**Acalypha australis** L. extract inhibits B16 melanoma cell metastasis through PI3K/AKT signaling pathway

Zhi-Zhong Wang, Tie-Shan Yi, Yu-Yang He, Qin Zhou, Bo Chen

1 College of Biology and Food Engineering, Huaihua University, Huaihua 418008, China. 2 Key Laboratory of Research and Utilization of Ethnomedicinal Plant Resources of Hunan Province, College of Biological and Food Engineering, Huaihua University, Huaihua 418008, China.

*These authors contributed equally to this work and are co-first authors.

**Corresponding to:** Bo Chen, College of Biology and Food Engineering, Huaihua University, No. 180 Huaidong Road, Hecheng District, Huaihua 418008, China. E-mail: hhuchenbo@163.com.

**Abstract**

**Background:** Melanoma is a deadly skin tumor resulting from the malignant transformation of melanocytes. It is highly malignant and invasive, with the highest mortality rate among skin cancers. *Acalypha australis* L. (AAL), a plant with dual medicinal and culinary purposes, is commonly regarded as an edible wild vegetable in southern China. Additionally, AAL has a long history of medicinal use in China, often employed for its hemostatic, anti-diarrheal, and anti-inflammatory properties. Modern pharmacology has demonstrated that AAL possesses functions such as weight loss, antimicrobial activity, antiviral effects, and treatment for ulcerative colitis. However, there is currently no research available regarding its effectiveness and mechanisms of action on melanoma.

**Methods:** In this investigation, we used methyl thiazolyl tetrazolium assay to detect cell viability, transwell assay to detect cell migration and invasion ability, and Western blot assay to detect relevant signaling pathways. **Results:** The present study reveals that 2 mg/mL AAL effectively suppresses the metastasis of B16 cells, while simultaneously triggering the expression of key apoptosis-related proteins, including Bcl-2, Bax, and cleaved caspased 3. Subsequent investigations demonstrate that AAL exerts this inhibitory effect via the PI3K/AKT signal transduction pathway, as evidenced by the observed deficits in Ras, AKT, p-AKT, and PI3K expression levels.

**Conclusion:** These findings indicated that AAL could be a valuable therapeutic option for reducing the metastatic potential of B16 melanoma cells.

**Keywords:** Acalypha australis L; melanoma; PI3K/AKT pathway
Introduction

Nature has provided mankind with a range of compounds that can help alleviate diseases. As science has progressed, more and more natural products with medicinal properties have been discovered [1, 2]. Plants and microorganisms have been the primary sources of these drugs [3, 4]. Acalypha australis L. (AAL) belongs to the Euphorbiaceae family and the genus Acalypha. It is an annual herbaceous plant. The whole plant or aerial parts of AAL are used in traditional Chinese medicine (TCM). It is believed to have the effects of clearing heat, detoxification, promoting diuresis, resolving food stagnation, and astringency for stopping bleeding. It is commonly used for conditions such as enteritis, external hemorrhage, eczema, dermatitis, and snake bites. It serves as the main ingredient for Xiancai Huanglian capsules, a class-three new drug in China. Modern pharmacological studies have found that AAL has multiple benefits. Firstly, the methanol extract of AAL can regulate the inflammatory response of RAW 264.7 macrophages stimulated by lipopolysaccharide and lipopolysaccharide-induced septic mice by inhibiting the activation of NF-κB [5]. Secondly, AAL significantly inhibits body weight gain in obese mice, reduces serum cholesterol, triglycerides, and low-density lipoprotein cholesterol levels, and increases the level of high-density lipoprotein cholesterol. AAL also significantly improves lipid accumulation in the liver and subcutaneous adipose tissue [6]. Additionally, AAL exerts a protective effect against dextran sulfate sodium-induced ulcerative colitis in mice by inhibiting the expression levels of NF-κB pathway-related proteins and reducing the release of inflammatory factors [7]. AAL possesses various medicinal effects in TCM. However, it is necessary to utilize modern scientific technologies to prove its target and mechanism of action, providing scientific evidence for further utilization of AAL. Therefore, further exploration is needed regarding the medicinal effects and mechanisms of action of AAL.

Melanoma is an extremely dangerous form of skin cancer, responsible for a significant number of fatalities. The World Health Organization reports that approximately 132,000 new cases of melanoma are diagnosed each year, and this number is on the rise globally [8]. While melanoma only represents 1% of all skin cancer cases, it is the most lethal form due to its highly malignant and aggressive nature [9]. Over the past decade, advancements in early diagnosis, screening, and surgical treatments, along with targeted therapies and immunotherapies, have significantly decreased the death rate from melanoma. Despite advancements in treatment, the five-year survival rate for patients diagnosed with metastatic melanoma remains alarmingly low at only 16% [10, 11]. In China, melanoma patients are often diagnosed in the middle and late stages, leading to a short average survival time [12].

Currently, the primary clinical treatment for melanoma is local surgery. Other treatment options include radiotherapy, chemotherapy, targeting, and immunotherapy. However, these treatments have limitations, such as drug resistance and toxic reactions in radiotherapy and chemotherapy. Resistance to targeted drugs and immunotherapy, as well as the high cost of treatment, are limiting factors that hinder the improvement of efficacy to some extent [13]. Hence, it is imperative to create novel chemical structures to treat melanoma and comprehend their modes of operation. Our study evaluate the impact of AAL on B16 melanoma cells and elucidate its mechanism of action. Our findings indicate that AAL effectively suppressed the growth and migration of B16 cells with an IC₅₀ of 1.42 mg/mL. The anticancer properties of AAL are attributed to its ability to induce apoptosis. In order to explore the potential of AAL as an anti-metastasis agent, we conducted experiments on cell migration and invasion. Our study demonstrated that AAL has a significant inhibitory effect on the migration and invasion of B16 cells. This effect is achieved by targeting the PI3K/AKT pathway. Our findings suggest that AAL has the potential to be a promising treatment for metastatic melanoma.

Materials and methods

Antibodies and reagents

The antibodies used in this study were obtained from Abcam (Cambridge, UK). Specifically, antibodies for Bcl-2, Bax, cleaved caspase-3, Ras, AKT, p-AKT, PI3K, and glyceraldehyde-3-phosphate dehydrogenase were acquired. Fetal bovine serum was obtained from Gibco (Gaithersburg, MD, USA), while Roswell Park Memorial Institute-1640 and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma-Aldrich (Burlington, MA, USA).

AAL sample preparation

The AAL extract sample was prepared using TCM decoction method. The decoction solution was concentrated by rotary evaporator and freeze-dried, and the resulting powder was dissolved in dimethyl sulfoxide. The solution was then prepared as a 1 g/mL stock concentration, and diluted to the target concentration. Finally, the solution was filtered through a 0.22 mm filter membrane before conducting the experiment.

Cell culture

The B16 cells utilized in this study were procured from American Type Culture Collection (Rockville, MD, USA) and cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum and 1% (w/v) penicillin (Sangon, Shanghai, China). The cells were incubated at 37°C in a CO₂ incubator (5% CO₂, 95% humidity) for 24 hours before being subjected to varying concentrations of AAL (1, 5, or 10 mg/mL) and collected at specific time intervals for further analysis.

B16 cell viability and colony-formation assay

Cell viability assay MTT was performed to detect cell viability of B16 cells. Briefly, 96-well plates were used for seeding, at a concentration of 2×10⁴ cells per well. After culturing at 37°C for 24 h, these cells were subjected to treatment with the AAL at varying concentrations for 48 h. 10 mL of MTT were added to each well, and the plate was further incubated for 1 h at 37°C, and then absorbance at 450 nm was recorded. Clonal formation experiment: after digestion of pancreatic enzymes of cells in logarithmic growth phase, the cell blast suspension was re-suspended and counted; each experimental group was inoculated with 500 cells/hole in 6-well plates. The culture was continued until 14 days, during which the fluid was changed every 3 days and the cell state was observed. After cloning, the cells were added with different concentrations of AAL. After 48 h, the cells were photographed under the microscope, and then washed with phosphate-buffered saline (PBS) once, and 1 mL 4% paraformaldehyde was added to each well for 30–60 min, and washed with PBS once. Add 1 mL crystal violet dye solution to each well and stain cells for 10–20 min. PBS washed the cells several times, dried them, and photographed them.

Cell migration and invasion assays

The cell migration test was performed using a 24-well transwell chamber. Cells were added to the upper cavity in a serum-free medium (2×10⁵ cells/pore). After 18 hours, the number of cells that migrated through the membrane to the lower lumen was counted. To perform the invasion assay, Matrigel was added to the transwell membrane chamber, incubated for 24 hours, and then seeded with cells. After 24 hours, the number of cells migrating through the membrane was counted. Migration and invasion tests in triplicate. The migrations and invasions of B16 cells in 5 random fields were counted and expressed as relative cell numbers. We then conducted cell migration and invasion assays using previously described methods [14].

Western blot analysis

Utilized primary antibodies for Bcl-2, Bax, cleaved caspase-3, Ras, AKT, p-AKT, PI3K, and glyceraldehyde-3-phosphate dehydrogenase at a 1:1000 dilution. To perform statistical analysis, we used Quality-One software (BioRad Laboratories, Richmond, CA, USA) to analyze the
detected bands via densitometry.

**Statistics**
The experiments were performed in 5 data was shown as the mean ± standard deviation and percentages (%). Student’s t-test were used to determine major differences among the groups. A P value < 0.05 was taken as statistically significant.

**Results**

**Effect of AAL on B16 cell viability**

In order to examine the impact of AAL on the viability of B16 cells, we performed an MTT assay on B16 cells that had been exposed to different concentrations of AAL (1, 5, 10 mg/mL). Our results showed that AAL decreased B16 cell viability in a dose-dependent manner. Figure 1A and 1B illustrated that the IC₅₀ of AAL towards B16 cells was 1.42 mg/mL. Additionally, AAL exhibited a selectivity greater than nine times towards normal HEK293 cells and human skin fibroblasts (Figure 1C and 1D). In order to further evaluate the impact of AAL on B16 cell proliferation, the colony formation assay was employed (Figure 2A and 2B). The findings of this study suggested that the rate of colony formation of B16 cells decreases in a dose-dependent manner as the concentration of AAL increases (1, 5, 10 mg/mL).

**AAL inhibited migration and invasion of B16 cells**

The migration ability of tumor cells is a critical factor in the process of tumor metastasis. In this study, we investigated the effect of AAL on the migration ability of B16 cells through a wound healing test. Our results showed that compared to the control group, the migration rate of B16 cells was significantly reduced after treatment with 5 mg/mL and 10 mg/mL AAL (Figure 3A and 3B). Specifically, 5 mg/mL and 10 mg/mL AAL inhibited cell migration by 65.8% and 82.3%, respectively (Figure 4A and 4B). In relation to the invasion assay, it was observed that the invasive capacity of B16 cells was significantly reduced by 60.2% and 78.6% when treated with 5 mg/mL and 10 mg/mL AAL respectively (Figure 4C and 4D). These findings suggested that AAL has the potential to regulate the metastatic activity of B16 cells.

**AAL triggers apoptosis in B16 cells**

AAL induces apoptosis in B16 cells through the activation of the intrinsic apoptotic pathway. Specifically, AAL increases the expression of pro-apoptotic proteins such as Bax, while decreasing the expression of anti-apoptotic proteins like Bcl-2. Additionally, the cleavage of caspase 3 indicates the activation of the caspase cascade, a key event in the apoptotic process. Overall, these findings provided insight into the mechanism of action of AAL in inducing apoptosis in B16 cells (Figure 5).

**AAL inhibits activation of the PI3K/AKT signaling pathway**

In cancer cells, various signaling pathways are activated, and these pathways are crucial targets for anticancer drugs. The PI3K/AKT pathway is a crucial pathway that is closely associated with the cell cycle and plays a vital role in regulating cell ability. To explore this further, we examined the effects of AAL on the PI3K/AKT pathway. Our findings revealed that 2 mg/mL AAL significantly reduced the expression of Ras, AKT, p-AKT, and PI3K proteins (Figure 6), suggesting that AAL may inhibit the PI3K/AKT signaling pathway.

**Figure 1 The anti-melanoma bioactivity of AAL.** (A & B) The cytotoxicity profile of AAL on B16 cells. (C) The cytotoxicity profile on HEK293 cells. (D) The cytotoxicity profile on HFF-1 cells. Each concentration was tested five times for accuracy. AAL, Acalypha australis L.

**Figure 2 The crystal violet staining method was used to count and analyze the number of colonies formed.** (A) The clonogenic assay was used to evaluate the ability of B16 cells to form colonies after treatment with varying concentrations of AAL. (B) Quantitative results are illustrated for clonogenic assay. Data represent the mean ± standard deviation, n = 5. **P < 0.001 vs. Control; ***P < 0.001 vs. Control. AAL, Acalypha australis L.
Figure 3 The effects of AAL in different concentrations on B16 cell migration ability. (A) Representative images from the wound healing assay. (B) Migration rates calculated by measuring the distance of the gap at different concentrations. ***P < 0.001 vs. Control. AAL, Acalypha australis L.; NS, no significance.

Figure 4 Effect of AAL on the migration and invasion of B16 cells. (A & C) Representative fields of migration and invasion cells on the membrane. (B & D) Average migration and invasion cell number per field. Migration and invasiveness were determined by counting cells in eight randomly selected microscopic fields per well. Data represent the mean ± standard deviation, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group. AAL, Acalypha australis L.

Figure 5 Effect of AAL on the expression of proteins involved in the apoptosis of B16 cells. (A) B16 cells were treated with AAL for 24 h and then subjected to Western blot analysis using antibodies against various known apoptotic factors. GAPDH was used as the loading control. Data represent the mean ± standard deviation, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group. AAL, Acalypha australis L.; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Discussion

As the incidence of melanoma continues to rise, the treatment of this disease is becoming increasingly challenging. While target drugs and immune drugs have been introduced to the market and have provided significant assistance in treating melanoma, issues such as drug resistance, high cost, and patient sensitivity have become more apparent [13]. Therefore, there is an urgent need for the development of new melanoma drugs to address these challenges. TCM believes that AAL possesses various medicinal effects and has a history of thousands of years of medicinal use in China. Recent pharmacological studies have shown that AAL has anti-inflammatory, antiviral, and other effects.

Inhibiting cell growth is a crucial aspect of inducing cell death through anticancer drugs. Additionally, apoptosis is a significant approach for combating cancer as it can also aid in inhibiting cell growth in cancer cells. As the deregulation of apoptosis is a defining characteristic of all cancer cells, inducing apoptosis has been recognized as a standard and highly effective strategy in anticancer therapy [15]. Therefore, we investigated the potential of AAL to inhibit cell proliferation and induce apoptosis in B16 cells. Our findings from the MTT and wound healing assays revealed that AAL effectively inhibited the growth and migration of B16 cells in a dose-dependent manner. These results provide evidence that AAL induces apoptosis as a mechanism for destroying B16 cells.

The intrinsic pathway of apoptosis is controlled by various members of the Bcl-2 protein family and inhibitors of apoptosis [16]. Among these, Bcl-2 is a crucial anti-apoptotic protein that inhibits apoptosis by obstructing the activation of the inner mitochondrial permeability transition pore [17]. This obstruction ultimately prevents the release of pro-apoptotic mitochondrial contents, such as cytochrome c [18]. In our study, we discovered that treating B16 cells with AAL resulted in a decrease in the levels of Bcl-2. Furthermore, AAL was found to increase the activation of Bax and cleaved caspase-3 in B16 cells, indicating the occurrence of apoptosis. These findings indicate that AAL-induced apoptosis in B16 cells is likely caused by changes in the levels of anti-apoptotic proteins, as well as the activation of Bax and caspases-3. This mechanism may also play a role in AAL-induced apoptosis in B16 cells. It is worth noting that the PI3K/AKT pathway, which is a key regulator in cell proliferation, growth, and apoptosis, may also be involved in this process [19]. Therefore, one of the primary strategies for cancer chemotherapy involves inhibiting the PI3K/AKT pathway [20, 21]. With this in mind, we conducted research to examine the impact of AAL on this pathway in B16 cells. Our study found that AAL treatment resulted in a reduction of Ras, AKT, p-AKT, and PI3K expression in B16 cell lines. These findings indicate that AAL not only inhibits cell migration and invasion but also regulates apoptosis via the PI3K/AKT pathway.

In summary, our study has demonstrated that AAL has the ability to inhibit the PI3K/AKT pathway, resulting in potent anticancer effects against B16 cells. This is achieved through the induction of apoptosis, as well as the inhibition of cell migration and invasion. These results strongly suggest that AAL has great potential as a bioactive phytochemical for the treatment of melanoma.

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