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Original Article

The antitumor effects and mechanisms of the novel compound Alocasia B against gastric cancer cells

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Abstract: Purpose: *Alocasia cucullata* (Lour.) G. Don is an antitumor herb, and Alocasia B is a novel molecule from it, indicating signs of antitumor activity. In this study, Alocasia B was analyzed to evaluate its effects and mechanisms against gastric cancer and was identified as the key chemical component of *A. cucullata*. Methods: A cell line panel with 12 gastric cancer cells was screened to determine the cytotoxic properties of Alocasia B using an MTT assay. MGC-803 was selected for further evaluation of the cell cycle and apoptosis by flow cytometry. The protein expressions of p-Akt, Akt, Bax, Bcl-2, p-p53, p53 and caspase-3 were estimated by western blotting. Results: Alocasia B was found to be cytotoxic towards 12 gastric cancer cell lines presenting IC50 values from 12.8 to 93.4 μg/ml. MGC-803 was the most sensitive cell line and was inhibited by Alocasia B in both dose- and time-dependent manners. Apoptosis and G0/G1 phase arrest were induced by Alocasia B. Alocasia B could trigger multiple signaling pathways, including the upregulation of Bax, p-p53, caspase-3 and the downregulation of p-Akt, Bcl-2. In an *in vivo* study, Alocasia B also significantly impeded tumor growth in the xenograft model. Conclusion: Taken together, Alocasia B exhibited cytotoxic activity against gastric cancer both *in vitro* and *in vivo* through multiple cancer-related signaling pathways. This study provides evidence that Alocasia B is a chemotherapeutic candidate for antitumor agent development.

Keywords: Tumor, *Alocasia cucullata* (Lour.) G. Don, IC50, p53, Bcl-2, Akt

Introduction

Alocasia cucullata (Lour.) G. Don (Chinese taro), belonging to Araceae family, is a kind of herbal medicine [1]. It's recorded that *A. cucullata* has the effects of clearing heat, detoxicating, detumescence, and analgesia in several Chinese herbal medicine works, such as *Compendium of Materia Medica*, *Dictionary of Medicinal Plants,* and *National Chinese Herbal Medicine* [2]. It's widely distributed, easy to obtain, and has been a well-known ethnic medicine of the Zhuang nationality with a more than two-thousand-year history. Our previous study [3] has shown that the extract of *A. cucullata* has antitumor effects and can induce apoptosis on human gastric cancer cells. However, the critical chemicals from *A. cucullata* are still unknown.

Various studies have shown that apoptosis is correlated to the regulation of many signaling pathways and apoptosis-related proteins. The Akt signaling pathway, p53, and mitochondrial-associated apoptosis proteins play important roles in apoptosis and tumor development [4]. Akt can inhibit apoptosis in a variety of ways. The reduction of p53, a tumor suppressor gene, can lead to a failure to suppress tumor growth. Also, the mitochondria-related apoptosis process participates in inducing apoptosis. In the previous study [3], it was demonstrated that the extract of *A. cucullata* could decrease Akt, Erk and Bcl-2 expression, as well as induce caspase 3/7 activity. This paper aims to identify the novel compound Alocasia B from *A. cucullata* as the critical chemical attributing to tumor inhibition, and also to explore the underlying mechanisms of this compound.

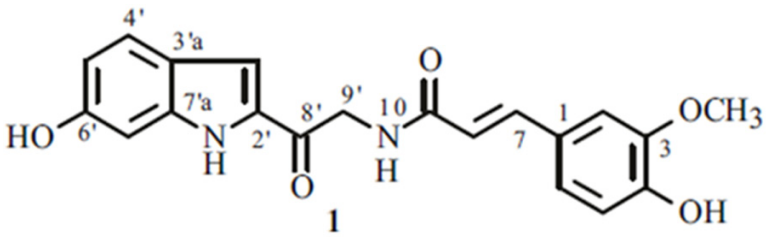

Figure 1. Two-dimensional representation of Alocasin B.

Materials and methods

Cell lines and animals

Human gastric cancer cell lines, MKN-45, AGS, Hs 746T, NCI-N87, KATO III, SNU-1, SNU-5, SNU-16, BGC-823, MGC80-3, SGC-7901 and HGC-27 were purchased from the American Type Culture Collection (ATCC, USA) and cultured in DMEM medium (Hyclone, USA) and supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO_2. 6-week old Balb/c nude mice were purchased from Shanghai SIPPR-B&K Laboratory Animal Corp., Ltd. (Shanghai, China) and maintained in pathogen-free conditions. All animal assays followed the guidelines of the Laboratory Animal Center of Chinese Academy of Science for the use of animal subjects.

Isolation and purification of Alocasia B

Alocasia B was purified according to the steps described in a previous study [5]. Briefly, small pieces of *A. cucullatai* (5 kg) were extracted three times with 50% EtOH, followed by vacuum freeze drying to obtain crude residue (200 g). The crude residue was partitioned with n-butanol. Then n-butanol fraction was eluted through column chromatography on silica gel and separated into four fractions. Fraction C was rechromatographed on octadecylsilane into six fractions. Then fraction C-3 was selected for chromatograph on Sephadex LH-20 to get Alocasia B, which is a yellow powder. The molecular weight of Alocasia B was detected as 364 Da (**Figure 1**).

MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

2000 gastric cancer cells were seeded into each well of a 96-well plate and treated for serials dosage of Alocasia B on the next day. After the indicated duration, 10 μl of MTT solution (Sigma-Aldrich, USA) were added and incubated for 1.5 hours. After dropping the supernatant, MTT was solubilized using 100 μl of DMSO and read at 595 nm.

Western blot

Alocasin B was added to MGC803 cells and incubated for 72 h. Total proteins were extracted in an ice-cold RIPA lysis buffer (Beyotime, China) containing a 1% protease inhibitor cocktail and a phosphatase inhibitor cocktail (Selleck, China). The samples were centrifuged at 12,000 rpm for 20 min at 4°C. The concentrations of total protein were measured by a BCA assay kit (Beyotime). Then an equal mass of protein lysis was loaded onto SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). Then the membrane was blocked by 5% non-fat milk in a TBST buffer and incubated with primary specific antibodies overnight at 4°C, followed by secondary indicated antibodies conjugated with HRP for 2 h incubation at room temperature. Then a substrate reagent was added onto the membrane and incubated for 30 min, then it was stopped by stop buffer (Millipore). Primary antibodies against the following proteins were used: p-Akt (Ser473) (#4060, Cell Signaling), Akt (#9272, Cell Signaling), Bcl-2 (#2872, Cell Signaling), Bax (#2774, Cell Signaling), p-p53 (Ser15) (#9286, Cell Signaling), p53 (#9282, Cell Signaling) and cleaved Caspase-3 (#9661, Cell Signaling).

Cell cycle and apoptosis study

For the cell cycle analysis, 2×10^5 cells were seeded into each well of a 6-well plate and treated with the indicated concentration of Alocasin B. After 48 h, the cells were washed with PBS and trypsinized, and then then were pelleted by centrifugation. After being washed with PBS, the cells were resuspended and fixed overnight at -20°C in 70% ethanol. For the cell cycle assay, the cells were stained with a propidium iodide (PI) solution (Lianke Bio, China) and subjected to flow cytometry analysis. For the apoptosis assay, the cells were stained with Annexin V-FITC and PI according to the manufacturer's protocol. The cells were analyzed on a flow cytometer (BD Bioscience, CA, USA).

Table 1. Summary of Alocasia B IC50 values againtst twelve human gastric cancer cells for 72 h with treatment by MTT assays

Human Gastric Cancer Cell Line	IC50 (μg/ml)
MKN-45	19.4±3.4
AGS	20.3±5.1
Hs 746T	17.7±2.2
NCI-N87	23.4±3.9
KATO III	22.1±7.1
SNU-1	80.2±10.1
SNU-5	93.4±9.3
SNU-16	76.2±7.5
BGC-823	19.7±9.5
MGC80-3	12.8±2.1
SGC-7901	20.3±8.2
HGC-27	25.3±9.6

Data were presented as the mean ± SD of three independent experiments.

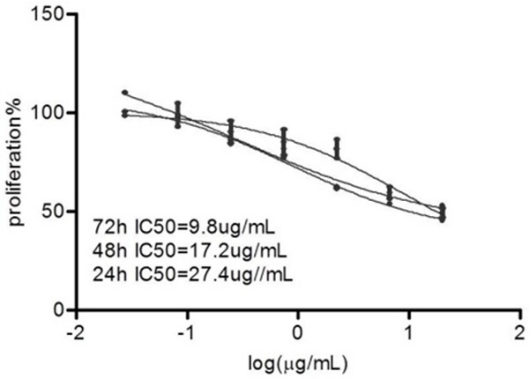

Figure 2. Viability inhibition by Alocasin B in MGC-803 cells. MGC-803 cells were treated with serial dosages of Alocasin B at 24, 48 and 72 h. IC50 was calculated by OD value from MTT. Values were expressed as the mean ± SD.

Anti-tumor activity of Alocasin B in vivo in xenograft mice

Balb/c nude mice were subcutaneously injected in their right axillas with 2×10^6 cells in Matrigel with a 1:1 volume ratio. After about one week, while palpable nodules were achieved, the mice were randomly assigned to four groups and were treated with different agents. PBS and 0.05 g/kg of cyclophosphamide were used to treat subcutaneously into the tumors for the vehicle and positive groups, respectively. 0.05 g/kg and 0.1 g/kg of Alocasin B dissolved in PBS were used to treat subcutaneously into the tumors for the low dose and high dose groups, respectively. All treatments were administered every other day. Then the mice were sacrificed, and the tumors were weighed at 21 days.

Statistical analysis

The data are presented as the means ± SD. The statistical comparisons of the means were performed using a two-tailed, unpaired Student's *t* test. The experiments were repeated at least three times. *P* values less than 0.05 were considered significant.

Results

Alocasia B showed broad-spectrum gastric cancer cell proliferation-suppressive activity

Alocasia B was first used to test its proliferation-suppressing activity on gastric cancer cells with dose-response growth curves at 72 h on a large panel of gastric cancer cells. The half maximal inhibitory concentration (IC50) was obtained against each cell line and the results are listed in **Table 1**. Among all the gastric cell lines, Alocasia B showed the inhibition range from 12.8 μg/ml to 93.4 μg/ml. MGC-803 showed the most sensitivity to Alocasia B, but SNU-1, SNU-5 and SNU-16 showed more resistance. Overall, Alocasia B had the ability to suppress the growth of a wide range of gastric cancer cells.

Furthermore, MGC-803 was used to investigate the anti-proliferative effects and mechanisms of Alocasia B. As shown in **Figure 2**, at 24, 48, and 72 hours, Alocasia B was able to significantly reduce the viability of MGC-803 in a dose-dependent manner, as assessed by MTT. And the IC50 values decreased with a rise in treatment duration, showing a potency of 9.8 μg/ml, 17.2 μg/ml, and 27.4 μg/ml at 24, 48, and 72 h, respectively. The result proved the cytotoxic ability of Alocasia B.

G0/G1 phase cell cycle arrest in Alocasia B-treated MGC-803 cancer cells

Due to the anti-proliferative effect, the inhibition of cell cycle progression was evaluated at different concentrations of Alocasia B. The number of MGC-803 cells at the G0/G1 phase increased with a rise in the concentration of Alocasia B, suggesting the G0/G1 phase arrest occurs in a dose-dependent manner (**Figure 3**). In addition, although a decreasing trend of

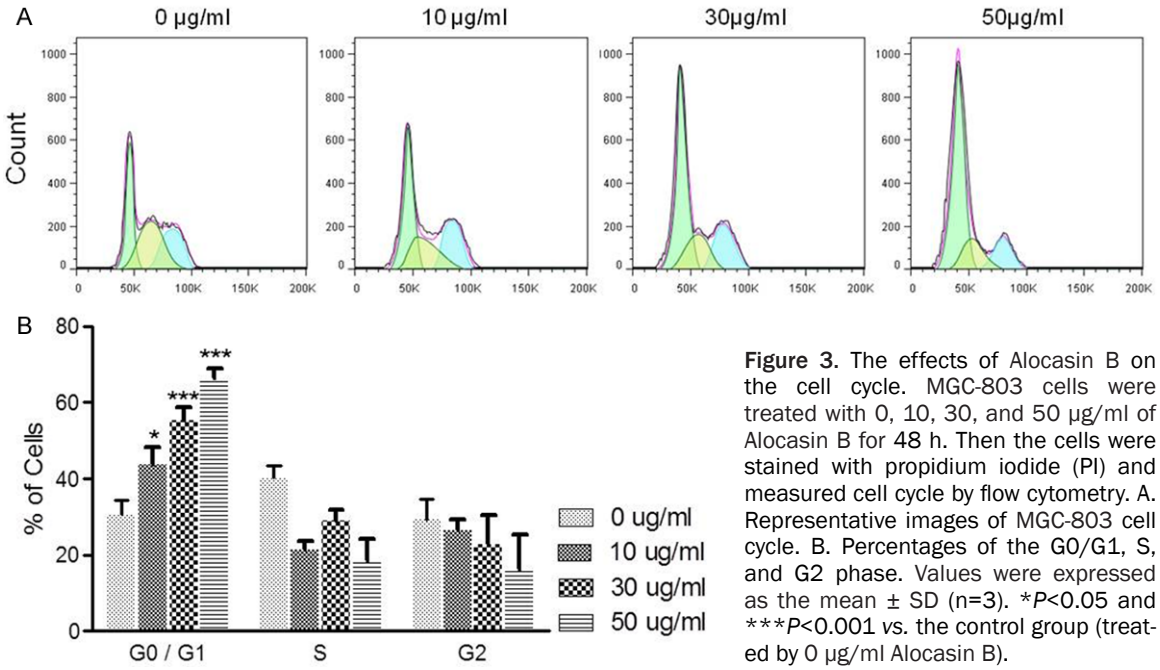

Figure 3. The effects of Alocasin B on the cell cycle. MGC-803 cells were treated with 0, 10, 30, and 50 μg/ml of Alocasin B for 48 h. Then the cells were stained with propidium iodide (PI) and measured cell cycle by flow cytometry. A. Representative images of MGC-803 cell cycle. B. Percentages of the G0/G1, S, and G2 phase. Values were expressed as the mean ± SD (n=3). **P*<0.05 and ****P*<0.001 *vs.* the control group (treated by 0 μg/ml Alocasin B).

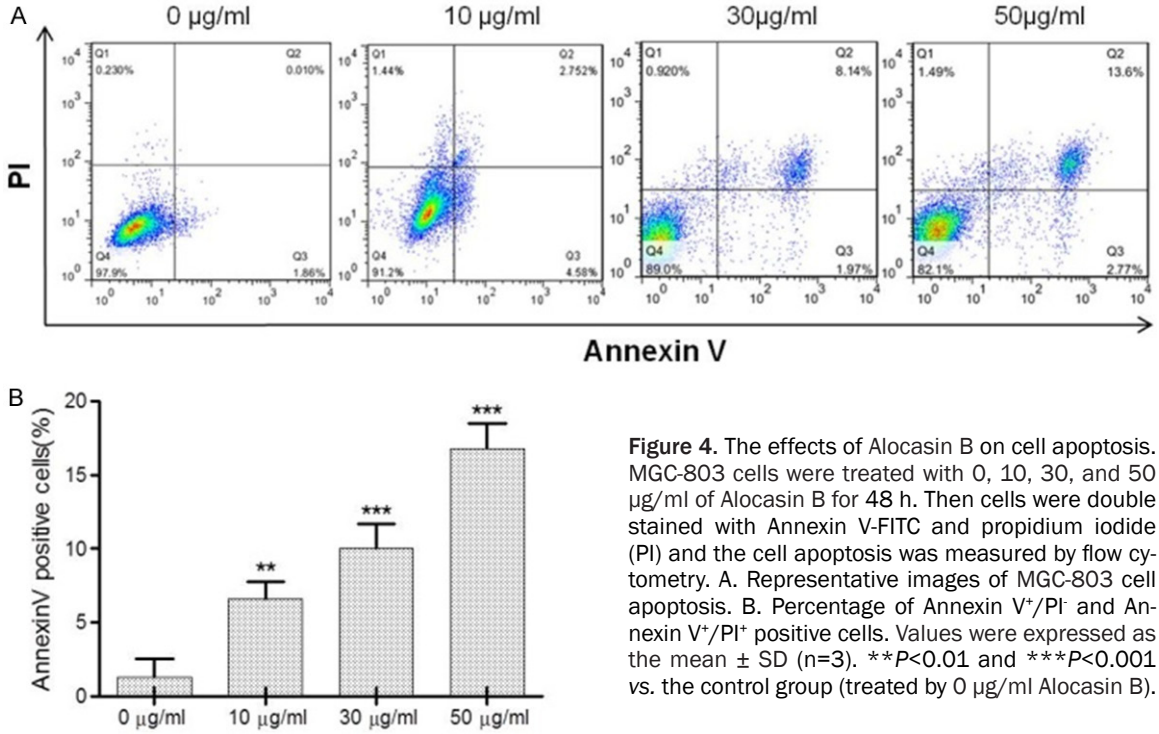

Figure 4. The effects of Alocasin B on cell apoptosis. MGC-803 cells were treated with 0, 10, 30, and 50 μg/ml of Alocasin B for 48 h. Then cells were double stained with Annexin V-FITC and propidium iodide (PI) and the cell apoptosis was measured by flow cytometry. A. Representative images of MGC-803 cell apoptosis. B. Percentage of Annexin V^+/PI^- and Annexin V^+/PI^+ positive cells. Values were expressed as the mean ± SD (n=3). ***P*<0.01 and ****P*<0.001 *vs.* the control group (treated by 0 μg/ml Alocasin B).

the G2 phase was observed, the difference between the groups was not significant (P>0.05).

Apoptotic effect of Alocasia B on MGC-803 cancer cells

After 48 h of cytotoxic treatment on MGC-803 cells by various concentrations of Alocasia B, the cells were stained with Annexin V-FITC and PI dyes to show the apoptosis occurring using the flow cytometry method. As shown in **Figure 4A**, dots, representing cells, are distributed into four quadrates based on cell status: viable ($Annexin\ V^-/PI^-$), early apoptosis ($Annexin\ V^+/PI^-$), late apoptosis ($Annexin\ V^+/PI^+$), and necrosis ($Annexin\ V^-/PI^+$) quadrates. Cells distributing in

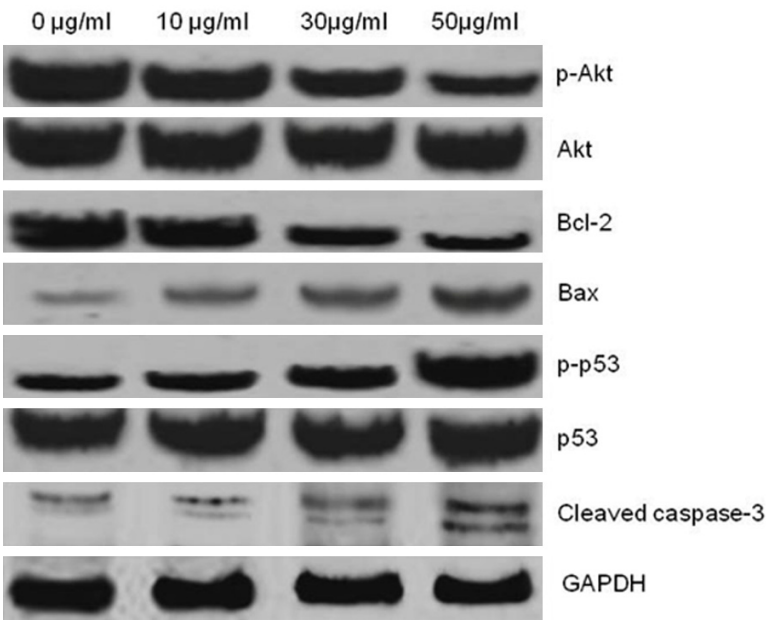

Figure 5. Detection of the effects of Alocasin B on molecule expressions in multiple signaling pathways via western blot assays. MGC-803 cells were treated with 0, 10, 30, and 50 μg/ml of Alocasin B for 48 h. Then p-Akt, Akt, Bcl-2, Bax, p-p53, p53, cleaved caspase-3 and the housekeeping protein GAPDH were detected by western blot. Representative protein bands were shown.

sum of early and late apoptosis increased with a rise in a higher concentration of Alocasia B (**Figure 4B**), suggesting that Alocasia B induced cell apoptosis.

Alocasia B induced upregulation of Bax, p-p53, caspase-3, and the downregulation of p-Akt, Bcl-2

To clarify the detailed mechanisms of how Alocasia B exerted cell apoptosis and an anti-proliferative effect, western blot assays were conducted to measure the expressions of the apoptosis-related proteins (**Figure 5**). A previous study indicated that PI3K/Akt is the signaling pathway for tumor growth [6]. The level of Akt and p-Akt were measured in the MGC-803 cells treated with various concentration of Alocasia B. Our results showed that p-Akt decreased with a rise in Alocasia B concentration. The mitochondrial-dependent pathway was particularly investigated for the regulatory effect of Alocasia B. p53 [7], Bax, and cleaved caspase-3 [8] were identified since these are potential candidates in apoptosis induction. Our results showed an elevated level of phosphorylation p53 upon Alocasia B suppression, yet no significant change in total p53 was observed at any concentration of Alocasia B. The expression of the pro-apoptotic protein Bax was up-regulated, while the MGC-803 cells were treated with increased Alocasia B. The expression of the anti-apoptotic protein Bcl-2 was shown to be down-regulated. Due to the activation of the upstream molecules in the mitochondrial-dependent pathway, we measured the expression change of caspase-3 protease, which played a crucial role in cell apoptosis [9]. Western blot result showed that the caspase-3 level was notably increased in the MGC-803 cells treated with Alocasia B, compared to the control cells that received no treatment (**Figure 5**). All these results indicated that Alocasia B induced pro-apoptosis signaling pathways in the MGC-803 cells.

Alocasia B suppressed the growth of gastric tumors

To determine the effects of Alocasia B on gastric tumors *in vivo*, MGC-803 cells were subcutaneously injected into the nude mice. After treatment with Alocasia B (s.c.) for 3 weeks, the tumor weights were measured (**Figure 6A**). The results showed that both dosages of Alocasia B could inhibit tumor weight significantly compared to the vehicle treatment group ($P<0.05$) (**Figure 6B**).

Discussion

Currently, with the development of antitumor research, great breakthroughs have been made in the treatment of malignant gastric cancer tumors. Drug therapy is one of the most effective ways to treat gastric cancer. However, gastric cancer is susceptible to resistance to the current, commonly used agents, so it is necessary to develop new antitumor substitutes [10]. Our previous study [3] found that *A. cucullata* has a broad-spectrum antitumor activity that inhibits the proliferation *in vitro* of gastric, breast, myelogenous leukemia, and hepatic cancer cells, particularly showing the strongest inhibitory effect on gastric cancer. However, the

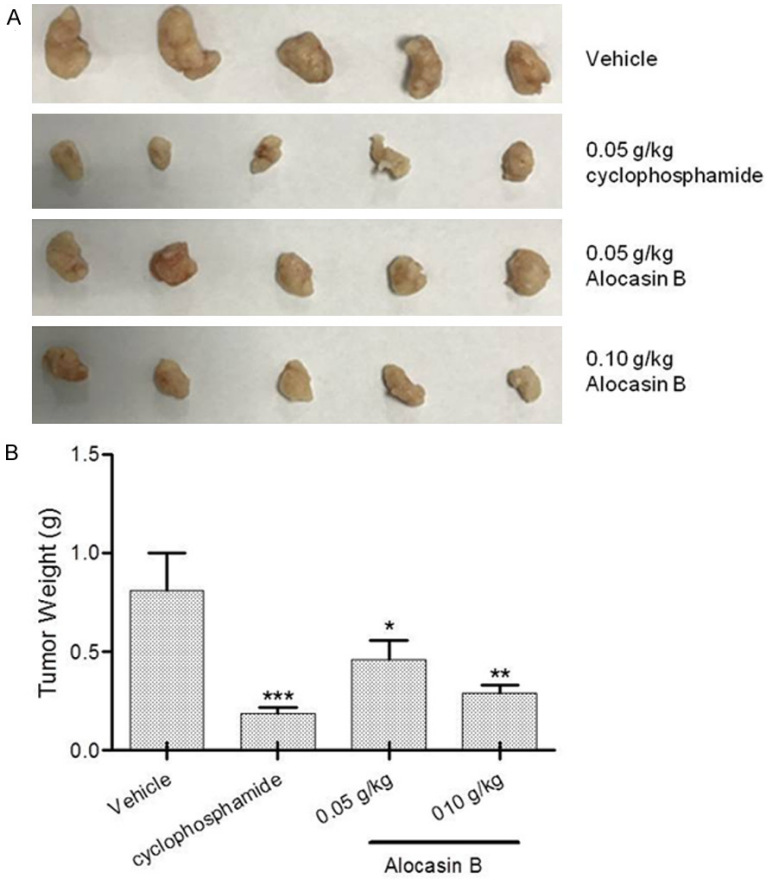

Figure 6. Inhibition effects of Alocasin B on tumor growth in nude mice. MGC-803 cells were injected to the right axillas of nude mice. Alocasin B was injected into tumors every other day for 21 days. (A) The presented tumors and their weights (B) were compared. Values were expressed as the mean ± SD (n=3). $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* vehicle group.

chemical composition of *A. cucullata* for tumor suppression is still unclear. Then we further isolated several lignanamides from *A. cucullata* [5]. And among them, Alocasin B showed the strongest cytotoxicity against leukemia and colorectal cancer cells and showed the strongest antioxidant and inhibitive effect to tyrosine kinase. This present research is part of a continuous study to identify the activity of Alocasin B against gastric cancer.

Cell proliferation and apoptosis are key biological events in tumor progression [11]. When MGC-803 cells were treated with an increased concentration of Alocasin B, proliferation suppression was observed with the elevated inhibition rate. Consistent with the MTT assay, the cell cycle assay verified the G0/G1 phase arrest of MGC-803 cells by Alocasin B, compared to the control cells. According to the results of the Annexin-V/PI dual staining assay, we found that Alocasin B could increase the percentage of late- and early-apoptotic cells in a dose-dependent manner. Therefore, proliferation inhibition by Alocasin B might occur as a result of both its cell cycle arrest and its pro-apoptotic effects.

The progression of gastric cancer involves complicated signaling pathways [12], which can promote cancer cell proliferation and inhibit apoptosis. And since Alocasin B is a novel compound, multiple signaling pathways were detected. p53 is a very crucial tumor suppressor, regulating downstream genes in cell cycle arrest and apoptosis through transcription [13]. In various cancers, p53 is always found inactivated, lost, or mutated [14]. The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays a vital role for cancer cells in countervailing apoptosis led by various kinds of apoptotic stimuli [15, 16]. Routine drugs used in clinics always inhibit Akt hyperactivation [17]. In the mitochondrial apoptosis pathway, members of Bcl-2 family act as the key regulators, with the representative proapoptotic member of Bax, and the representative anti-apoptotic member of Bcl-2 [18]. Bax perforates mitochondria to efflux cytochrome-c, which is a vital trigger to induce downstream apoptosis cascades [19]. Bcl-2 acts as a blocker to bind with Bax to impede its function. Thus, the balance of Bcl-2 and Bax is the crucial step in transferring upstream death signals to be carried out. So, to carry this out, caspase proteases selectively cleave individual protein molecules to induce cell apoptosis [20]. In addition, it was found that Akt can prevent apoptosis through phosphorylation to activate Bcl-2, to inactivate Bax [21, 22].

When treated with Alocasin B, p-p53, and caspase-3, Bax was increased, while p-Akt and Bcl-2 were decreased, all of which indicated

multiple signaling pathways were triggered by Alocasin B. Alocasin B can inhibit the anti-apoptotic PI3K/Akt signaling pathway, enhance the proapoptotic promotor p53, and regain the balance of Bax/Bcl-2. As shown by its proliferation inhibition *in vitro* and its interaction with multiple signaling pathways, Alocasin B can inhibit tumor growth.

Conclusion

In summary, Alocasin B is a novel compound from *A. cucullata* that can inhibit the proliferation and induce the apoptosis of human gastric cancer cells *in vitro* and *in vivo*, with the potential influencing expressions of the key pathway molecules of p53, Akt, Bax/Bcl-2 balance, and the release of caspase-3.

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Disclosure of conflict of interest

None.

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