

RESEARCH ARTICLE

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Habb-e-Asgandh Suppresses Cell Proliferation and Induces Apoptosis through Mitochondria Dysfunction in Multiple Myeloma Cells (RPMI8226)

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Abstract

Objective: This study was conducted to assess the anti-neoplastic properties of Habb-e-Asgandh in multiple myeloma cells (RPMI8226). **Methods:** Multiple myeloma cells (RPMI8226) were cultured according to the ATCC's instruction. The anti-proliferative effect of HeA was assessed by MTT assay and proliferating cell nuclear antigen (PCNA) activity. Cell cycle analysis, cellular apoptosis, and mitochondria membrane potential analysis was done by flow cytometry. Total antioxidants, migratory potential, angiogenesis and inflammatory biomarkers were also estimated after treatment of RPMI8226 with HeA. **Results:** LD30 and LD50 dose of HeA was 0.3mg/ml and 0.5mg/ml respectively determined by MTT assay and also confirmed by a reduced PCNA activity. Cell cycle analysis of RPMI8226 cells revealed that sub-G0/G1 phase increases upon treatment with HeA alone or in combination with lenalidomide. Annexin V-FITC/PI is used to detect early apoptosis, late apoptosis and necrotic cells and results showed that percentage of apoptotic cells increased in RPMI8226 cells after treatment with HeA. Also, HeA induces loss of mitochondria membrane potential (MMP) in MM cells in-vitro as measured by cationic JC1 dye staining. Upon treatment, the abnormal overexpression of oncogenic protein, AKT serine/threonine kinase has also been reduced. Furthermore, anti-oxidants level also increased while migratory potential, angiogenesis and inflammation decreased in multiple myeloma cell line upon treatment with HeA. **Conclusion:** Collectively, our results demonstrated that integrative therapy of habb-e-asgandh efficiently eliminates the need to use higher dose of lenalidomide for multiple myeloma treatment.

Keywords: Habb-e-Asgandh- multiple myeloma- anti-neoplastic- Mitochondria- Apoptosis

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Introduction

Multiple Myeloma (MM), a disease of malignant plasma cells, which is 1% of all neoplastic diseases and takes second place in occurrence in haematological malignancies (10-15%) (Abramson, 2018). Several novel potential therapeutic agents such as immunomodulatory drugs, proteasome inhibitors, cell surface molecule specific antibodies and autologous stem-cell transplantation (ASCT) are used for the treatment of juvenile patients with multiple myeloma (Nishida and Yamada, 2019; Wudhikarn et al., 2020). Various invitro studies have been conducted for discovery of novel therapeutic agents for treatment of multiple myeloma such as tanshinone I which modulates telomerase activity and expression of shelterin complex thereby inducing apoptosis (Kumar et al., 2018). As explored in our previous study, anti-angiogenic and antitumor activity of cyclooxygenase-2 could be targeted as new therapeutic approach for treatment of multiple myeloma patients (Khan et al., 2013). Despite the numerous attempts to treat MM, current therapeutics still

have limitations and side effects that impair the patient's quality of life. To overcome these limitations, one strategy is to combine new agents from natural compounds with standard therapy to reduce adverse effects. One such study has been conducted by our lab using Cinnamon extract as a potent anti-cancer agent as it modulates angiogenesis and cyclooxygenase in myeloma cells (Khan et al., 2016). In this current study, we are exploring novel polyherbal drug i.e. Habb-e-Asgandh (HeA) as plausible therapeutic target for multiple myeloma.

Habb-e-Asgandh (HeA) is a herbal formula as described in Unani Pharmacopeia of India is a concoction of eight medicinal herbs *Trachyspermum ammi*, *Withania somnifera*, *Argyrea speciosa*, *Piper longum*, *Ficus religiosa*, *Zingiber Officinale*, *Asparagus racemosus*, *Curculigo orchoides*. Name of the herbal extract i.e. Habb-e-Asgandh is officially considered according to National Formulary of Unani Medicine (NFUM).

Bioactive compounds present in Ashwagandha exerts antioxidant, anti-inflammatory, and immunomodulatory activities and hence can be used for treatment of many

diseases such as arthritis, impotence, amnesia, anxiety, cancer, neurodegenerative and cardiovascular diseases (Mirjalili et al., 2009; Upadhyay et al., 2011; Baliga et al., 2013; Rasheed et al., 2013; Ojha et al., 2014). In a prostate cancer cell line, ashwagandha proves to downregulate various pro-inflammatory gene expression, including interleukins (IL-6, IL-1 β , IL-8, HSP 70, and STAT2) and upregulates p38/MAPK-kinase expression (Srinivasan et al., 2007; Nishikawa et al., 2015; Rah et al., 2015).

The aim of the study was to investigate the anti-cancer properties of Habb-e-Asgandh (HeA) on multiple myeloma cells. We verified the effects of HeA on RPMI 8226 cells on (1) Cell proliferation, (2) Cell cycle progression, (3) Apoptosis, (4) mitochondrial dysfunction, (5) Cell migration, (6) cytokine production, and (7) antioxidant capacity of HeA by Oxygen Radical Absorbance Capacity (ORAC) assay.

Materials and Methods

HeA Formulation

The HeA has been formulated as described in Table 1 using a soxhlet apparatus. The individual components of the formulation HeA were procured from the Aligarh city (Baradwari) in the month of August 2019 and were properly identified according to the botanical and Unani literature. These were also authenticated by the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi with the voucher no. NISCAIR/ RHMD/ Consult/2020/3646-47-12 to 3646-47-19. The solubility of HeA has been tested in various solvents and 1X phosphate buffer saline (PBS) was found to have better solubility and hence, used for in vitro experiments.

GC-MS Characterisation of HeA

0.1g of HeA was dissolved in 1ml of DMSO followed by filtration using 0.22 μ syringe filter and used for GCMS analysis. The phytocomponents were separated in zebron (Zb-5) column with column length 30 metres in GCMSQP2010, SHIMADZU instrument.

Cell Maintenance of RPMI 8226 cell line

The ethical approval for the cell line work was obtained from the Institutional Ethics Committee of AIIMS, New Delhi (IEC-539/02.12.2016).

RPMI 8226 cells were purchased from ATCC and cultured according to the manufacturer's instruction. The cells were also authenticated by STR typing in 2018 by DNA Forensics Laboratory, New Delhi, India. RPMI 8226 cells were maintained in complete RPMI1640 (Himedia, catlog no. AL162S) medium containing 10% Fetal Bovine Serum (Himedia, catalog no. RM9954) in an incubator at 37°C with 5% CO₂. When cell confluency reached 70% cells were incubated in complete medium for 24 h and then treated with different concentrations of HeA for 48 h. Mycoplasma testing (Abcam, catalog no. ab289834) was done in RPMI8226 cells and culture was found to be negative.

Measurement of cellular metabolic activity by MTT Assay

The cytotoxic effect of HeA was assessed by MTT assay. In brief, 1 x 10⁴ cells were seeded in 96-well plate and incubated overnight at 37°C with 5% CO₂. Cells were then treated with different concentrations of HeA for different time points (24, 48, 72 h). After respective time period, 10 μ l of 5mg/ml Thiazoyl blue tetrazolium bromide (MTT) (catalog no. M5655, CAS No. 298-93-1, Sigma Aldrich) was added to each well including control and incubated at 37°C with 5% CO₂ for 4 h. After 4h incubation, when formazon crystals (purple colour) was clearly visible, plate was centrifuged at 1500 rpm for 10 minutes. Then, supernatent was carefully removed and settled crystals were dissolved in DMSO (solubilisation solution) and plate was gently shaken for 20 minutes at room temperature. The absorbance was then measured at 570nm with reference wavelength of 630nm in a microtiter plate reader. LD30 and LD50 doses of drug were determined at which cells were 70% and 50% viable, respectively.

Detection of Cell Cycle Arrest

RPMI8226 Cells at 60-70% confluence were treated with LD30 and LD50 doses of HeA (HeA30, HeA50), LD30 and LD50 doses of Lenalidomide (standard drug) (L30, L50) and combinatorial doses of HeA30+L30, HeA30+L50, HeA50 + L30, HeA50 + L50 for 48 h. The cells from respective wells were then collected, fixed with 70% ethanol, washed and incubated with RNase A and propidium iodide (catalog no. P4170, CAS No. 25535-16-4, Sigma Aldrich) for 30 minutes at room temperature in dark. The cells were then analysed in flow cytometry.

Detection of Cellular Apoptosis (FITC Annexin V Apoptosis Detection Kit with PI, Catalog No. 640914, Bio legend)

Drug treated cells were resuspended in 100 μ l of Annexin V Binding buffer, 4 μ l Annexin-V-FITC dye and 8 μ l PI were added to cells, followed by incubation of 20 minutes and analysed by flow cytometry.

Detection of depolarization of mitochondrial membrane

RPMI8226 Cells were harvested 48 h post drug treatment and stained with 2 μ M of JC1 dye solution (catalog no. T4069, CAS no. 3520-43-2, Sigma Aldrich) and incubated for 30 minutes at 37°C in dark. The stained cells were analysed by flow cytometry.

Cell Migratory Potential

1.5 X 10⁵ drug treated RPMI8226 cells in 150 μ l in serum free media were added in the trans-well insert of 24-well migration plate. 500 μ l of complete culture media (with 10% FBS) was added to the lower well of migration plate, followed by an overnight incubation at 37°C with 5% CO₂. The percentage of migrated cells was calculated.

Oxygen Radical Absorbance Capacity (ORAC) Assay

Anti-oxidant potential of HeA was performed by ORAC Assay Kit (catalog no. ab233473, Abcam) and was performed as per the manufacturer's guidelines. To perform this assay, the ELISA plate reader was equilibrated

to 37°C. 25µl of samples (treated cell lysates) or Trolox standard was added to the respective wells, followed by addition of 150µl working fluorescein solution. The plate was kept at 37°C incubator for 30 minutes. Then 25µl free radical initiator working solution was added and fluorescence kinetics was started and continued for 60 minutes. Then, standard curve was plotted between trolox concentration and Net AUC to determine the equation that was used to calculate the trolox equivalent of the sample.

Immunoblotting

After 48 h of drug treatment, cells were harvested and lysates were prepared in RIPA lysis buffer and protein concentration of the respective lysates was quantified using Bradford assay. The denatured proteins were separated by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membrane was incubated with the respective primary and secondary antibodies. Chemiluminescence of the blots was observed in Azure Biosystems c280 and intensity of the bands was quantified using ImageJ software.

Enzyme Linked Immunosorbent Assay (ELISA)

A high sensitive sandwich ELISA kit was employed for the estimation of the level of Th1 (IL2), Th2 (IL-4, IL-10) and pro-inflammatory (IL-6) to investigate the anti-inflammatory role of HeA in cell culture medium supernatant. Standards and samples were added to the pre-coated micro-titre plate provided in the kits and

followed with steps as given in the protocol. Concentration of the analyte is directly proportional to the intensity of final yellow coloured product. The optical density was then measured and plotted against the concentration to get a standard curve and a linear equation which can further be used for estimation of concentration in unknown sample.

Statistical Analysis

The data analysis was done using GraphPad Prism 6.0. All data results were presented as mean + SD. Different groups were compared using Student's t-test. Differences with a probability value of * $p < 0.05$ were considered statistically significant.

Results

GC-MS Characterisation of HeA

The GC-MS chromatogram of HeA aqueous extract recorded a total of 60 peaks corresponding to bioactive compounds that were recognised by relating their peak retention time, peak area (%), height (%), and mass spectral fragmentation patterns to that of the known compounds described by National Institute of Standards and Technology (NIST) library. These compounds majorly include class of siloxanes, hydrolylamines, heterocyclic organic compounds, cyclical aldehyde, acyclic olefins, sesquiterpene, amino acid carbamates, phthalates, aminosterol, niflumic acid, urocanic acid. GC-MS fingerprint chromatogram (Figure 1S) along with list of bioactive phytoconstituents (Table 1S) is provided in

Table 1. HeA Composition

S.No.	Plant Common Name	Plant Botanical Name	Plant Part Used	Weight (g)
1	Ajwain Desi	Trachyspermum ammi	Fruit	20
2	Ashwagandha	Withania somnifera	Root	40
3	Bidhara	Argyrea speciosa	Root	40
4	Peepla Mool	Piper longum	Stem	20
5	Peepal Kalan	Piper longum	Fruit	20
6	Zanjabeel	Zingiber officinale	Rhizome	40
7	Satawar	Asparagus racemosus	Tuberous root	40
8	Musli Siyah	Curculigo orchiodes	Rhizome	20
9	Gur	Jaggery	--	50

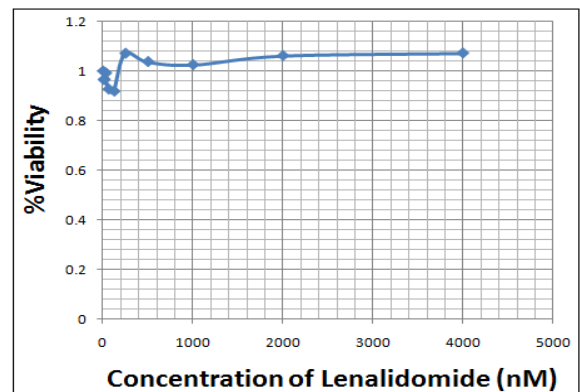
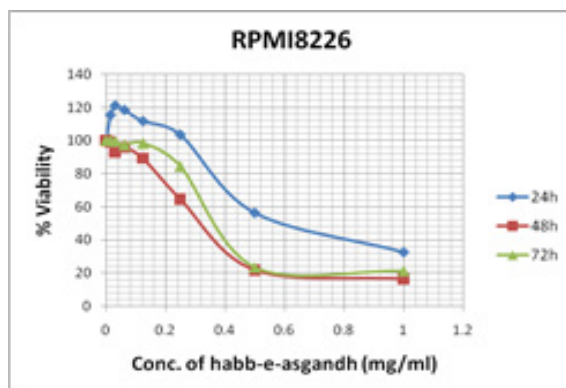


Figure 1. Graphical Representation of % Viability of RPMI8226 Cells after Treatment with Different Doses of habb-e-asgandh and Standard Chemotherapeutic Drugs (Lenalidomide).

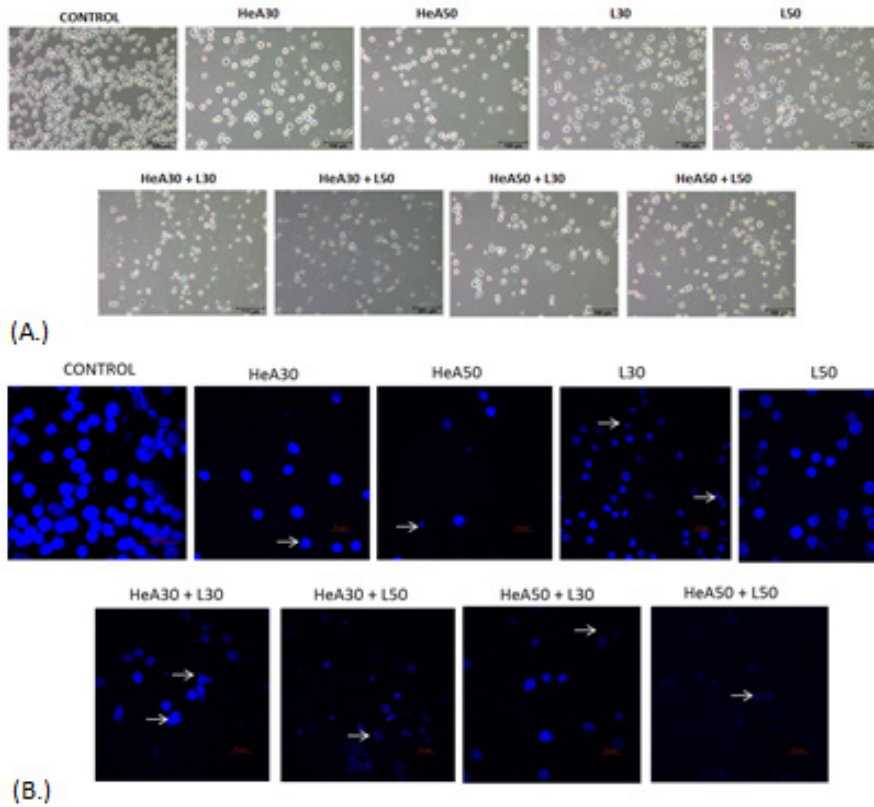


Figure 2. Morphology of Habb-e-Asgandh and Lenalidomide Treated RPMI8226 Cells. (A), Phase contrast microscopic images of RPMI8226 cells treated with Habb-e-asgandh alone or in combination with lenalidomide exhibited reduction in number of cells and altered morphological attributes. [HeA: Habb-e-Asgandh]; (B), Confocal microscopic images at 60X of DAPI stained RPMI8226 cells showed morphological distortion and nuclear fragmentation.

supporting information.

Habb-e-Asgandh regulates cellular metabolic activity

To initiate the study of identifying anti-tumor potential of HeA, firstly, multiple myeloma (MM) cell line (RPMI8226) was treated with HEA and standard

chemotherapeutic drug (Lenalidomide for MM) for 48 hours to determine sub-lethal dose (LD30) and lethal dose (LD50) of these drugs using MTT assay (Figure 1 and Table 1). The standard drug, lenalidomide was not showing any cytotoxicity at any concentration, hence, as

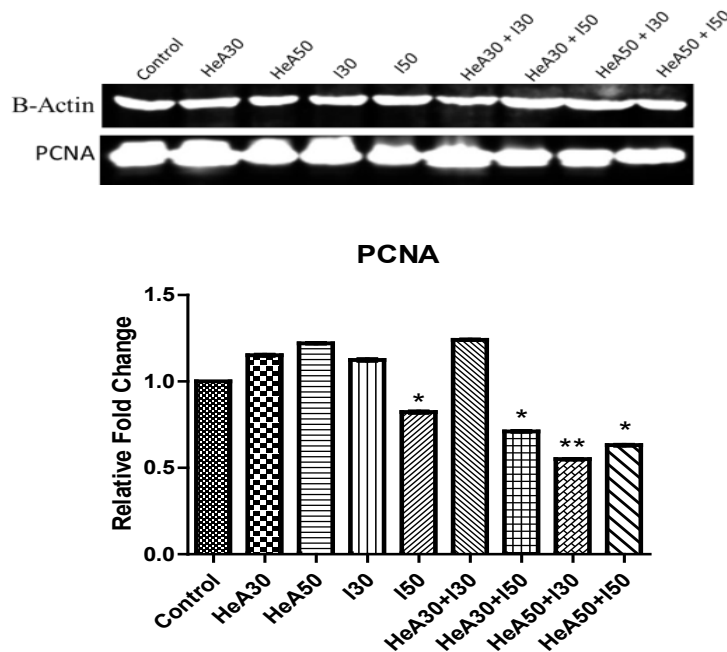


Figure 3. PCNA Expression in RPMI8226 Cells upon Treatment with HeA Alone and Combinatorial Treatment with Standard Lenalidomide Drug. Asterisks represents significance level with respect to control (*p<0.05, **p<0.01).

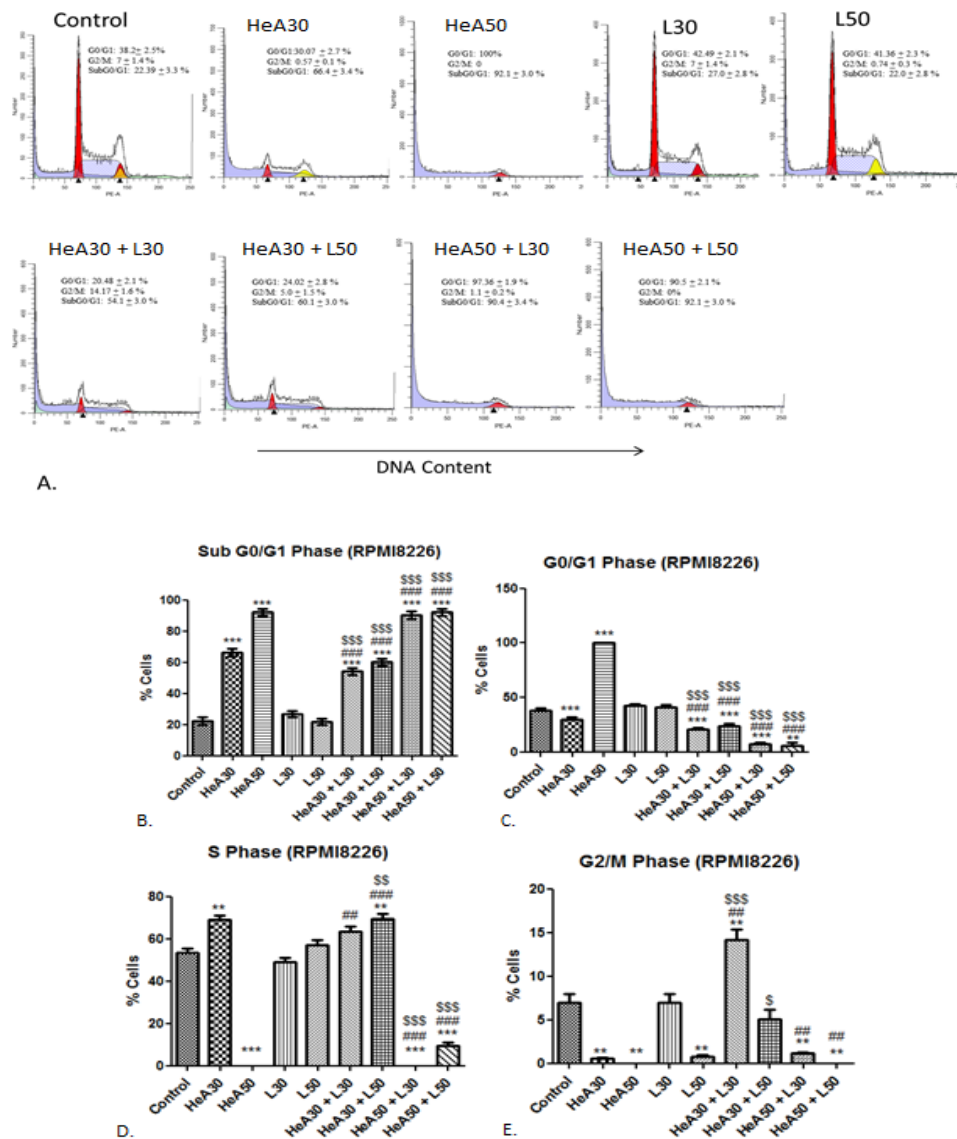


Figure 4. Cell Cycle Distribution of RPMI8226 Cell Treated with Habb-e-Asgandh alone or in Combination with Lenalidomide. A, Representative image showing distribution of RPMI8226 cells in different phase of cell cycle upon Habb-e-Asgandh treatment alone or in combination with lenalidomide; B, Habb-e-asgandh alone or in combination with lenalidomide induces significant accumulation of Sub G0/G1 population; C, Habb-e-asgandh treatment alone or in combination with lenalidomide significantly reduces the G0/G1 population of RPMI8226 cells; D, S phase population of RPMI8226 cells in indicated treatment groups; E, Significant accumulation of G2/M cell population upon combination treatment of Habb-e-asgandh with lenalidomide. * represents significance wrt controls. # represents significance wrt L30 and \$ represents significance wrt L50 (*P<.01, **P<.001 and ***P<.001). [HEA: Habb-e-Asgandh; L: Lenalidomide]

per the published literature, two doses of 5 μ M and 10 μ M were chosen for further experiments. All the experiments were performed in triplicates on these doses.

Morphological changes in myeloma cells (RPMI8226) upon treatment with Habb-e-Asgandh and standard chemotherapeutic drug

The phase contrast microscopy images in Figure 2 showed the combinatorial effect of drug treatment on cell toxicity and morphology. The cells appeared with visibly distorted morphologies and membrane blebs as shown in Figure 2 (A). The confocal microscopy of DAPI stained RPMI8226 cells also reveals morphological distortion and nuclear fragmentation as shown in Figure 2(B).

Habb-e-Asgandh regulates proliferation and cell cycle in RPMI8226 Cells

HeA reduced cell proliferation in a dose-dependent manner (Figure 1). We compared the Proliferating Cellular Nuclear Antigen (PCNA) between the RPMI8226 cells treated with different doses of HeA (HeA30, HeA50); Lenalidomide (L30, L50) and combinatorial treatment with HeA + Lenalidomide (HeA30+L30; HeA30+L50; HeA50+L30; HeA50+L50). Upon treatment we found the decrease in PCNA activity of RPMI8226 cells as shown in Figure 3.

Then, we confirmed the cell cycle arrest in RPMI8226 Cells. HeA and combinatorial treatment increases the relative proportion of cells in sub G0/G1 phase. It also

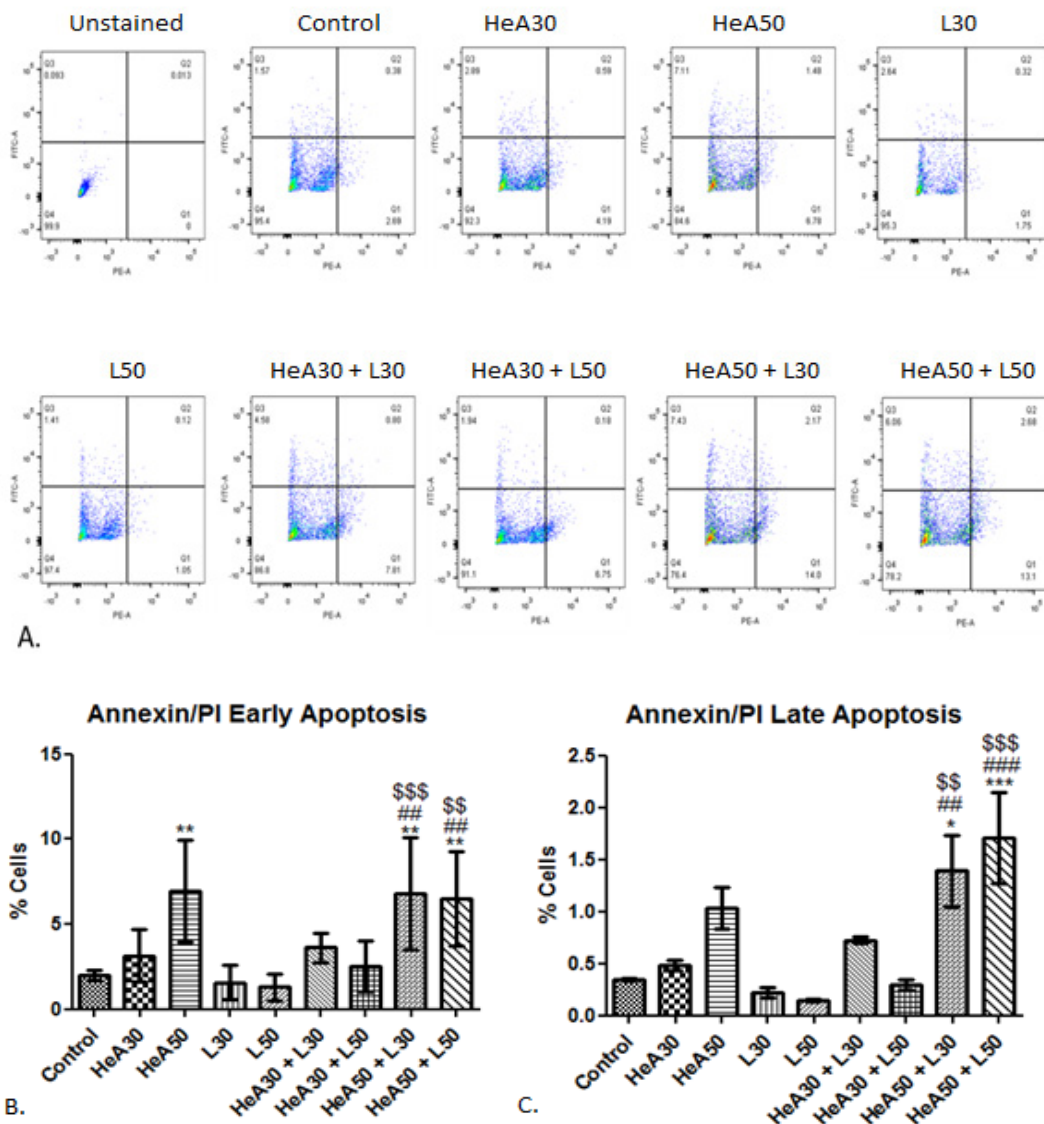


Figure 5. Apoptosis in RPMI8226 Cells Treated with habb-e-asgandh Alone or in Combination with Lenalidomide: A, Representative image showing Annexin V and PI stained RPMI8226 cells post Habb-e-asgandh and lenalidomide treatment; B-C, Significant increase in early apoptotic and late apoptotic cells upon combination treatment of RPMI8226 cells with habb-e-asgandh and Lenalidomide.

increases the proportion of cells in G0/G1 phase upon treatment with HeA50, HeA50 + L30 and HeA50 + L50, therefore indicating the cell cycle arrest at the G0/G1 phase. Treatment with HeA alone or combinatorial treatment significantly reduced the proportion of cells in G2/M phase as shown in Figure 4.

HeA accelerates apoptosis in RPMI8226 cells

Followed by cell cycle analysis by PI staining, the mode of cytotoxicity of Habb-e-Asgandh was investigated, i.e., the cell death caused by drug treatment occurred by apoptosis or necrosis. For this, Annexin-V-FITC/PI assay was performed. In this assay, viable cells do not show any fluorescence, early apoptotic cells show only green fluorescence while late apoptotic cells show dual fluorescence. It has been observed that both early and late apoptotic cells increase upon treatment with Habb-e-Asgandh and standard drugs alone at

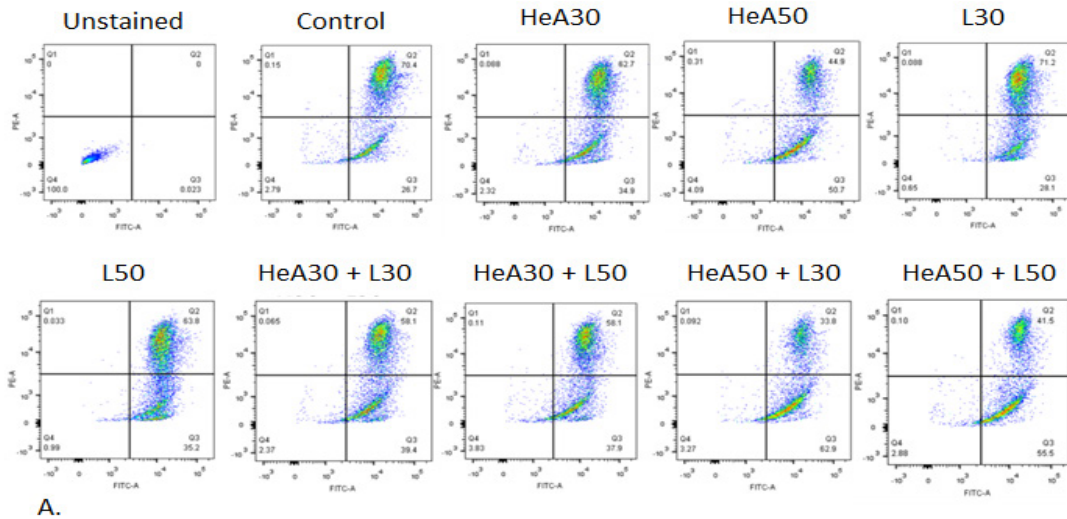
dose dependent manner in RPMI 8226 cells, however, proportion of apoptotic cells further increase when both the drugs were given in combination as shown in Figure 5.

HeA hampers mitochondrial homeostasis in RPMI8226 cells

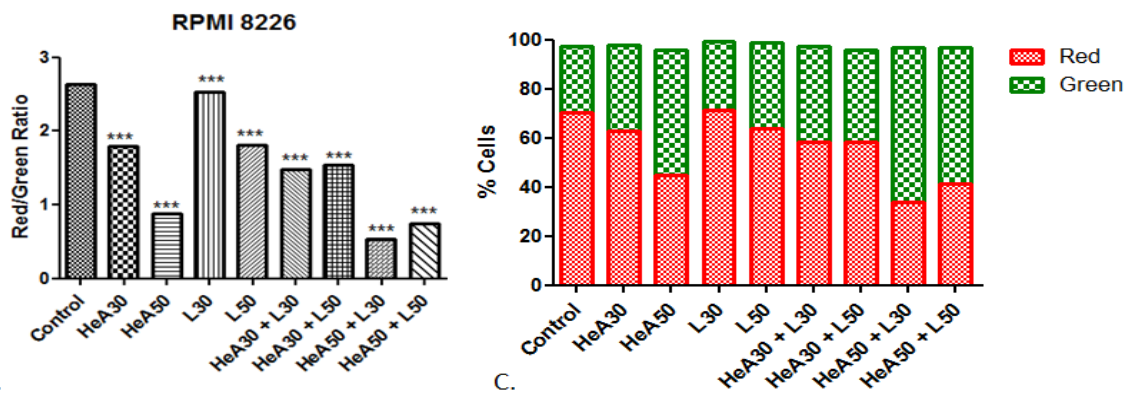
We assessed the relative mitochondrial membrane potential (MMP) using JC1 dye. HeA treatment alone or in combination with standard Lenalidomide drug increased the loss of MMP compared to control RPMI8226 cells as shown in Figure 6 (A, B, C).

HeA reduces the migratory potential of myeloma cells

An essential step towards the invasive potential of cells is cell migration. Results indicate that Habb-e-asgandh in combination with standard drugs substantially reduced the migratory ability of RPMI8226 cells (Figure 7). Therefore, by regulating the progress and migration of cancer cells,



A.



B.

C.

Figure 6. Loss of Mitochondria Membrane Potential. A, Representative image showing RPMI8226 cells with dissipated $\Delta\Psi_m$ as evidenced from increase in cell population higher green fluorescence of JC1 dye; B, Bar graphs depicting significant decrease in Red/Green fluorescence of RPMI8226 cells treated with Habb-e-asgandh and lenalidomide; C, Increase in RPMI8226 cell population with green fluorescence with concomitant decrease in red fluorescence upon Habb-e-asgandh and lenalidomide treatment. Asterisks represents significance wrt controls. (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.001$) [HEA: Habb-e-Asgandh; L: Lenalidomide].

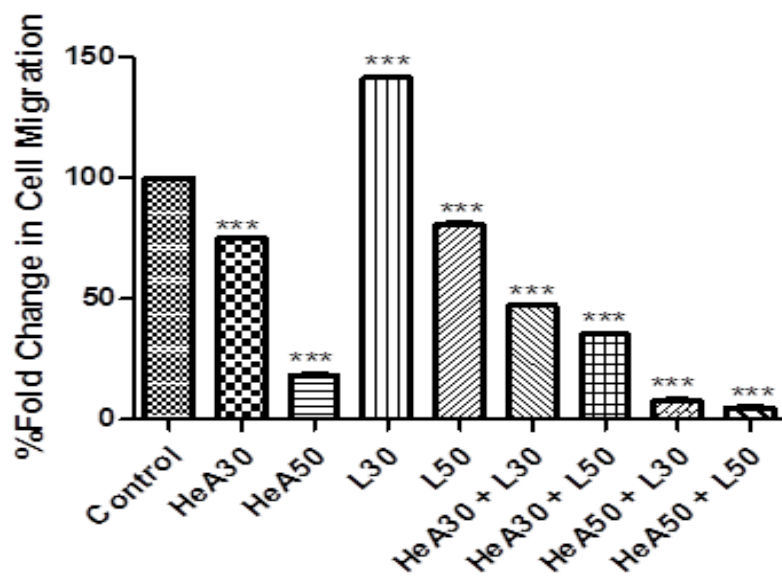


Figure 7. Migration of RPMI8226 Cells is Reduced upon Habb-e-asgandh Treatment. Habb-e-asgandh alone or in combination with standard drugs significantly reduced migratory ability of RPMI8226 cells. Asterisks represents significance with respect to controls. (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.001$). [HEA or H: Habb-e-Asgandh; L: Lenalidomide]

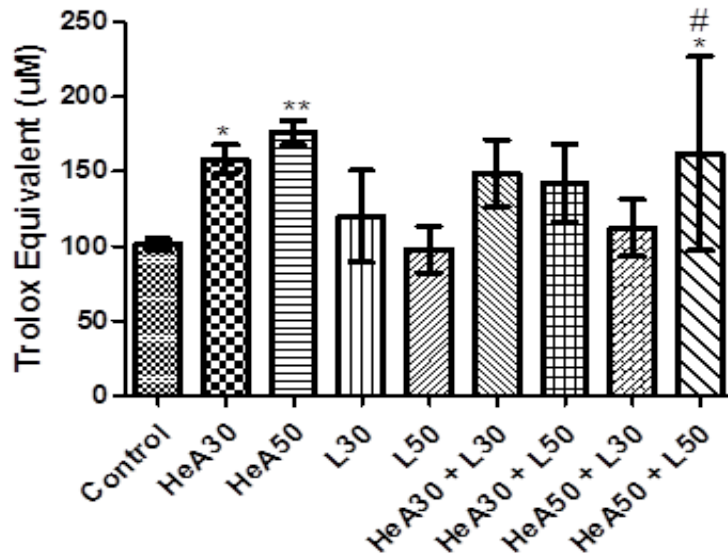


Figure 8. Oxidative Status of RPMI8226 Cells Treated with Habb-e-asgandh. Habb-e-asgandh exhibits significant effect on ORAC of RPMI8226 cells when treated with combination of Habb-e-Asgandh and lenalidomide. * represents significance wrt controls. # represents significance wrt L30. (*P<0.01, **P<0.001 and ***P<0.001) [HEA: Habb-e-Asgandh; L: Lenalidomide]

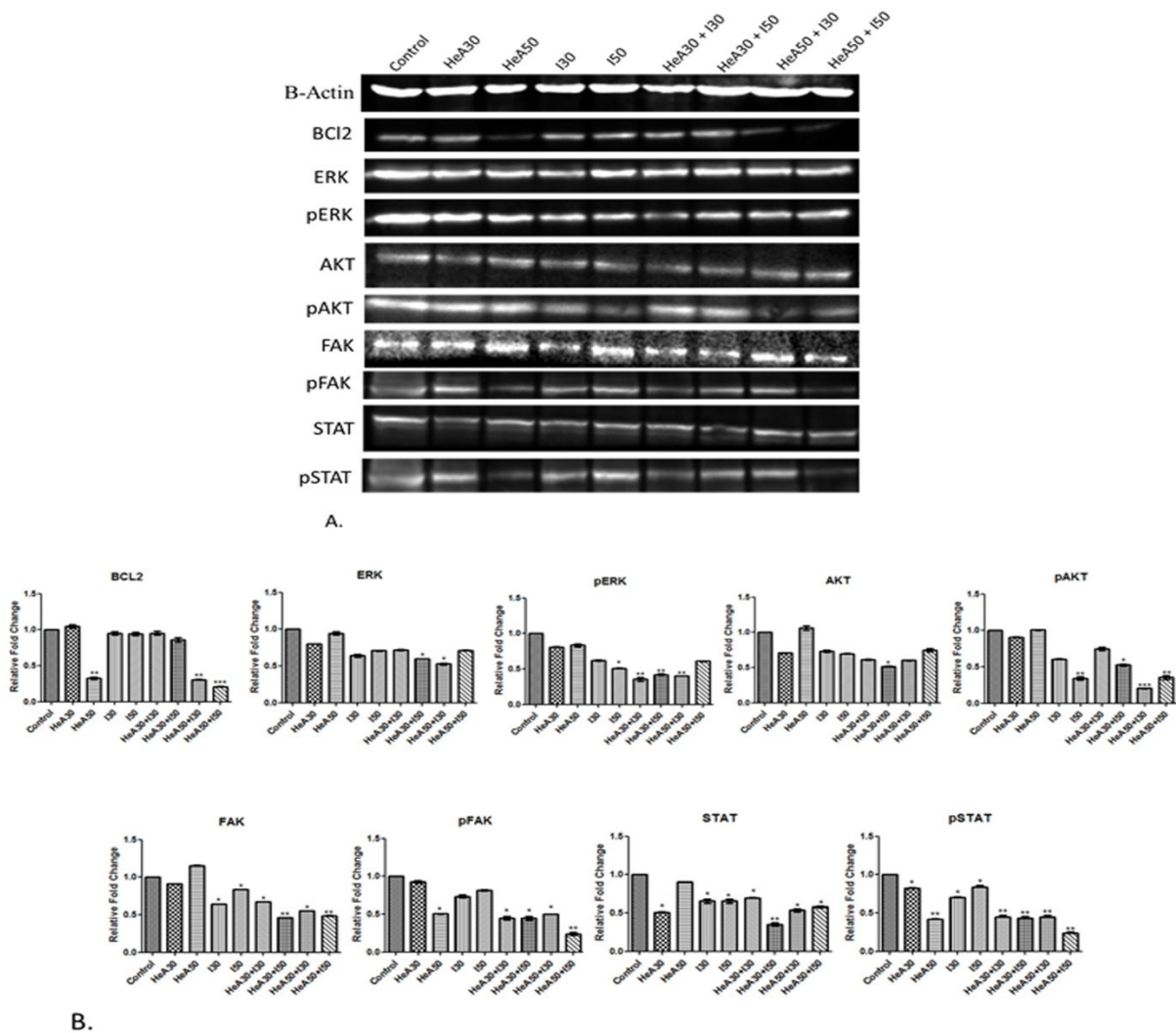


Figure 9. HeA Shows anti-neoplastic Activity by Regulating the Expression of Proteins that Mediates Many Aspects of Cellular Immunity, apoptosis, cell proliferation and differentiation, cellular adhesion and spreading processes. Asterisks represent significance with respect to control RPMI8226 cells. (*P<0.01, **P<0.001 and ***P<0.001)

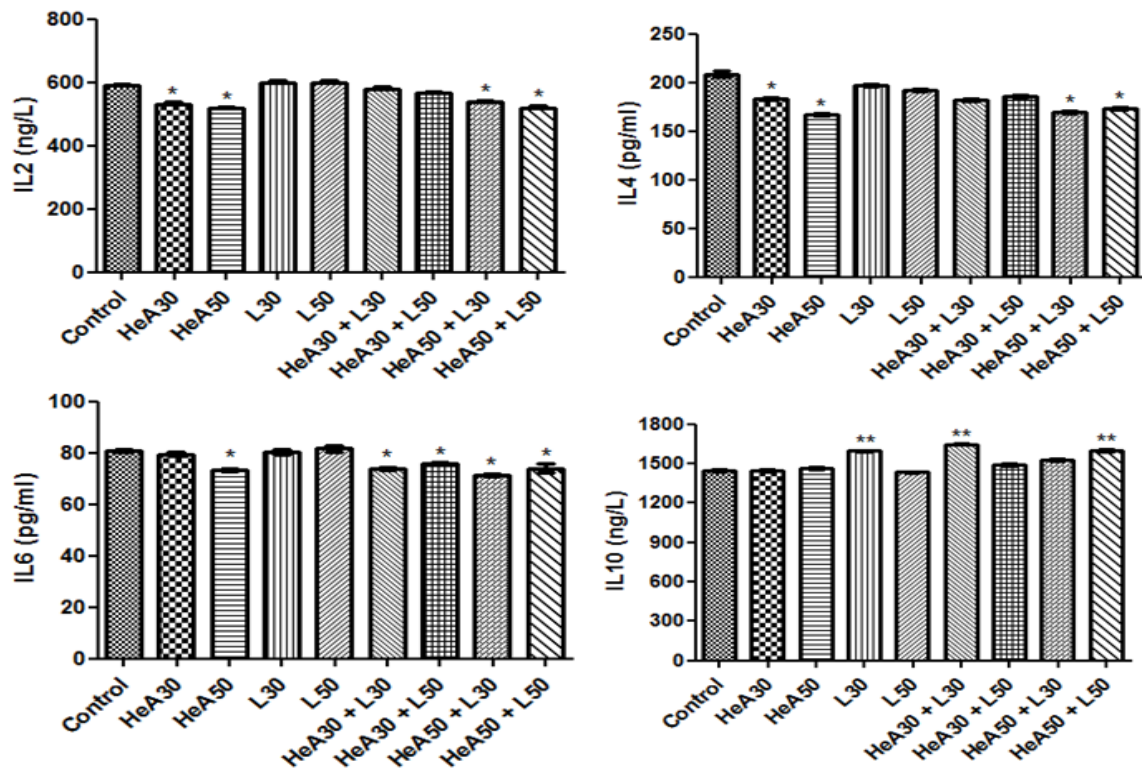


Figure 10. HeA Significantly Alters the Th1 (IL2), Th2 (IL4, IL10) and Pro-inflammatory (IL6) Cytokines.

integration of HeA with standard lenalidomide may acts as the anti-metastatic herbal drug.

ROS generation is induced by HeA in RPMI8226 cells

ORAC assay was performed to determine the anti-oxidant potential of Habb-e-Asgandh alone or in combination with standard drug in myeloma cells. Firstly, standard curve was plotted with the known concentration of anti-oxidant (Trolox) supplied in the kit to obtain a linear equation. This equation was then used to determine the anti-oxidant concentration in the unknown samples.

The results showed that the levels of trolox equivalent which is a measure of anti-oxidant in RPMI8226 cells treated with higher concentrations of Habb-e-asgandh were significantly higher. HeA induced significant ROS generation in RPMI8226 cells. These data indicates that one of the possible mode of anti-cancer activity of Habb-e-asgandh against RPMI8226 cell line is intracellular ROS generation (Figure 8).

HeA regulates expression of various proteins leading to activation of apoptosis pathway

To confirm the phosphorylation of signalling proteins affected by HeA treatment, we conducted western blot analysis of various signalling proteins. HeA dose-dependently down regulated the expression of Bcl2 protein which leads to activation of programmed cell death. Also, HeA treatment alone or in combination with standard drug suppressed the expression of proteins like ERK, pERK, AKT, pAKT, STAT, pFAK (Figure 9).

HeA effects production of cytokines in RPMI8226 cells

Treatment of RPMI8226 cells with Habb-e-Asgandh

alone or in combination with standard drugs affects inflammation. For this, the ELISA kits was used for estimation of the level of Th1 (IL2), Th2 (IL-4, IL-10) and pro-inflammatory (IL-6) and the equation obtained was then used to determine the concentration in the cell supernatant after treatment (Figure 10). Inflammatory cytokines IL2, IL4 and IL6 were found to be decreased and anti-inflammatory cytokine i.e. IL-10 level was significantly increased.

Discussion

Multiple Myeloma (MM) has been known debilitating diseases from many centuries across the world and numerous chemotherapeutic agents are being used for their treatment. In spite of having surfeit of treatment regimen, relapse of disease after a short period of remission is of great concern. Hence, partial recovery along with toxic side effects of chemotherapeutic drugs in patients prompts to identify a potent drug with least toxicity. In Indian traditional medicine, herbal medicines are being used for the treatment of diseases including cancer (Patterson et al., 2007).

In this present study, translational application of polyherbal Unani formulation, Habb-e-Asgandh has been explored. GC-MS Characterization of HeA shows presence of various organic compounds, siloxanes, alkanes, long chain fatty acids, amino acid carbamates, flavanones, oxysterols, aromatic carboxylic acid, hydroxycinnamic acid as its bioactive constituents which showed anti-tumor, pro-apoptotic, anti-inflammatory, anti-proliferative and anti-metastatic activities in previous research as listed in Table 2S (Harada et al., 2002; Lin

et al., 2010; Yin et al., 2013; Mattarei et al., 2015). HeA decreased cell proliferation, PCNA expression and MMP in multiple myeloma cells (RPMI8226). Also, Oxygen Radical Absorbance Capacity (ORAC) has been found to be increased in RPMI8226 cells after treatment with HeA which shows increase in ROS production. Thus, HeA induced mitochondrial dysfunction and anti-proliferative effects on RPMI8226 cells by regulating signalling pathways. Therefore, in this study, we elucidated anticancer effects of HeA on RPMI8226 cells and their cellular mechanisms.

Ashwagandha has also been evaluated in oncology settings. In an open-label study, 100 patients with breast cancer were given a standardized extract (2 g every 8 hours) throughout chemotherapy. Researchers reported reduction in chemotherapy-induced fatigue ($P < 0.001$ with Piper Fatigue Scale; $P < 0.003$ with Schwartz Cancer Fatigue Scale) along with improvement in quality of life ($P < 0.001$) (Biswal et al., 2013). Larger trials are needed to validate these findings, establish an optimal dose, and determine long-term safety. In addition, microarray analysis revealed that Ashwagandha repressed proinflammatory gene expression, including interleukin-6 (IL-6), IL-1 β , IL-8, heat shock protein 70, and STAT2, and induced p38/MAPK-kinase expression in a prostate cancer cell line (Aalinkeel et al., 2010). The root extracts were shown to exert cytotoxic effects in lung, colon, central nervous system, and breast cancer cell lines (Jayaprakasam et al., 2003). Active constituent of Ashwagandha demonstrated anticancer effects in leukemia cells (Malik et al., 2007) as well as in estrogen receptor-positive and estrogen receptor-negative breast cancer cells, via inducing apoptosis and decreasing tumor size (Stan et al., 2008).

Ashwagandha was proved to be selectively cytotoxic to cancer cells by ROS signalling (Widodo et al., 2010). Complementary to the prior study, in this research, we found that habb-e-asgandh (HeA) caused cytotoxicity of myeloma cells in vitro in dose dependent manner. Ashwagandha causes G2 and M phase cell cycle arrest in human breast cancer cells (Stan et al., 2008), similarly, upon cell cycle analysis, this has been observed that sub-G0/G1 phase increased upon treatment with HeA alone or in combination with standard drug, Lenalidomide, suggesting the cell death caused by the formulation and inducing cell cycle arrest at G2/M check point thereby increasing percentage of cells in G2 phase. The mode of cytotoxicity has been assessed by Annexin V-FITC/PI assay which showed that percentage of apoptotic cells got increased in RPMI 8226 cells after treatment with habb-e-asgandh. In addition, mitochondrial membrane potential (MMP) determination by JC1 assay showed the loss of MMP after treatment. Furthermore, anti-oxidants level also increased while migratory potential, angiogenesis and inflammation decreased in myeloma cells upon treatment with HeA. In combination with standard chemotherapeutic drugs (lenalidomide), habb-e-asgandh further affected the morphology of RPMI8226 cell line, induces cell cycle arrest at G1/S check point leading to increase in cell number in G1 phase of cell cycle, persuading cellular apoptosis, increasing

cytokine production and inhibiting cell migration in myeloma cells.

Conclusively, our study is the first to demonstrate the anticancer effects of Habb-e-Asgandh on RPMI8226 cells and to elucidate their cellular mechanisms and action on mitochondrial function by activating signalling pathways via ROS generation and MMP depolarization. Also, we confirmed that HeA suppressed proliferation via ROS generation in multiple myeloma cancer cells. HeA in combination with lenalidomide showed promising cytotoxicity in vitro. Importantly, HeA could be used as an adjuvant therapy with lower dose of lenalidomide as it showed favourable anti-proliferative effect, thereby, effectively eliminating the need to use higher dose of lenalidomide which could further reduce side effects related to standard therapy. Although our results are limited to an in vitro evaluation, this study may provide valuable insights regarding the underlying molecular mechanisms for future in vivo studies.

Supporting Information

GC-MS chromatogram of HeA (Figure 1S) and list of bioactive phytoconstituents (Table 1S) along with their biological relevance (Table 2S) are available as supporting information.

Author Contribution Statement

AV, NG and AS designed the research study. AV performed the research and analyzed the data. NG provided assistance in few experiments. AV and AS drafted the manuscript. SN prepared the formulation, Habb-e-Asgandh as per unani guidelines. AS supervised the research, contributed essential reagents or tools, critically reviewed and edited the manuscript. All authors have read and approved the content of the manuscript.

Acknowledgements

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Ethics Approval

The ethical approval for the cell line work was obtained from the Institutional Ethics Committee of AIIMS, New Delhi (IEC-539/02.12.2016).

Conflict of Interest

There is no conflict of interest to disclose.

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