as besides induction of point mutations, a compound can induce changes in chromosome number (polyploidy or aneuploidy) or in chromosome structure (breaks, deletions, rearrangements)[18-19]. The certain set of various in-vitro/in-vivo tests have been suggested by the regulatory authorities which can be used to detect the possibilities of a chemical compound being a genotoxic material.

SuvarnaBhasma supreme ayurvedic product can be prepared by different methods as mentioned in Ayurvedic texts[1, 2, and 3]. The gold is treated with extract of Kanchanar Bauhinia Variegata for shodhanand compounds like mercury eg. mercury sulfide (Shodhana and Marana process)prior to the incineration is used which is considered to improve the efficacy and reduce the toxicity of SuvarnaBhasma[19]. The use of the heavy metal in ayurvedic medicines has always been a point of concern and controversial. These products cannot be exported to EU (European Union) because of limits of heavy metals as they are considered contaminants. As a consequence of this the government of India has taken an initiative to document the safety and efficacy of these products.

The present study has been carried out to determine genetic toxicity and mutagenicity of SuvarnaBhasma.The Micronucleus assay and Comet assay which were carried out in an in-vivo system using Swiss mice as an animal model at the dose levels of 3, 15 and 30 mg/kg bw showed no effect on frequency of MN-PCEs and the PCE/NCE ratio in mice bone-marrow cells and also no significant difference in the tail movement olive, tail movement, tail length and percentage DNA damage in lymphocytes of both male and female mice.

In Ames assay the mutagenic response was not observed in any of the tester strains at the various concentrations of SuvarnaBhasma in presence and absence of S9 fractions.

The results obtained in an *in-vivo* MN assay complemented results of In vitro chromosome aberration assay. SB did not cause any DNA damage.

These results documented the safety of Suvarna Bhasma in terms of genotoxic and mutagenic activity. The SuvarnaBhasma preparation evaluated in this study is safe not only at the human therapeutic dose but also at 10times higher than the TD .Therefore, this Suvarna Bhasma preparation can be utilized safely in humans for its therapeutic benefits.

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7. Conflict of Interest

None Declared.

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