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Ultradiluted Homeopathic Medicines Cause Apoptosis in RPMI-8226 Multiple Myeloma Cells *in vitro*: a Pilot Study

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Homeopathy

Abstract

Background Multiple myeloma (MM) is the second most common type of cancer among hematological malignancies and is difficult to treat. Although controversial in nature, homeopathy's effects have been tested on a wide range of cancer cell types *in vitro*, as well as clinically. However, homeopathic medicines have yet to be tested in MM cells. In this preliminary study, we investigated the effects of *Arsenicum album*, *Hecla lava*, *Carcinosinum* and *Carboneum sulphuratum* 200C on a human MM cell line.

Methods The RPMI-8226 MM cell line was cultured *in vitro* for up to 96 hours and treated with each of four homeopathic preparations. The spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometric Annexin V-PE/7-actinomycin D (7-AAD) and propidium iodide (PI) staining were each used to examine cell viability, apoptosis and cell cycle, respectively.

Results The MTT assay showed that all four homeopathic preparations reduced cell viability over time when compared to the control group cells, especially at 72 and 96 hours whereby only 50% of cells remained viable. Similarly, after 96 hours of treatment, the proportion of viable cells was significantly decreased and the proportion of early apoptotic (Annexin-V-PE +/7AAD-) cells was significantly increased for all four homeopathic preparations. Based on the PI-staining cell cycle data, cells treated with *Hecla lava* and *Carboneum sulphuratum* showed a statistically significant accumulation in the sub-G0/G1 phase of the cell cycle ($p < 0.05$).

Conclusion This is the first study to demonstrate that each of four homeopathic medicines causes apoptosis in a MM cell line. Further exploration of the potential of *Arsenicum album*, *Hecla lava*, *Carcinosinum* and *Carboneum sulphuratum* as a complementary therapeutic option in MM is warranted.

Keywords
- homeopathy
- cytotoxicity
- apoptosis
- cell cycle arrest
- multiple myeloma

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Introduction

Multiple myeloma (MM) is a type of hematological cancer characterized by uncontrolled clonal proliferation due to the malignant transformation of plasma cells in the bone marrow microenvironment. MM accounts for 1.3% of all malignancies and 15% of hematological neoplasms.[1] Although the discovery of new drugs with different mechanisms of action has recently changed treatment approaches for MM, survival has been limited to 6 to 10 years, depending on the age of the patient. Unfortunately, resistance to these new anti-cancer drugs due to genetic abnormalities and epigenetic differences remains a challenge, meaning that new treatment strategies for this type of cancer are still needed.[2–4]

Homeopathy is one of the complementary and alternative medical (CAM) treatment methods in which potentized micro- and even nanodoses of natural substances are used at ultramolecular dilutions.[5,6] Homeopathy was invented by Samuel Hahnemann in the 19th century and is widely used around the world, especially in France, Germany, India, South America, the United Kingdom and the United States of America.[7] There are experimental animal[8–12] and human[6,13–15] studies showing the effectiveness of homeopathically prepared medicines in many cancer types, suggesting a rich potential for developing new anti-cancer treatments. However, in some circles, this treatment approach has been claimed to be due solely to the placebo effect[16] because it is often used at ultra-high dilutions and the mechanism of action of homeopathic preparations is unknown.

However, experimental evidence does exist to support the potential of homeopathic medicines in treating cancer cells *in vitro*, where the placebo effect is absent. For example, a number of studies have demonstrated that homeopathic medicines have cytotoxic properties,[2,10,17–19] are able to induce apoptosis[2,5,17–19] and can alter cell cycle stages[5,17–19] in a range of cancer cell types. In summary, both *in vitro* and *in vivo* studies in the literature show that homeopathic medicines with different potencies have cytotoxic and apoptotic effects on cancer types, as well as producing reduction in tumor size and increased survival rate.

Despite this body of evidence, the potential usefulness of homeopathic medicines in MM patients has been presented only in a congress communication that reported the effects of *Hecla lava*.[4] No research has been found on the molecular and biological effectiveness of homeopathic medicines in MM cells *in vitro*. Thus, the aim of this pilot study was to provide the first information about the anti-cancer activities of homeopathic medicines on MM cells at the molecular level. Four homeopathic medicines were selected at the 200C potency: *Arsenicum album* and *Carcinosinum*, as they have both been previously well studied and characterized *in vitro*[17,20]; *Hecla lava* because it is the only medicine to date tested in MM patients[4]; and *Carboneum sulphuratum*, which has not been studied at the molecular level before but has been cited as potentially useful in reducing the negative effects of cancer treatment.[20]

Methods

Preparation of Homeopathic Medicines

In Turkey, homeopathic medicines are generally given to patients in the form of medicated starch globules dissolved in water. For this reason, in our study, such a homeopathic preparation was used in preference to liquid remedies or medicating potencies. Thus, a stock solution for experimental use was prepared from starch globules previously medicated by spraying with the homeopathic medicines *Arsenicum album* (200C), *Hecla lava* (200C), *Carcinosinum* (200C) and *Carboneum sulphuratum* (200C; Helios Homeopathy Ltd., UK), which were then dissolved in sterile distilled water at 10 mg/100 µL. This stock solution was centrifuged to remove particulate starch and used immediately. All homeopathic medicines were prepared fresh for each experiment.

Cell Line and Cell Culture

The human multiple myeloma (MM) cell line RPMI-8226 was procured from the American Type Culture Collection (ATCC, Manassas, VA, United States) and cultured at 1×10^6 cells/mL in complete medium (RPMI 1640 medium supplemented with L-glutamine (Bio-Ind, United States), 10% fetal bovine serum (Bio-Ind, United States), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, United States)). Cells were grown at 37°C with 5% CO_2 in an incubator.[21]

Cell Viability Assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, United States) method as directed by the manufacturer. In brief, RPMI-8226 cells were seeded into 96-well plates at a density of 1×10^4 cells/well in a final volume of 100 µL complete medium. Cells were treated with a single dose of each individual homeopathic preparation or control at the start of the culture. Given that distilled water was used as the solvent for the homeopathic preparations, an equal volume of distilled water was used as the vehicle (negative) control. In each experiment, the highest concentration of each remedy and distilled water was not more than 0.5%.[21]

At the appropriate timepoint (24, 48, 72, or 96 hours), the cells were incubated with 5 mg/mL MTT solution for an additional 4 hours at 37°C. The tetrazolium salt was then dissolved in a solution containing sodium dodecyl sulphate (Sigma, United States) to solubilize the formazan, and the plates were incubated at 37°C overnight. The optical density (OD) of each sample was assessed using a spectrophotometric plate reader (Biotek, United States) at 550 nm and a reference wavelength of 690 nm. The OD of control cells was used as 100% viability, and the percentage of cell viability in each treated group was calculated accordingly.

Flow Cytometric Analysis of Apoptosis

RPMI-8226 cells (1×10^5 cells/well) were seeded into 24-well plates and incubated for 96 hours in complete medium with each homeopathic medicine or control. The cells were then harvested by gentle pipetting and washed twice with phosphate buffered saline (PBS; Merck, Germany). The cells

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were then centrifuged and labeled for Annexin V-PE and 7-Aminoactinomycin D (7-AAD) according to the manufacturer's protocol (BioLegend, United States). In brief, cells were re-suspended in 100 µL 1xBB (Binding Buffer) with equal parts Annexin V-PE (5 µL) and 7-AAD (5 µL) and incubated at room temperature for 15 min in the dark. Following incubation, the cells were washed with PBS again, a further 400 µL 1XBB was added, and apoptosis was assessed using flow cytometry (Accuri C6; BD Biosciences, United States), where forward scatter and side scatter were first used to identify single cells.[21]

Cell Cycle Analysis

RPMI-8226 cells (5×10^5/well) were seeded into 6-well plates incubated for 96 hours in complete medium supplemented with each homeopathic medicine or control. The cells were then harvested by gentle pipetting, washed once in cold PBS and fixed with 75% ice-cold ethanol (Merck, Germany) at 4°C for 30 minutes. The fixed cells were then washed twice in PBS, the supernatant discarded and the cells treated with 50 µL RNase (Sigma, United States; 100 µg/mL) for 15 min at 37 °C. Propidium iodide (PI; Sigma, United States; 200 µL of 50 µg/mL stock) was then added to the cells and incubated for 15 minutes at 4 °C in the dark. The DNA content of the cells was determined by flow cytometry, as above.

Statistical Analysis

All experiments were repeated three times, each in triplicate. Mean ± SD (standard deviation) was used for all experimental data. For statistical analysis, Graph Pad Prism 8 software (Graphpad Software Inc, CA, United States) was used. One-way analysis of variance followed by Dunnett's post-test was performed to compare the differences between the control and the treated groups within the multiple samples. Statistical significance was set at $p < 0.05$.

Results

Cytotoxic Effects of Homeopathic Medicines on Multiple Myeloma Cells

The MTT assay was used to assess the impact of homeopathic medicines at 200C on the viability of the RPMI-8226 MM cell line cultured *in vitro* for 24, 48, 72 and 96 hours. The viability of the control cells (exposed to distilled water vehicle only) was set as 100%. Cell viability was reduced by all four homeopathic medicines at each timepoint tested, reaching statistical significance by 24 hours, 48 hours and effectively being halved by 72 and 96 hours ($p < 0.05$; ►**Fig. 1A,B**).

Homeopathic Medicines Induce Apoptosis in Multiple Myeloma Cells

RPMI-8226 MM cells were treated with each homeopathic medicine for 96 hours to investigate the type of cell death induced (i.e., apoptosis or necrosis). As a result, compared to control cells, the proportion of viable (PE-/7AAD-) cells was significantly reduced ($p < 0.05$), while the proportion of early apoptotic (PE+/7AAD-) cells was significantly increased ($p < 0.05$) after treatment with each homeopathic medicine (►**Fig. 2A,B**).

Cell Cycle Arrest in Multiple Myeloma Cells

Based on the cytotoxic response of the RPMI-8226 MM cells to the homeopathic medicines tested, the concomitant

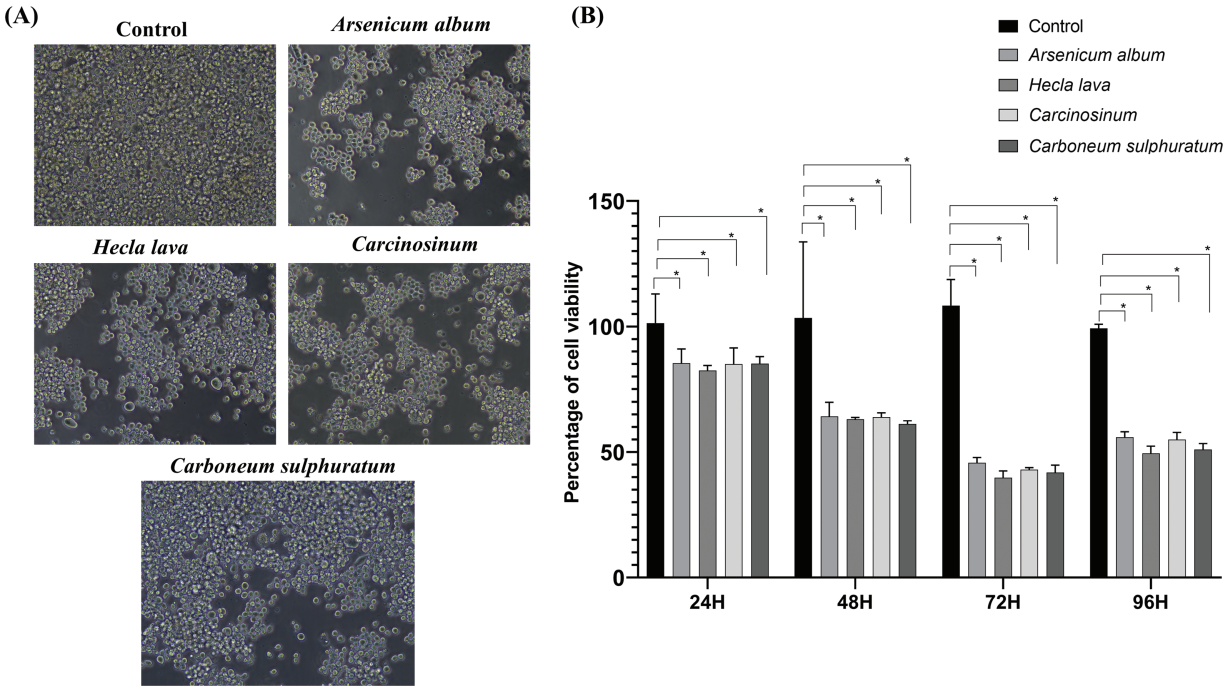

Fig. 1 (A) Phase contrast microscopy visualization of RPMI-8226 MM cell morphology treated with homeopathic medicines compared to control at 96 hours. (B) MTT assay analysis to determine the impact of *Arsenicum album*, *Hecla lava*, *Carcinosinum* and *Carboneum sulphuratum* (200C) on cell viability. Each homeopathic medicine was administered to RPMI8226 MM cells (1×10^4/mL) for 24, 48, 72 and 96 hours. ($^*p < 0.05$ shows significant differences from the control group).

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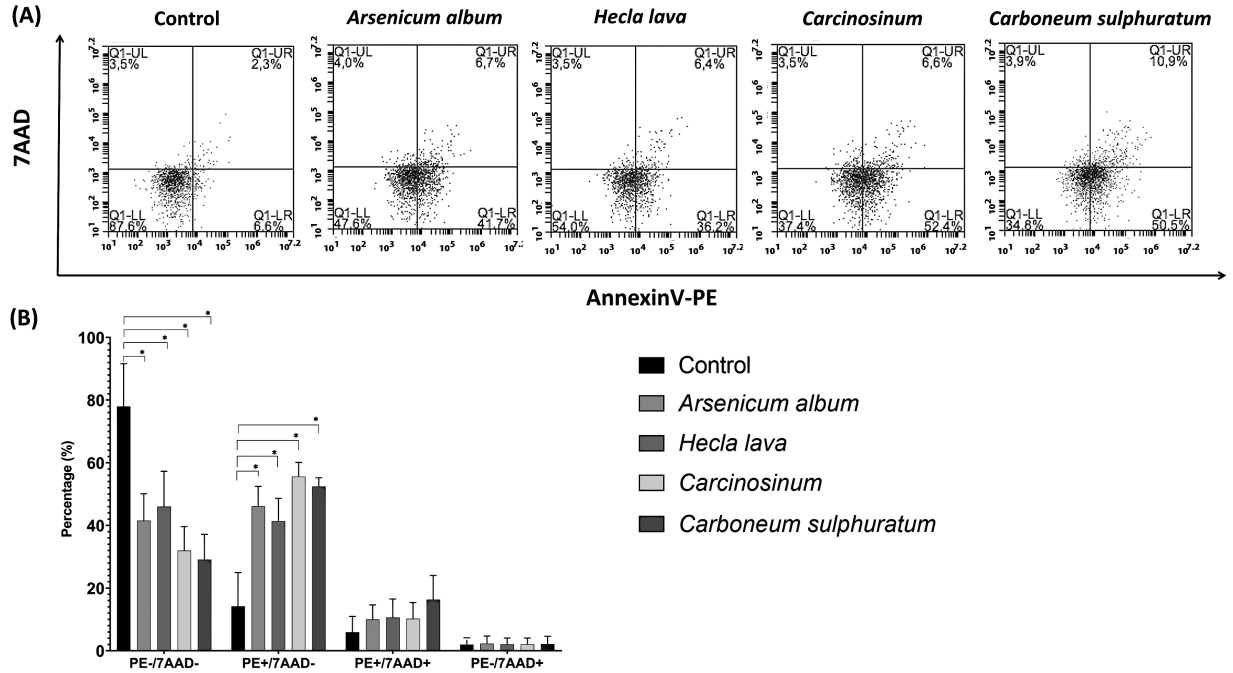

Fig. 2 (A) Representative flow cytometric dot plots showing the staining patterns for Annexin V-PE and 7-AAD markers of apoptosis and necrosis in MM cells treated with distilled water control or homeopathic medicines for 96 hours. On each dot plot the lower left quadrant shows viable cells (PE-/7AAD-); the lower right quadrant shows early apoptotic cells (PE +/7AAD-); the upper right quadrant shows late apoptotic cells (PE +/7AAD +); and the upper left quadrant shows necrotic cells (PE-/7AAD +). (B) Bar chart representation of percentage (%) MM cells with each PE/7-AAD staining pattern as assessed by flow cytometric dotplot ($^*p < 0.05$ shows significant differences from the control within each staining pattern).

effects on cell cycle progression were assessed at 96 hours by flow cytometry. According to the results, while the cell cycle arrest rate of the control group cells in the sub-G0/G1 phase was 37.55% (± 1.061), the arrest rates of cells treated with *Arsenicum album, Hecla lava, Carcinosinum* and *Carboneum sulphuratum* in the sub-G0/G1 phase increased to 40.1% (± 2.207), 49.2% (± 3.341), 40.667% (± 7.047) and 48.3% (± 2.524), respectively (representative histograms are shown in ►**Fig. 3A**). However, statistically significant arrest in the sub-G0/G1 phase was seen only for *Hecla lava* and *Carboneum sulphuratum* treatments ($p < 0.05$), not for *Arsenicum album* and *Carcinosinum* ($p > 0.05$) (►**Fig. 3B**).

Discussion

Examining a potential medical agent's cytotoxicity is generally accepted to be the first step in learning how to employ it as a cancer chemotherapeutic in drug development studies.[18] Inducing apoptosis in cancer cells is one of the main goals of cancer treatments, especially with chemotherapy. Any substance or agent that can selectively suppress the proliferation of malignant cells by modulating the abnormal signal transduction pathway involved in the cell cycle and/or the apoptosis mechanism is considered as an important chemotherapeutic resource in anti-cancer studies.[22] Though homeopathy has recently been used as a CAM method, especially in cancer treatments, the use of these remedies is still viewed with suspicion due to the perceived scarcity of scientific data on how the anti-cancer effects of homeopathic remedies on cancerous tissues or cells are realized.[23] For this reason, any demonstration of induction of apoptosis in malignant cells by homeopathic preparations and/or suppression of malignant proliferation by induction of apoptosis are extremely important in CAM.

In their *in vitro* and *in vivo* study, MacLaughlin et al in 2006 investigated the cytotoxic effects of *Sabal serrulata* homeopathic remedy on PC-3 and DU-145 human prostate cancer cell lines and MDA-MB-231 human breast cancer cell lines and emphasized that *Sabal serrulata* had a cytotoxic effect on human prostate cancer cell lines but was not effective on breast cancer.[10] Moreover, in the same study, they could not see the effects of homeopathically prepared *Thuja occidentalis* and *Conium maculatum* on prostate or breast cancer, either in animal experiments or in cytotoxicity analyses. Similarly, Arora et al in 2013 investigated the cytotoxic effects of homeopathically prepared *Sarsaparilla, Ruta graveolens* and *Phytolacca decandra* in different potencies and mother tinctures on kidney, colon and breast cancer cell lines, regardless of dose.[2] In their study, they showed that cytotoxic effects of the homeopathic preparations in the mother tincture were maximal in all three cell lines; moreover, they reported that the cytotoxicity rates decreased from 30C to 10M potency. When taken together, this evidence suggests that not every homeopathic remedy will be effective in every type of cancer. For this reason, the choice of homeopathic medicines should be based on evidence, especially when being considered as a CAM treatment in cancer care.

Thus, we tested four carefully selected homeopathic medicines at a potency of 200C in MM cells specifically, in order to establish some preliminary data as to the potential usefulness of *Hecla lava* and *Carboneum sulphuratum*, as well as confirming previously reported findings for *Arsenicum*

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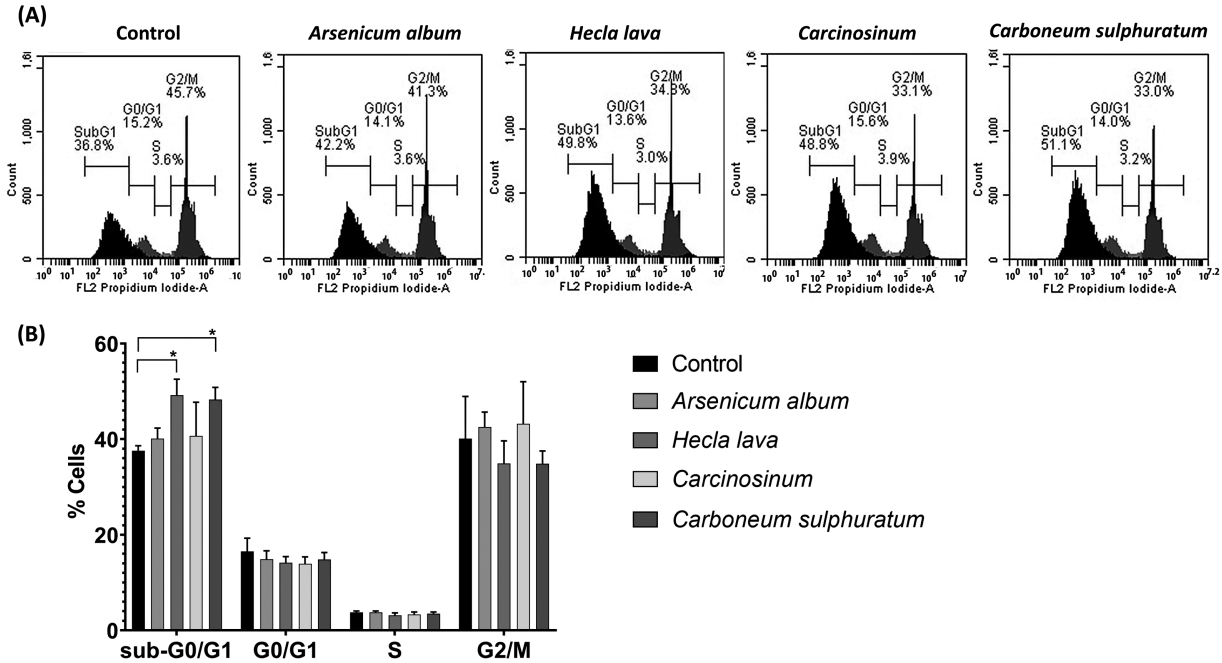

Fig. 3 (A) Representative histograms of flow cytometric PI-staining cell cycle analysis of MM cells treated with homeopathic medicines for 96 hours. (**B**) Summary histogram of proportion of MM cells in each cell cycle phase ($^{*}p < 0.05$ shows significant differences from the control group).

album and *Carcinosinum* tested in other cancer cell lines. Whilst we found that all four medicines significantly reduced MM cell viability and induced apoptosis at 96 hours, there was a differential effect seen between medicines in terms of cell cycle arrest: only *Hecla lava* and *Carboneum sulphuratum* showed a significant effect, suggesting a degree of specificity in their mode of action in MM cells and highlighting novel findings.

Arora & Tandon, in their study investigating the anti-cancer activity of *Ruta graveolens* 30C and mother tincture (MT) in human colon cancer cell lines (COLO-205), found that MT of *Ruta graveolens* stopped the cell cycle at the G2/M checkpoint.[18] Therefore, they thought that with this arrest, the cells entered an intrinsic apoptotic pathway, with pro-apoptotic genes being upregulated and anti-apoptotic genes being downregulated. In the study by Arora et al, in which they showed cytotoxic effects of *Sarsaparilla, Ruta graveolens* and *Phytolacca decandra* homeopathic medicines on kidney, colon and breast cancer cell lines, they also provided a morphological demonstration of apoptotic effects, reporting that the different potencies resulted in apoptotic blebs, chromatin condensation and DNA fragmentation in all three malignant cell lines[2]: cell death thus occurred via apoptosis pathways.

In 2010, Frenkel et al showed that breast cancer cells treated with *Carcinosin* and *Phytolacca* were arrested mostly in the sub-G0/G1 phase, especially at 72 and 96 hours, and this effect was not observed in untreated control cells.[17] The researchers explained this as resulting from altered expression of cell cycle regulatory proteins, including downregulation of phosphorylated Rb and upregulation of the CDK inhibitor p27, activation of caspase-7 and cleavage of the apoptosis-associated proteins poly (ADP-ribose) polymerase (PARP).

Although we have not yet investigated the gene and/or protein level in our study on the MM cell line, we have found flow cytometry evidence for apoptotic and cell cycle arrest effects of the medicines we used. Our findings are consistent with the results of Frenkel et al.[17] In our study, we showed that all four homeopathic medicines used had an apoptotic effect on MM cells, and two of these medicines stopped the cycle in the sub-G0/G1 phase rather than the G2/M phase. Chromosomal DNA fragmentation and entry of cells into the apoptotic pathway are indicators that a cell has entered the sub-G0/G1 phase of the cell cycle.[24] Therefore, we believe that, among our findings, cells arrested specifically in the sub-G0/G1 phase undergo apoptosis due to the fragmentation that occurs in their DNA.

In the study by Sikdar et al in 2014, which investigated the cytotoxic and apoptotic effects of *Condurango* 6C and 30C on human lung cancer cells, it was reported that the homeopathic remedy killed more than 50% of lung cancer cells at 48 hours, that apoptosis was induced by a caspase-3-mediated signaling cascade, and that *Condurango*-treated cells were arrested in the sub-G0/G1 phase of the cell cycle.[5] In our study, we found that the time period in which more than half of the MM cells were killed was 72 and 96 hours. These differences may be explained by the choice of potency. Arora et al, in their work,[2] showed the cytotoxic effects of homeopathic remedies for MT and potencies varying from 30C to 10M and reported that remedies at each potency showed significant cytotoxic effects —but the highest effect was for MT. However, they reported that cytotoxicity decreased at the same rate as potencies from 30C to 10M. Therefore, the longer duration of cytotoxicity we found in our study compared with that of Sikdar et al may be because they worked at 6C and 30C potencies,[5] whereas the remedies we used were at 200C potency.

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Conclusion

In this study, we demonstrated—for the first time in the literature—the cytotoxic, cell cycle arrest and apoptotic effects of the homeopathic medicines *Arsenicum album, Hecla lava, Carcinosinum* and *Carboneum sulphuratum* at 200C potency on MM cells. In addition, the *in vitro* activity of *Carboneum sulphuratum* against a cancer cell line has been demonstrated for the first time here. Our results agree with other studies supporting the anti-cancer properties of homeopathic medicines. Further exploratory studies of the potential of these medicines as an adjunct to the conventional treatment of MM are warranted. For our part, we plan to investigate how these medicines might affect the expression of genes and proteins involved in cell cycle arrest and apoptosis.

Highlights

- Homeopathic remedies inhibit the proliferation of RPMI-8226 multiple myeloma cells.
- RPMI-8226 multiple myeloma cells treated with *Hecla lava* and *Carboneum sulphuratum* are arrested in the sub-G0/G1 phase of the cell cycle.
- Homeopathic remedies induce apoptosis in RPMI-8226 multiple myeloma cells.

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Conflict of Interest

None declared.

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