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ORIGINAL ARTICLE

Herbal extract of *Artemisia vulgaris* (mugwort) induces antitumor effects in HCT-15 human colon cancer cells via autophagy induction, cell migration suppression and loss of mitochondrial membrane potential

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Summary

Purpose: *Artemisia vulgaris (A.vulgaris) belonging to family Compositae, commonly known as mugwort, has been used as a medicinal herb in Chinese traditional medicine for treatment of diseases. Studies have reported a diversity of activities for this plant which include antiseptic, antispasmodic, antigastric, anticancer and nervous system diseases. However, the anticancer activity of A.vulgaris in HCT-15 human colon cancer cells has not been scientifically validated. Therefore the present study aimed at evaluating the anticancer activity of methanolic extract of A.vulgaris against HCT-15 human colon cancer cell line.*

Methods: *Cell cytotoxicity effects of the extract were evaluated by MTT cell viability assay, while clonogenic assay assessed the effects on cancer cell colony formation. Effects on reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) were evaluated by flow cytometry. In vitro wound healing assay was used to evaluate the effects on cell migration. To confirm autophagy, we evaluated the expression of several autophagy-associated proteins using Western blot assay.*

Results: *Results indicated that the methanolic extract of A.vulgaris exhibited an IC_{50} value of 50 μg/ml and exerted its cytotoxic effects in a dose-dependent manner. Moreover, it was observed that the extract inhibits colony formation and induces autophagy dose-dependently. The underlying mechanism for the induction of autophagy was found to be ROS-mediated MMP and significant inhibition of cell migration potential of colon cancer cells at the IC_{50} was observed.*

Conclusion: *These results strongly stress that the methanolic extract may prove a source for the isolation of novel anticancer lead molecules for the management of colon cancer.*

Key words: *Artemisia vulgaris, autophagy, colon cancer, ROS*

Introduction

Artemisia is a large genus consisting of small herbs and shrubs commonly observed in northern temperate regions. *Artemisia* belongs to the family *Compositae*, comprising 1,000 genera and over 20,000 species. The *Artemisia* family itself is a large family mostly found in Asia, Europe and North America [1]. Among Artemisia species, *A. vulgaris* is commonly used as a medicinal herb in Chinese traditional medicine. Studies have reported a diversity of activities for this plant which include antiseptic, antispasmodic, gastric, anticancer and nervous system diseases [2,3]. However, the anticancer activity of this important herb has not evaluated against colon cancer. Colon cancer is the second most widespread cancer among women and the third widespread cancer in men around the world. It arises from a single or a blend of chromosomal instability usually triggered by aneuploidy and loss of heterozygosity [4,5]. Although there are several treatment options currently

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available, however, these treatments have several drawbacks which include, but are not limited to, frequent relapse, development of drug resistance and effect on the quality of life of patients [6].

Natural products are considered important for the development of new anticancer lead molecules. Owing to their lower side effects they have gained considerable attention in the recent past [7,8]. In the present study, A.vulgaris extract was evaluated for its anticancer activity against colon cancer cell line HCT-15 and the possible underlying mechanism was determined. The extract induced cytotoxicity in colon cells by promoting autophagy through ROS-mediated alterations in MMP ($\Delta\Psi_m$) and suppression of cell migration. Taken together, our results indicate that A.vulgaris may prove a source of potential natural anticancer molecules against colon cancer.

Methods

Collection of plant material and extract preparation

The plant material was collected at the experimental farm in Hunan, China and authenticated by the taxonomist in Changsha University of Science and Technology. The aerial parts of A.vulgaris were subjected to washing under running tap water to remove the surface contamination. The plant material was dried in air under shade and then cut into pieces and then to powder using a mechanical blender. Dried root powder was packed in a Soxhlet apparatus and extracted at 60-65°C for 4-5 hrs. Extracts were obtained using pure methanol as solvent. The extracts were stored at 4°C for 24 hrs and then filtered through Whatman No. 4 filter paper and evaporated to dryness under vacuum and stored at 4°C till further analysis.

Chemicals and reagents

The following chemicals were used in the present study: RNase A triton X-100 dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis,MO,USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz,CA,USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics (ampicillin and streptomycin) were obtained from Invitrogen Life Technologies (Carlsbad,CA, USA).

Cell culture conditions

Colon cancer cell line HCT-15 was procured from Cancer Research Institute of Beijing, China, and was maintained in DMEM supplemented with 10% FBS and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in an incubator at 37°C (5% CO_2 and 95% air).

Determination of IC_{50} by MTT assay

The antiproliferative effect of the methanolic extract on cancer cell line HCT-15 was evaluated by MTT assay. HCT-15 cells were grown at $1x10^6$ cells per well in 96-well plates for 12 hrs and then exposed to 0, 10, 25, 50, 100, 150 and 200 µg/ml methanolic extract dose for 48 hrs. To each well, MTT solution (20 µl) was added. Prior to the addition of 500 µl of DMSO, the medium was completely removed. To solubilize MTT formazan crystals, 500 µl DMSO was added. ELISA plate reader was used for the determination of optical density.

Clonogenic assay

Exponentially growing HCT-15 cancer cells were collected and calculated with a hemocytometer. Platting of the cells was done at 200 cells per well and kept for 48 hrs to allow the cells to adhere, and then different doses (0, 25, 50 and 100 µg/ml) of the methanolic extract were added. After treatment, the cells were again kept for incubation for 6 days, washing was done with PBS and methanol was used to fix colonies. Afterwards, colonies were stained with crystal violet for about 30 min before being counted under light microscope.

Transfection with LC3-mCherry expression vector

HCT-15 cells were seeded at a density of 3.75×10^4 cells/well in 24-well plates and permitted to adhere for 24 hrs. Transfection with the LC3-mCherry vector was done using Lipofectamine (Invitrogen) according to manufacturer's instructions. HCT-15 cells were incubated with media having 5% FBS for the first 4 hrs of transfection; afterwards, incubation media were replaced by new media with 10% FBS. After 24 hrs of transfection, cells were collected and exposed for 48 hrs to (i) blank medium only and (ii) methanolic extract of *A. vulgaris* (at IC_{50} concentration). Afterwards, the cells were treated with 4% paraformaldehyde and then fixed in PBS. The cells were then finally examined under a fluorescence microscope.

Determination of apoptosis-related proteins expression

HCT-15 cells were seeded in 6-well plates at the density of 1.5×10^5 cells/well and incubated at 37°C for 24 hrs. The cells were then exposed to the methanolic extract of A.vulgaris at its IC_{50} concentration. Untreated cells were also included as control. Following 48 hrs of treatment, cells were collected and lysed for quantification of proteins and expression analysis.

Autophagy inhibitor treatment

HCT-15 cells were seeded at the density of 1.5×10^5 cells/well in 6-well plates and permitted to adhere for 24 hrs. Cells were then exposed to 15 µg/mL of the lysosomal inhibitors E-64d and Pepstatin A for 1 hr and then administered IC_{50} concentration of the extract for 48 hrs. Untreated cells were kept as control. Protein expression analysis was carried out by Western blot analysis.

Protein expression by western blotting analysis

The methanolic extract administered cells were harvested and lysed. The protein concentrations of the lysates were quantified by BCA assay using specific antibodies. β-actin was used as control. From each sample

equal amounts of protein were loaded and separated by electrophoresis on a 12% denaturing SDS gel. Afterwards, the proteins were electroblotted on polyvinylidene difluoride membranes (0.45 µm pore size).

Evaluation of ROS and MMP

HCT-15 cells were platted at a density of 2×10^5 cells/well in a 6-well plate, kept for 24 hrs and treated with 0, 25, 50 and 1000 µg/ml methanolic extract for 72 hrs at 37°C in 5% CO_2 and 95% air. Thereafter, cells from all samples were collected, washed twice by PBS and re-suspended in 500 µl of DCFH-DA (10 µM) for ROS estimation and DiOC6 (1 µmol/l) for MMP at 37°C in the dark for 30 min. The samples were then examined instantly using flow cytometer as previously described [9].

Wound healing assay

HCT-15 cells were seeded at 5×10^4 cell density in 96-well plates and then allowed overnight to adhere. As the cells reached 80% confluence, a wound was scratched across each well by wound maker device. Afterwards, the cells were washed with PBS to remove the detached cells.

Statistics

All experiments were carried out in triplicate and presented as representative images or average values ± SD. One-way ANOVA and Tukey's test were used for statistical analyses with the help of Graphpad prism 7. P values were considered significant at $p<0.01$, $p<0.001$ and $p<0.0001$.

Results

Cytotoxic potential of methanolic extract of A.vulgaris on HCT-15 cell line

The growth inhibitory role of methanolic extract on HCT-15 cells was detected by treatment of these cells with varied methanolic extract concentrations. Methanolic extract displayed potent antiproliferative effect against HCT-15 cells with an IC_{50} 50 µg/ml (Figure 1a). In the colony formation assay, we observed that methanolic extract treated cells showed reduced number of colony formation in a dose-dependent manner (Figure 1b).

A. vulgaris extract induced autophagy in HCT-15 cell line

After transfecting AGS cells with mCherry-LC3 expression vector, they were administered IC_{50} concentrations of the methanolic extract for 48 hrs. The autophagy experiment was carried out because the extract inhibited the growth of cells and triggered cell death. Therefore, we speculated that the extract might be triggering autophagy in HCT-15 cells in a concentration-dependent manner. The results of the study indicate that the

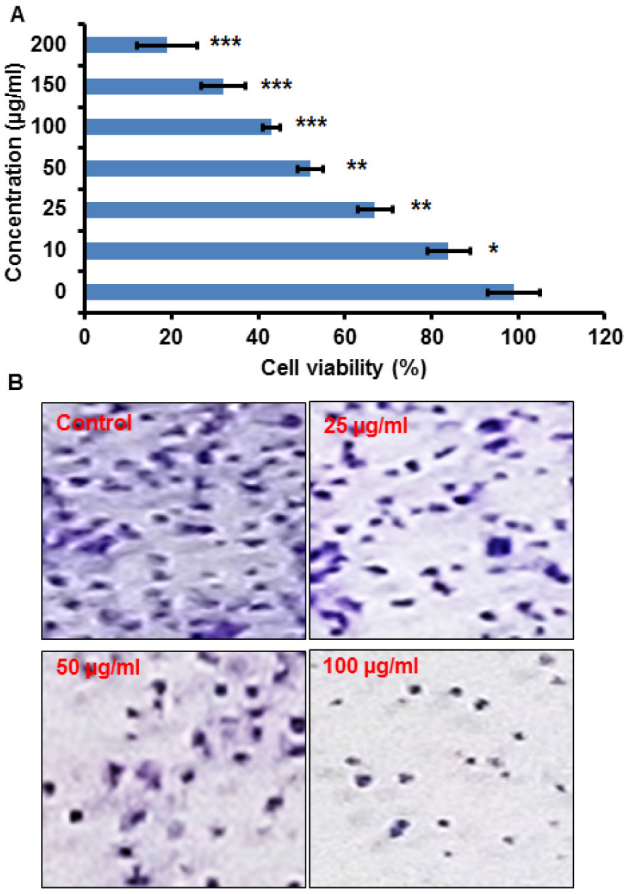

Figure 1. Effect of indicated doses of methanolic extract of *A.vulgaris* on **(A)** cell viability and **(B)** colony formation. All experiments were carried out in triplicate and the results are shown as mean ± SD. The Figure depicts that *A.vulgaris* extract inhibits cell viability and decreases colony formation potential of cancer cells in a concentration-dependent manner. *p<0.01, **p<0.001, ***p<0.0001.

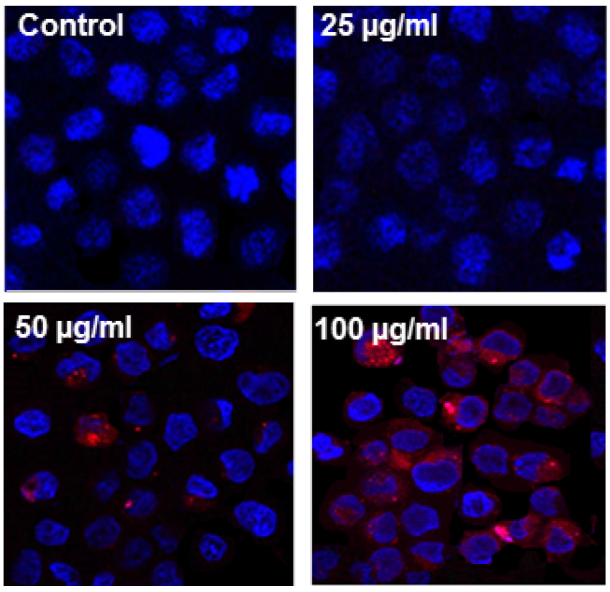

Figure 2. Induction of autophagy by methanolic extract at indicated concentrations. The Figure depicts that *A.vulgaris* extract induces autophagy in a concentration-dependent manner.

treatment with the extract induced the presence of LC3 within autophagic vacuoles (Figure 2). To confirm autophagy, we evaluated the expression of several autophagy-associated proteins. The results indicated that the treatment with the extract induced the expression of several autophagy-associated proteins as indicated in Figure 3. No change was observed in the expression of several proteins including Vps34, Beclin-1, and LC3-I. However expression of LC3-II was significantly increased in a time-dependent manner, while slight reduction in the levels of p62 were also observed. The capacity of the methanolic extract of A. vulagris was further confirmed by the use of autophagy inhibitors (E-64d/Pepstatin), however, the results indicated that the extract abridged the effect of the inhibitors (Figure 4).

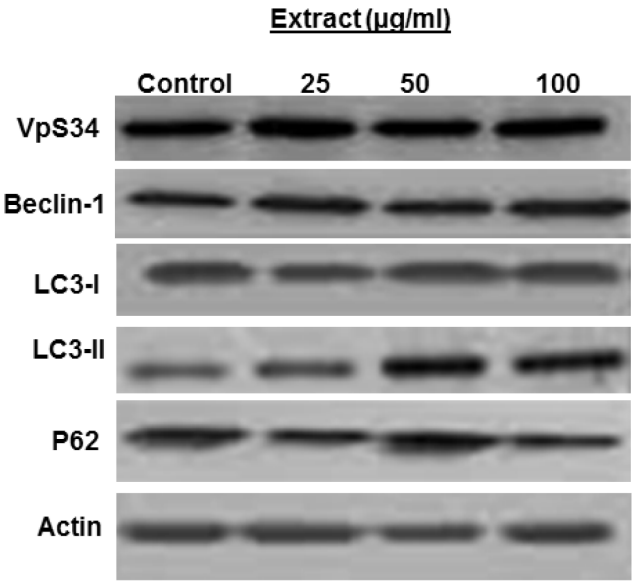

Figure 3. Expression pattern of autophagy-associated proteins by western blotting. The Figure shows that *A.vulgaris* extract alters the expression of autophagy-related proteins in a concentration-dependent manner.

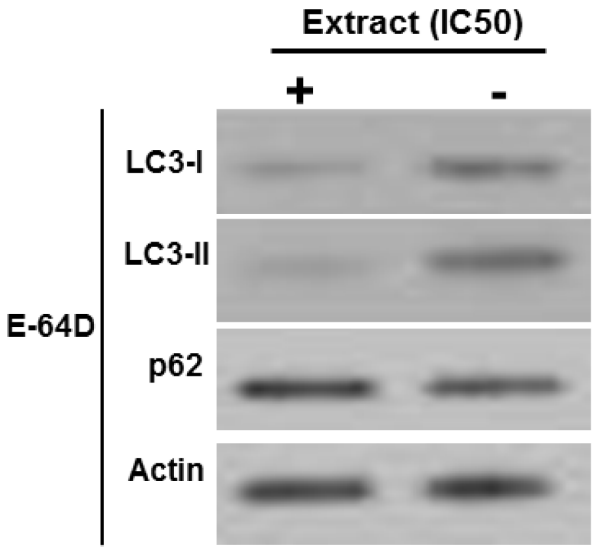

Figure 4. Methanolic extract of *A.vulgaris* abolishes the effect of autophagy inhibitors at IC_{50} concentrations. The Figure shows that A.vulgaris extract nullifies the effect of autophagy inhibitors on the expression of LC3-I and LC3-II at IC_{50}.

Methanolic extract of A. vulgaris induced ROS accretions in HCT-15 cells

The autophagic potential of methanolic extracts suggested that it might induce generation of intracellular ROS. Therefore, we calculated the ROS level at varied concentrations of methanolic extract for 48 hrs and observed that the intracellular ROS levels of treated cells increased to 225 % at 80 µM as compared to untreated cells (Figure 5a). Our results suggested that the methanolic extract can act as a potent source for activating ROS in HCT-15 cells to trigger autophagy.

Methanolic extract reduced the mitochondrial membrane potential

ROS generation causes mitochondrial dysfunction. It disrupts the outer mitochondrial membrane potential to release the death-promoting proteins . Therefore, we examined whether

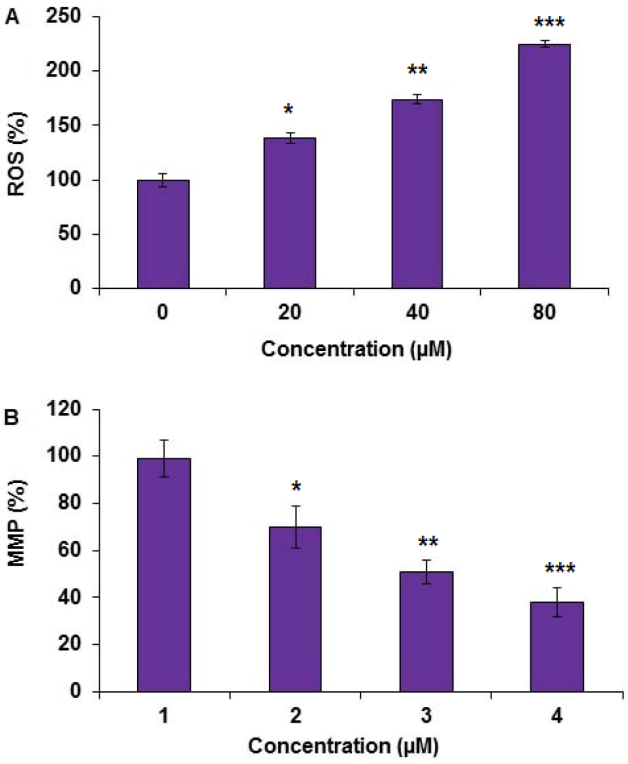

Figure 5. Effect of indicated concentrations of methanolic extract of *A.vulgaris* on **(A)** ROS and **(B)** MMP. The Figure shows that *A.vulgaris* extract increases ROS and decreases MMP in a concentration-dependent manner. The values were considered significant at *p<0.01, **p<0.001 and ***p<0.0001.

methanolic extract reduced the MMP in HCT-15cells treated with varied concentrations (0, 25, 50 and 100 µg/ml). Treated HCT-15 cells showed a significant reduction in MMP in a dose-dependent manner. The MMP reduced by 72% at 100µg/ml of methanolic extract as compared to untreated control (Figure 5b).

Methanolic extract affected cell migration in wound healing assay

We further investigated that if the methanolic extract of *A. vulgaris* can suppress the migration of HCT-15 cancer cells at the IC_{50} concentration by wound healing assay. The results of wound healing assay showed that the methanolic extract reduced the migratory capability of wound healing assay cells (Figure 6), while the control cells showed fairly good capacity to migrate. The treated cells showed less migration as depicted in Figure 6.

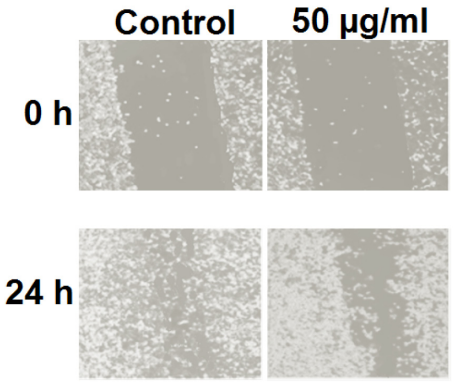

Figure 6. Wound healing assay depicting the effect of methanolic extract on cell migration (IC_{50}=50µg/ml). The Figure shows that *A.vulgaris* extract inhibits cell migration at IC_{50} after 24 hrs of incubation.

Discussion

Colon cancer is one of the leading causes of cancer related deaths across the globe and the treatment options for this type of malignancy are limited. Moreover, the currently available treatments have severe side effects and severely affect the patient quality of life [4,5]. Therefore, the present study aimed at determining the anticancer activity of the methanolic extract of *A. vulgaris* against colon cancer cells. The results indicated that the methanolic extract exerted significant anticancer activity against colon cancer cells in a dose-dependent manner with an IC_{50} of 50 µg/ml. Furthermore, it also reduced the colony formation of HCT-15 cells dose-dependably. The IC_{50} value of *A. vulgaris* is much lower than the IC_{50} value observed for several plant extracts. For instance, IC_{50} value of *Moringa oleifera* leaf water extract was 150 µg/ml (48 h) against HeLa derivative KB cells [10,11]. Similarly, *Cassia tora* methanolic leaf extract exhibited an IC_{50} value of 191 µg/ml against HeLa cells [12]. Therefore, these results suggest that the methanolic extract of A.vulgaris is a potential source of cytotoxic agents. The cytotoxic effect of methanolic A.vulgaris was reported later on to be due to the induction of autophagy. Expression of several of the autophagy associated proteins was evaluated and was found that the expression of only LC3-II was highly induced by the methanolic extract of A.vulgaris. Furthermore, the extract exhibited a strong potential to abridge the expression of autophagy inhibitors, providing a strong clue towards the role of this extract in the execution of autophagy.

Moreover, the results indicated that methanolic extract-treated cells displayed ROS-mediated MMP reduction. Therefore, the results suggest that the methanolic extract of A.vulgaris may induce autophagy through increasing intracellular ROS and reduction in MMP. Our results are in agreement with studies wherein several anticancer drugs have been reported to target cancer cells partly by accretion of high levels of ROS [13]. In addition, mitochondria play a key role in ROS generation [14-16]. For example, capsaicin disrupts MMP and mediates oxidative stress resulting in apoptosis in pancreatic cancer cells [9]. Colon cancer cells have very high capacity to migrate to other tissues and in our case the methanolic extract exhibited the capacity to inhibit the migration of such cells. Therefore, the inhibitory effect of methanolic extraction on colon cancer cells may prove crucial in the treatment and management of colon cancer.

Conclusion

Taken together, we conclude that the methanolic extract exhibits significant anticancer activity against colon cancer cells by inducing autophagy and inhibiting cell migration which is considered critical for anticancer agents. Thus, the methanolic extract may prove a very handy source for the isolation of anticancer molecules against colon cancer and therefore requires further research endeavors.

Conflict of interests

The authors declare no conflict of interests.

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