Research Paper

Anticancer and Immunomodulatory Properties of Herbomineral Formulation Abhrak Bhasma on Breast Cancer

K. S. SREE LAKSHMI AND GEETHA NANDAGOPALAN*

Department of Zoology, PSG College of Arts and Science, Coimbatore, Tamil Nadu 641014, India

Abhrak Bhasma (Shatputi) is a time tested classical Ayurvedic formulation, prepared from mica, as well as the juices of numerous other indigenous substances. In this work, we focus on the efficacy of Abharak Bhasma on anticancer activity and immunomodulatory effects using breast cancer Michigan Cancer Foundation-7 cell line and macrophage cell line Robert Abelson leukemia virus 264.7. The *in vitro* anti-cancer activities of the formulation were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The percentage of cell death against Michigan Cancer Foundation-7 cell breast cancer cells increases with an increase in the concentration of Abhrak Bhasma. A high percentage of cell death (49.22±1.504 %) was observed at the concentration of 200 µg/ml of Abhrak Bhasma. The corresponding half-maximal inhibitory concentration value of Abhrak Bhasma was found to be 193.2±10.72 µg/ml. There was a dose-dependent decrease in the nitrite level in Robert Abelson leukemia virus 264.7 incubated with Abhrak Bhasma at the concentration ranges from 50 to 200 µg/ml. A significant reduction in nitrite level was found to be about 489.3±35.92 at 200 µg/ml. Lipopolysaccharide (1 µg/ml) treated was served as a control with a maximum nitrite level of about 1873±83.59 µg. The present study reveals that the percentage of cell viability in the macrophage cell line decreases with an increase in the concentration of Abhrak Bhasma. The least viability of the cell was observed at the concentration of 200 µg/ml showing 53.85±1.211 %. Our study highlights dual qualities of Abhrak Bhasma as a potential drug against breast cancer and as an immunomodulatory drug showing anti-inflammatory properties.

Key words: Breast cancer, Abhrak Bhasma, cell viability, nitrite level, anti-inflammatory, MCF-7 breast cancer cell line, RAW 264.7 macrophage cell line

A common illness with widespread documentation is cancer. The predicted number of new instances of female breast cancer in 2020 is 2.26 million, making breast cancer as the most frequently diagnosed cancer globally[1]. Indian traditional remedies have a long history of successfully treating a variety of illnesses. It provides support for a sizable, rapidly expanding industry with yearly sales of 1 billion United States Dollar (USD)[2]. Most commercially available chemotherapeutics have major side effects, which put people's lives at stake[3,4]. To address this issue, innovative strategies with excellent biocompatibility and minimal cancer chemotherapy side effects are urgently required[5].

Bhasma is a class of herbometallic Ayurvedic remedies that include the puta (repeating cycles) technique of incineration and the repeated burning of various metals or their ores combined with decoction of various herbal items. These preparations are sometimes referred to as nanomedicines since the components of the finished product typically have sizes in the nanometer range[6,7]. In comparison to plant-based medications, bhasmas offer various advantages, including stability over longer time periods, lower doses, simple storage, quick accessibility and availability[8-10]. Several Bhasmas have been discovered to have various therapeutic benefits such as Heerak Bhasma (nanodiamonds-based Ayurvedic herbomineral preparations) possess immunostimulatory and tumor-killing qualities[11]; Manikya Bhasma functions as a nanomedicine and the cell death it causes inside cancer cells is characterized

***Address for correspondence**
E-mail: geethsen@gmail.com

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by mitochondrial-dependent apoptosis[12]. Anti-cancer[13-15], anti-inflammatory[16], analgesics[17] and immunomodulatory[18] actions were disclosed by Swarna Bhasma. It has been established that Rajatha Bhasma possesses anti-cancer and hepatoprotective effects[19,20].

Abhrak Bhasma is a red powder with particles between 100 and 1000 nm in size that is high in iron, calcium, potassium and other minerals[21-23]. There is proof of anti-diuretic[24], anti-stress, anti-inflammatory, immunomodulatory[25], adaptogenic[26], memory-enhancing[27], anti-cancer[28], anti-diabetic[29] and Deoxyribonucleic Acid (DNA) repair[30] role of Abhrak Bhasma. Even though lung, leukemia and prostate cancer cell lines exhibited anticancer activity, prostate cancer cell line revealed significant positive *in vitro* anticancer activity for Abhrak Bhasma[28]. Immunomodulation is a very general word that refers to any modifications to the immune system and might include the induction, expression, amplification or inhibition of any component or stage of the immune response[31]. Immunomodulators are biological or synthetic compounds that have the ability to activate, inhibit or modify any immune system function, including the innate and adaptive immune systems[32]. Recent scientific studies have focused on the potential therapeutic use of Ayurveda herbal remedies for modulating immune response. Rasayana includes immunomodulators with a range of chemical compositions and molecular sizes. They work by altering a number of immune system processes, including the activation and multiplication of macrophages, stimulation of phagocytosis, proliferation of lymphocytes producing T helper cells and activation of complement pathways. Immunostimulants have the potential to improve both the nonspecific and specific immune response to infection[33,34]. Anticancer activity on breast cancer cell lines and immunomodulatory effects of Abhrak Bhasma, have not yet been the subject of any studies. Hence in this work we have focused on the efficacy of Abhrak Bhasma on anticancer activity and immunomodulatory effects using Human breast cancer cell line (Michigan Cancer Foundation-7 (MCF-7)) and Macrophage cell line (Robert Abelson leukemia virus (RAW) 264.7).

MATERIALS AND METHODS

Abhrak Bhasma-Baidyanath (Nagpur) Abhrak Bhasma (Shatputi) is time tested classical Ayurvedic formulation, prepared from mica as well as the juices of numerous other indigenous substances.

Anti-cancer activity study:

10 mg/ml Abhrak Bhasma, MCF-7 breast cancer cell line, Fetal Bovine Serum (FBS), trypsin, Ethylenediaminetetraacetic acid (EDTA), haemocytometer, 1 % streptomycin, pencillin G, amphotericin B, glucose, 3-[4,5-dimethyl thiozole-2-yl]-2-5-diphenyl tetrazolium bromide (MTT), phosphate buffer saline pH 7.4, Dimethyl Sulfoxide (DMSO), microplate Enzyme-Linked Immunosorbent Assay (ELISA) reader.

Procedure:

10 mg of Abhrak Bhasma dissolved in 1 ml of distilled water (stock solution), was then further diluted in distilled water to attain the following concentrations of 10, 50, 100, 200 µg/ml Abhrak Bhasma. MCF-7 breast cancer cell line which was obtained from the National Centre for Cell Sciences (NCCS), Pune, cultured in a Minimal Essential Medium (MEM) with 10 % FBS, trypsin, EDTA, Glucose, 1 % streptomycin, pencillin G and amphotericin B, in a fully humidified atmosphere with 5 % CO_2 at 37°. MCF-7 breast cancer cell line was made to detach from the MEM using trypsin and EDTA to create single cell suspensions. Viable MCF-7 breast cancer cells in single cell suspension were counted using a haemocytometer. Viable MCF-7 breast cancer cells in single cell suspension diluted to a density of 1×10^5 cells/ml, using a medium containing 5 % FBS. 100 µl per well of the MCF-7 breast cancer cell suspension seeded were in 96-well plates at plating density of 10 000 cells/well and incubated at 37°, 5 % CO_2, 95 % air and 100 % relative humidity to allow for cell attachment. After 24 h of incubation, the cells were treated with 100 µl of different concentrations of the Abhrak Bhasma (10, 50, 100, 150 and 200 µg/ml). Following the treatment with Abhrak Bhasma, the plates were further incubated for 48 h at 37°, 5 % CO_2, 95 % air and 100 % relative humidity. The medium without samples served as control and triplicate maintained for all used concentrations.

Modified MTT assay:

The effect of Abhrak Bhasma on the viability of MCF-7 breast cancer cell line were determined by modified MTT assay[35]. 100 µl of cell suspensions in growth medium plated in 96-well microtiter plate at concentrations of 1×10^4 cells/well and incubated for 48 h at 37° in a humidified incubator. After 48 h, 0.1 % DMSO was added to the incubated cells suspension and further incubated for 72 h at 37°. Suspension was

washed with pH 7.4 phosphate buffer. 20 µl of MTT (5 mg/ml) added to all wells in the plate and incubation continued for 4 h at 37° which resulted in the formation of formazan. Solution in all wells was aspirated. 100 µl buffered 0.1 % DMSO added to dissolved formazan and plates shaken well for 5 min. Optical density measured using a microplate Enzyme-Linked Immunosorbent Assay (ELISA) reader at 540 nm, with 0.1 % DMSO as control. Cytotoxicity of the Abhrak Bhasma was obtained by comparing the absorbance (inferred from ELISA) between the control and samples. The percentage inhibition calculated using the formula

Percentage cell viability=A540 of treated cells/A540 of control cells×100 IC_{50} calculated from dose-response curves.

Immunomodulatory study:

Abhrak Bhasma, macrophage cell line RAW 264.7, FBS, sodium nitrite solution, trypsin EDTA, 1 % streptomycin, penicillin-streptomycin (10 %) , 1 µl lipopolysaccharide (LPS) (1 µg/ml), DMSO, Microplate (ELISA) reader, centrifuge, lysis buffer (0.1 M Tris Hydrochloride (Tris HCl), 0.25 M EDTA, 2 M NaCl, 0.5 % Triton X-100), sulphosalicylic acid, Vortex mixer, Griess reagent, sodium nitrite solution.

Procedure:

Cell culture, measurement of cell viability: Macrophage cell line RAW 264.7 was obtained from National Center for Cell Science (Pune, India). Cell line was cultured in Dulbecco's modified eagle medium supplemented with FBS (10 %) containing penicillin-streptomycin (10 %) at 37° in a humidified atmosphere containing 5 % CO_2. Cells were plated at a density of 1×10^4 cells/well in 25 or 75 cm^2 flasks, or in 96-well plate overnight. RAW 264.7 were grown to 60 % confluence followed by activation with 1 µl LPS (1 µg/ml). LPS stimulated RAW cells were exposed with different concentration (50, 100, 200 µg/ml) of Abhrak Bhasma and incubated for 24 h. After 24 h of incubation, the cells were digested and centrifugation was done at 6000 revolutions per minute (rpm) for 10 min. Supernatant was discarded and cells were then re-suspended in 200 µl of cell lysis buffer (0.1 M Tris HCl, 0.25 M EDTA, 2 M NaCl, 0.5 % Triton X-100). The samples were then kept at 4° for 20 min. After incubation, the immunomodulatory response was performed by estimating nitrite levels in the cell lysate.

Estimation of cellular nitrite level:

The level of nitrite was estimated by the method of Lepoivre *et al*.[36]. To 0.5 ml of cell lysate, 0.1 ml of sulphosalicylic acid was added and vortexed well for 30 min. The samples were then centrifuged at 5000 rpm for 15 min. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µl of the supernatant, 30 µl of 10 % sodium hydroxide (NaOH) was added, followed by 300 µl of Tris HCl buffer and mixed well. To this, 530 µl of Griess reagent was added and incubated in the dark for 10-15 min and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained. The bar graph represents values with standard error.

RESULTS AND DISCUSSIONS

In vitro anti-cancer evaluation of Abhrak Bhasma on the cell death against MCF-7 breast cancer cell line was performed at varying concentration ranging from 10 to 200 µg/ml. The result obtained from the study reveals that the percentage of cell death of MCF-7 breast cancer cell line increases with increase in concentration of Abhrak Bhasma. High percentage of cell death was observed at the concentration of 200 µg/ml as 49.22±1.504 %. Followed by this at 100 µg and 50 µg shows 33.45±4.865, 20.31±2.559, similarly at 10 µg/ml it shows 5.718±0.9802 % cell death in MTT assay (fig. 1). The corresponding IC_{50} value of Abhrak Bhasma was found to be 193.2±10.72 µg/ml. The morphology of cells treated with Abhrak Bhasma in the present study showed significant cell shrinkage/ rounding and formation of apoptotic cell bodies due to cellular plasma membrane deformation, apoptotic cell death and cytoplasm shrinkage (fig. 2). Furthermore, Jha *et al*.[12] reported that Manikya Bhasma showed significant reduction in the number of cells in the S-phase (proliferation phase with active DNA synthesis). It was observed that the cytotoxicity of the Bhasma has made the cancer cells unable to recover from the cellular damages and could not revert back to their original phase, similar mechanisms might have caused the above said cellular damages in the present study. The present study suggests that Abhrak Bhasma has an immunomodulatory effect on RAW 264.7 cells, as evidenced by the observed dose-dependent decrease in nitrite levels and macrophage cell viability. LPS (1 µg/ml) treated well was served as a control with maximum nitrite level of about 1873±83.59 µg. The formulation Abhrak Bhasma at the dose of 50 µg/ml shown a decrease in nitrite level of about 1422±66.3 µg, similarly at the concentration of 100 µg/ml it was 831.3±85.76 µg. At

higher concentrations of Abhrak Bhasma, there was a significant decrease in nitrite levels, with the maximum decrease 489.3±35.92 µg observed at 200 µg/ml (fig. 3). These results suggest that Abhrak Bhasma may be able to modulate the production of Nitric oxide (NO), a key signaling molecule involved in the immune response. NO generated by inducible NO Synthase (iNOS) is vital in inflammation and immune system control[37]. The reduction in nitrite level by Abhrak Bhasma suggest the anti-inflammatory properties as reported in *Quercus infectoria* by Wan-Nor-Amilah *et al*.[38]. The result obtained from the study reveals that the percentage of cell viability of macrophage cell line decreases with increase in concentration of Abhrak Bhasma. The least viability of cell was observed at the concentration of 200 µg/ml shows 53.85±1.211 % (fig. 4 and fig. 5). The present study indicates that the Abhrak Bhasma displays promising potential *in vitro* anti-cancer activity on breast cancer cell line MCF-7 and immunomodulatory effects in macrophage cell line RAW 264.7. This suggests that, in addition to its effectiveness against prostate cancer, it may also be useful in the treatment of breast cancer. However, further *in vivo* studies are needed to gain a better understanding of the potential effects of Abhrak Bhasma.

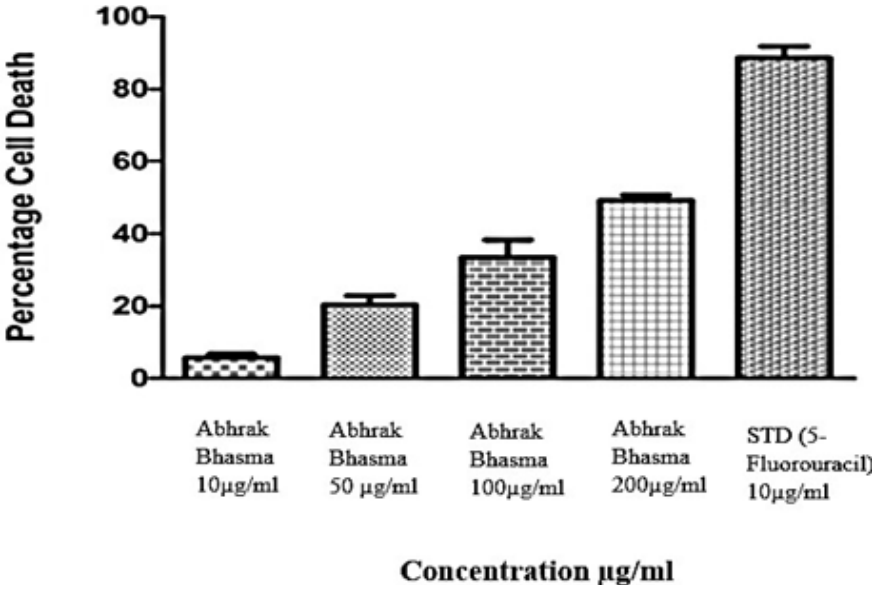

Fig. 1: Bar diagram showing anti-cancer activity for various concentrations of Abhrak Bhasma and standard 5-fluorouracil on cell death of MCF-7 cancer cell line

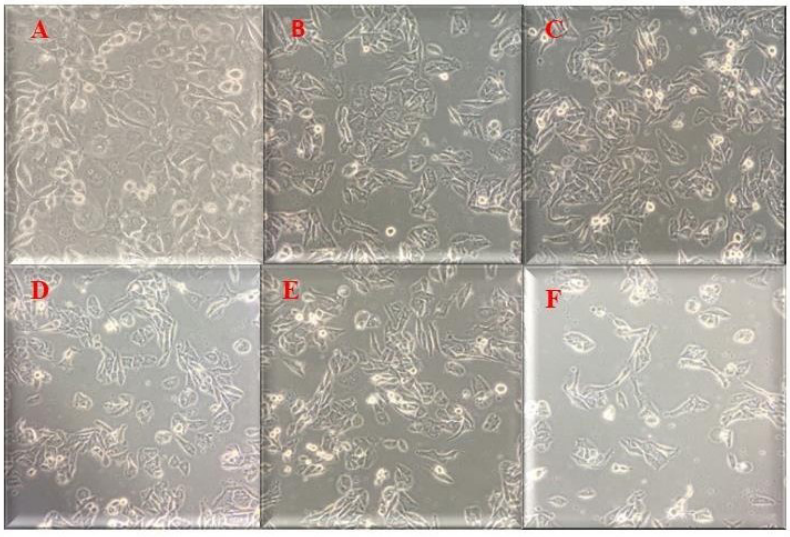

Fig. 2: Anti-cancer activity for various concentrations of Abhrak Bhasma on cell death of MCF-7 cancer cell line, (A): MCF7 control cells; (B): Abhrak Bhasma 10 µg/ml; (C): Abhrak Bhasma 50 µg/ml; (D): Abhrak Bhasma 100 µg/ml; (E): Abhrak Bhasma 200 µg/ml and (F): Standard (STD) 5-fluorouracil 10 µg/ml

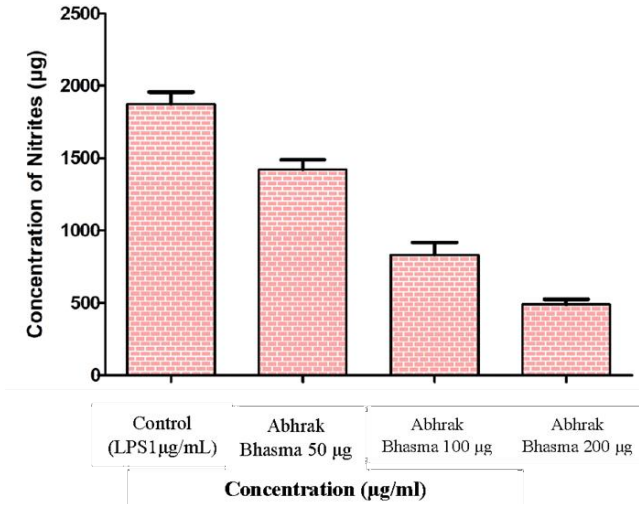

Fig. 3: Effect of various concentrations of Abhrak Bhasma on nitrite level in macrophage RAW 264.7 cell line

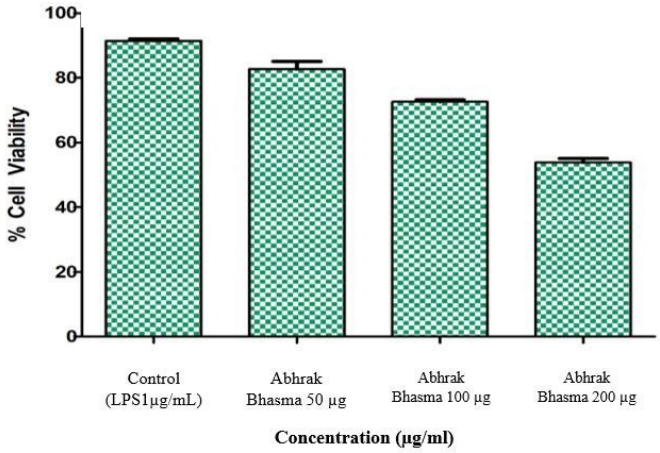

Fig. 4: Effect of various concentrations of Abhrak Bhasma on cell viability in macrophage RAW 264.7 cell line

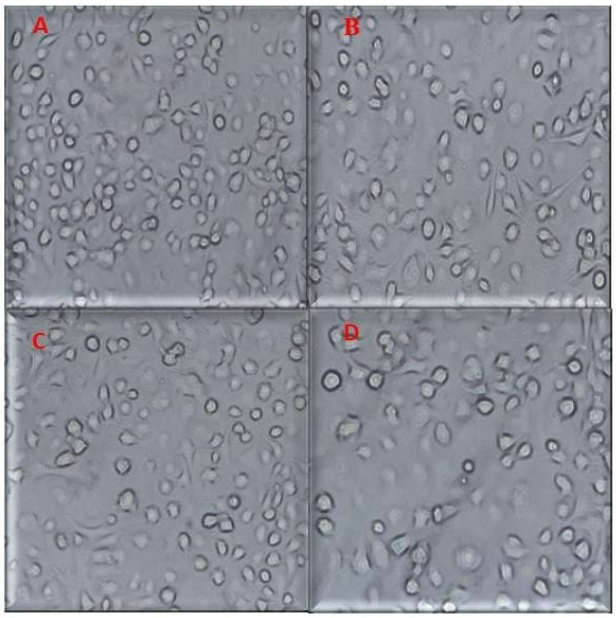

Fig. 5: Effect of various concentrations of Abhrak Bhasma on cell viability in macrophage RAW 264.7 cell line; (A): LPS induced proliferation in macrophage cell line RAW 264.7; (B): Abhrak Bhasma 50 µg; (C): Abhrak Bhasma 100 µg and (D): Abhrak Bhasma 200 µg

Conflict of interest:

The authors declared no conflict of interests.

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