

RESEARCH ARTICLE

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A Polyherbal Ashwagandha Formulation Exhibits Adjunctive Antitumor Efficacy Against U266 Myeloma Cells by Multi-Strategic Cytotoxic Effects: An Experimental Approach

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Abstract

Background: The present study explored the molecular mechanism of herbal (Unani) drug Habb-e-asgandh as anti-tumorigenic adjuvant therapy experimentally in U266 cells and its role in treatment of Multiple myeloma. The formulation of Habb-e-asgandh is investigated alone or as a combinatorial therapy with standard drug lenalidomide to check for its efficacy against U266 myeloma cells for prevention of drug relapse and resistance. **Methods:** We performed the following assays on singly or in combination of Habb-e-asgandh-Lenalidomide treated U266 cells. The cytotoxicity evaluation done by MTT assay, we studied cell cycle kinetics by Propidium Iodide staining, mitochondrial apoptosis analysis by Annexin V/PI dual staining and JC1 staining assays. Further, anti-oxidative potential was assessed by ORAC assay and cytokine levels estimation of anti-inflammatory (TNF-alpha and IL6) and anti-angiogenic (VEGF and Ang-2) markers were done by ELISA. **Results:** The myeloma U266 cells when treated with Habb-e-asgandh alone or in combination with standard drug lenalidomide showed cytotoxicity in dose dependent manner with promising effects at 0.4 mg/ml (IC₃₀) and 1.5 mg/ml (IC₅₀) inhibitory concentrations. The formulation treated cells showed modulation in cell cycle kinetics patterned by sub G₀/G₁ population accumulation. Furthermore, it induced mitochondrial apoptosis mainly at half maximal inhibitory concentration and in combinatorial combinations. Significantly elevated oxidative capacities (p<0.05) and reduced levels of angiogenic and pro-inflammatory markers were observed. Multiple mechanism based inhibition by Habb-e-asgandh in co-treatment with lenalidomide against myeloma cells is indicated. **Conclusion:** Habb-e-asgandh formulation possess anti-tumorigenic efficacy against multiple myeloma. The adjunctive Habb-e-asgandh formulation with standard chemotherapeutic drug may prove to be a potent anti-myeloma agent in interventional therapy for Multiple myeloma if further studied in future avenues.

Keywords: Multiple myeloma- Habb-e-asgandh- anti-tumorigenic- adjuvant therapy- cancer therapeutics

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Introduction

Multiple myeloma is a myeloproliferative disorder concerning plasma cells accounting for approximately 15 % of all hematological malignancies and with severe end stage complications (Rajkumar, 2022). Combination therapies most prominently used for multiple myeloma treatment still lack in enhancing survival years of the patient due to drug relapse and severe disease refractory elicitation. This provides the stimulus to search for efficient treatment modality to reduce side effects of the current scheme of clinical management of multiple myeloma. Natural plant based products have been long been used in cancer therapeutics due to its efficacy and activity against cancer by various mechanism (Agbarya et al., 2014). Moreover, cost effectiveness and minimal side effects on combination favors these agents to be explored for malignancy and treatment for multifactorial

disease like cancers. Our group had previously studied the Habb-e-asgandh *in vitro* for treatment chronic myelogenous leukemia (CML), and was found to be efficacious for the disorder (Gupta et al., 2022). Although, Individual constituents like *Withania Somnifera* Dunal of this formulation has been explored and found to be possessing antitumor action against numerous types of cancers (Yadav et al., 2010; Lee, 2016; Rai et al., 2016; Saggam et al., 2020; Seresht et al., 2019). Our experimental focus is to decipher molecular mechanism for inhibition of cancer and explore therapeutic modality using Habb-e-asgandh for targeting U266 myeloma cells *in vitro* while its effects in multiple myeloma RPMI 8226 cell line have already being studied by our group recently (Vashist et al., 2022).

The aim of the study is to check the efficacy and potential as an anti-proliferative agent in totality of polyherbal Ashwagandha formulation, Habb-e-asgandh

(HeA) in U266 multiple myeloma cells. Further, the study spanned evaluation of our test drug HeA in combination with standard chemotherapeutic immunomodulatory drug lenalidomide for their anti-tumorigenic effects. In turn, the anti-cancer capacity of the Habb-e-asgandh formulation would be investigated to study its role on strategic hallmarks of cancer including apoptosis regulation, cell cycle analysis, and effect on angiogenesis, oxidative stress and inflammation. Therefore, collective analysis of the above roles through various assays will pave the way of molecular mechanisms involved in its functionality against myeloma cells in model cell line *in vitro* in cancer therapeutics.

Materials and Methods

Cell lines and culture

The multiple myeloma cell line U266 (human origin) procured from ATCC was harvested, characterized by STR typing and maintained in RPMI-1640 Media (catlog no. AL162S Himedia, India) supplemented with 15% (v/v) Fetal Bovine Serum (heat inactivated) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, United States) was added as antibiotic solution at 37°C and 5% CO₂. The ethical approval obtained for cell line through Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi (IEC-539/02.12.2016).

Habb-e-asgandh Formulation

The detailed composition, procurement and laboratory extraction of the herbal drug Habb-e-asgandh has been mentioned in a previous literature report of validated investigation done by our group to observe effect of HeA on leukemic disorder, CML (Gupta et al., 2022). Total 5g of herbal extract was obtained through Soxhlet technique. The Habb-e-asgandh extract with viscous semi-solid consistency was dissolved in Phosphate Buffer Saline (PBS) solution to make a stock formulation of 25 mg/ml concentration. The stock was further diluted in working media to a requisite concentration as per the assay carried out. The Habb-e-Asgandh formulation is a light brownish color solution. The characterization of the extract of HeA has been carried out by a prior laboratory research which deciphered role of this medicinal drug in RPMI8226 myeloma cells (Vashist et al., 2022).

Cell Viability assay

Cells were seeded in 96-well plate and harvested for 24 hrs (37°C, 5% CO₂) to obtain a density of 1.5×10^4 cells/ml. The ranges of concentration from 0 to 2.4 mg/ml HeA Formulation (in triplicate) were added and kept for three time point- 24 h, 48 h and 72 h incubation under culture microenvironment. 10 µl of stock of 5 mg/ml of (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) MTT (catalog no. M5655, Sigma Aldrich) prepared in PBS was added to each well and incubated for 4 h at above mentioned culture conditions. After completion of 4 h, centrifuged the plates at 425g for 7 mins (Thermo Scientific Legend XT/XF, U.S.) at room temperature to obtain purple crystal precipitate and supernatant was discarded from side. Further, addition

of 100 µl of dimethyl sulfoxide (DMSO) was done in each well for dissolution of purple colored formazan precipitate. The absorbance was measured at 570 nm with 630 nm as reference wavelength using micro plate reader (Bio-Tek, U.S.). Similar treatment was done taking standard chemotherapeutic immune modulatory drug lenalidomide at a concentration range of 2.5 to 40 µM. The percentage viability of the cells after treatment with HeA were obtained by comparison to untreated control and standard therapeutic drug thus, calculated cytotoxicity of HeA in terms of percentage inhibition to evaluate selected inhibitory concentration (IC₃₀) and half maximal inhibitory concentration (IC₅₀).

Propidium Iodide DNA binding assay

U266 cells were cultured in 15% serum containing media treated with HeA formulation at 0.4 and 1.5 mg/ml (IC₃₀ and IC₅₀ obtained by MTT assay respectively) as well as 5 µM and 10 µM lenalidomide for 48 h. Cells were harvested to an approximate count of 2×10^5 cells/ml, washed with PBS (at 4°C). Further, treated U266 cells fixed with ice cold 70% ethanol and kept on ice for 30mins or kept overnight before proceeding for next PBS washing step. Addition of 1% RNase and further 1% PI proceed with incubation at 4°C in dark conditions and were analyzed with FACS Canto (BD Biosciences) Flow cytometer. The study of distribution of stages of cell cycle was done using BD FACSDiva software (Becton, Dickinson and Company). Later, relationship between cell cycle phases obtained and their relative DNA content histogram were plotted using ModFit LT version 5.0 software.

Annexin V-FITC/PI Dual staining assay

U266 cultured and harvested to a population density 2×10^5 cells/ml were incubated with HeA formulation solution for 48 h duration (as discussed above in section). Cells were then washed with ice-cold IX PBS solution, pelleted and suspended in 1X Annexin Binding buffer containing 4 µl of Annexin-FITC stain (catalog No. 640914, Bio legend) and 8 µl of PI dye per tube (working solution of 100 µg/mL) and were left for incubation in dark at RT for a duration of 10-15 mins. After incubation, a PBS wash is given and cells were resuspended in PBS buffer and applied to FACS Canto (BD) Flow cytometer at approximate fluorescence emission of 530 nm and >575 nm.

Mitochondrial membrane potential assessment

U266 cells cultured and harvested to a population density of 2×10^5 cells/ml were incubated with HeA formulation solution for 48 h duration. Myeloma cells were washed with 1X PBS buffer, pelleted and suspended in 200 µl of JC1 solution. (JC1 dye is a cationic dye that was procured as a lyophilized powdered of 5.0mg vial from Sigma Aldrich. The powdered form was dissolved in 1ml DMSO to obtain a stock solution of 7.5 mM and was diluted in DMSO to make intermediate concentration of 200 µM, thus finally diluted in 1X PBS to make a working concentration of 2 µM to be used). After addition of JC1, incubated at 37°C for 30 mins in dark and ahead

of incubation PBS washed. The sample were acquired by FACS (FACSCanto) as mentioned and the variation in Red: Green (aggregated: monomeric) form ratio is directly correlated as the measure of change in mitochondrial membrane potential.

Enzyme Linked Immunosorbent assay (ELISA)

The commercially available ELISA kits with high sensitivity were used to estimate the levels of four biomarkers (two inflammatory markers: TNF- α and IL-6 and two angiogenic molecules: VEGF and ANG-2). Vascular endothelial growth factor (VEGF), Interleukin- 6 (IL-6), angiopoietin-2 (ANG-2) and tumor necrosis factor alpha (TNF α) were measured in the cell culture supernatant of HeA treated U266 myeloma cells at IC₃₀ and IC₅₀ concentration as well as for the combination of Habb-e-Asgandh and standard drug lenalidomide. The kits used for experimental estimation were supplied by G-Biosciences, USA which was based upon principal of Sandwich enzyme immunoassay through colorimetric assay. The reading was acquired through measure of absorbance at 450 nm. The level of the unknown cytokins were extrapolated and measured from the standard reference plot between different standard drug concentrations and respective Optical Densities.

Oxygen Radical Absorbance Capacity Assay

High sensitivity oxygen radical absorbance capacity (ORAC) assay was used for assessment of oxidative tendency of the HeA treated myeloma cell line model. The kit utilized and assay was performed following manufacturer's guidelines for measurement of oxidative capacity from the test and standard chemotherapeutic drug treated U266 cell culture lysate were supplied by Abcam (Catalog no. ab233473). The lysate and trolox sample was added to 96 well plate equilibrated at room temperature prior to start of assay. This is followed by addition and mixing of fluorescein solution, the plate was incubated at 37°C for 30 min. Then, freshly prepared free radical initiator solution is added and mixed. Immediately the fluorescence intensity kinetics of both standard as well as samples was acquired at excitation and emission wavelength of 480 nm and 520 nm respectively by a multimode microplate reader (Tecan SPARK) for a duration of 60 mins at an interval of every 1 min. The

principle of assay is evaluated as the fluorescence intensity is directly related to the concentration of antioxidant and the values are assessed as by graphically plotted the net area under the curve (AUC) to the trolox antioxidant standard.

Statistical Analysis

Graphpad Prism 5.0 was used for statistic assessment; the presented data was displayed as mean \pm SD for all measured variables. Each experimental assay was performed in triplicate. One-way ANOVA test was used to statistically analyze the differences in values between different parameters of treated and untreated cells. Each assay was repeated two to three times. A p-value of <0.05 was considered statistically significant.

Results

HeA Formulation impedes U266 cells proliferation in a concentration dependent manner

The evaluation of the anti-tumorigenic potential of HeA on myeloma cell line U66 was determined by viability plots and analyzed in terms of percentage inhibition of myeloma cells. Also, the comparative inhibition of the polyherbal drug and standard chemotherapeutic drug Lenalidomide for U266 cells were observed.

The observation inferred that the proliferative capacity of the myeloma cells (U266) was reduced upon treatment with Habb-e-asgandh in a dose-dependent manner (Figure 1) with a greater progression in anti-proliferative on MM cells with increased doses of HeA. After 48 h of treatment, the IC₃₀ and IC₅₀ values of HeA on MM cell line model came out to be at 0.4 mg/ml and 1.5 mg/ml respectively compared with untreated control. While the cytotoxic effect of HeA on MM cell line model was found to be efficacious on the higher side of concentration range of HeA value. For the standard drug, lenalidomide no cytotoxic effect was obtained at any concentration range, thus previously literature reported reference doses of 5 μ M and 10 μ M were taken as respectively sub inhibition concentrations (IC₃₀ and IC₅₀) for further assays.

Re-phasing of Cell cycle profile by Habb-e-asgandh alone or in combination with standard drug

In 48 h incubated drug treatment groups, there was

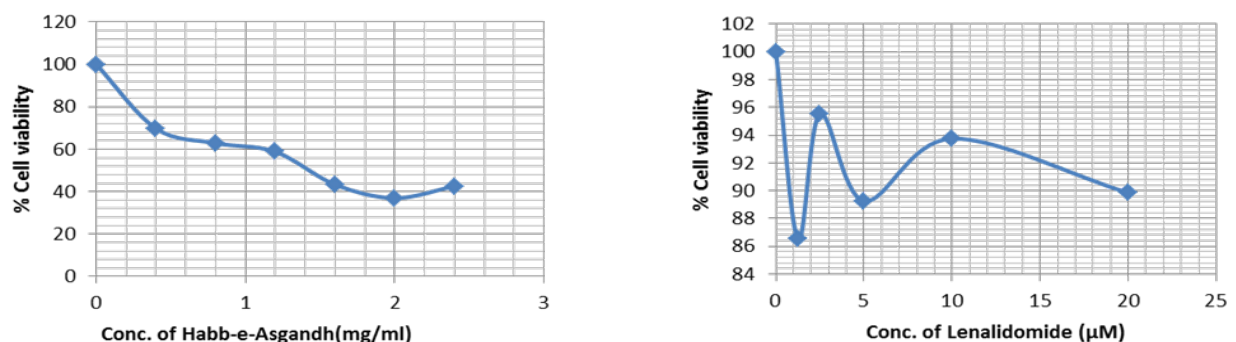


Figure 1. Evaluation of Sub Maximal and Half Maximal Inhibitory Concentration of Habb-e-asgandh and Standard Drug Lenalidomide Post Treatment in U266 Myeloma Cells. MTT assay was performed after 48 h to determine inhibitory concentrations of HeA (IC₃₀=0.4 mg/ml; IC₅₀=1.5 mg/ml) in U266. The results showed decrease in cell viability in a dose dependent manner.

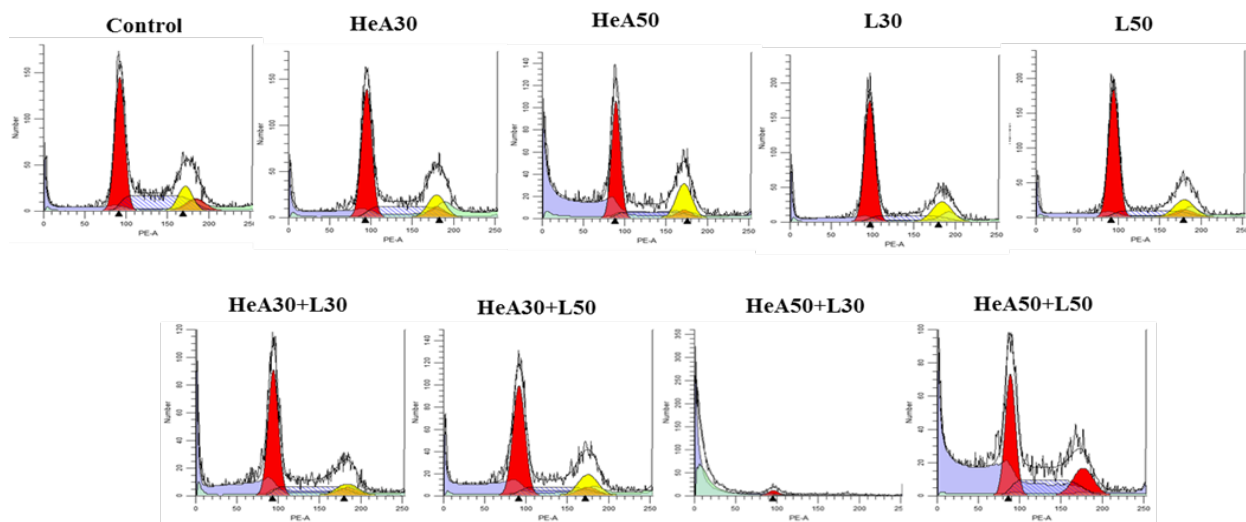


Figure 2. Re-Phasing of Cell Cycle by Habb-e-asgandh Alone or in Combination with Standard Drug Lenalidomide Representative Flow Cytometry Profile Showing Distribution of U266 Cells in Different Phase of Cell Cycle after 48 h upon Habb-e-Asgandh Treatment Alone or in Combination with Lenalidomide.

an overall stage wise alteration in the cell cycle. On Habb-e-asgandh treatment alone or in combination with lenalidomide, an alteration or rather increase in the G0/G1 population of U266 cells was seen which was not significant. While, the alteration in arrested sub G0/G1

population on treatment of HeA formulation alone or in combination with standard drug reported as significant accumulation of sub G0/G1 staged cells compared to untreated control cells. The maximal percentage increase in arrest (sub G0/G1) phased cell population was found in

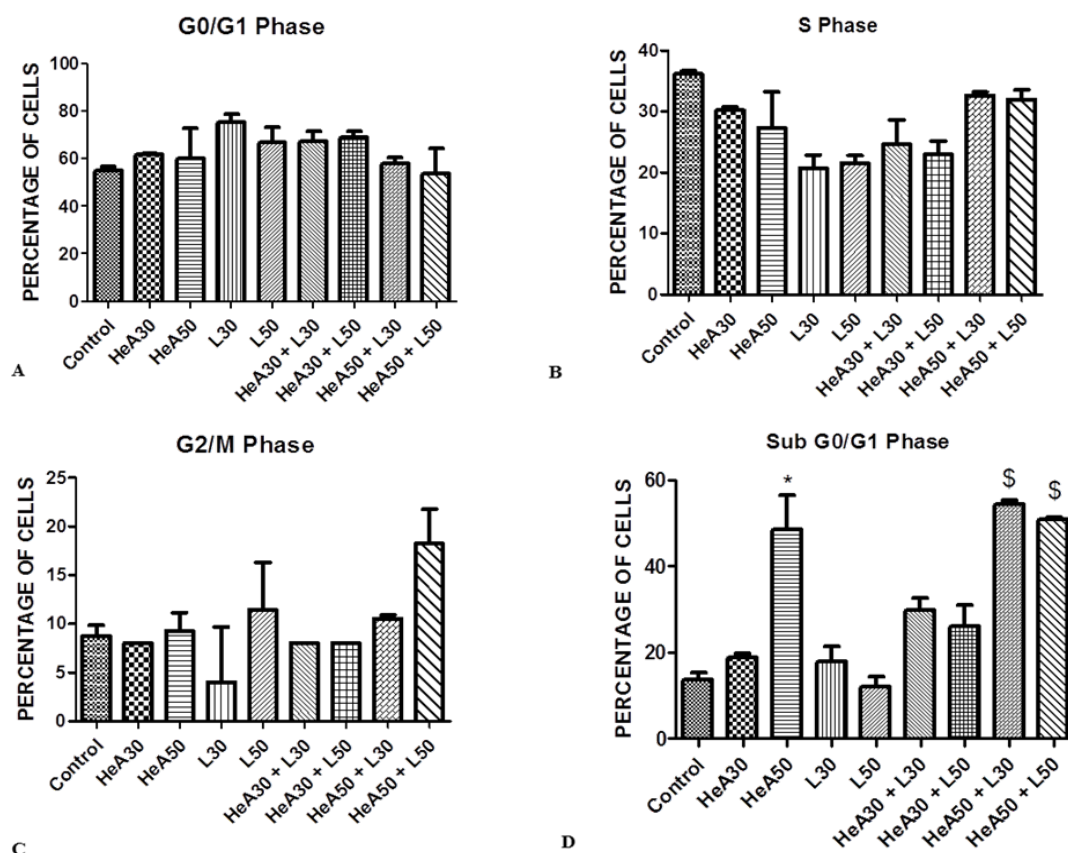


Figure 3. Bargraphs Indicates A) Habb-e-asgandh treatment alone or in combination with lenalidomide alters or rather increases the G0/G1 population of U266 cells in non-significant manner. B) Percent variation of S phase population of U266 cells is in indicated treatment groups. C) Pattern of accumulation of G2/M cell population upon Habb-e-asgandh treatment. D) The alteration in arrested sub G0/G1 population on treatment of HeA formulation alone or in combination with standard drug reported as significant accumulation of sub G0/G1 staged cells. Here * symbol represents significance ($p < 0.05$) w.r.t. controls. While \$ symbol represents significance ($p < 0.05$) with respect to standard drug doses L30 or L50. [HeA: Habb-e-Asgandh; L: Lenalidomide]

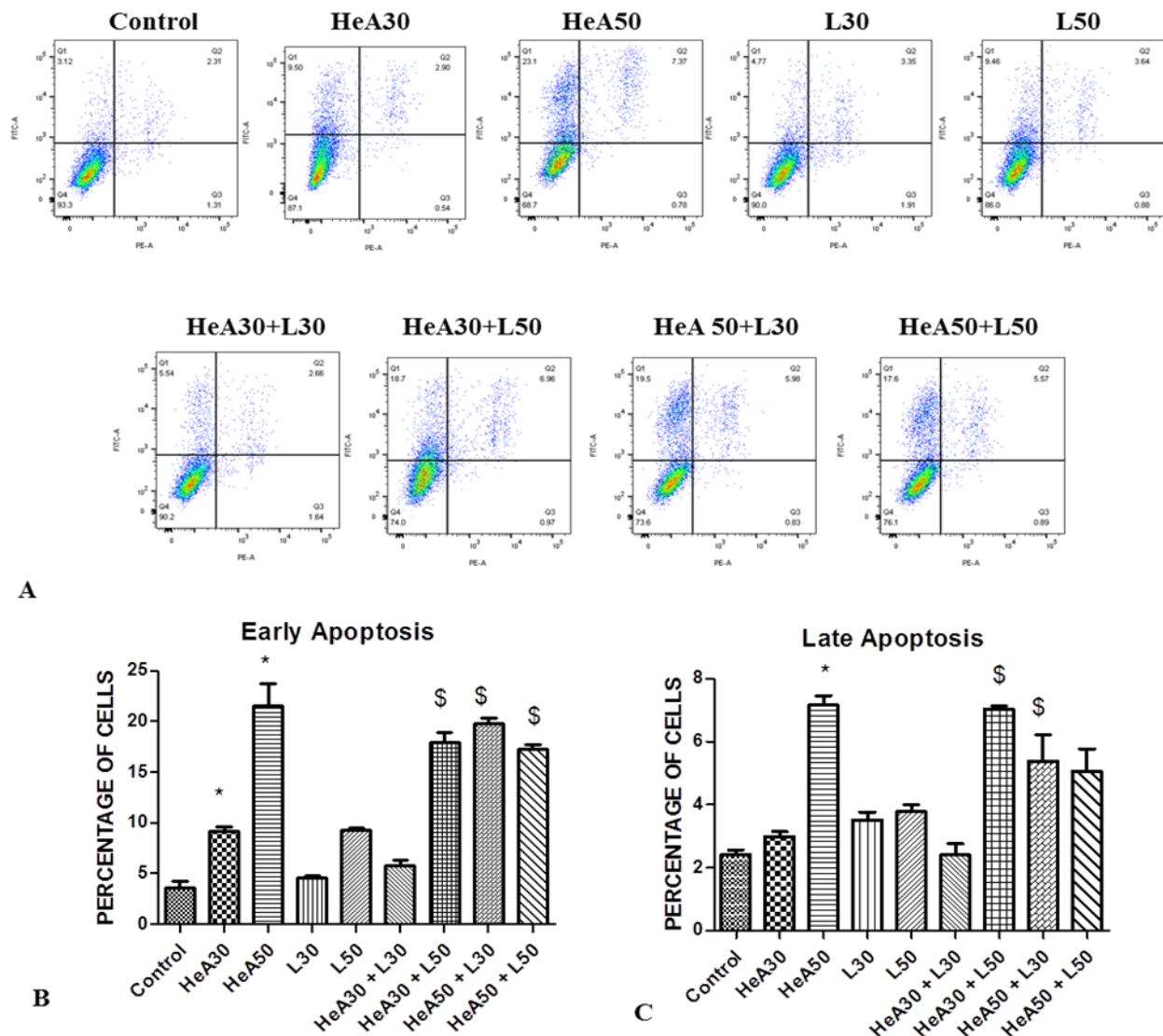


Figure 4. Habb-e-Asgandh Induces Apoptosis in U266 Cells Alone or in Combination with Standard Drug Lenalidomide. Representative flow cytometry profile showing Annexin V and PI stained U266 cells post Habb-e-asgandh and lenalidomide treatment. B-C) Significant increase in early apoptotic and late apoptotic cells upon Habb-e-asgandh and lenalidomide treatment of U266 cells. * symbol represents significance ($p < 0.05$) w.r.t. controls. While \$ symbol represents significance ($p < 0.05$) with respect to standard drug doses L30 or L50. [HeA: Habb-e-Asgandh; L: Lenalidomide]

1.5 mg/ml HeA treated with respect to untreated control. Similar increases in the sub G0/G1 population were found in combinatorial combinations with respect to referred standard drug dosage (Figures 2 and 3).

Habb-e-Asgandh induces mitochondrial apoptosis in U266 cells with potentiation of anti-tumor effects in drug combination

After observing PI staining herewith inferred towards the occurrence of cell death. The next experimental phase was to decipher the mode of cell death of the herbal drug treated U266 cells through Annexin V/PI assay. This method primarily indicates early apoptotic cell death patterns.

The classification of the stages of apoptosis was reported as a measure of only green fluorescence positive (early apoptotic cell) and later also with dual fluorescence positive (late apoptotic) cells were present.

The finding indicates cellular proportion distributed in early phase of apoptosis increase by treatment with HeA in a dose dependent manner. Meanwhile, the population of apoptotic cells with maximal significant increase was observed in solely HeA treated IC_{50} fraction compared to untreated U266 control cells. This surge in apoptotic count of alone HeA treated IC_{50} fraction was found to be of almost comparatively equivalent to increase in HeA IC_{50} –lenalidomide sub IC_{30}/IC_{50} treated combinations (Figure 4). This deciphered Habb-e-Asgandh given in combination with lenalidomide would potentiate the apoptotic effect of the standard drug for the treatment of MM.

Habb-e-Asgandh impedes mitochondrial membrane permeability of U266 cells

To further investigate disruption in mitochondrial membrane potential (MMP) using JC1 dye as a key

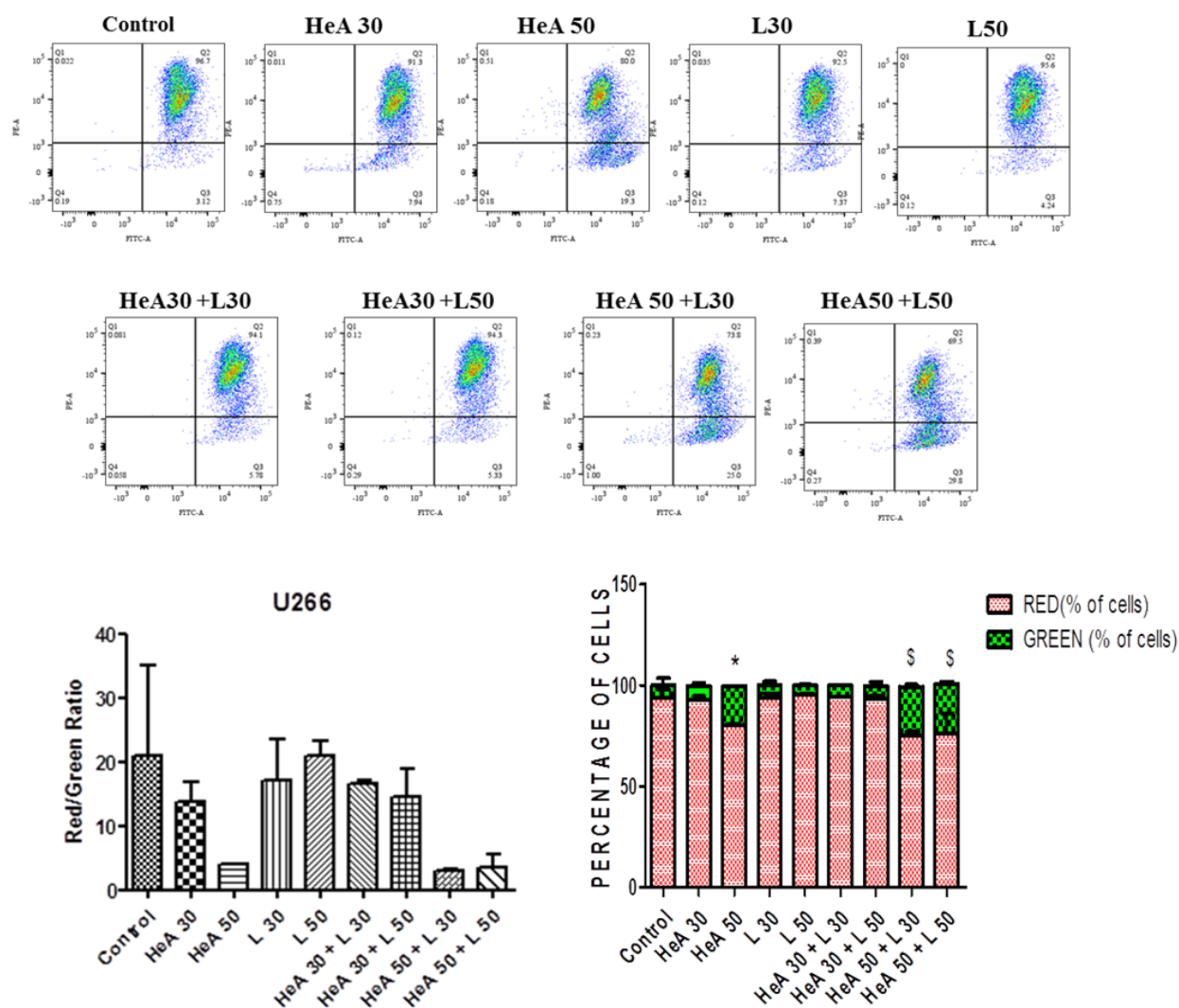


Figure 5. Habb-e-Asgandh Impedes Mitochondrial Membrane Permeability of U266 Cells Alone or in Combination with Standard Drug Lenalidomide. Representative flow cytometry profile showing U266 cells with dissipated $\Delta\Psi_m$ as evident from increase in cell population higher green fluorescence of JC1 dye. A) Bar graphs showing repressed Red/Green fluorescence ratio of U266 cells treated with Habb-e-asgandh and lenalidomide. B) Significant increase in U266 cell population with green fluorescence with concomitant lowering in red fluorescence upon Habb-e-asgandh and lenalidomide treatment. * symbol represents significance ($p < 0.05$) w.r.t. controls. While \$ symbol represents significance ($p < 0.05$) with respect to standard drug doses L30 or L50. [HeA: Habb-e-Asgandh; L: Lenalidomide].

event in induction of apoptosis. A significant increase in cellular proportion with dissipates MMP after HeA IC_{50} treated dose as compared untreated control. Also, there is significantly enhanced U266 cell population with green fluorescence with concomitant decrease in red fluorescence upon Habb-e-asgandh and lenalidomide co-treatment as compared to sub IC_{50} and IC_{50} only lenalidomide treated fraction (Figure 5).

Morphological depiction of Habb-e-Asgandh treatment on U266 myeloma cells

The occurrence of apoptosis as a mode of cytotoxicity in Habb-e-Asgandh treated cells was additionally confirm by acquired images of the cultured U266 cells by phase contrast microscopy in Figure 6. The morphology of HeA treated U266 cells after 48 h incubation were seen with the presence of visible distortions and membrane blebbing as implicated as a characteristic morphological feature in

case of apoptosis. Also, reduction in cellular population was observed in treated experimental group as compared to vehicle treated cells.

Effect of Habb-e-Asgandh on oxidative capacity of U266 myeloma cells

To further explore, if Habb-e-asgandh utilizes anti-oxidative capacity (usually possessed by phyto compounds) as a mode of action against myeloma cells. Furthermore, to check whether co-treatment of HeA-lenalidomide effect the oxidative potential of treated myeloma, ORAC assay was performed. The findings values were reported as trolox equivalent concentration (in μM) tested against various experimental combinations. The levels of ORAC in U266 cells treated with higher concentration (IC_{50}) of Habb-e-asgandh alone were moderately elevated compared to control sample with the difference found to be statistically significant (Figure 7). The ORAC levels

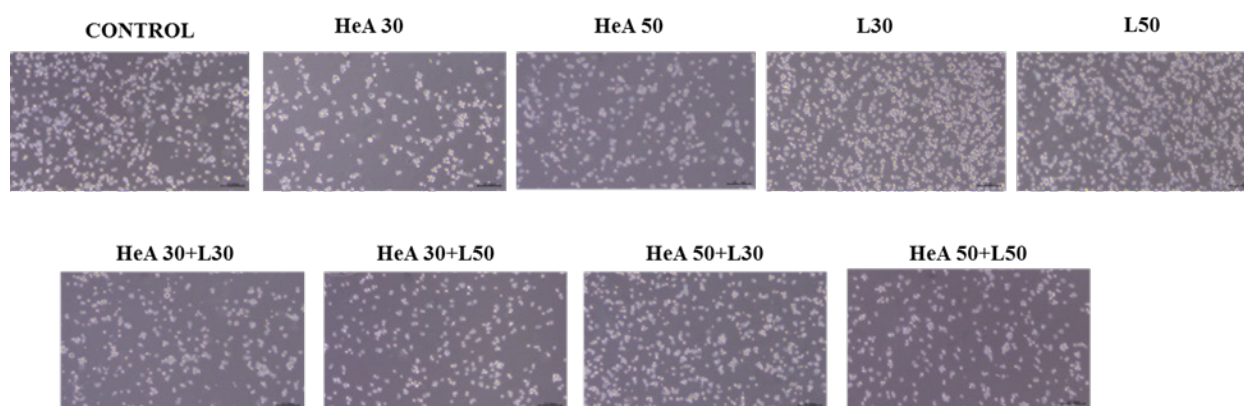


Figure 6. Morphological Depiction of Habb-e-Asgandh Treatment on U266 Myeloma Cells. Acquired images of the Habb-e-asgandh and Lenalidomide treated U266 cells by phase contrast microscopy images at 20X magnification and scale of 200µm depicting reduction in cellular population [HeA: Habb-e-Asgandh; L: Lenalidomide]

were also significant ($p < 0.05$) in HeA with standard drug lenalidomide combinations thus, concluding the drug and combination is found to be efficacious in inducing anti-oxidative status in treated U266.

Reduced pro-inflammatory and angiogenic markers levels after Habb-e-asgandh treatment

The sole or combinatorial effect of HeA with lenalidomide was studied by testing levels of pro-inflammatory (IL-6, TNF α) and angiogenic (VEGF and Ang-2) cytokine markers in cell culture supernatant of test drug treated U266 cells by ELISA. For both IL-6 and TNF- α estimated levels, were decreased in all HeA treated U266 culture supernatant samples either alone or in combination (Figure 8).

For angiogenic marker VEGF, estimated values

were significantly lessened ($p < 0.05$) in U266 cells when treated alone or in Lenalidomide and Habb-e-asgandh combination (Figure 9). Similar results were obtained for Angiopoietin 2; its levels are significantly reduced in drug HeA treated samples except for fraction IC₅₀ Habb-e-asgandh co-treated with IC₅₀ drug concentration lenalidomide U266 cells in which statistically significant increase in Ang- 2 were found.

Discussion

Therapeutic management of multiple myeloma patients is daunting due to development of drug resistant, relapse, refractory nature of disease thus, decreasing the survival rate in the patient. (Gulla and Anderson, 2020). The present scenario demands the need of drug trials of safer alternatives with current standard regime as combination drug adjuvant therapy which aims at reducing relapse, and thus, improving quality and survival years of patients. Herbal medicine may offer such safer alternatives in treatment of cancer immune therapeutics.

Herbal drugs and plant based traditional medicine has been utilized by large proportion from long for medical management. Currently, there is enhanced reappearance in utilization of such plant based alternatives (Sen and Chakraborty, 2017). The benefit of our exploration may provide on *in vitro* validation is minimizing drug-drug interactions in combination therapy thus, enhancing safety and cost effective treatment. Cinnamon extract examined against multiple myeloma has shown promising results by varying expression of genes involved in angiogenesis and cyclooxygenase (Khan et al., 2016). *Withania Somnifera* Dunal has been shown to possess anticancer and anti-proliferative activity against multiple cancer cell lines (Yadav et al., 2010). While its anti-cancerous potential is suggestive though as it is mixture of numerous medicinal herbs with some of its individual constituent like *Withania somnifera*, *Zingiber officinale*, *Trachyspermum ammi* having found to be cytotoxic against various types of cancers (Yadav et al., 2010; Lee, 2016; Rai et al., 2016; Saggam et al., 2020; Seresht et al., 2019). Moreover, our research group reported anti-leukemic activity of Habb-e-asgandh against CML experimentally

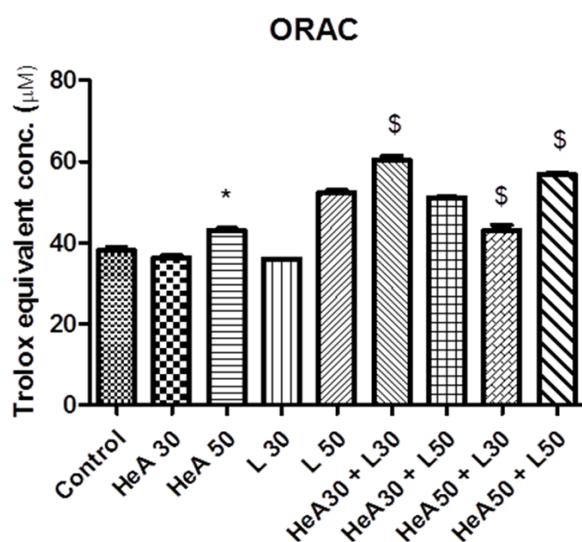


Figure 7. Effect of Habb-e-Asgandh Alone or in Combination with Standard Drug Lenalidomide on Oxidative Capacity of U266 Myeloma Cells. Elevated ORAC values of HeA and standard drug treated U266 cells indicates efficaciousness of drug and its combination with lenalidomide in inducing anti-oxidative effects in treated U266 cells. * symbol represents significance ($p < 0.05$) w.r.t. controls. While \$ represents significance ($p < 0.05$) with respect to standard drug doses L30 or L50. [HeA: Habb-e-Asgandh; L: Lenalidomide]

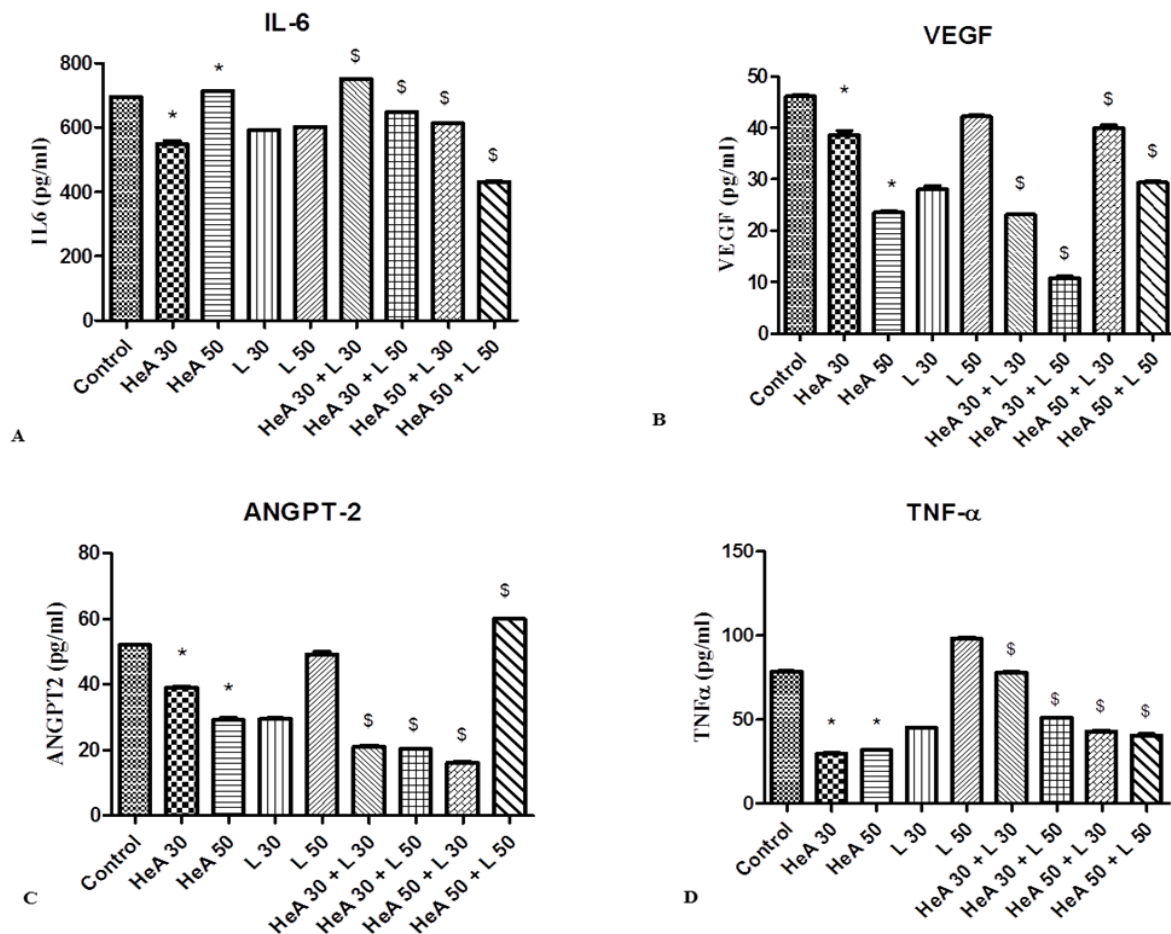


Figure 8. Reduced pro-Inflammatory and Angiogenic Markers Levels after Habb-e-asgandh Treatment Alone or in Combination with Lenalidomide. [HeA: Habb-e-Asgandh; L: Lenalidomide]

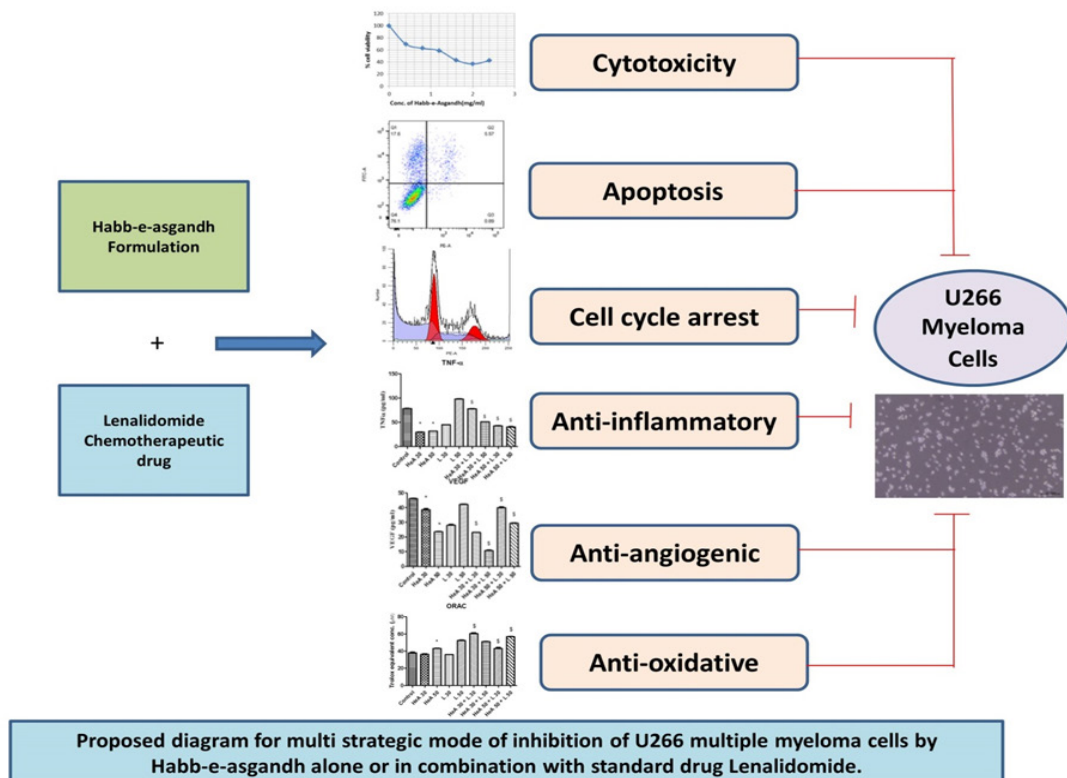


Figure 9. Image Indicates Broad and Multi-Directed Mode of Action of Habb-e-asgandh Formulation Alone or in Combination Lenalidomide on U266 Myeloma Cells (Graphical abstract).

(Ali et al., 2014; Gupta et al., 2022). The present study provides the insight to cover alternative combinatorial therapeutic modality using Habb-e-asgandh for targeting U266 myeloma cells *in vitro* while its effects in multiple myeloma cells RPMI 8226 have already being studied in our group recently (Vashist et al., 2022). Hence, in our current study scheme the polyherbal Habb-e-asgandh has been investigated for its efficacy against MM cell line U266 specifically and its modulating effect on generalized tumor cell characteristics and related molecular mechanism.

In our scientific venture indicates potent anticancer efficiency of Habb-e-asgandh alone or in combination with lenalidomide against U266 cells *in vitro*. Our test drug resulted in anti-proliferative effects and cell death through apoptosis induced cytotoxicity with alteration in mitochondrial membrane potential. Further, Habb-e-asgandh treatment had shown surge in oxidative stress and decrease in inflammatory and angiogenic capacity on U266 myeloma cells. Thus, it indicates multi-strategic approach utilized by the herbal drug against U266 myeloma cells.

Withanolide D, a component of *W. Somnifera* found to have cytotoxic and cytostatic effect on multiple myeloma cell lines (Issa et al., 2017). The extract of *Zataria multiflora* after 24 h showed decrease in cell viability in a dose dependent manner in U266 cells (Anani et al., 2020). Our observation was in coherence with as per the similar literature reported finding as indicated. It has been observed that after 48 hrs of HeA treatment alone or in combination with lenalidomide, the U266 myeloma cell proliferation was inhibited in concentration dependent manner. For IC_{50} value obtained, the efficaciousness of HeA was obtained on the higher end of concentration range. This might be due to induction of cell death through apoptosis which was confirmed on further investigation.

A derivative of *Piper longum* Linn. ROS generation mediated anticancer activity, also reported sub G1 and G0-G1 accumulation in cell cycle arrest on treatment in prostate cancer cells (Lee et al., 2013). Similarly, slight increase in arrested G2/M phased cell in myeloma RPMI 8226 treated versus untreated cell line was seen in a herbal mushroom, *Ganoderma lucidum* (Müller et al., 2006). *Argyrea nervosa* treatment cell cycle arrest G2/M against cells of oral cancer (Subramanyam et al., 2021). There was a significant increase in arrested population i.e. accumulation of sub G0/G1 cells number in both HeA treatment alone or in combination group observed on PI staining. This inferred towards cell cycle phase specific inhibition pattern characteristically obtain on Habb-e-asgandh treatment on U266 cells.

Suppression of Tumor through apoptosis induction and change in membrane permeability by *Piper longum* Linn. was seen in case of human ovarian cancer cells (Si et al., 2018). There was a dose dependent increase in early apoptotic cells population which took Annexin V/FITC stain observed. The equivalent measured surge of early apoptotic cells population in HeA treated IC_{50} alone and with HeA IC_{50} -lenalidomide IC_{30}/IC_{50} combination may conclude apoptotic effect observed independent of combining lenalidomide inhibitory concentration,

which may or may not indicate synergistic apoptotic development.

The depolarization in mitochondrial membrane with HeA treatment was seen in concentration dependent manner which was much more pronounced in combination treated samples. This is indicative of the role of mitochondrial membrane pathway of apoptosis induction. The observation signifies association between apoptosis and loss of mitochondrial membrane potential by chemotherapeutic drugs (Vyas et al., 2021). In a recent study, *Argyrea nervosa* and its active ingredient causes MMP disruption and apoptotic changes in colorectal colon cancer cells *in vitro* (Rasool et al., 2021). The enhancement in degree of apoptosis and anti-proliferative effects collectively with herbal drug treatment may indicate cytotoxicity induced through apoptosis.

Meanwhile, moderately high ORAC levels at IC_{50} value of Habb-e-asgandh which came out to be statistically significant when compared to control. Also, high ORAC values obtained in standard drug combination treated group due to increase reactive oxygen species (ROS) generation. In both alone HeA or in combination treated U266 cells, significant reduction in pro-inflammatory and angiogenic marker infer marked decline in phenomenon of angiogenesis and inflammation after Habb-e-asgandh treatment. *Viscum cruciatum* Sieb traditional medicine extract evident to be effective against hematological tumors, similar to our findings to significant decline in level of markers U266 Multiple myeloma cell lines compared to untreated cells (Assaf et al., 2013).

To summarize, our study analyzed effect of Habb-e-asgandh treatment on U266 myeloma cells that is enhanced cytotoxicity, apoptosis induction and loss of MMP, decreased levels of cytokines markers and elevated oxidative stress. The multiple inhibitory modes against tumorigenic activity in HeA treated U266 myeloma cell line and ability to potentiate action with improved efficacy with standard drug *in vitro* can pave way for Habb-e-Asgandh to act as an adjuvant with standard chemotherapeutic regime for better future outcome.

This manuscript is followed in coherence with the MIRDA standard reporting recommendation (Ahmad et al., 2022).

In conclusion, traditional and herbal drugs have been of enormous importance in inhibition of cancer. We studied the anti-tumorigenic capacity of Habb-e-asgandh, a plant based polyherbal drug formulation against multiple myeloma cells U266 *in vitro*. Our present study deciphered the dose dependent cytotoxicity and anti-proliferative effects of the herbal drug and its combination with standard drug, though it lacked in evaluating degree of synergism with chemotherapeutic regimen and focus on variation in crude cancer characteristics. Further, the ability of HeA to act as an anti-apoptotic agent and its role in potentiation of effects against experimental U266 myeloma cells in combination with standard therapy at varied doses. Also, the test drug was found to have oxidative, inflammatory and angiogenic properties against U266 cell line. Our study is an overall experimental profiling and validation demonstrating the multi-strategic anti-myeloma action of Habb-e-asgandh against U266

in vitro.

Author Contribution Statement

Shraddha Kapoor: Data curation, Methodology, Formal analysis, Writing - original draft; Nidhi Gupta: Conceptualization, Project administration, Writing - review and editing; Alpna Sharma: Funding acquisition, Conceptualization, Resources management, Investigation, Supervision, Writing - review and editing. The content of the manuscript is read and accepted by all the authors.

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Ethical Declaration

The ethical approval obtained for cell line through Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi (IEC-539/02.12.2016).

Conflict of interest

The authors declare that they have no conflict of interest.

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