

Inhibitory Activity of Fruits Extracts of *Antidesma bunius* on the Proliferation and Migration of MDA-MB-231 Breast Cancer Cells

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Abstract In this research, we investigated the inhibitory effect of the ethanolic (EtOH) extract of bignay (*Antidesma bunius*) fruit on the viability of breast cancer cells (MDA-MB-231) by CCK-8 assay. The results showed that the half inhibitory concentration (IC₅₀) was 219 µg/mL after the cells were treated with EtOH extract for 72 hours. The EtOH extract was sequentially extracted by petroleum ether (PET), ethyl acetate (EtOAc), and n-butanol (nBuOH). Among them, the EtOAc fraction showed higher anti-proliferative activity than the other fractions. Wound healing experiment showed the EtOAc fraction inhibited the migration of MDA-MB-231 cells and significantly delayed the transition of the G1 to S phase compared to control. UHPLC-MS/MS analysis showed that in addition to citric acid and phenolic acids (3-Hydroxybenzoic acid, aspirin, fetic acid), EtOH extract of bignay fruit contains active ingredient like coumarin, genistin, amentoflavone, and luteolin 7-galactoside. The anti-proliferation activity of amentoflavone against MDA-MB-231 cells was also confirmed.

Keywords: breast cancer, ethno-medicine, flavonoid, tropical fruits

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1. Introduction

Bignay (*Antidesma bunius* (L.) Sprengel) also known as Wu-Yuer-Cha in China and Mao-Luang in Thailand, belongs to Euphorbiaceae family, Phyllanthoideae subfamily [1]. There are about 170 plant species contained in the genus *Antidesma* and are distributed in tropical regions of the eastern hemisphere [2]. The plants of genus *Antidesma* are important ethno-medicinal plants in Thailand, the Philippines, and Indonesia [3]. The entire plant of *A. bunius* is of medicinal value due to its antioxidant, anticancer and antidiabetic activities [4]. There are 16 species of *Antidesma* genus plants in China, mainly distributed in Guangdong, Guangxi, Fujian, Guizhou, Yunnan and Hainan provinces. It often grows as a wild plant or is used as a green ornamental tree. The Chinese traditional medicine books "Color Atlas of Commonly Used Chinese Herbal Medicines" records that the roots, fruits, and leaves of *A. bunius* has the medicinal activities of convergence, quenching diarrhea, quenching thirst and promoting blood flow. Edible fruits of *A. bunius*

are used to make juice or red wine rich in phytochemicals with enhanced health-promoting properties [5]. Despite the presence of phenolic acids, flavonoids and anthocyanins in the fruit of *A. bunius* [6], its anticancer effect has not been fully elucidated.

Breast cancer is the kind of cancer with the highest incidence in women in the world today, and about 2 million new cases and 500,000 deaths in 2018 [7]. The MDA-MB-231 cell line is a well-known malignant breast cancer model, due to its high invasiveness and motility and its ability to develop tumors and metastasize in *in vivo* assays in rodents [8]. Currently, chemotherapy is the most effective treatment for cancer-suffering patients, and there is a constant need to find new therapeutic drugs and treatment methods to fight this disease. Paclitaxel, originally extracted from the bark of *Taxus brevifolia*, is a drug commonly used to treat breast and ovarian cancer, but it has the disadvantages of low water solubility and large adverse reactions [9].

Thus, research dedicated to finding novel compounds to inhibit any of the acquired features of cancer cells are of high value. In addition to this topic, our *in vitro* research indicates that the fruit extract of *A. bunius* prevents breast

cancer cell proliferation and migration. This result provides an empirical basis for using fruits of *A. bunius* as food with anticancer effects.

2. Materials and Methods

2.1. Plant Material

Fruits of *A. bunius* were collected from Wanning, China and were certified in August 2019 by the botanist Rongtao Li from the Institute of Medicinal Plants (Hainan Branch), Chinese Academy of Medical Sciences. The red colored fruit (2 kg) were dried in an oven at 40°C for 72 h. Later, it was grounded and extracted with 80% ethanol (EtOH) for 24 h. The sample was sonicated 3 times (30 min each time) in a sonicator by maintaining 25°C bath temperature. Extract was dried by rotary evaporation at 40°C and then by using a freeze dryer. Stock solution was prepared by dissolving 0.2 g of the dried extract in 2 mL DMSO, filtered with a filter (0.22 µm) and stored at -20°C until use.

In a second phase of extraction, the stock solution was dissolved in ultra-pure water, and sequentially extracted using petroleum ether (30-60°C boiling range, PET), ethyl acetate (EtOAc), and n-butanol (n-BuOH). Each fraction was separately freeze-dried to obtain PET, EtOAc, n-BuOH and water extracts.

2.2. Cell Line

Human breast cancer cell line (MDA-MB-231) was received from Kunming Cell Bank, Chinese Academy of Sciences (Kunming, China) and was cultivated with RPMI 1640 media (Gibco, USA) containing 10% fetal bovine serum (BOSTER, Wuhan, China) and 1% streptomycin/penicillin (Biosharp, Hefei, China). 25 mM HEPES (Yuanye, Shanghai, China) was added to cell culture when performing experiment with the fruit extract. Cultures were maintained in humidified incubator (Thermo, USA) at 37 °C with 5% CO₂.

2.3. Cell Viability Assay

Cells were seeded at the rate of 2000 cells per well in 96-well microplate. After 24 h, media containing different concentrations of *A. bunius* fruit extract was added. As a control, media with 0.1% DMSO was used. After treatment, cell viability was assessed by using the Cell Counting Kit-8 (CCK-8) (BOSTER, China) following the company's recommended protocol. Briefly, media was gently removed after 72 h of treatment, cells were washed with phosphate-buffered saline (PBS) and then 90 µL of medium plus 10 µL of CCK-8 were added. The cell culture plate was put back in the incubator for 2 h. Finally, the absorbance was determined at 450 nm with a microplate reader (Thermo, USA). To calculate the relative cell viability, the following formula was used:

$$\text{Cell viability} = \frac{\left[\frac{\text{Absorbance reading (control)}}{-\text{Absorbance reading (treatment)}} \right]}{\text{Absorbance reading (control)}}$$

2.4. UHPLC-MS/MS Analysis of EtOH Extract

Extracting solution (methanol: water = 1: 1, with internal standard) was added in 2 mg of extract. LC-MS/MS analysis were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 µm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). The mobile phase was 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH = 9.7) (A) and acetonitrile (B). The analysis was carried with gradient elution as follows: 95% B for 0-0.5 min; 95-65% B for 0.5- 7.0 min; 65-40% B for 7.0-8.0 min; 40% B for 8.0-9.0 min; 40-95% for 9.0-9.1 min and 95% B for 9.1-12.0 min. The temperature of the column was 30°C. The temperature of the auto-sampler was 4°C and the volume of the injection was 2 µL.

The QE HFX mass spectrometer with acquisition program (Xcalibur, thermo), was used to acquire MS/MS spectra in the information-dependent acquisition (IDA) mode. The ESI source conditions were set as following: Aux gas flow rate as 25 Arb, sheath gas flow rate as 30 Arb, capillary temperature 350°C, MS/MS resolution as 7500, full MS resolution as 60000, spray Voltage as 3.6 kV (positive) or -3.2 kV (negative), collision energy as 10/30/60 in NCE mode.

Using Proteo Wizard, the raw data was converted to mzXML for processing with an in-house software developed using R and based on XCMS for peak detection, extraction, alignment and integration. Then an in-house MS2 database (Biotree DB) was applied in metabolite annotation. Subsequently, an in-house MS2 database (Biotree DB) was used for metabolite annotation. The annotation cutoff was fixed at 0.3.

2.5. Wound-healing Activity

In vitro cell migration can be calculated by a scratch assay that is appropriate, inexpensive and commonly used to mimic cell migration during *in vivo* wound healing [10]. MDA-MB-231 cells (2.2×10^5) were seeded per well in 24-well plates for 24 h before performing a wound with a 200 µL sterile tip. Cells were gently washed with PBS to remove floating cells, and media containing 2.5% fetal bovine serum (FBS) with and without fruit extract was added. Media was removed and cells were fixed after staining with 0.1% crystal violet (Yuanye, Shanghai, China). Pictures of the same field were taken at 0 and 24 h. Cell-free areas were determined using ImageJ software. The % relative migration was calculated with the following formula:

$$\% \text{Relative migration} = \frac{\left[\frac{(\text{Area between cells}_{0h})}{-(\text{Area between cells}_{24h})} \right]}{\text{Area between cells}_{0h}} \times 100$$

2.6. Flow Cytometry

Each well of 6-well plate was seeded with 5×10^5 MDA-MB-231 cells, and cultivated with 50 µg/mL EtOH extract. After 24 h of treatment, cells were detached with

trypsin without EDTA, washed twice with pre-cooled PBS. Then, cells were fixed with 70% ethanol, and refrigerated overnight at -20°C . Later, cells were washed twice with pre-cooled PBS, and $2\ \mu\text{L}$ ribonuclease (RNaseA) at a concentration of $1\ \text{mg/mL}$ was added, and kept at 37°C for 30 min, then $400\ \mu\text{L}$ propidium iodide (PI) staining solution (KeyGen, JiangSu, China) was added and kept in the dark for 30 minutes. Flow cytometry (BD FACS Calibur, USA) detected amount PI per cell and the Modfit LT 5.0 software (Variety Software Home, USA) calculated the percentage of cells in each cell cycle phase.

2.7. HPLC Analysis of EtOAc Extract

The EtOAc extract and amentoflavone (Yuanye, Shanghai, China) standard were analyzed by Agilent 1200 HPLC (Agilent, USA) with an UV detection, SB-C18 column, using methanol (A) and 0.5% formic acid aqueous solution (B) as mobile phases, and gradient elution (0~20 min, 40~60% A; 20~40 min, 80% A; 40~60 min, 40% A). The flow rate and injection volume were $0.4\ \text{mL/min}$ and $10\ \mu\text{L}$, respectively.

2.8. Statistical Analysis

All experiments were repeated more than 3 times. One-way ANOVA and Dunnett's multiple comparisons test were applied by using GraphPad Prism version 8.0.1.

3. Results

3.1. Antiproliferative Activity of Ethanolic Extract

Altogether $14\ \text{g}$ of extract powder were obtained from $0.29\ \text{kg}$ dried fruits of *A. bunius* by ethanol extraction. EtOH extract was dissolved in DMSO, diluted to a certain concentration in DMEM medium, and tested for its proliferation inhibitory activity on

breast cancer cell lines (MDA-MB-231). The results are shown in Figure 1. The anti-proliferative activity of EtOH extract showed a dose-dependent effect. The IC_{50} of the EtOH extract of *A. bunius* fruits against MDA-MB-231 cells was determined to be $219\ \mu\text{g/mL}$ after treatment for 72 h.

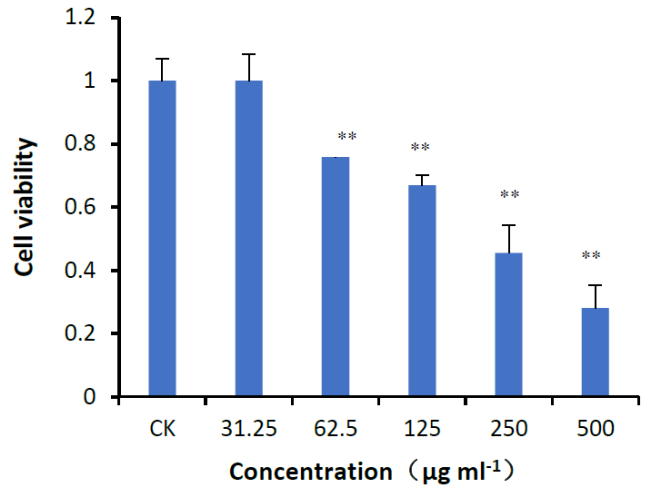


Figure 1. Effect of EtOH extract of *A. bunius* fruit on the viability of MDA-MB-231 cells

3.2. UHPLC-MS/MS Analysis of EtOH Extract

The 30 top content compounds are listed in Table 1. Most of these compounds were detected in the negative MS and only two compounds were detected in the positive MS (Figure 2). The compound in highest concentration was citric acid, which is the main acid in fruits like orange, passion fruit and pineapple. There are other phenolic acids like 3-hydroxybenzoic acid, 4-hydroxycinnamic acid, ferulic acid, aspirin, 2,3-dihydroxybutanedioic acid, itaconic acid, gallic acid, terephthalic acid and vanillic acid. Coumarin and flavonoids like genistin, amentoflavone and luteolin 7-galactoside are also in high content in EtOH extract and may play an important anticancer activity.

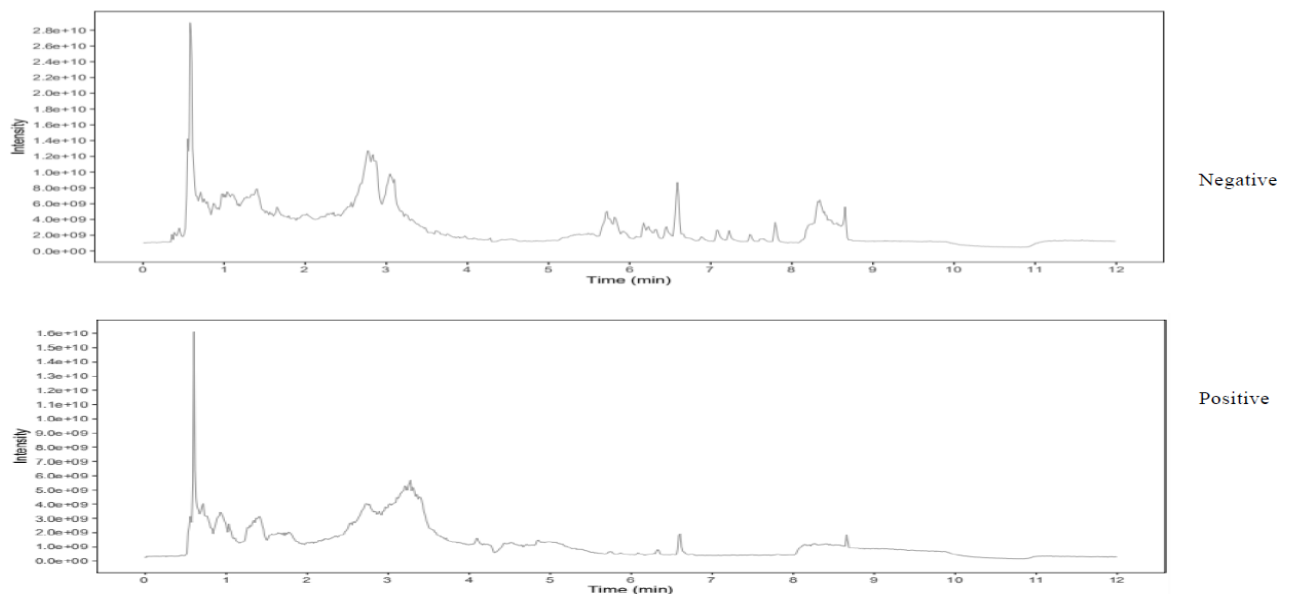
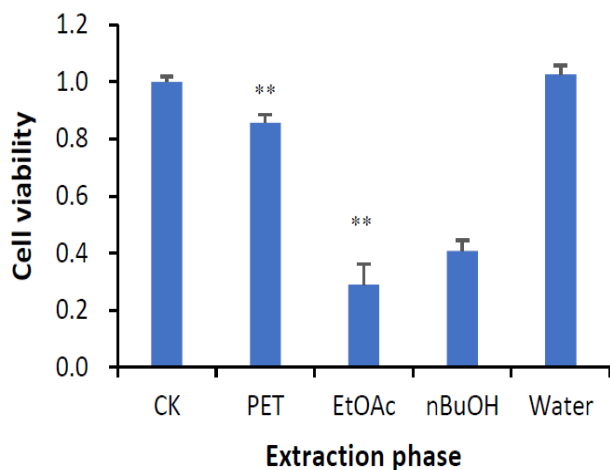


Figure 2. UPLC MS/MS analysis of EtOH extracts of *A. bunius* fruit

Table 1. Identified main compounds in ethanolic extract of *A. burius* fruit using UPLCMS/MS

No.	MS2 name	MS2 score	rt	mz	type	Super Class	Area%
1	Citric acid	0.9941	516.89	191.0189	-	Organic acids and derivatives	19.76
2	3-Hydroxybenzoic acid	0.9917	35.48	137.0235	-	Benzenoids	9.50
3	Coumarin	0.5256	183.57	145.0287	-	Phenylpropanoids and polyketides	7.87
4	Genistin	0.9531	158.49	431.0987	-	Phenylpropanoids and polyketides	5.53
5	4-Hydroxycinnamic acid	0.9243	180.20	163.0396	-	Phenylpropanoids and polyketides	5.05
6	Aspirin	0.9904	112.33	179.0344	-	Benzenoids	4.35
7	Amentoflavone	0.9420	33.75	537.0827	-	Phenylpropanoids and polyketides	3.77
8	Fertaric acid	0.4985	349.60	325.0562	-	Phenylpropanoids and polyketides	3.65
9	Mesylate	0.9357	119.48	94.9800	-	Organic compounds	3.57
10	2,3-Dihydroxybutanedioic acid	0.9942	434.55	149.0084	-	Organic oxygen compounds	3.51
11	Itaconic acid	0.9968	64.63	129.0187	-	Lipids and lipid-like molecules	3.09
12	Gallic acid	0.9527	477.04	169.0134	-	Benzenoids	2.85
13	Terephthalic acid	0.9855	125.75	165.0189	-	Benzenoids	2.83
14	4-Hydroxybenzeneacetoneitrile	1.0000	163.54	132.0449	-	Benzenoids	2.77
15	Vanillic acid	0.9715	177.66	167.0345	-	Benzenoids	2.76
16	4-Hydroxybenzoic acid	0.9981	186.89	137.0238	-	Benzenoids	2.37
17	Abcsicic acid	0.8276	85.21	263.1290	-	Lipids and lipid-like molecules	2.25
18	cis-Aconitic acid	0.9803	468.52	173.0087	-	Organic acids and derivatives	1.53
19	3-Hydroxymethylglutaric acid	0.9466	389.14	161.0447	-	Lipids and lipid-like molecules	1.44
20	But-2-enoic acid	1.0000	468.52	85.0287	-	Lipids and lipid-like molecules	1.40
21	Luteolin 7-galactoside	0.9060	216.47	447.0927	-	Phenylpropanoids and polyketides	1.36
22	Isoferulic acid	0.8679	34.82	193.0501	-	Phenylpropanoids and polyketides	1.21
23	Maleic acid	0.9981	295.00	115.0030	-	Organic acids and derivatives	1.09
24	Methyl vanillate	0.8092	35.48	181.0499	-	Benzenoids	1.06
25	4-Hydroxystyrene	0.9991	180.20	119.0496	-	Benzenoids	1.05
26	trans-Ferulic acid	0.9791	349.60	193.0501	-	Phenylpropanoids and polyketides	1.01
27	1,2,3-Trihydroxybenzene	0.9945	325.53	125.0236	-	Benzenoids	1.00
28	Cosmosin	0.9896	176.01	433.1130	+	Phenylpropanoids and polyketides	0.86
29	Peonidin-3-glucoside	0.9257	139.49	463.1224	+	Phenylpropanoids and polyketides	0.74
30	Choline	0.9997	291.34	104.1070	-	Organic nitrogen compounds	0.74

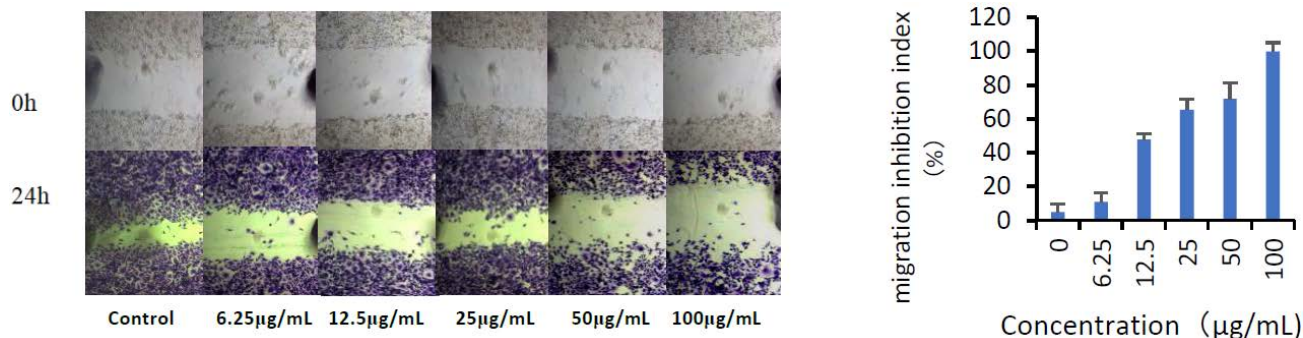
**Figure 3.** Effect of different solvent extracts of *A. burius* fruit on the viability of MDA-MB-231 cells

3.3. Antiproliferative activity of Fractions

200 $\mu\text{g/mL}$ of PET, EtOAc, n-BuOH and water extracts were tested to evaluate their inhibitory activity on the viability of MDA-MB-231 cells. Among these fractions, EtOAc extract exhibited the highest inhibition of cell viability and the aqueous phase showed the lowest activity (Figure 3).

3.4. Effect on Cell Migration

In the wound healing process, cell migration and proliferation play a significant role. The effect of EtOAc extract on the migration of MDA-MB-231 cells was also examined. Migration of these cells was interrupted by increasing concentration of EtOAc extract in a dose dependent manner (Figure 4). Results showed that treatment with 100 $\mu\text{g/mL}$ EtOAc extract strongly inhibited the motility of MDA-MB-231 cells towards the wound (Figure 4).

**Figure 4.** Effect of EtOAc extract of *A. burius* fruit on the migration of MDA-MB-231 cells

3.5. Effect on Cell Cycle

The 50 µg/mL EtOAc extract significantly increased the proportion of cells in G0/G1 phase after 24 h of treatment compared to control (Figure 5). The proportion of cells in G0/G1 phase was 58% in the control group while in the EtOAc treated extract reached to 63% ($p < 0.01$). On the other hand, the cell proportion in S phase was 37% when treated with EtOAc compared to 32% in the control group ($p < 0.05$). This result shows that the EtOAc extract phase of *A. bunius* fruit accumulates MDA-MB-231 cells in the G0/G1 phase.

3.6. HPLC Analysis of EtOAc Extract and Amentoflavone

From the HPLC analysis of the EtOAc extract fraction

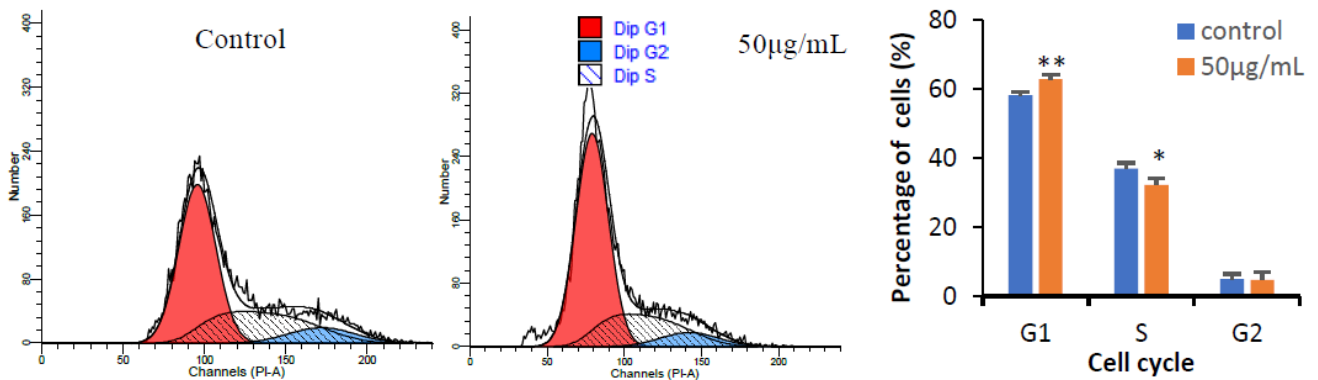


Figure 5. Effect of EtOAc extract of *A. bunius* fruit on cell cycle of MDA-MB-231 cells

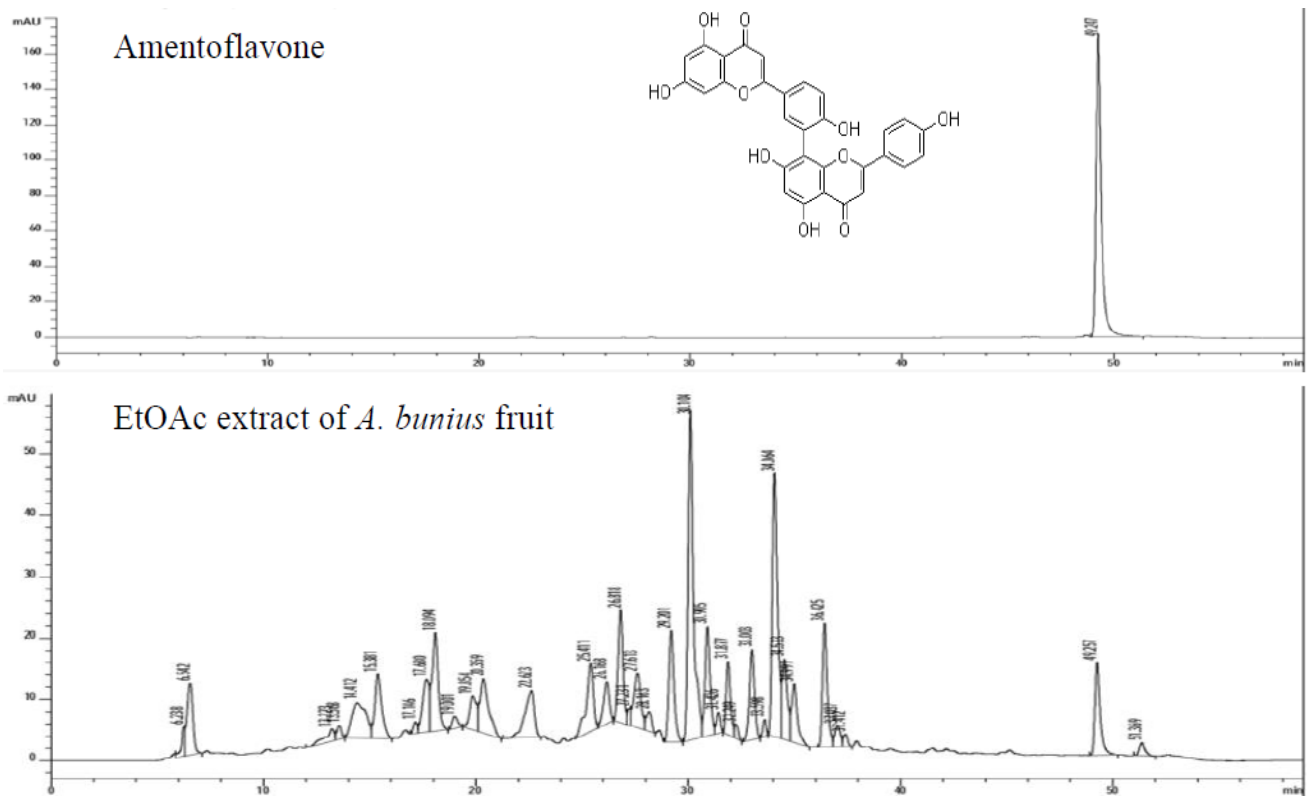


Figure 6. HPLC total ion chromatogram of EtOAc extract of *A. bunius* fruit

of *A. bunius*, the component that peaked at 49.2 min was isolated and identified as amentoflavone ($C_{30}H_{18}O_{10}$) by mass spectrometry. Molecular Weight of amentoflavone was 538.4579 (Figure 6).

3.7. Amentoflavone Inhibited the Viability of MDA-MB-231 Cells

Five different concentrations of amentoflavone (31.25, 62.5, 125, 250, 500 µg/mL) were tested to inhibit the viability of MDA-MB-231 cells. It was observed that the amentoflavone IC_{50} against breast cancer cell's viability was 192 µg/mL after treatment for 72 h. The results showed significant inhibition of the cancer cells at highest concentration i.e., 500 µg/mL. A slight non-significant increase on cell viability was observed with concentrations of amentoflavone lower than 100 µg/mL (Figure 7).

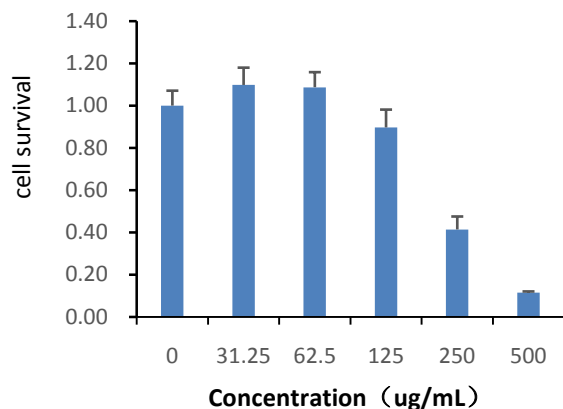


Figure 7. Anti-proliferation of MDA-MB-231 cells by amentoflavone

4. Discussion

Breast cancer accounts for 7%-10% of all malignant tumors and triple-negative breast cancer (TNBC) accounts for about 15-20% of breast cancer [11]. Intervention of traditional Chinese medicine at different stages of breast cancer treatment can effectively improve the cancer patients, life quality and reduce the rate of recurrence and metastasis [12]. Previous studies have shown that crude methanol extracts from leaves and fruits of *A. bunius* have cytotoxic activity against *Artemia salina* [5]. Matured leaves of *A. bunius* have been used against snakebite and young leaves are boiled and used in syphilis and skin disorders in India [13]. Ethanolic fruit extracts of *A. bunius* were used as herbal drug in diabetes therapy [14].

It was found that the polyphenolic compounds in fruit of *A. bunius* are mainly procyanidin B2, procyanidin B1, (+)-catechin, (-)-epicatechin, rutin and tran-resveratrol cyanidin-3-O-glucoside, and the contents of gallic acid, (-)-epicatechin, and (+)-catechin decreased during fruit ripening, and *A. bunius* fruit possess the highest antioxidants at over ripe stage [6]. In this study, anthocyanins with wine red color mainly concentrate in water fraction, which showed no anti-proliferative activity to MDA-MB-231 cells. In our work, the high concentration of phenolic acid in our extract implies the red fruits we collected were not fully ripened, since the fully ripened fruit shows dark red color. The relationship between anti-cancer activity and the concentration of polyphenols needs to be further studied.

The G1 phase of the cell cycle involves mechanisms that allow gene expression for regular cell functioning and evaluation of DNA damage before the duplication of the genetic material in the S phase. The results of this study show that fruit extract of *A. bunius* delays MDA-MB-231 cells in the G1 phase that correlates with the decrease proportion of cells in the S phase. Altogether with the decrease in cell survival caused by exposure to *A. bunius* suggest that the extract inhibits cell proliferation by blocking or inhibiting cells in the transition from G1 to S phase. Probably, signaling pathways of cyclin D and cyclin E (regulators of entering the S phase) could be possible targets of *A. bunius* fruit extract.

MDA-MB-231 cells represent a great model of aggressive breast cancer since they have a high level of

motility. Thus, finding compounds that preclude their cell motility are of great interest. Clinically, preventing cancer cell motility could retard cancer aggressiveness. As showed in this work, the extract of *A. bunius* fruit inhibits the migration of MDA-MB-231 cells, pointing to a plausible use of this fruit extract as an anticancer agent.

Flavonoids are a large group of polyphenolic compounds present in a variety of fruits and vegetables with many biological properties. The flavonoids in the fruit extract of *A. bunius* such as coumarin, genistin, amentoflavone and luteolin 7-galactoside have already been proved to have multiple biological activities [15,16,17]. Amentoflavone is a naturally occurring biflavonoid compound abundant in *Selaginella tamariscina*, with anti-inflammatory [18], anti-oxidative [19], anti-microbial [20], anti-apoptotic [21], anti-radiation [22], antitumor [12], neuroprotective [23,24] and cyano-bacterial killing effects [25]. Previously, the authors confirmed the anti-proliferative effect of the ethanolic extract of leaves of *Antidesma montanum* on the MDA-MB-231 cells and found that amentoflavone was present in this extract [26].

The molecular mechanism underlying amentoflavone have been previously studied [27]. Amentoflavone inhibits TNF- α , VEGF, MMP-9, MMP-2, IL-6, and IL-1 β expression and secretion by blocking NF- κ B activation. [28]. The blockade of fatty acid synthesis triggered SK-BR-3 breast cancer cell apoptosis [21], and by regulating the Hedgehog/Gli1 signaling pathway in SUM159 stem cells of breast cancer and also inhibits tumor formation [29]. Amentoflavone has also been suggested as a potential adjuvant agent to boost the anti-cancer effect of doxorubicin [30].

5. Conclusion

The ethanol extract of the fruit of bignay (*A. bunius*) possess anti-proliferative and anti-migratory effect on MDA-MB-231 breast cancer cells. There are abundant phenolic acids and flavonoids in the ethanol extract of bignay fruit. The main active substance concentrates in the ethyl acetate fraction, and it delays the G1 phase of MDA-MB-231 cells, probably causing apoptosis that could explain the reduced cell survival. Importantly, amentoflavone was identified as one active ingredient in fruit of *A. bunius*.

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