



Original Research Article (Experimental)

# Manikya Bhasma is a nanomedicine to affect cancer cell viability through induction of apoptosis

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## ABSTRACT

**Background:** Ayurveda is an ancient medicine system practiced in the Indian sub-continent. Ayurvedic Bhasma is incinerated herbo-metallic/mineral preparations that consist of the particles in the range of nano/micrometers with therapeutic effects against different diseases. *Manikya Bhasma* (MB) is composed of purified ruby, orpiment, and purified arsenic sulfide.

**Objective:** This study was conducted to identify the potential of MB as a nanomedicine that can be used for the treatment of cancer.

**Materials and methods:** Biophysical characterization to determine the morphology and composition of bhasma particles was done using several techniques such as DLS, FTIR, FETEM, FESEM, EDX, and XRD. Cell viability assays were conducted to identify the cytotoxic effect of MB against different cancer cell lines and also to determine the mode of death caused by MB.

**Results:** The biophysical characterization of MB indicates that it is crystalline with a particle size of 70 nm. MB exhibits anticancer activity against MDAMB-231, HeLa, HCT-116, DLD-1, MG-63 cancer cells with an  $IC_{50}$  in the range of 105–155  $\mu\text{g/mL}$ . MB induces oxidative stress in cancer cells, which in turn affects their cell-cycle with an accumulation of cells in the G1-phase. Also, apoptosis induced by MB involves loss of mitochondrial membrane potential, the release of Cyt c, activation of caspases, and DNA degradation.

**Conclusion:** Our study highlights the dual potential of MB as a nano-carrier to deliver the drugs and exerting cytotoxic effects against cancer cells.

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## 1. Introduction

Cancer is one of the deadliest diseases of the present century and the second leading cause of deaths worldwide [1,2]. There are several treatment strategies available for cancer treatment such as radiotherapy and chemotherapy, but these therapies kill cancer cells along with neighboring healthy cells in tissue and probably be responsible for severe pathological outcomes in patients. As a result, there is a continuous hunt to identify treatment strategies which, not only compromise cancer cell's death but also may provide healing effects in patients. Ayurveda is an ancient medicine system practiced in the Indian subcontinent [3,4]. Its basic philosophy of treatment relies on providing healing effects to the person

and also it allows the body to fight against pathological effects developed by diseases. This approach of treatment is based on maintaining a suitable balance between the physical, mental, and spiritual functions of the human body [5]. In Ayurveda, an enormous number of herbs are used that possess the anticancer activity, such as garlic (*Allium sativum*), bhunimb (*Andrograpis paniculata*), green tea (*Camelia sinensis*), amalaki (*Emblica officinalis*), mulethi (*Glycyrrhiza glabra*), and tulsi (*Ocimum tenuiflorum*) [6].

Bhasma is the metallic/non-metallic preparation documented in Ayurveda with therapeutic effects against different diseases [7,8]. *Yashada Bhasma* (zinc based) and *Naga Bhasma* (lead-based) are used for the treatment of diabetes [9–12], *Mandura Bhasma* (iron-based), is used for the treatment of anemia, jaundice, edema as well as skin diseases [13], *Swarna Bhasma* (gold-based) is used for the treatment of solid malignancies (lung, liver, gall bladder, pancreas, and colon) [14,15] and *Tamra Bhasma* (copper-based) is used for the treatment of jaundice, abdominal disorders, and anemia [16,17]. Metallic preparations are used as anticancer sources in traditional

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medicines throughout the world [18]. Synthesis of bhasma involves an elaborate process termed as “*Bhasmikiranana*” [16]; and the objective of these processes is to transform the toxic metal/non-metal into nontoxic form with enriched therapeutic value as well as enhanced efficacy [19,20]. During this process zero-valent metal state gets converted to a higher oxidation state and the toxic nature of the metal oxide is destroyed while rendering medicinal properties in it [16]. The end product of bhasma preparation are residues of metals/minerals. Ayurvedic Bhasma is composed of toxic elements such as Lead, Mercury, Arsenic, which gives rise to safety, quality, and efficacy issues related to its use. A preliminary toxicity study on *Hartala Bhasma* (arsenic-based) [21], *Lauha Bhasma* (iron-based) [22], *Yashada Bhasma* (zinc-based) [10], *Tamra Bhasma* (copper-based) [23,24], *Swarna Bhasma* (gold-based) [14] validate their non-toxic and safe nature. Bhasma synthesis involves two major steps. First, the raw mineral used is detoxified using animal and plant-based byproducts. Also, it homogenizes the mixture and removes any form of adulteration present. Further, it is exposed to repeated cycles of incineration or calcination, that converts it into ashes [25,26]. This process transforms, the heavy, rough, and hard minerals into soft and smooth powder, also it converts the macro-sized particles into micro/nano-sized as confirmed by several spectroscopic and microscopic studies [27]. The bhasma obtained at the end, has very higher absorption and assimilation in the human body.

*Manikya Bhasma* (MB) is the incinerated powder of purified ruby, orpiment, and sulfide of arsenic [28]. MB is used in immunomodulation, and it affects various enzymatic and hormonal cycles [29]. According to Ayurveda, *Manikya Bhasma* possesses several properties like an appetizer, heart, and brain tonic [30,31].

In the present study, biophysical characterization of MB was done using several spectroscopic and microscopic techniques such as DLS, FETEM, FESEM, EDX, and XRD, to study the size, morphology, and composition of particles present in MB. Further, cell viability assay was used to explore the biological effects of MB against different cancer cell lines. MB is causing a reduction in cellular viability of MDAMB-231, HeLa, HCT-116, DLD-1, MG-63 cancer cells with an  $IC_{50}$  in the range of 105.73–155.47  $\mu\text{g/ml}$ . The cytotoxic activity of MB exists in aqueous extract, and cancer cells follow apoptosis as a mode of death. MB is inducing oxidative stress in cancer cells, which in turn affects their cell-cycle with an accumulation of cells in the G1-phase. Also, Apoptosis induced by MB involves loss of mitochondrial membrane potential, the release of Cyt-c, activation of caspases, and DNA degradation. Hence, our study highlights the dual potential of MB as a nanocarrier to deliver the drugs and exerting cytotoxic effects against cancer cells.

## 2. Materials and methods

### 2.1. Chemicals

*Manikya Bhasma* was obtained from local Baidyanath store in Guwahati city, acridine orange, propidium iodide, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), agarose, 2,7-dichlorofluorescein diacetate (DCFH-DA), dulbecco's modified eagle's medium were purchased from Sigma Aldrich (St. Louis, MO, USA). RNase A, Proteinase K, DMSO, Foetal Bovine Serum (FBS), Penicillin-Streptomycin (100X) antibiotic solution, Phosphate Buffer Saline (PBS), sodium azide, trypan blue, and trypsin were obtained from HiMedia (Mumbai, India). Ethylenediaminetetraacetic acid (EDTA), ethanol, sodium chloride, was purchased from Merck, Germany. Anti-Cyt-c antibodies, Mitotracker Red, and JC-1 dye were obtained from BD-Biosciences (San

Jose, USA). The caspase-9 colorimetric kit was obtained from Invitrogen Corporation (Waltham, USA). All the cell culture plates and dishes were purchased from Corning, Lowell, MA, USA. MDAMB-231, DLD-1, HCT-116, MG-63, HeLa cancer cell lines were procured from National Centre for Cell Sciences, Pune, India. All other reagents and chemicals were of analytical grade purity.

### 2.2. Extract preparation of Manikya Bhasma

MB (200 mg) powder was taken and dispersed in 10 mL of autoclaved milli-Q water, which was further subjected to ultrasonication in a bath sonicator for 30 minutes it was then centrifuged at 6000 rpm for 10 min at RT, and the supernatant or pellet fraction was collected separately for cell viability assay using MTT assay.

### 2.3. Dynamic light scattering (DLS) analysis of MB

Aqueous MB extract (1 mg/mL) was prepared in milli-Q water, and measurement of the hydro-dynamic diameter of bhasma was done. DLS experiment was recorded on Zetasizer Nano ZS (Malvern Instrument) equipped with a 633 He–Ne laser, using 173° non-invasive backscattering detector configuration. The sample was recorded for ten scans at 25 °C, and the intensity-weighted hydrodynamic diameters were plotted.

### 2.4. Field emission scanning electron microscopy (FESEM) analysis of MB

Aqueous MB extract (1 mg/mL) was drop-casted on the coverslip covered with the aluminium foil and allowed to dry overnight. It is coated by ultrathin electrically conducting gold and palladium mixture alloy by sputter coating. Images from random fields were obtained at a magnification between 1kX– 50kX using FESEM (Make: Zeiss, Model: Sigma 300).

### 2.5. Energy dispersive X-Ray (EDX) analysis of MB

For EDX analysis FESEM instrument (Make: Zeiss, Model: Sigma) was used, which gave the elemental composition of *Manikya Bhasma*.

### 2.6. FTIR analysis of MB

KBr pellet was prepared by mixing sample and KBr in the ratio of (1:100). Then the sample was kept in the pellet holder, and the spectrum was recorded on IR affinity-1S Shimadzu.

### 2.7. Field emission transmission electron microscopy (FETEM) analysis of MB

*Manikya Bhasma* was drop casted on to the Cu-coated TEM grid and kept for air drying for 24 h. Further samples were analyzed by the JEOL 2100UHR-TEM.

### 2.8. Powder X-Ray diffraction analysis of MB

*Manikya Bhasma* powder was kept on to a quartz sample holder and spread uniformly using a glass cover, and diffraction pattern was recorded in X-ray Diffractometer (Rigaku, Smartlab X-Ray Diffractometer) at 45 kV and 200 mA with copper filament giving X-Ray of 1.54 Å wavelength. Particle size was determined using Scherrer's eq (1) as given below,

$$d = \frac{K\lambda}{\beta \cos\theta} \quad (1)$$

where K is a constant having the value of 0.9, and  $\beta$  is full width half maximum (FWHM),  $\theta$  is Bragg's angle,  $\lambda$  is the wavelength of Cu-K $\alpha$  radiation. A Match software version 3.7.0.124 was used for the analysis of results.

## 2.9. Cell culture and treatments

MDAMB-231 (human breast cancer cells), HeLa (human cervical cancer cells), MG-63 (human osteosarcoma cells), DLD1 and HCT-116 (human colon cancer cells) were cultured in DMEM:F12, supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic solution (100 units/mL penicillin and 100  $\mu$ g/ml streptomycin sulfate). Cells were grown at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Ten thousand cells were seeded overnight before the day of experiments in 200  $\mu$ L complete media. On the day of the experiments, cells were washed twice with cell culture grade phosphate buffer saline (PBS) and subjected to various treatments in serum-free medium as described before [32,33].

## 2.10. Cell viability assay

The MTT assay was used to measure the cellular viability as described before [32,33]. Ten thousand cells were seeded in a 96 well plate in the total volume of 0.2 mL DMEM: F12 complete medium. After overnight incubation, cells were washed twice with cell culture grade phosphate buffer saline (PBS) and were subsequently incubated with *Manikya Bhasma* (0–1000  $\mu$ g/mL) for 48 h in 0.2 ml of serum-free medium. After the treatment period, cells were washed twice with PBS and incubated with 100  $\mu$ L of MTT (0.5 mg/mL) solution for 4 h at 37 °C with 5% CO<sub>2</sub>. Then, MTT solution was removed, and the formazan crystals were dissolved in 100  $\mu$ L cell culture grade DMSO. The optical density was determined using a spectrophotometer (SpectraMax M2) at 570 nm and 660 nm (to subtract scattering effects of crystals). Cells treated with serum-free medium alone were considered as 100% viable and used to express the results of MB treated cells. Images of cells during treatment were taken from random fields using the Cytell cell imaging system (GE Healthcare).

## 2.11. Cellular toxicity study

Blood was withdrawn in anticoagulant containing tubes from a healthy volunteer after informed consent inside a biosafety cabinet under aseptic conditions. Blood was centrifuged at 750 g for 5 min at room temperature. Plasma was separated and a buffy coat was isolated from the RBC pack and re-suspended in DMEM containing 10% serum and 1% antibiotic cocktails. The cells were allowed to adhere overnight and unattached cells were removed by washing. In each well, 10,000 cells were plated into 96 wells plate and treated with MB (0–1000  $\mu$ g/mL) for 48 h and cell viability was measured by MTT assay.

## 2.12. Cell cycle analysis of *Manikya Bhasma*

Cells were seeded in a six-well plate (around 3–4 lakh cells/well) for 12–16 h. Further cells were treated using IC<sub>50</sub> (105.7  $\mu$ g/mL) concentration of MB prepared in the serum-free medium for 24 h, and cell-cycle analysis was performed as described previously [32,33]. The distribution of cells in different phases was analyzed by Modfit software (BD Biosciences, USA) [34].

## 2.13. *Manikya Bhasma* induces cell death in cancer cells due to apoptosis

Cells were seeded in a six-well plate (around 3–4 lakh cells/well) for 12–16 h. Further cells were treated using IC<sub>50</sub> (105.7  $\mu$ g/mL) concentration of MB prepared in the serum-free medium for 24 h, and apoptosis was studied as described previously [32,33].

## 2.14. Intracellular ROS measurement

Cells were seeded in a six-well plate (around 3–4 lakhs cells/well) for 12–16 h. Further cells were treated using IC<sub>50</sub> (105.7  $\mu$ g/mL) concentration of MB prepared in the serum-free medium for 12 h, and intracellular ROS was analyzed as described previously [32,33,35].

## 2.15. Caspase 9 colorimetric assay

Cells were grown on 10 cm well plates overnight. The next morning cells were treated with MB at IC<sub>50</sub> concentrations for 24 h in serum-free media. Post-treatment, the cells were washed twice with sterile PBS. Then the caspase-9 assay was carried out as per the manufacturer's instructions according to the Invitrogen caspase-9 colorimetric kit, (Cat no: KHZ0101).

## 2.16. JC-1 assay for mitochondrial membrane potential measurement

Cells were seeded (10,000 cells per well) in 96 well plates for 12–16 h next, it was treated with MB for 24 h at 37 °C in serum-free media. Cells treated with the serum-free medium were considered as control. Post-treatment, cells were washed twice with sterile cold PBS and incubated with JC-1 dye (1X in 1X assay buffer) according to kit manufacturer's protocol (BD-biosciences Cat. No.551302) for 20 min at 37 °C, 5% CO<sub>2</sub> incubator. Finally, cells were gently washed once with PBS and then taken for imaging in the cytell cell imaging system (GE Healthcare).

## 2.17. DNA-fragmentation assay

Two lakh ( $2 \times 10^5$ ) cells were seeded in a six-well plate for 12–16 h, next the cells were treated with the MB for 24 h at 37 °C in serum-free media. Cells treated with the serum-free medium were considered as control. DNA fragmentation analysis was performed as described previously [32,33,35]. Fragments of DNA were visualized under UV-light and images were captured with a BIO-RAD chemidoc™ Imaging system.

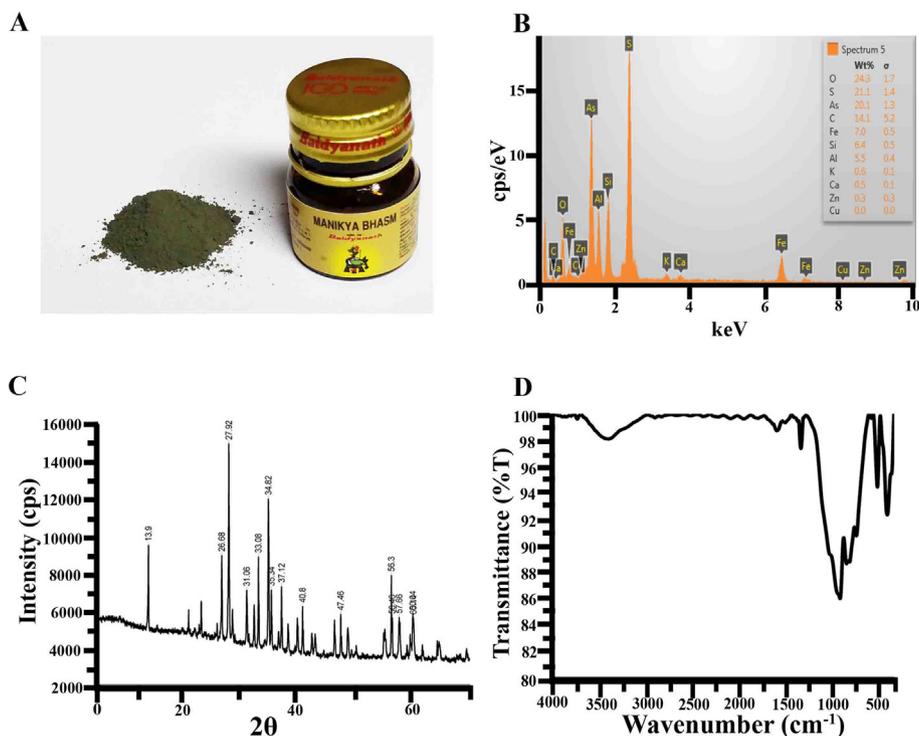
## 2.18. Immunolocalization to study Cyt-c release

MG-63 cells were seeded (10,000 cells per well) in 96 well plates overnight. The next morning, cells were treated with the IC<sub>50</sub> concentration of *Manikya Bhasma* for 24 h in serum-free media. Cyt-c localization in control or treated cells was performed as described previously [32,33]. Finally, the cells were washed twice with PBS and taken for imaging in the Cytell cell imaging system (GE Healthcare).

## 3. Results

### 3.1. Biophysical characterization of *Manikya Bhasma*

*Manikya Bhasma* is a dark green color powder, which possesses a pungent odor (Fig. 1A). It is a formulation that improves circulation, works as an immuno-modulator, and can be used as a memory enhancer, appetizer, cardio as well as brain tonic [29]. *Manikya Bhasma* is constituted of purified ruby, orpiment, and purified



**Fig. 1. Biophysical Characterization of Manikya Bhasma** (A) Dark green color powder of *Manikya Bhasma* commercially available in market (B) EDX analysis of *Manikya Bhasma* showing the presence of Carbon (C), Oxygen (O), Sulphur (S), Arsenic (As), Aluminium (Al), Silicon (Si), Iron (Fe) along with Potassium (K), Calcium (Ca) and Zinc (Zn). (C) Powder X-Ray Diffraction pattern of *Manikya Bhasma*. (D) FTIR spectra of *Manikya Bhasma*, peak at  $3436\text{ cm}^{-1}$  was due to the presence of moisture otherwise there is a complete absence of a significant peak in the region of  $4000\text{--}1500\text{ cm}^{-1}$  indicates the minimal presence of functional groups in *Manikya Bhasma*.

sulfide of arsenic [28]. In orpiment, arsenic is present in trisulfide form while purified arsenic sulfide is the disulfide form of arsenic [31]. It is an ayurvedic herbal medicine prepared by the repeated calcination of minerals. The preparation process involves the major step of the detoxification of raw minerals by the herbal juices [31]. Elemental analysis of *Manikya Bhasma* by FESEM-EDX, revealed the presence of considerable amounts of Carbon (C), Oxygen (O), Sulphur (S), Arsenic (As), Aluminium (Al), Silicon (Si), Iron (Fe) along with Potassium (K), Calcium (Ca), Zinc (Zn) (Fig. 1B & Table 1). The presence of a considerable amount of Carbon (14.1%) and Oxygen (24.3%) might be due to the repeated cycle of calcination, during the preparation of bhasma. The calcination process plays an important role in the deposition of carbonaceous material. During calcination which is carried out in closed assembly of earthen crucibles, volatile components of organic materials released from the herbs get transformed into a gaseous form that gets attached to crystalline particles through weak binding. Also the presence of

Iron (7.0%), Aluminium (5.5%), Arsenic (20.1%), Silicon (5.4%) indicates the use of minerals such as Arsenic Sulphide, ruby, and orpiment in the preparation of *Manikya Bhasma*. The presence of a significant amount of iron in MB is due to two reasons firstly, the use of iron mortar pestle for grinding of minerals and the use of various animal and plant-based byproducts during bhasma preparation. To know about the nature of the *Manikya Bhasma* (crystalline or amorphous), X-Ray diffraction was used. XRD study of the *Manikya Bhasma* showed the presence of distinct high-intensity peaks, which indicates the crystalline nature of the bhasma (Fig. 1C). The average crystallite size was found to be  $\sim 70\text{ nm}$  as calculated by Scherrer's formula given in Eq (1) below:

$$d = \frac{K\lambda}{\beta \cos\theta} \quad (2)$$

where K is a constant having the value of 0.9, and  $\beta$  is full width half maximum (FWHM),  $\theta$  is Bragg's angle,  $\lambda$  is the wavelength of Cu-K $\alpha$  radiation. Phase matching of the XRD peaks was done using match software version 3.7.0.124, and it shows matching with different minerals such as almandine ( $\text{Al}_2\text{Fe}_3\text{O}_{12}\text{Si}_3$ ), Iron Pyrite ( $\text{FeS}$ ), Arsenic trioxide ( $\text{As}_2\text{O}_3$ ), and quartz ( $\text{SiO}_2$ ). The elemental analysis from XRD correlates well with the elemental analysis from EDX.

Since *Manikya Bhasma* is functionally active against several disease conditions, it is crucial to explore the presence of a chemically reactive group present in MB. FTIR analysis of *Manikya Bhasma* by the KBr pellet method as described in the materials and methods section was done to determine the presence of the chemically reactive group, whereas no significant peaks in the region of  $4000\text{--}1500\text{ cm}^{-1}$  indicate the absence of functional groups in *Manikya Bhasma* (Fig. 1D). Along with biophysical characterization, the size and morphology of *Manikya Bhasma* might play a significant role in its activity.

**Table 1**  
Semi-Quantitative Elemental analysis of *Manikya Bhasma*.

S. No.	Element	Wt%	At%
1.	S	21.1	15.6
2.	As	20.1	6.4
3.	Si	5.4	7.4
4.	Al	5.5	4.9
5.	Fe	7.0	3.0
6.	C	14.1	27.9
7.	O	24.3	36.0
8.	K	0.6	0.4
9.	Ca	0.5	0.3
10.	Zn	0.3	0.1

MB was subjected to EDX analysis to estimate the presence of different elements in bhasma.

### 3.2. Manikya Bhasma has nanomedicine like characteristics

Size, surface morphology, elasticity, stiffness, and other properties of Ayurvedic *Bhasma*, which determines its therapeutic outcomes. Dynamic Light Scattering analysis of *Manikya Bhasma* using the Zeta Sizer Nano ZS instrument as described in the materials and methods section revealed that its hydrodynamic diameter is in the range of 800–1200 nm and Poly Dispersity Index (PDI) was found to be 0.370 (Fig. 2A). FESEM micrographs of the *Manikya Bhasma* showed the aggregated morphology of the bhasma particles, having a size of around 1–2  $\mu\text{m}$  (Fig. 2A). Whereas the FETEM micrograph confirmed the agglomeration of the *Manikya Bhasma* particles, although, the individual particles were spherical and were having a size in the range of 60–70 nm (Fig. 2C). Since *Manikya Bhasma* particles were having a size  $\sim 70$  nm as determined by FETEM and XRD, so it indicates that the bioactivity of MB might be due to the particle size and hence MB is possessed with nanomedicine like characteristics.

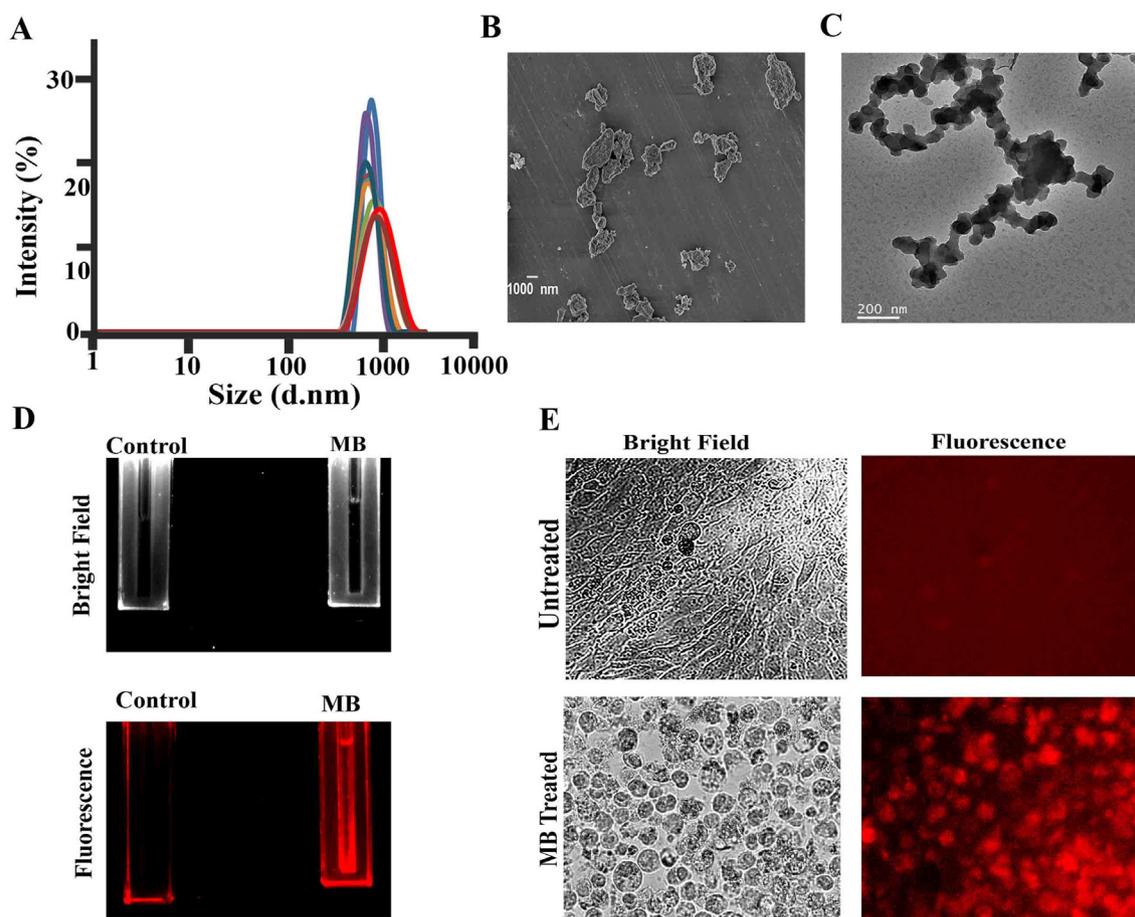
### 3.3. Manikya Bhasma is readily taken up by mammalian cells

As *Manikya Bhasma* possess therapeutic activity [28], which directs its immediate uptake by the cells. Firstly, it was checked if *Manikya Bhasma* possesses fluorescence activity which can be used

to track its up-take by the mammalian cells. It was found that the *Manikya Bhasma* (1 mg/mL) gives intense fluorescence in the far-red region, whereas no significant fluorescence was found in the control “buffer” kept in a quartz cuvette (Fig. 2D). As *Manikya Bhasma* is giving fluorescence in the far-red region, we have incubated MG63 with MB (100  $\mu\text{g}/\text{mL}$ ) for 12 h and fluorescence from cells was recorded in the Cytell cell imaging system. MG-63 (osteosarcoma cells) cells were giving bright red fluorescence compared to untreated cells (Fig. 2E). It indicates the association of MB with the cells, but more in-depth studies are required to explore the location of MB within the cell. The current experiment highlights that MB is interacting with cells, and it may give biological outcomes.

### 3.4. Manikya Bhasma induces cytotoxicity in cancer cells

*Manikya Bhasma* has several therapeutic uses; it is a herbo-metallic drug that can alter cellular metabolism by preventing proliferation and metastasis of cancer cells. To determine the role of *Manikya Bhasma* as anticancer nanomedicine, MG-63 (osteosarcoma) cells were treated with *Manikya Bhasma* (0–1000  $\mu\text{g}/\text{mL}$ ) for 48 h, and cell viability was determined using MTT reduction assay as described in the materials and methods section. The plot between cell viability (%) and the concentration of *Manikya Bhasma* is



**Fig. 2. Manikya Bhasma is fluorescent, nanosize, and readily been taken-up by the mammalian cells:** (A) Size distribution by intensity curve of *Manikya Bhasma* showing its hydrodynamic diameter is in the range of 800–1200 nm (B) FESEM Micrograph of *Manikya Bhasma*. The particles of *Manikya Bhasma* forms aggregates in the range of 1–2  $\mu\text{m}$  (scale: 1  $\mu\text{m}$ ). (C) FETEM micrograph of *Manikya Bhasma* showing the particles are highly aggregated and the individual particles are spherical in shape and were having the size in the range of 60–70 nm (scale 200 nm). (D) *Manikya Bhasma* is fluorescent in nature. *Manikya Bhasma* dispersed in milliQ water was taken in a quartz cuvette and visualized using chemidoc™ Imaging system, milliQ water was taken as control. (E) *Manikya Bhasma* is readily been taken-up by cells. Observation of *Manikya Bhasma* treated Osteosarcoma (MG-63) cells using the cytell imaging system (GE Healthcare), treated cells showing red fluorescence indicated its uptake by the cells.

used to calculate  $IC_{50}$ . MB is reducing the cell viability of MG-63 cells in a dose-dependent manner with an  $IC_{50}$  of  $105.73 \pm 6.15 \mu\text{g/mL}$  (Fig. 3A). Also, *Manikya Bhasma* is effective against other cancer cell lines too, such as breast cancer (MDAMB-231) cells, cervical cancer (Hela) cells, colon cancer (DLD1, HCT-116) cells and cell viability was measured by MTT reduction assay (Table 2). Interestingly, it is reducing the cell viability of primary WBCs isolated from fresh blood with an  $IC_{50}$  of  $873.81 \pm 14.12 \mu\text{g/mL}$ . A shift of five folds in  $IC_{50}$  towards primary cells indicates the utility of the MB as a promising anticancer agent (Table 2). Morphology of *Manikya Bhasma* treated cells appeared highly disrupted due to shrinkage of cytoplasm and distortion of cellular plasma membrane compared to untreated cells (Fig. 3A).

### 3.5. *Manikya Bhasma* activity lies in supernatant

*Manikya Bhasma* is giving cytotoxicity against the cancer cell lines, but it is not clear whether activity lies in crude powder or aqueous extract. *Manikya Bhasma* was dispersed in sterile milliQ water and fractionated following the scheme given in Fig. 3C. Cell Viability of MG-63 after treatment with residue and supernatant fractions was calculated using MTT reduction assay as given in the materials and methods section. It was found that the supernatant fraction is affecting the cellular viability compared to pellet fractions (Fig. 3D). Hence, we can conclude that the activity of *Manikya Bhasma* lies in the supernatant fraction i.e. the water-soluble fraction as compared to the pellet fraction.

**Table 2**

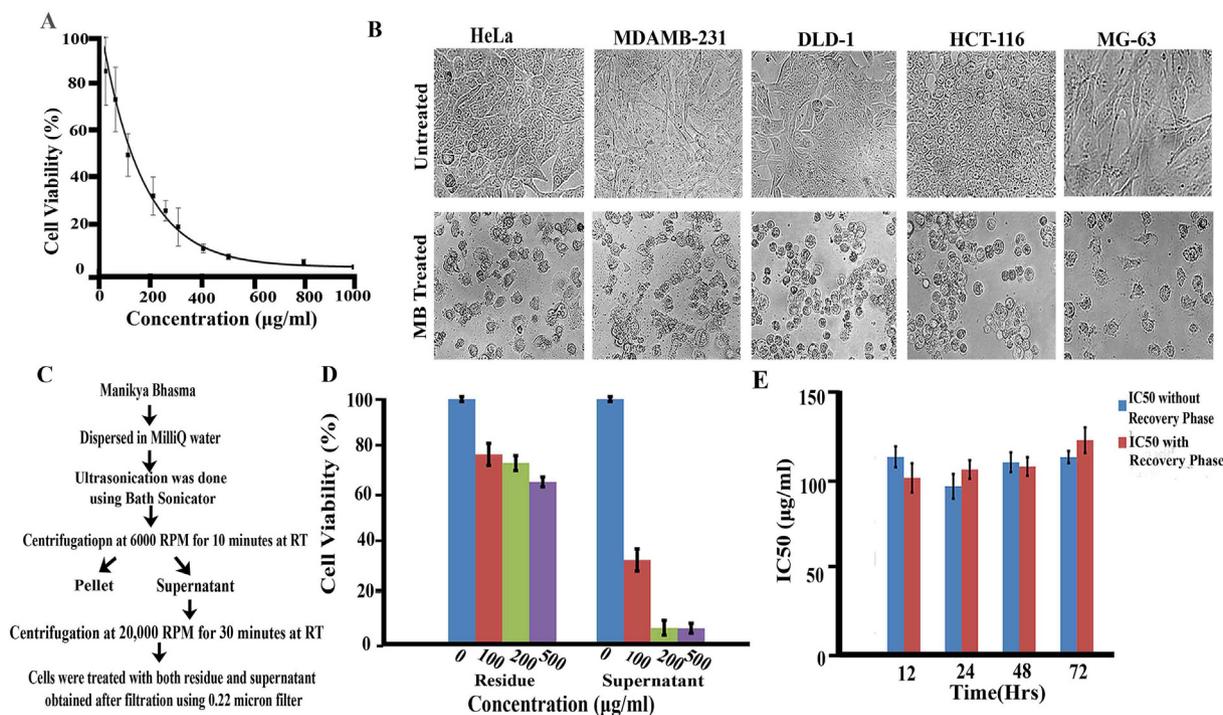
Effect of *Manikya bhasma* on cellular Viability of different cancer cells.

S. No.	Cancer Cell lines	Type	$IC_{50} \pm SD$ ( $\mu\text{g/mL}$ )
1	MDAMB-231	Human breast cancer cells	$117.06 \pm 9.09$
2	HeLa	Cervical cancer cells	$126.43 \pm 5.85$
3	MG-63	Human osteosarcoma cells	$105.73 \pm 6.15$
4	DLD1	Human colon cancer cells	$151.15 \pm 5.85$
5	HCT-116	Human colon cancer cells	$107.81 \pm 4.09$
6	WBCs	Human Blood primary cells	$873.81 \pm 14.12$
7	Triphala <sup>#</sup>	Human breast cancer cells	$172.4 \pm 2.67$
8	Mitomycin C <sup>&amp;</sup>	Human breast cancer cells	$12.42 \pm 1.31$

Different Cancer cells and WBCs isolated from fresh blood were treated with MB (0–1000  $\mu\text{g/mL}$ ) for a period of 48 hs and cellular viability was measured by MTT assay as described in “Material and Methods”. <sup>#</sup>The MDA-MB-231 cells treated with Triphala was included as a positive anticancer ayurvedic formulation whereas cells treated with Mitomycin C<sup>&</sup> was included as an anticancer drug to compare the results with MB.

### 3.6. *Manikya Bhasma* kills the cells with no recovery

All the cancer strategies available have the major disadvantage of recurrence. Usually, once the treatment is removed, there are higher chances of recurring cancer. To determine if *Manikya Bhasma* induces cell death in cancer cells without reversion, MG-63 cells were treated with *Manikya Bhasma* (0–500  $\mu\text{g/mL}$ ) for different time intervals (12 h, 24 h, 48 h, and 72 h). Post-treatment, cells were allowed to recover and grow in complete media for another 48 h. The  $IC_{50}$  values from both conditions (Cells without recovery or with



**Fig. 3. *Manikya Bhasma* induces a reduction in cellular viability in cancer cells.** (A) *Manikya Bhasma* is reducing the cellular viability of cancer cells in a dose-dependent manner. Osteosarcoma MG-63 cancer cells were treated with different concentrations of *Manikya Bhasma* (0–1000  $\mu\text{g/mL}$ ) prepared in serum-free media and viability was determined by MTT reduction assay.  $n = 3$  (B) Images of untreated and treated cancer cells with *Manikya Bhasma* (aqueous extract) prepared in incomplete media taken by Cytell cell imaging system (GE Healthcare) (C) **Fractionation scheme of *Manikya Bhasma*.** *Manikya Bhasma* was dispersed in sterile milliQ water, and the ultrasonication was done using bath sonicator for 30 minutes, next it was centrifuged at 6000 RPM for 10 min. Further fractionation was done by centrifugation of collected supernatant at 20,000 RPM for 30 min, both residue and supernatant fraction were filtered using a 0.22-micron filter before testing them in cell-based assay system. (D) **The bioactivity of *Manikya Bhasma* lies in the supernatant.** Osteosarcoma (MG-63) cells treated with different concentrations (100  $\mu\text{g/mL}$ , 200  $\mu\text{g/mL}$ , and 500  $\mu\text{g/mL}$ ) of residue and supernatant fraction obtained during fractionation. Standard Deviation was calculated from the data of two independent ( $n = 2$ ) experiments. (E) ***Manikya Bhasma* is killing the cancer cells.** MG-63 cells were treated with *Manikya Bhasma* (0–500  $\mu\text{g/mL}$ ) for different time intervals (12 h, 24 h, 48 h, and 72 h) with or without an additional recovery phase of 48 h in complete medium. The  $IC_{50}$  values of MB in both conditions were given as a bar diagram. Standard Deviation was calculated from the data of three independent ( $n = 3$ ) experiments.

recovery to grow in complete medium) were calculated. Interestingly, IC<sub>50</sub> comparison in both conditions indicate no survival of cells even after the removal of MB from the culture medium and providing pro-growth supporting conditions (Fig. 3E and Table 3).

### 3.7. *Manikya Bhasma* induces oxidative stress inside cancer cells

Further investigation was done to determine the disturbance of cellular metabolism and physiology of cancer cells treated with *Manikya Bhasma*. MG-63 cells were treated with *Manikya Bhasma* (105.7 µg/mL) for 12 h, and intracellular ROS levels were determined using a fluorescent probe DCFH-DA dye as described in the materials and methods section. Fluorescent imaging of cells gives bright green fluorescent signals present inside the treated cells (Fig. 4A) whereas flow cytometric analysis of treated cells exhibits significant accumulation of ROS inside the treated cells compared to untreated cells (Fig. 4B).

### 3.8. *Manikya Bhasma* disturbs the cell cycle progression in cancer cells

Usually, mammalian cells progress through four stages in the cell cycle to divide and increase their number. In the Gap 1 (G1) phase, cells increase its size, DNA synthesis occurs in S-phase and Gap 2 (G2) prepare cell to divide through Mitotic (M) and cytokinesis. In the case of cellular stress, cellular division, as well as cell-cycle, was getting affected through multiple mechanisms [32,33]. MG-63 cells after treatment with *Manikya Bhasma* were quantified for a number of cells in different stages of the cell cycle using flow cytometry, and it was visualized in the 2D histogram (cell number vs DNA content). In untreated MG-63 cells, 38.85% of cells were present in the G1 phase, 18.74% in the S phase, and 42.41% cells in the G2 phase (Fig. 4C). Whereas MG-63 cells treated with *Manikya Bhasma* exhibited 70.71% cells in G1 phase, 12.69% cells in S phase, and 16.60% in G/M phase (Fig. 4C). Cell cycle analysis indicates that the *Manikya Bhasma* reduces the number of cells in the S-phase (the stage with active DNA synthesis and required for proliferation) with a significant increase in the G1 phase (the phase with low metabolic activity).

### 3.9. MB induces cell death in cancer cells due to apoptosis

Apoptosis is a programmed cell death that regulates the development of multicellular organisms and tissues by removing damaged and abnormal cells. Cells undergoing apoptosis shows multiple biochemical and morphological features, which may vary depending on the type of apoptotic inducer and time for which the apoptosis is observed. Dual staining is done using acridine orange and propidium iodide as described in the materials and methods section to determine the distribution of live/apoptotic cells. Untreated MG-63 cells were mostly green fluorescent, as live cells were stained with acridine orange, while the treated cells showed significant red fluorescence due to the staining of dead cells by the

propidium iodide (Fig. 5A). Flow cytometric analysis of double-stained cells and subsequent quadrant analysis gave the information about the cell populations into healthy (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic/dead (upper left) phases. Untreated cells showed mostly healthy cells (~92%), which were evident from reduced staining of propidium iodide (Fig. 5B). *Manikya Bhasma* treated cells showed a significant increase of cells in the apoptotic (early and late) or death phases. In the case of MG-63 cells, the proportion of cells in the early apoptotic was 21.25%, and late apoptotic was 26.01%, and healthy cells were reduced to 36.01% (Fig. 5B). The flow cytometric analysis, as well as the fluorescent imaging, indicates that the cell death caused by *Manikya Bhasma* follows the apoptotic pathway rather than the necrotic pathway for cell death.

Apoptosis is characterized by distinctive changes in the morphology of the nucleus, which includes chromatin condensation and fragmentation, overall cell rounding/shrinkage, and formation of apoptotic cell bodies. Apoptosis in cells can be induced by two pathways; death receptor pathway or mitochondrial pathway. MG-63 cells were treated with *Manikya Bhasma* (105.7 µg/mL) in serum-free media and stained with JC-1 as per the manufacturer's protocol and observed by the Cytell cell imaging system (GE Healthcare). Untreated MG-63 cells were found to be healthy with an orange fluorescence that indicated the accumulation of the dye aggregates inside the mitochondrion (Fig. 5C). Whereas *Manikya Bhasma* treated cells were showing green fluorescence, spread evenly throughout the cytosol with little orange fluorescence (Fig. 5C). It indicates the leaky behavior of mitochondria and disruption of mitochondrial membrane potential; as evident by no accumulation of JC-1 dye aggregates inside the mitochondria (Fig. 5C). We further explored the release of Cytochrome-c from mitochondria in *Manikya Bhasma* treated cells. Fluorescence microscopy was used to visualize cytochrome-c inside cells. The location of mitochondria was identified by Mitotracker red dye which localizes inside the mitochondrion irrespective of any mitochondrial membrane potential. Immunostaining in untreated cells shows that signal of Cytochrome-c was highly specific to distinct locations inside the cell. It was found that the Cytochrome-c signal (green) was co-localizing with the mitotracker signal (red). It indicates the presence of Cytochrome-c inside the intact mitochondria in untreated cells (Fig. 5D). *Manikya Bhasma* treated cells, Cytochrome-c was almost evenly distributed throughout the cytosol; was not co-localizing with the mitotracker signals (Fig. 5D). The immunostaining data indicate that there was the release of Cytochrome-c from mitochondria into the cytosol in *Manikya Bhasma* treated cells. Caspases are intrinsically involved and are activated downstream in the mitochondrial pathway of apoptosis, especially after mitochondrial depolarization and Cyt-c release.

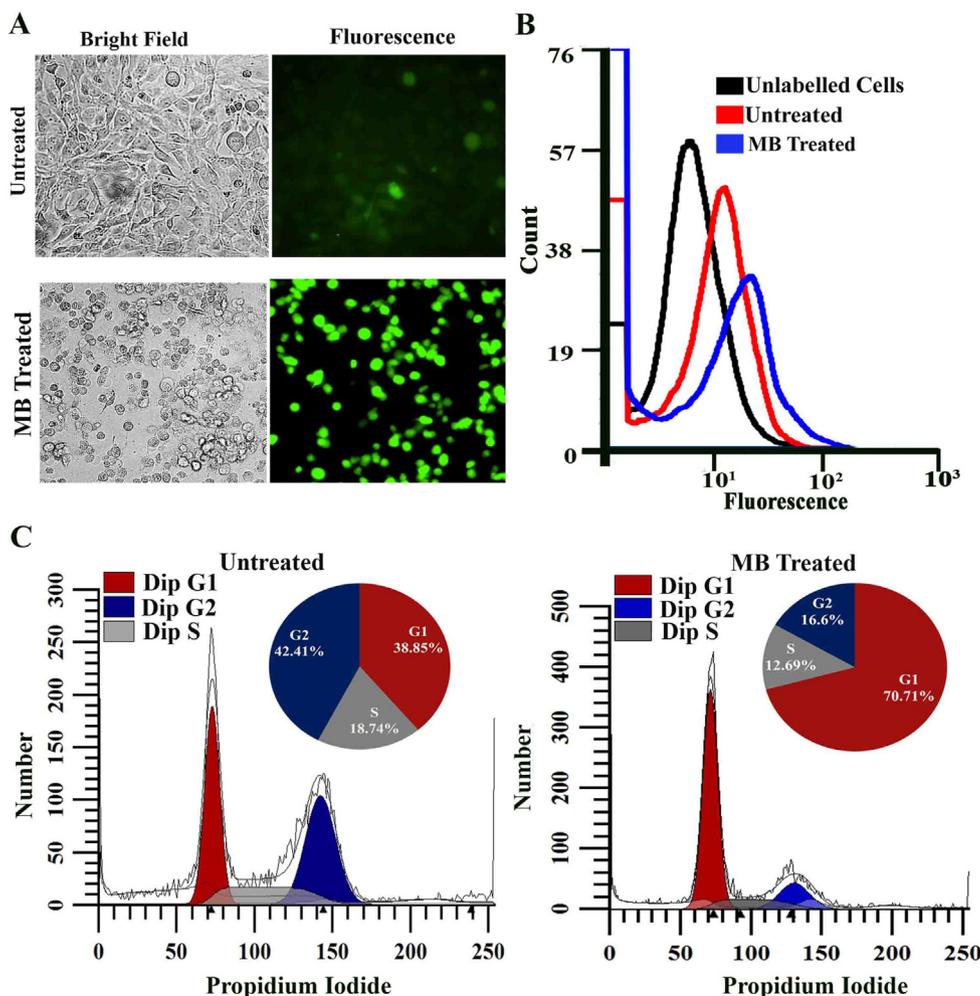
Caspase 9 is an initiator caspase that is involved in driving organized events of apoptosis. Hence, caspase 9 was assayed as a consequence of mitochondrial depolarization and Cyt-c release on the treatment of MG-63 for 24 h. For caspase-9 colorimetric assay MG-63 cells treated with *Manikya Bhasma* (105.7 µg/mL) displays a

**Table 3**

MB kills the cancer cells with no recovery.

Time period (Hr)	IC <sub>50</sub>	IC <sub>50</sub> with 48hr Recovery	Change in IC <sub>50</sub> (%)
12	110.751 ± 5.8956	98.838 ± 8.19653	10.9
24	94.404 ± 6.9172	103.582 ± 5.2510	8.7
48	107.7 ± 5.57	105.281 ± 5.254	1.86
72	110.6 ± 3.4058	120.235 ± 7.2401	8.3

Different Cancer cells were treated with MB (0–1000 µg/mL) for a period of 48 h and cellular viability was measured by MTT assay as described in "Material and Methods". In few reactions, cells were treated with indicated time points and then allowed to recover for additional time period of 48 h in complete medium. IC<sub>50</sub> was calculated in both conditions and compared.



**Fig. 4.** *Manikya Bhasma* induces oxidative stress and dysregulates cell cycle in cancer cells: (A) *Manikya Bhasma* (105.2  $\mu\text{g}/\text{mL}$ ) treated osteosarcoma (MG-63) cells incubated with DCFDHA showed bright green fluorescence in comparison to untreated cells confirming the generation of ROS inside the cells. (B) Flow cytometric analysis of untreated Osteosarcoma (MG-63) cells with or without *Manikya Bhasma* (105.2  $\mu\text{g}/\text{mL}$ ) treatment indicates a significant amount of ROS was produced inside cancer cells.  $n = 2$  (C) *Manikya Bhasma* is disturbing the cell-cycle of cancer cells. *Manikya Bhasma* (105.7  $\mu\text{g}/\text{mL}$ ) treated or untreated Osteosarcoma (MG-63) cells were stained with propidium iodide and analyzed in FL-2A channel (excitation: 488 nm, emission: 585/42-nm). In untreated osteosarcoma cells (MG-63),  $38.85 \pm 1.37\%$  were present in G1 phase,  $18.74 \pm 3.32\%$  MG-63 in S phase, and  $42.41 \pm 1.94\%$  cells in G2 phase. Whereas cells treated with *Manikya Bhasma* exhibited  $70.71 \pm 0.219\%$  MG-63 cells in G1 phase,  $12.69 \pm 0.473\%$  MG-63 cells in S phase, and  $16.6 \pm 0.254\%$  in MG-63 cells in G2 phase. A representative data of 3 independent experiments were presented,  $n = 3$ .

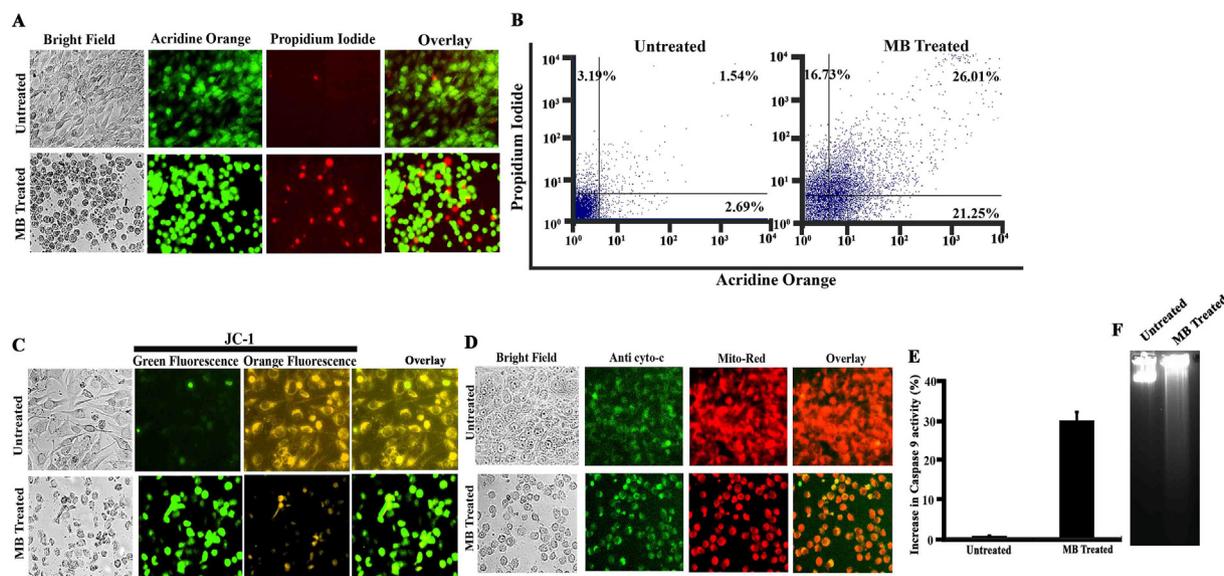
$29.6 \pm 2.1\%$  increase in caspase-9 activity (Figure: 5E). This finding confirms that *Manikya Bhasma* treated MG-63, which shows a higher amount of caspase 9 as compared to the untreated cells, and it indicates that apoptosis induced by *Manikya Bhasma* follows the mitochondrial pathway. MG-63 cells were treated with *Manikya Bhasma* (105.7  $\mu\text{g}/\text{mL}$ ) and genomic DNA was isolated as described in the materials and methods section. Control cells gave intact genomic DNA with no evidence of smear or laddering pattern (Fig. 5F). In contrast, the DNA of cells treated with *Manikya Bhasma* shows characteristic smear formation (Fig. 5F).

#### 4. Discussion

Cancer is the major cause of death across the world and various strategies are developed to combat this disease [1]. The use of chemotherapeutic drugs for the treatment of cancer has several problems like the development of multidrug resistance and severe pathological side-effects [36]. Ayurveda is an ancient science dealing with the host defense machinery to combat different types of diseases. The basic philosophy of Ayurveda is to boost the host

machinery to rectify the disturbance in cellular physiology to restore homeostasis [5]. This approach has a significant advantage for the treatment of cancer and also to reduce the side effects. There is an enormous number of ayurvedic herbs that have anticancer activity, such as garlic (*A. sativum*), green tea (*C. sinensis*), and tulsi (*Ocimum tenuiflorum*) [6]. Rasa Shastra is a branch of Ayurveda that deals with the metallic/mineral preparations termed as bhasmas. Ayurvedic bhasmas are incinerated herbo-metallic/mineral preparation that possesses nanomedicine like characteristics [25,37]. Preparation of Ayurvedic bhasma involves two major steps such as detoxification and incineration of metal/minerals and the end products obtained are non-toxic, safe with increased therapeutic effects [5,20]. *Manikya Bhasma* is an ayurvedic bhasma that is synthesized using purified ruby, orpiment, and arsenic sulfide and possesses tremendous therapeutic value [28].

The current study primarily explored the potential of *Manikya Bhasma* as nanomedicine like characteristics with anticancer activity. Biophysical and chemical characterization of bhasma reveals that MB has 70 nm particles which are crystalline (Fig. 1). From the FESEM, FETEM, and DLS analysis of *Manikya Bhasma*, it



**Fig. 5. *Manikya Bhasma* causes cell death in cancer cells by apoptosis through the mitochondrial pathway. (A) *Manikya Bhasma* is inducing apoptosis in cancer cells.** MG-63 cells treated with *Manikya Bhasma* (105.7 µg/ml) and dual staining was done by acridine orange and propidium iodide. Untreated cells show more green fluorescence due to the presence of live cells, whereas treated cells show red fluorescence due to the staining of dead cells by propidium iodide. The fluorescence imaging was done using a cytecell cell imaging system (GE Healthcare). **(B) Flow cytometry analysis of dual stained MG-63 cells by BD FACS Caliber.** A representative data of 2 independent experiments were presented, n = 2. **(C) *Manikya Bhasma* is disturbing mitochondrial membrane potential in cancer cells.** MG-63 cells were treated with *Manikya Bhasma* (105.7 µg/ml) in serum-free media for 24 h, and were stained with JC-1 dye and observed using the Cytell cell imaging system (GE Healthcare). *Manikya Bhasma* treated cells show a decrease in mitochondrial membrane potential as indicated by green fluorescence and loss of orange fluorescence. **(D) *Manikya Bhasma* treatment causes the release of Cyt-c from Mitochondria.** MG-63 cells were treated with *Manikya Bhasma* (105.7 µg/ml), in serum-free media for 24 h. Post-treatment, cells were fixed using acetone and methanol (1:1) and further stained for cytochrome-c using anti-Cyt-c antibodies. The location of mitochondria in cells was determined by staining the cells with mitotracker red as given in the materials and methods section. Labeled cells were observed in the Cytell cell imaging system (GE Healthcare). Untreated cells seem healthy with the cytochrome-C signal overlapping with the mitotracker signal. High-resolution imaging detected the release of Cyt-c from *Manikya Bhasma* treated cells. **(E) *Manikya Bhasma* treatment causes up-regulation of caspase activity in cancer cells.** *Manikya Bhasma* treated Osteosarcoma (MG-63) cells show the  $29.6 \pm 2.1\%$  increase in the level of caspase 9 activity as compared to untreated cells. Standard deviation was calculated from the data of three independent (n = 3) experiments. **(F) *Manikya Bhasma* mediated caspase activation in-turn causes degradation of genomic DNA.** MG-63 cells treated with *Manikya Bhasma* (105.7 µg/ml) show smear formation, however, the genome is intact in the case of untreated cells.

was found that its particles were spherical and were highly aggregated. Also, *Manikya Bhasma* was found to reduce the cellular viability of cancer cell lines in a dose-dependent manner. Interestingly, the cytotoxic effect of *Manikya Bhasma* was found to be irreversible as cancer cells cannot recover from cellular damages induced by bhasma. This is an important property to explore the utility of MB in over-coming the relapses in cancer [30]. Cell death in cancer cells can follow either apoptosis or necrosis pathway [32,33]. These apoptosome granules containing cytoplasmic constituents serve as excellent opsonins and are effectively phagocytosed by our immune cells. In the current study, it was found that the cell-death mediated by *Manikya Bhasma* is apoptotic in nature.

## 5. Conclusion

This study concludes that *Manikya Bhasma* works as nano-medicine and the cell death induced by the *Manikya Bhasma* inside the cancer cells follows mitochondrial-dependent apoptosis. *In vivo* studies and clinical trials can be done to check the potential of *Manikya Bhasma* as an anticancer drug. Higher-level studies have to be conducted to determine the combined effect of *Manikya Bhasma* along with conventional anticancer therapies.

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## Conflict of interest

None.

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