Original Article

Cytotoxic Activity Against Liver Cancer and Cholangiocarcinoma Cells of *Artemisia vulgaris* L. Extract and Cirsimaritin, Its Isolated Compound

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Abstract

Introduction: Liver cancer is the most common cancer in Thailand. *Artemisia vulgaris* L. is one of Thai herbs used in many Thai traditional cancer recipes. The objective was to investigate cytotoxic activity against hepatocellular carcinoma (HepG2), cholangiocarcinoma (KKU-M156) cells of *Artemisia vulgaris* L. Extract, and its compound.

Methods: The SRB assay was used to determine the cytotoxic activity. Bioassay guided isolation was used for isolating cytotoxic compound.

Results: The ethanolic extract of *A. vulgaris* L. showed good cytotoxic activity against HepG2 and KKU-M156 (IC_{50} =13.36 ± 0.45 and 17.07 ± 0.95 µg/mL, respectively), while it also showed cytotoxicity against normal cell lines (HaCaT, IC_{50} = 27.30 ± 3.71 µg/mL). Vacuum liquid chromatography (VLC) in (Chloroform: Methanol) fraction showed the best cytotoxic. Cirsimaritin, the isolated compound from the active fraction showed better cytotoxic activity against HepG2 than KKU-M156 (IC_{50} =1.82 ± 0.63 and 21.01 ± 0.84 µg/mL, respectively), while it had no cytotoxicity against normal cell lines (HaCaT) (IC_{50} >100 µg/mL). This compound showed selective cytotoxicity against HepG2 with selective index (SI) value of 54.94.

Conclusions: Cirsimaritin showed selective cytotoxic activity against liver cancer cells with the IC_{50} value less than 4 µg/mL and the SI value more than 50. In regards to NCI criteria which consider that the value of IC_{50} of a good cytotoxic compound must be less than 4 µg/mL, therefore, it has the potential to be developed as an anti-cancer drug for liver cancer cells. It also demostrated selective cytotoxic activity against liver cancer cells better than cholangiocarcinoma.

Keywords: Cancer, SRB assay, *Artemisia vulgaris* L., Bioassay guided isolation, Cirsimaritin

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Introduction

Liver cancer is the first leading cause of death in the Thai male population with the highest mortality rate in cancer patients.[1] Thai herbal medicine is the first choice of Thai people for taking care of themself from the past to the present. *Artemisia vulgaris* L. has bitter taste and is widely used in Thai traditional drug recipes for antipyretic, and hepatotonic. The bitter herbs in Thai traditional medicine (TTM) are principally used as antipyretic drug, hepatotonic drug, antimalarial, laxative detoxification and cancer treatment.TTM principle believes that bitter herb can affect bitter organ such as liver or bile duct because bitter taste herbs help fire element to do better work. Fire elements are essential to body metabolism and detoxification. Thus, the bitter herbs should be helpful in cancer treatment. According to the National List of Essential Medicine 2017, *A. vulgaris* L. (Kod-Julalumpa as Thai name; mugword or common wormwood as Englisg names) was one of the ingredients in 13 formulas and used against many symptoms[2] such as fever, phlegm, asthma, improve the wind element, burp, nourishing blood, nourishing bones.[3]

Many previous studies on *A. vulgaris* L. had confirmed the cytotoxic properties and relative activities on cancer treatment including their antioxidant and anti-inflammation. For example, its whole plant extract showed free radical scavenging activity against DPPH and reactive hydroxyl radical.[4] Its methanol extract showed the total phenolic content of 117.14 ± 0.47 mg of gallic equivalent/g of extract and total flavonoid content of 79.47 ± 0.77 mg of rutin equivalent/g of extract.[5] Its whole plant extract also exhibited anti-inflammatory response by inhibiting the activity of prostaglandins synthesizing enzyme.[6] The mechanism for the anti-inflammatory effect of camphor in *A. vulgaris* L. was shown by production inhibition of interleukin-4, interleukin-2, and TNF-α.[7] It also showed cytotoxic activity against HCT-15 human colon cancer cells and decreased cell viability of SW-480 colon cancer cells.[8] The 70% ethanolic extract of *A. vulgaris* L. also showed cytotoxicity against PANC-1 human pancreatic cancer cells with IC_{50} value of 12.5 µg/mL.[9] Low concentration of the essential oil of *A. vulgaris* L. also inhibited the growth of HL-60 cells in MTT assay.[10] Two glucosides (vulgarosides A and vulgarosides B) were isolated from the aerial parts of *A. vulgaris* L. exhibited cytotoxicity against five human cancer cell lines, including KB (epidermoid carcinoma), HepG2 (hepatocarcinoma), MCF7 (breast carcinoma), SK-Mel-2 (melanoma), and LNCaP (prostate cancer) by the SRB assay.[11] From these evidences, *A. vulgaris* L. has the potential to be used for treatment of liver cancer. However, there was no report on cytotoxicity against cholangiocarcinoma or KKU-M156.

For these reasons, the objective of this research was to investigate cytotoxic activity against hepatocellular carcinoma (HepG2), cholangiocarcinoma (KKU-M156) of the ethanolic extract of *A. vulgaris* L. (AVE) and its isolated compound using the bioassay guided technique, as well as to compare cytotoxicity on normal cell as human keratinocyte cells (HaCaT).

Methods

Materials and Methods

Chemicals and Reagents

Column chromatography (CC) and vacuum liquid chromatography (VLC) were carried out on silica gel (Merck, Darmstadt, Germany) type 60 (70 - 230 mesh) for column chromatography and 230 - 400 mesh for vacuum liquid chromatography, respectively). Precoated plates of silica gel (Merck, Kieselgel 60 F254, 0.25 mm) were used for analytical TLC. The spots on TLC were detected under UV light at 254 and 365 nm, then by heating at 120°C after spraying with acidic anisaldehyde reagent (SigmaAldrich, St Galen, Switzerland). Analytical grade ethanol and dimethyl sulfoxide (DMSO) were purchased from RCI LabScan (Bangkok, Thailand). Hepatocellular carcinoma cell lines (HepG2: ATCC® HB-8065™) were purchased from American Type Culture Collection (ATCC®, VA, USA). Cholangiocarcinoma cell lines (KKU-M156) was obtained from Dr. Piti Aungaree-withaya and Prof. Dr.Veeraphol Kukongviriyapan, Faculty of Medicine, Khon-Kaen University. Normal human keratinocyte immortal cell line (HaCat) was purchased from CLS cell line service (No. 300493-SF). Fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco® (OK, USA). Minimum essential medium (MEM), Dulbecco Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium 1640 (RPMI-1640), penicillin-streptomycin (P/S), and phosphate buffer saline (PBS) were purchased from

Biochrom (MA, Germany). Nutrient Mixture F-12 Ham medium (HAM F-12) and sulforhodamine B (SRB) were purchased from Sigma (MO, USA).

Plant Material

The aerial part of *A. vulgaris* L. was collected from Phetchaburi Province in Thailand in 2018. The voucher specimen was identified as SKP 051 01 22 01. The verification of the plant names was done by the herbarium specimen, which prepared, and deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand.

Extraction and Isolation

The aerial part of *A. vulgaris* L. (1 kg) was sliced and dried at 50°C in an oven and ground to powder. The dried powder of each plant was macerated with 95% ethanol for 3 days/3 times. After filtration all filtrates were combined and concentrated to dryness under reduced pressure, obtaining an 18.54%w/w yield of crude extract.

Crude ethanolic extract of *A. vulgaris* L. (AVE) (50 g) was separated by vacuum liquid chromatography (VLC) on silica gel by using hexane as eluent and increasing polarity with chloroform and methanol successively, to give five fractions as follows: hexane (2000 mL), $CHCl_3$: hexane (1:1, 2000 mL), $CHCl_3$ (2000 mL), MeOH: $CHCl_3$ (1:1, 2000 mL), and MeOH (2000 mL), respectively. Each fraction was dried by a rotary evaporator to yield F1 (0.0008 g), F2 (1.255 g), F3 (4.04 g), F4 (12.575 g), and F5 (1.49 g), respectively. The percentage of the yield as % w/w of starting weight of crude extract was showed in Table 1. According to the cytotoxic activity against HepG2 and KKU-M156 cell lines by the SRB assay, the F4 fraction was selected for the bioassay-guided isolation as it showed the highest percentage of the yield as 25.15% and also exhibited the highest cytotoxicity against all types of cancer cell lines (HepG2; IC_{50} = 18.64 ± 2.69 µg/mL, and KKU-M156; IC_{50} = 15.69 ± 1.08 µg/mL), it had no cytotoxicity against normal-cell lines (HaCaT).

Fraction F4 (5.00 g) was subjected to column chromatography (CC) on silica gel (150 g) using a gradient of solvents as follows; EtOAc: Hexane (2:8, 500 mL), EtOAc: Hexane (3:7, 500 mL), EtOAc: Hexane (4:6, 500 mL), EtOAc: Hexane (1:1, 500 mL), EtOAc: Hexane (6:4, 500 mL), EtOAc: Hexane (8:2, 500 mL), EtOAc (200 mL), EtOAc: MeOH (7:3, 300 mL), EtOAc: MeOH (1:1, 300mL), and finally MeOH (100 mL). Each 20 mL fraction was collected for each eluting solvent and combined fractions, followed by TLC examination (silica gel/Hexane: EtOAc (7:3) and detected with acidic anisaldehyde spray to give eleven fractions (fr.1 - fr.11). Then, fr.5 (0.0583g) was recrystallized with MeOH to give compound 1 as a yellow crystal (0.0124g) which showed only one spot on TLC in three different solvent systems with Rf = 0.15 in hexane: $CHCl_3$ (1:9), Rf = 0.28 in hexane: EtOAc (7:3), and Rf = 0.64 in Hexane: $CHCl_3$ (6:4).

Compound 1: 1H NMR (400 MHz, CDCl3): 3.93 (3H, s, 6-OCH3), 3.97 (3H, s, 7-OCH3), 6.55 (1H, s, H-3), 6.59 (1H, s, H-8), 6.97 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.82 (2H, d, J = 8.8 Hz, H-2', H-6'), 12.77 (1H, s, 5-OH).

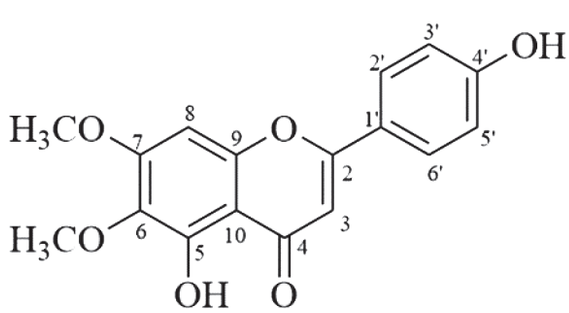

Figure 1 The structure of compound 1 isolated from the aerial part of *Artemisia vulgaris* L.

***In Vitro* Assay for Cytotoxic Activity by SRB Assay**[12]

Human Cell Lines

Cancer cell lines was used in this experiment as human liver hepatocellular carcinoma (HepG2) (ATCC® HB-8065™), human cholangiocarcinoma (KKU-M156) and the normal human keratinocyte cell line HaCaT was grown in an incubator with 5% CO_2 at 37°C in MEM Medium 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin (P/S). According to their growth profiles, the optimal plating densities of each cell line were determined (3×10^3, 3×10^3, and 8×10^3 cells/well for HepG2, KKU-M156 and HaCaT cells, respectively) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay.

Preparation of *A. vulgaris* L. Extract and Compounds for Cytotoxicity Testing

Samples were initially dissolved in sterile dimethyl sulfoxide (DMSO) and prepared at a concentration of 10 mg/mL for extract, and 1 mg/mL for compounds. The extracts were diluted in a medium to obtain 4 final concentration ranges of 1 - 100 mg/mL, and pure compound ranges of 0.1 - 50 mg/mL. The final mixture was used for treating cells containing no more than 1% of the solvent, the same as in the solvent control well. Paclitaxel and vincristine were used as the positive control.

Sulforhodamine B (SRB) Assay

The sulforhodamine B (SRB) assay was used to estimate cell viability numbers indirectly by staining total cellular protein with the SRB. In brief, cells at the exponential growth phase were detached with 0.25% trypsin-EDTA to make single-cell suspensions. The viable cells were counted by trypan blue exclusion using a hemocytometer 100 µL/well of cell suspensions in 96-well microtiter plates and incubated to allow cell attachment. After 24 hours the cells were treated with various concentrations of the extracts. The plates were incubated for the selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells were washed with the medium. And 200 µL of fresh medium was added to each well. The plates were incubated for a recovery period of 72 hours. On the seventh day of the culture period, cells were fixed with 100 µL per well of ice-cold 40% trichloroacetic acid (TCA), incubated at 4°C for 1 hour in the refrigerator and washed with tap water to wash non-viable cells, so viable cells were fixed as a monolayer in each well. Then, 50 µL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min; then the plates were washed with 1% acetic acid until only dye adhering to the cells was left. The dry plates, and 100 µL of 10 mM Tris base (tris-hydroxy methyl) aminomethane, pH 10.5 was added to each well to dissolve the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (4 replicates) was read on a Microplate reader at 492 nm as an indication of cell number. Cell survival was measured as a percentage of absorbance compared with the control (non-treated cells). The IC_{50} values were calculated from the Prism program. According to the National Cancer Institute guidelines or NCI,[13] the extract with an IC_{50} value < 20 µg/mL and the compounds with IC_{50} values < 4 µg/mL were considered active.

Results and Discussion

Structure Elucidation

The bioassay-guided fractionation of AVE was used for the isolation of one flavonoid (Cirsimaritin) (Figure 1). Compound 1 was isolated as a yellow crystal, its 1H NMR (400 MHz, $CDCl_3$) spectral data showed the chemical shift (δ) of proton at 3.93 (3H, s, 6-OCH_3), 3.97 (3H, s, 7-OCH_3), 6.55 (1H, s, H-3), 6.59 (1H, s, H-8), 6.97 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.82 (2H, d, J = 8.8 Hz, H-2', H-6'), and 12.77 (1H, s, 5-OH). The COSY technique was used to confirm compound 1 as a pure compound. Compound 1 was also demonstrated to be pure by using TLC and HPLC, comparison with three different solvent systems of varying polarity and detection with acidic anisaldehyde spray.[13] The Rf values were 0.15 in Hexane: $CHCl_3$ (1:9), 0.28 in Hexane: EtOAc (7:3), and 0.64 in Hexane: $CHCl_3$ (6:4). Then it was subjected to single crystal x-ray diffraction technique. It was elucidated to be chemically named as 4',5-Dihydroxy-6,7-dimethoxyflavone 7-Methylcapillarisin ($C_{17}H_{14}O_6$), which was in accordance with previous spectral.[14] Observation by GC-MS (Figure 2) showed seventeen peaks. There were two main peaks at RT 32.09 and RT 41.14, respectively.

Cytotoxic activity

The cytotoxicity against hepatocellular carcinoma (HepG2) and cholangiocarcinoma (KKU-M156) and an isolated compound were determined using SRB assay, AVE showed good cytotoxic against HepG2 and KKU-M156 (IC_{50} = 13.36 ± 0.45 and 17.07 ± 0.95 µg/mL, respectively), while it also showed cytotoxicity against normal cell lines (HaCaT; IC_{50} = 27.30 ± 3.71µg/mL). Vacuum liquid chromatography (VLC) of F4 (Chloroform: Methanol) fraction showed the best cytotoxic against HepG2 and KKU-M156 (IC_{50} = 18.64 ± 2.69 and 15.69 ± 1.08 µg/mL, respectively), while this fraction had no cytotoxicity against normal cell lines (HaCaT). Cirsimaritin compound was isolated from F4 fraction by bioassay guided isolation technique, it also showed good cytotoxic against HepG2 and KKU-M156 (IC_{50} = 1.82 ± 0.63, and 21.01 ± 0.84 µg/mL, respectively), while it had no cytotoxicity against normal cell lines (HaCaT) (Table 1).

Table 1 Cytotoxic activity of crude extract, VLC fractions, and isolated compound (IC_{50}, µg/mL) against two human cancer cells and one normal cell by using SRB assay at exposure time 72 h (n=3)

Sample	%Yield (w/w)	Cell lines [IC_{50} µg/mL ± SEM, (SI)]		
		HepG2	KKU-M156	HaCaT
F1	0.00016	NT	NT	NT
F2	2.51	48.45 ± 1.03 (> 2.06)	46.51 ± 3.45 (> 2.15)	> 100
F3	8.08	82.17 ± 2.15 (> 1.22)	> 100	> 100
F4	25.15	18.64 ± 2.69 (> 5.36)	15.69 ± 1.08 (> 6.37)	> 100
F5	2.98	24.78 ± 1.045 (> 4.04)	20.25 ± 2.18 (> 4.94)	> 100
AVE	18.54	13.36 ± 0.45 (2.04)	17.07 ± 0.95 (1.60)	27.30 ± 3.71
Cirsimaritin (1)	0.248	1.82 ± 0.63 (> 54.95)	21.01 ± 0.84 (> 4.75)	> 100
Vincristine[a]	-	0.00615 ± 0.00 (0.0054)	0.00165 ± 0.00 (0.02)	0.000033 ± 0.00
Paclitaxel[a]	-	0.00009 ± 0.00 (0.02)	0.00002 ± 0.00 (0.01)	0.000002 ± 0.00

F1-F5 = VLC fractions; AVE = ethanolic extract of *Artemisia vulgaris* L.; SI as the ratio of IC_{50} (µg/mL) values of normal cell/IC_{50} (µg/mL) values of cancer cells. SI value ≥ 3 for classifying of a prospective anti-cancer sample.[26]
[a]Positive control.

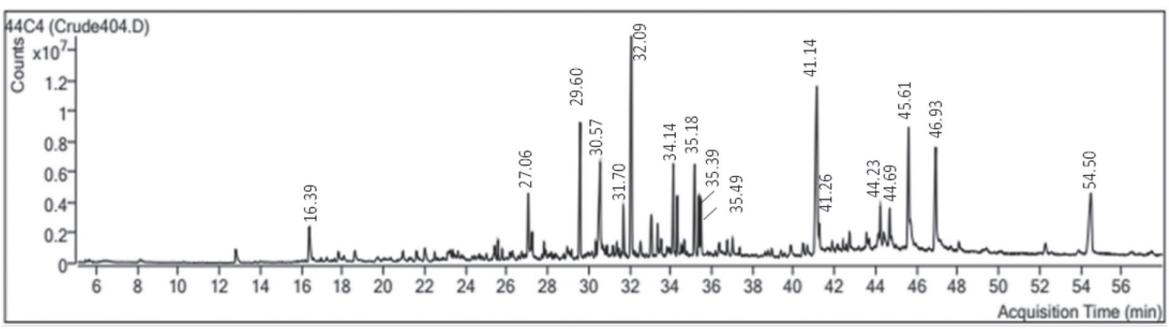

Figure 2 GC-MS chromatogram of the ethanolic extract of *Artemisia vulgaris* L.

Table 2 Chemical composition of volatile compound from ethanolic extract of *Artemisia vulgaris* L. analysed by using GC-MS

Retention time	Chemical composition	Formula	% Area
16.39	4,4-Dimethylcrotonolactone	$C_6H_8O_2$	1.62
27.06	Spathulenol	$C_{15}H_{24}O$	2.33
29.60	Ethylpalmitate	$C_{18}H_{36}O_2$	4.13
30.57	Glycerin	$C_3H_8O_3$	4.77
31.70	Ethyl hydrogen succinate	$C_6H_{10}O_4$	1.38
32.09	3-(5-Methyl-5-vinyltetrahydrofuran-2-yl) butan-2-ol	$C_{11}H_{20}O_2$	8.49
34.14	Ethyl linolate	$C_{20}H_{36}O_2$	2.29
35.18	Ethyl linolenate	$C_{20}H_{34}O_2$	2.29
35.39	2-Pyrrolidinecarboxylic acid-5-oxo-, ethyl ester	$C_7H_{11}NO_3$	1.65
35.49	Phytol	$C_{20}H_{40}O$	1.39
41.14	Palmitic acid	$C_{16}H_{32}O_2$	8.83
41.26	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	0.94
44.23	Stearic acid	$C_{18}H_{36}O_2$	1.37
44.69	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	1.24
45.61	Linoleic acid	$C_{18}H_{32}O_2$	4.66
46.93	Linolenic Acid	$C_{18}H_{30}O_2$	4.97
54.50	Vulgarin	$C_{15}H_{20}O_4$	4.79

As shown in Table 2 and Figure 2, the GC-MS chromatogram of AVE found many compounds which have ever been reported as cytotoxic, antitumor, or anticancer compounds. From the previous data, spathulenol exhibited cytotoxic activity against B16-F10, HepG2, K562 and HL-60 and PBMCs by using the alamar blue assay.[15] Ethylpalmitate exhibited significant hepatoprotective activity.[16] Ethyl linolenate effective ability to inhibit oxidants, cancer, and inflammation.[17] Ethyl ester was considered as an odor producing compound in many fruits benefits such as anticarcinogenic properties.[18] Phytol is potential dietary compounds for cancer prevention.[19] Palmitic acid decreased hepatocellular carcinoma cell membrane fluidity and limited glucose metabolism, and thus reduced cell proliferation and suppressed tumor growth and metastasis in mouse models.[20] Stearic acid diet in combination with cell therapy accelerates the recovering of hepatic dysfunction in a rat model of liver injury.[21] Trans-13-Octadecenoic acid showed the potential as an anti-inflammatory, anti-cancer, antioxidant.[22] Vulgarin have been reported to have anti-tumour as well as anti-inflammatory properties,[23] antioxidative, anti-allergic, nephroprotective, antimicrobial, anti-breast cancer, antidepressant properties and protective effects on type 1 diabetes.[24]

AVE showed cytotoxicity against liver cancer cell (HepG2) and cholangioma cell (KKU-M156) but it also showed cytotoxicity against normal cell by selective index (SI) value of 2.04 and 1.6. However, bioassay guided isolation was used to isolate active compounds with SRB assay, the active compound showed selective cytotoxic activity against liver cancer better than cholangiocarcinoma and surprisingly it had no cytotoxic activity against normal cell. The isolated compound was identified to be a flavonoid compound, cirsimaritin (4',5-dihydroxy-6,7-dimethoxyflavone or scrophulein). The criteria of National Cancer Institute (NCI) regards that the compound with cytotoxic value or IC_{50} must be less than 4 μg/mL and be selective with some types of cancer cell line without cytotoxicity against normal cell or the SI value of IC_{50} of normal cell/cancer cell more than 2 times. From these surprisingly results, cirsimaritin or scrophulein, a flavone or chemically known as 4',5-dihydroxy-6,7-dimethoxyflavone. This compound, cirsimaritin, showed high SI value against liver cancer as 54.94 and against cholangioma cell as 4.75

which were better than the two chemotherapeutic drugs, vincristine and paclitaxel (positive control; SI<1). This result was the first report of cirsimaritin which showed cytotoxic against cholangiocarcinoma, and it had found to be cytotoxic activity against liver cancer cell from *A. vulgaris* L. Cirsimaritin which was isolated from *Microtea debilis* have also been reported to be active against HepG2.[25]

The ethanolic extract of the aerial part of *A. vulgaris* L. showed cytotoxicity against HepG2 and KKU-M156 cell lines. Bioassay guided isolation was used to isolate active compounds with SRB assay. The F4 (MeOH: $CHCl_3$) fraction was separated by vacuum liquid chromatography (VLC) showed the highest percentage of the yield and the highest cytotoxicity against all types of cancer cell lines (HepG2 and KKU-M156) and had no toxicity against the normal-cell lines, HaCaT. The isolated compound was identified as Cirsimaritin, it showed better cytotoxicity against liver cancer cells than cholangiocarcinoma cell and was not cytotoxic to HaCaT cells, while the positive control, vincristine and paclitaxel, exerted strong cytotoxicity against normal cells. The GC-MS analysis of AVE showed several cytotoxic compounds which have been previously reported. AVE which was used as an antipyretic in cancer patients could have a potential for cancer treatment especially liver cancer. Cirsimaritin was its active compound which showed selective cytotoxic activity and should be further studied on cytotoxic mechanism and further developed as an anti-cancer drug because it had no cytotoxic activity against normal cell. This compound should be used for active cytotoxic marker of AVE. This study supports the use of this plant for treating liver cancer patients according to Thai traditional medicine principle, i.e. the bitter herbs are used for diseases of bitter organ such as liver cancer.

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All authors report no conflicts of interest relevant to this article.

Author Contributions

P. Maki carried out the experiments and wrote the draft manuscript with support from A. Itharat and P. Thongdeeying who are supervisor of P. Maki. A. Itharat is a project manager and provide funding of this project. W. Pipatrattanaseree verified the analytical methods. N.M. Davies provided grammatical revisions to the manuscript. All authors read and approved the final version of the manuscript.

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