

Withaferin A inhibits cell proliferation of U266B1 and IM-9 human myeloma cells by inducing intrinsic apoptosis

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Withaferin A, a withanolide obtained from *Withania somnifera* exhibits remarkable pharmacological properties. Withaferin A has been reported to exert cytotoxic effects against human multiple myeloma cells. Nevertheless, the in-depth understanding of the withaferin A induced antiproliferative effects against human myeloma cells is still unclear. The results showed that withaferin A inhibited the viability of six different myeloma cells with a lowest IC₅₀ of 9 μM against the U266B1 and IM-9 cell lines. Withaferin A inhibited the viability and colony formation of the U266B1 and IM-9 cells in a dose and time-dependent manner. The DAPI and annexin V/PI staining assays revealed that withaferin A exerts anticancer effects against the human myeloma cells via induction of apoptosis. The induction of apoptosis in U266B1 and IM-9 cells was associated with upregulation of Bax and cytochrome c, downregulation of Bcl-2 and activation of PARP, caspase-3 and caspase-9 cleavage. Additionally, withaferin A triggered the production of ROS in human myeloma cells indicative of ROS mediated apoptosis in human myeloma cells. The treatment of the U266B1 and IM-9 with ascorbic acid (antioxidant) could prevent the withaferin A mediated ROS production and the withaferin A induced antiproliferative effects. Collectively, the results show that withaferin A inhibits human myeloma cell proliferation via ROS mediated intrinsic apoptosis.

Keywords: myeloma, withanolide, Withaferin A, apoptosis, anti-cancer

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Abbreviations: ROS, reactive oxygen species; DAPI, 4',6'-diamidino-2-phenylindole; CCK-8, cell counting kit-8; DMSO, dimethyl sulfoxide

which is responsible for the frequent disease recurrence within noticeably short periods of diminution (Munshi & Avet-Loiseau, 2011). Recent years have witnessed a paradigm shift in the management of human myeloma. There is growing support that before assigning a particular treatment procedure for myeloma, the disease development and subsequent progression must be intensively explored for achieving better clinical results (Kumar, 2019). Moreover, the recent studies have envisaged how to target specific genetic abnormalities to avoid the treatment failures arising as a result of instances of development of drug resistance (Pinto *et al.*, 2020). In connection with this, there is an urgent need of examining the efficacy of natural products for development of novel drugs so as to enable ourselves to simulate a judicious drug combination against human myeloma in the future (Kumar, 2019). With the same objective, the anti-cancer effects of Withaferin A were investigated against human myeloma cells in the present study. Withaferin A is the most bio-active withanolide, highly oxygenated lactone from *Withania somnifera* known for antioxidant, anti-inflammatory, and anti-depressant properties (Singh *et al.*, 2011; Berghe *et al.*, 2012). The effectiveness of Withaferin A to serve as anti-cancer agent has been assessed against different types of human cancer cells including breast cancer, colon cancer, head and neck cancer, leukemia, ovarian cancer, multiple myeloma to name a few (Hassannia *et al.*, 2020). Withaferin has been shown to inhibit the proliferation of the human multiple and induce their cell differentiation (Issa & Cuendet, 2017). However, the in-depth underlying mechanism for the withaferin A induced antiproliferative effects against the human myeloma cells has not been explored. This study for the first-time reports that the anticancer effects of withaferin A against the human myeloma cells is due to ROS mediated intrinsic apoptosis.

INTRODUCTION

Multiple myeloma or myeloma is a malignant neoplastic disorder of blood plasma cells (Muccio *et al.*, 2016). It is characterized by the uncontrolled growth and accumulation of plasma cells in bone marrow (Szudy-Szczyrek *et al.*, 2016). The most frequent clinical symptoms associated with multiple myeloma include, but are not limited to, kidney injury, bone destruction and paraproteinemia (Kumar & Rajkumar, 2018). Myeloma is ranked as the second most common hematologic disorder and accounts for 10% of all the malignant blood disorders (Dicato, 2018). Besides, it makes up to 1 percent of all human cancers at global level (Kazandjian, 2016). Despite the great progress in the therapeutic regimes over few decades, the multiple myeloma is still incurable because of the molecular heterogeneity of malignant plasma cells

MATERIALS AND METHODS

Cell lines and culture conditions

The six multiple myeloma cell lines (MOLP-8, U266B1, RPMI-8226, KMS-11, IM-9 and JJN-3) were purchased from American Type Culture Collection (ATCC, USA). The myeloma cell lines were cultured using Roswell Park Memorial Institute medium (RPMI-1640, Gibco; Ireland). The culture medium was supplemented with 10% fetal bovine serum (FBS, Gibco; Ireland) and streptomycin (100 μg/mL)-1% penicillin (100 U/mL). The myeloma cells were maintained in a humidified atmosphere at 37°C with 5% CO₂. Normal plasma cells (NPCs) were extracted from peripheral blood smear

and cultured according to previously reported methods (Adham *et al.*, 2020).

Cell proliferation assay

After treatment with varying doses for 24 h or varying durations with withaferin A in 96-well plates, the cell proliferation of myeloma and normal plasma cells was analyzed using the cell counting kit-8 (CCK-8, Beyotime Institute of Biotechnology) assay. Herein, immediately after treating with Withaferin A, the cells (2×10^5 /well) were supplemented with 10 μ L CCK-8 reagent. Cells were again incubated for 2.5 h at 37°C. Using a microplate reader (Bio-Rad Laboratories, Inc.), the absorbance was measured at 450 nm to determine the cell proliferation.

Colony formation assay

For the analysis of colony formation, a total of 10^5 U266B1 or IM-9 cells were grown for 14 days in 6-well plates after being administered with varying Withaferin A doses for 24 h or with 9 μ M Withaferin A for 0, 12, 24, 48 or 96 h at 37°C. After two weeks when the colonies were visible, the supernatant was removed, and the colonies formed were washed thrice with phosphate-buffered saline (PBS). Then, the colonies were fixed with 70% ethanol for 25 min and subsequently stained using 0.1% crystal violet for 20 min. Photographs were taken with a camera and the Image J software (National Institutes of Health, USA) was used for estimating the number of colonies from each well and then percent colony formation was calculated.

Analysis of cell apoptosis

The U266B1 or IM-9 myeloma cells were incubated with 0, 4.5, 9.0 or 18 μ M of Withaferin A for 24 h at 37°C. Apoptotic signs were visualized using DAPI staining. Here, the variably treated cells along with the untreated U266B1 or IM-9 cells were PBS washed and then fixed with 70% ethanol for 20 min at room temperature. The cells were then permeabilized with Triton X-100 (0.1% in PBS) and stained with DAPI (1 μ g/mL in PBS) for 10 min in the dark at 37°C. Finally, the nuclear morphology was studied using the fluorescence microscope.

The apoptosis of U266B1 or IM-9 myeloma cells was also assessed using Annexin V-FITC/PI staining method. Briefly, post-treatment, the cells were collected through centrifugation. After trypsinization, the cells were re-suspended in 185 μ L binding buffer and then supplemented with 5 μ L Annexin V-FITC and 10 μ L PI. This was followed by a dark incubation of 25 min at room temperature. Finally, the apoptosis of myeloma cells was measured using flow cytometry.

Western blotting

The U266B1 or IM-9 myeloma cells were treated with different doses of withaferin A for 24 h at 37°C. Afterwards, the cells were harvested, and PBS washed. Total proteins were extracted from the myeloma cells using M-PERR Mammalian Protein Extraction Reagent and protease inhibitor (1:100). The Nano-Drop1000 spectrophotometer (Thermo Fisher Scientific) was used for the determination of total protein concentration. Then, 40 μ g of total protein were resolved by SDS-PAGE. The PAGE-gels were blotted to the PVDF membranes. 5% BSA/TBS-T was used for blocking the membranes. The membranes were incubated with primary antibodies

against desired proteins overnight. The human β -actin served as an internal reference protein. This was followed by the incubation of PVDF membranes with anti-mouse IgG HRP-linked secondary antibodies. Specific protein bands were detected by a colorimetric reaction using Luminata TM Classico Western HP.

Estimation of intracellular ROS-levels

The intracellular ROS levels from differentially treated U266B1 or IM-9 myeloma cells were quantified using H2DCFH-DA. The ROS oxidize the cell permeable non-fluorescent dye H2DCFH-DA to a highly fluorescent (green) 2',7'-dichlorofluorescein (DCF). The myeloma cells pre-incubated with 2 μ M H2DCFH-DA for 35 min. The cells were then administered with different withaferin A doses or DMSO (negative control) for 2 h. Post-treatment, the cells were harvested, PBS washed and then subjected to fluorescent microscopy. The relative ROS levels were determined using fluorescent spectrophotometer.

Transwell migration and invasion assays

The myeloma cell migration and invasion were analyzed using the transwell chambers either uncoated or Matrigel (BD Biosciences, USA) coated according to the manufacturer's instruction. For migration assay, U266B1 or IM-9 cells variably treated with withaferin A were grown in 150 μ L serum-free RPMI-1640 after treatment for 48 h at 37°C. The cells were placed into a fibronectin-coated polycarbonate membrane inserts of a transwell apparatus. Culture medium alone was added into the lower chamber. Following 24 h incubation at 37°C, the cells from the upper surface of the membrane were swabbed away with cotton while those adhering to its lower surface were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet solution for 25 min. The invasion of myeloma cells was also analyzed through similar procedure except that the transwell membranes were coated in advance with 30 mg/mL Matrigel.

Statistical analysis

Each experiment was performed using three replicates and final results were shown as mean \pm standard deviation (S.D.). All the statistical analyses were carried out using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). The Student's *t*-test was performed to assess differences between two treatment groups. The *p* values <0.05 were taken as the representative of statistically significant difference.

RESULTS

Withaferin A inhibited *in vitro* proliferation and colony formation of myeloma cells

Withaferin A, the most bio-active withanolide from *Withania somnifera*, is a highly oxygenated plant-based lactone (Figure 1A). A panel of six multiple myeloma cell lines (MOLP-8, U266B1, RPMI-8226, IM-9, KMS-11 and JJN-3) and the normal plasma cells (PCs) were administered with varying concentrations of withaferin A to evaluate its effects on the myeloma cell proliferation. It was found that withaferin A inhibited the proliferation of all the myeloma cancer cell lines (Table 1). However, the growth of U266B1 and IM-9 myeloma cell line was most severely affected with an estimated IC₅₀ of

Table 1. Proliferation of different myeloma and normal plasma cells was determined by CCK-8 assay and presented as IC₅₀ values

S. No.	Cell line	IC ₅₀ (μM)
1	MOLP-8	18
2	U266B1	9
3	RPMI-8226,	24
4	IM-9	9
5	KMS-11	12
6	JJN-3	18
7	PCs	74

9 μM (Fig. 1B and 1C). Thus, U266B1 and IM-9 cells were used for further study. On the other hand, when the normal PCs were co-incubated with different doses of withaferin A for 24 h at 37°C, the decline in cell pro-

liferation was not very prominent and evident from the IC₅₀ of 74 μM that was observed for the normal plasma cells (Fig. 1D). Furthermore, the U266B1 and IM-9 myeloma cell growth was inhibited in time-dependent manner when the cancer cells were administered with 9 μM withaferin A for varying incubation periods (Fig. 1E). Withaferin A also decreased the colony formation from U266B1 and IM-9 cancer cells in a dose-dependent manner (Fig. 1E). Collectively, the results signify the anti-proliferative action of withaferin A against the human myeloma cells without much effect on the normal cell growth.

Withaferin A induced apoptosis in myeloma cells

To look for the possible mechanism for the cytotoxic action of withaferin A, U266B1 or IM-9 myeloma cells were treated for 24 h with 0, 4.5, 9.0 or 18.0 μM of withaferin A. The differently treated myeloma cells were DAPI stained, and it was observed that the with-

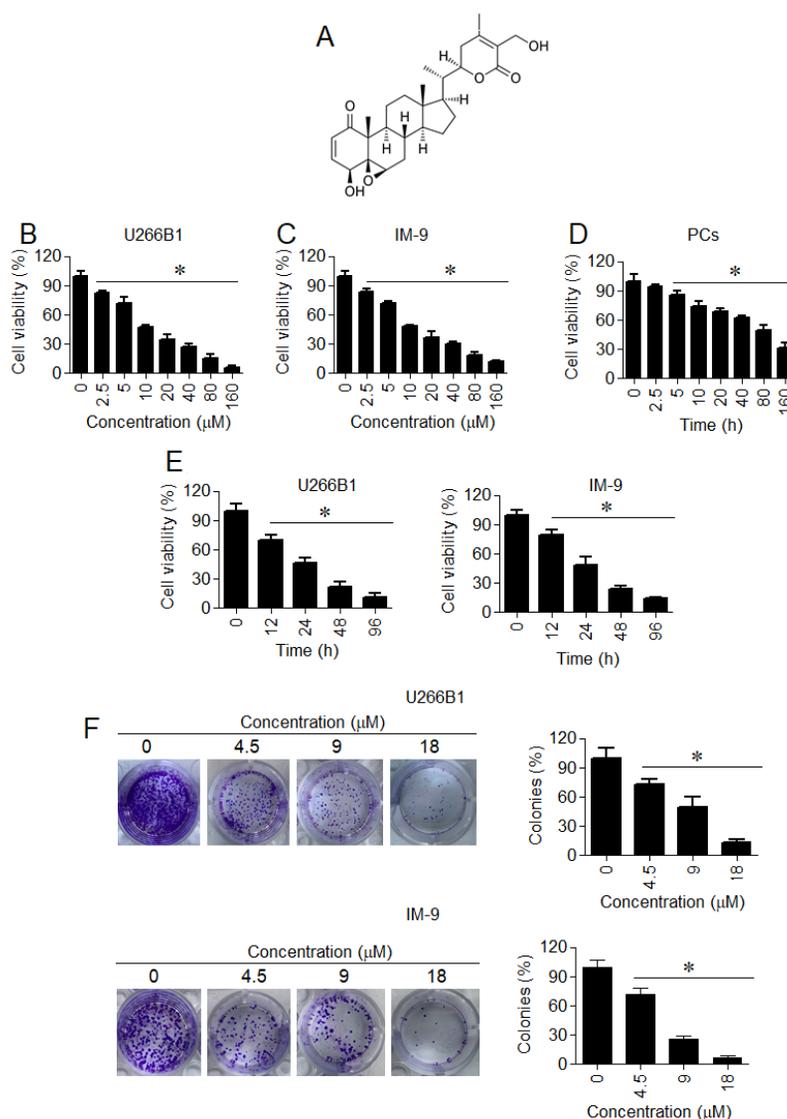


Figure 1. Withaferin inhibits *in vitro* myeloma cell proliferation.

(A) Chemical structure of Withaferin A (B) analysis of effects of withaferin A on proliferation of U266B1 myeloma cells by CCK-8 assay (C) analysis of effects of withaferin A on proliferation of IM-9 myeloma cells by CCK-8 assay (D) analysis of effects of withaferin A on proliferation of normal PCs by CCK-8 assay (E) analysis of effect of withaferin A administration for different durations on proliferation of U266B1 or IM-9 myeloma cells by CCK-8 assay (F) assessment of colony formation of U266B1 or IM-9 myeloma cells pre-administered with different doses of withaferin A. Experiments were performed in triplicates and presented as mean \pm S.D. (* P <0.05).

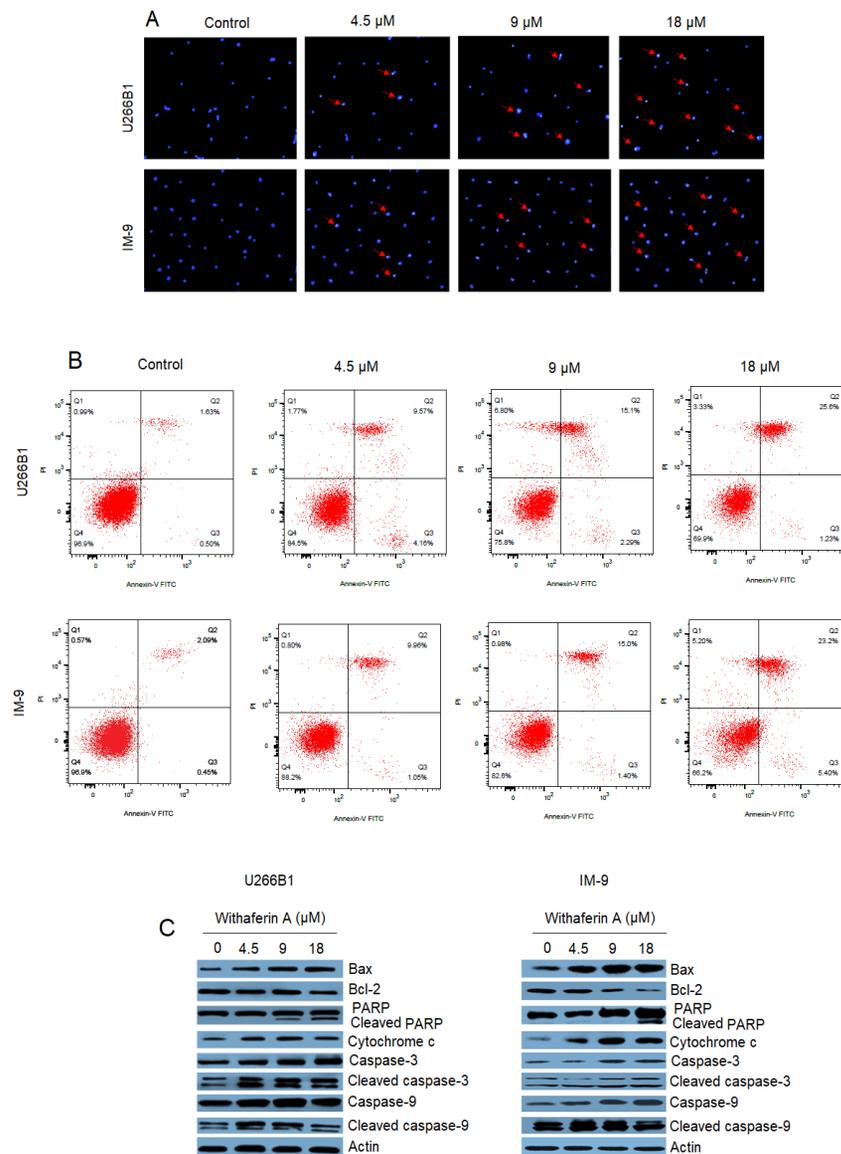


Figure 2. Withaferin induces apoptosis in myeloma cells.

(A) DAPI staining for the analysis of nuclear morphology of U266B1 or IM-9 cancer cells administered with varied concentrations of Withaferin A (B) Annexin V-FITC/PI staining for the analysis of apoptosis of U266B1 or IM-9 cancer cells administered with varied concentrations of Withaferin A (C) western blotting of apoptosis marker proteins from U266B1 or IM-9 cells administered with varied concentrations of Withaferin A. Experiments were performed in triplicates.

aferin A treated U266B1 or IM-9 myeloma cells exhibited signs of apoptosis evidenced by nuclear disintegration when analyzed by fluorescence microscopy and the signs were very prominent at higher treatment concentration (Fig. 2A). Annexin V-FITC/PI staining was used for further confirmation. The flow cytometry results indicated that the percentage of apoptotic myeloma cells increased with the increase in Withaferin A concentration (Fig. 2B). The percentage apoptotic U266B1 cells increased from 2.13 in control to 26.83 at 18 μM withaferin A. Similarly, the percentage of IM-9 apoptotic cells increased from 2.53 to 28.6% at 18 μM withaferin A. The induction of apoptosis in myeloma cells was finally confirmed by western blotting of apoptosis marker proteins. The expression levels of Bax and Cytochrome *c* proteins were increased while that of Bcl-2 protein decreased with increasing Withaferin A treatment doses (Fig. 2C). Treat-

ment of Withaferin A was found to trigger the cleavage of caspase 3, caspase 9 and PARP proteins. The expression of caspase 3 was slightly increased while PARP and caspase 9 expression levels remained unaffected. The results show that withaferin A induced apoptosis in myeloma cells to inhibit their growth, *in vitro*.

Withaferin A increased ROS-levels in myeloma cells

To assess the effect of Withaferin A on intracellular ROS generation, the U266B1 or IM-9 myeloma cells treated with 0, 4.5, 9.0 or 18 μM Withaferin A along with untreated cancer cells were probed with the non-fluorescent dye H2DCFH-DA, which when oxidized gets converted into green, fluorescent DCF. The intensity of green, fluorescent signal increased with the increasing withaferin A concentration, which showed

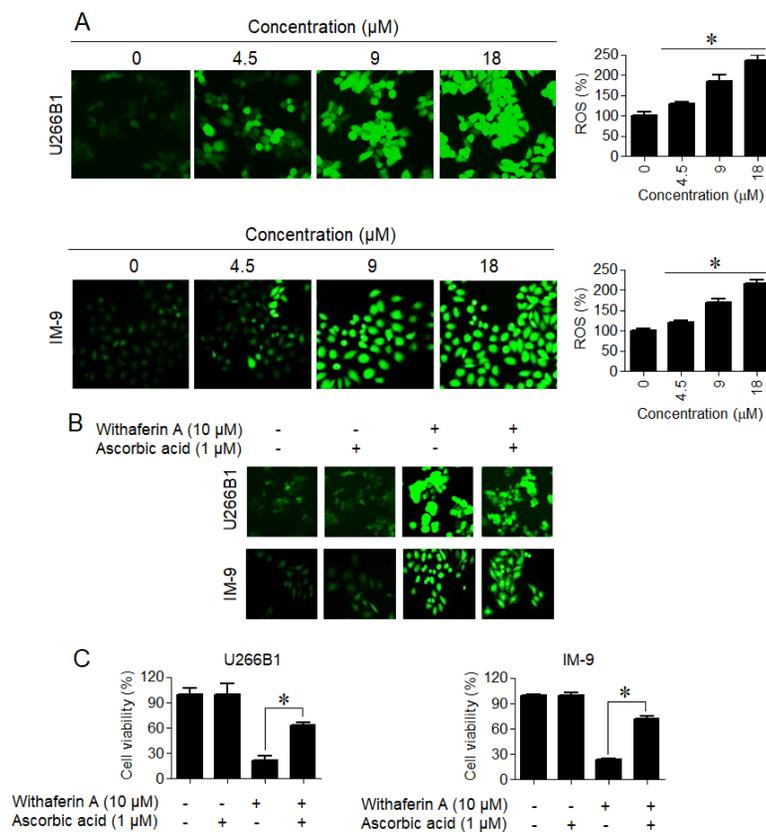


Figure 3. Withaferin A treatment led to ROS accumulation in myeloma cells.

(A) Fluorescence based analysis of ROS levels of U266B1 or IM-9 cancer cells treated with varied concentrations of Withaferin A (B) analysis of relative ROS generation in U266B1 or IM-9 myeloma cells administered with Withaferin A and/or ascorbic acid (C) analysis of relative percentage of viability of U266B1 or IM-9 myeloma cells administered with Withaferin A and/or ascorbic acid. Experiments were performed in triplicates and presented as mean \pm S.D. (* $P < 0.05$).

that withaferin A augmented the generation of ROS in myeloma cells (Fig. 3A). The fluorescence intensity of DCF was used for estimation of the relative ROS levels of myeloma cells differentially treated with Withaferin A. The intracellular ROS levels were shown to be more than 2-fold higher at 18 μ M Withaferin A treatment (Fig. 3B). The administration of Withaferin A (10 μ M) treated U266B1 or IM-9 cancer cells with 1 μ M ascorbic acid, a ROS-scavenger, significantly declined the DCF fluorescence (Fig. 3C). Moreover, the ascorbic acid supplementation attenuated the anti-proliferative effects of withaferin A and enhanced the myeloma cell viability, considerably (Fig. 3D). The results thus indicate that withaferin A increased intracellular ROS generation and possibly induced apoptosis in myeloma cancer cells via this ROS accretion.

Withaferin A abridged the migration and invasion of myeloma cells, *in vitro*

The anti-cancer effects of withaferin A on U266B1 or IM-9 cells were also studied in terms of its effect on the cell migration and invasion, *in vitro*. Notably, the U266B1 or IM-9 myeloma cells were administered with exceptionally low concentrations of withaferin A (up to 8 μ M) to over-rule its cytotoxic effects for studying their migration and invasion. The migration of myeloma cells was reduced in dose-dependent fashion (Fig. 4A). The percentage of cell migration was less than 30% at 8 μ M withaferin A concentration in comparison to the normal untreated myeloma cells. Withaferin A treatment simi-

larly affected the cell invasion and the latter decreased by more than 70% when myeloma cells were administered with 8 μ M Withaferin A (Fig. 4B). Withaferin A might thus exhibit an anti-metastatic action against myeloma cells. Collectively, the results suggest that withaferin A induces ROS mediated apoptosis in myeloma cells (Fig. 5) and also inhibits cell migration and invasion.

DISCUSSION

Natural products are considered as a precious source for prevention and treatment of human ailments since times immemorial (Mousa, 2017). Cancer is still categorized as one of the dreadful clinical disorders threatening the human survival (Xia *et al.*, 2017). A large number of naturally occurring chemical compounds have been shown to exhibit considerable therapeutic effects against different types of cancer cells (Cragg & Pezzuto, 2016). Plant derived metabolites like taxol, vincristine, vinblastine etc. are some of the important chemotherapeutic agents presently used against human cancer (Iqba *et al.*, 2017). Withaferin A is the first plant-based anti-cancer withanolide, which was extracted way back in 1967 (Lee & Choi, 2016). There are several reports that withaferin A exhibits anti-proliferative properties and inhibits the cancer cell growth both *in vitro* and *in vivo* (Kakar *et al.*, 2017). Withaferin A has been shown to exert its anti-cancer effects through the induction of apoptotic cell death and/or cell cycle arrest by targeting diverse molecular pathways (Tang *et al.*, 2020). The present study

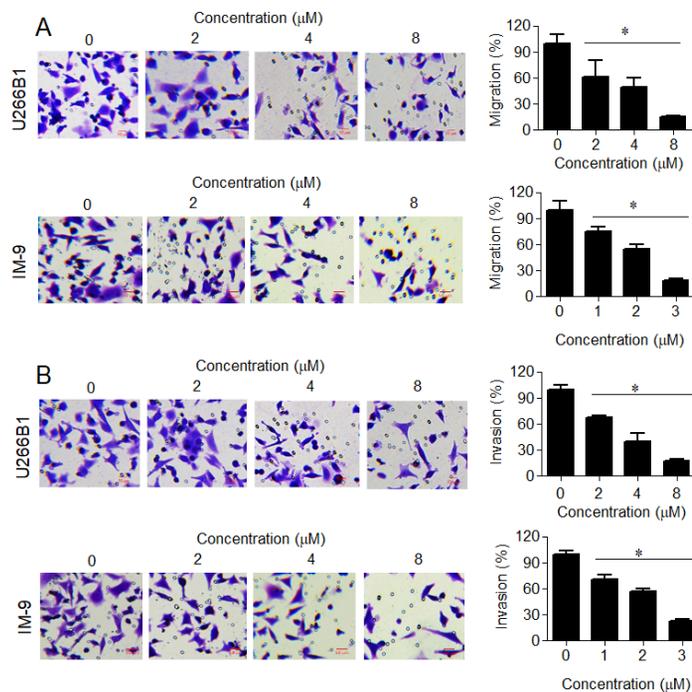


Figure 4. Withaferin A restrained migration and invasion of myeloma cells, *in vitro* (A) Analysis of migration of U266B1 or IM-9 myeloma cells differentially administered with Withaferin A by transwell migration assay (B) analysis of invasion of U266B1 or IM-9 myeloma cells differentially administered with Withaferin A by transwell invasion assay. Experiments were performed in triplicates and presented as mean \pm S.D. (* P <0.05).

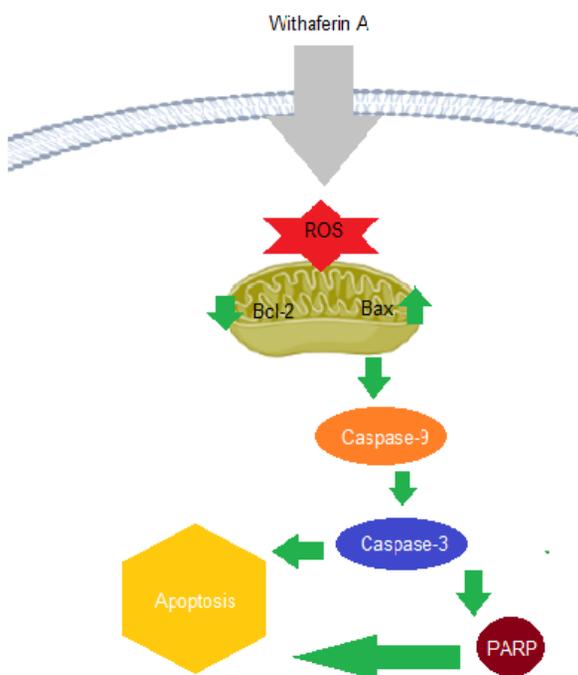


Figure 5. Model for the withaferin A induced apoptosis in myeloma cells.

Withaferin A triggers the accretion of ROS which triggers alteration of the apoptosis related proteins (Bcl-2, Bax, Caspase-9 and -3 and PARP) eventually activating intrinsic apoptosis.

connoted the dose-dependent and selective growth inhibitory property of withaferin A against the malignant plasma cells with little effect on the normal plasma cell viability, suggesting withaferin A as a molecule of choice for drug design against the human myeloma (Ding & Chen, 2018). Previously, the targeting of signal transducer and activator of transcription 3 (STAT3) was elucidated as the possible mechanism mediating the anti-cancer effects on the human myeloma cells via the apoptosis induction *in vitro* (Yco *et al.*, 2014). The induction of apoptosis in myeloma cells was also confirmed by the results of the current study. Withaferin A triggered the activation of caspases that are otherwise present as inactive zymogens inside the cells (Shi, 2004). In addition, the apoptotic signal was modulated through Bax and Bcl-2 proteins (Naseri *et al.*, 2015). Additionally, the myeloma cells exhibited higher cleaved PARP-1 levels, which have been shown to promote necrotic cell death (Eguchi *et al.*, 1997). The caspase activation and subsequent PARP cleavage might be resulting from the release of mitochondrial cytochrome *c* by withaferin A (Cai *et al.*, 1998). The results of the present study suggested that the induction of apoptosis in myeloma cells was prompted *via* ROS-accretion. Similar mode of apoptosis induction has already been confirmed for withaferin against the human breast cancer cells (Hahm *et al.*, 2010). ROS mediated induction of apoptosis in cancer cells is also true for other natural products and has been valued as potent anti-cancer strategy (Fulda, 2011). Withaferin A has been proven to have an anti-metastatic potential against the ovarian and breast cancer cells (Kakkar *et al.*, 2014, Thaiparambil *et al.*, 2011). Consistently, the migration and invasion of myeloma cells was also remarkably inhibited by withaferin A treatment in the present study. Summing up, the *in vitro* study results indicate the therapeutic potential of withaferin A against the human myeloma. However, same needs to be confirmed in animal systems *in vivo*.

CONCLUSION

Collectively, withaferin A exhibits selective cytotoxic effects against the myeloma cells without affecting the normal plasma cell viability. The antiproliferative effects of withaferin were found to be due to its ability to induce ROS mediated intrinsic apoptosis. The study suggests the therapeutic potential of withaferin A against human myeloma. The semi-synthetic chemistry approaches might be helpful in enhancing the effectiveness of Withaferin A in the future.

Conflict of interest

All authors declare that there is no conflict of interest.

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