Artemisia vulgaris inhibits BCR/ABL and promotes apoptosis in chronic myeloid leukemia cells

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Abstract. In previous research, the authors demonstrated that the methanol extract of *Artemisia vulgaris* (AVM) has the ability to inhibit chronic myeloid leukemia (CML) cell proliferation. The aim of the present study was to determine and clarify the mechanism of action of AVM. BCR/ABL activation is present in >90% of CML cases. As a result, cells expressing different forms of BCR/ABL were recruited for the present study, including K562 (human wild-type) or TCCY-T315I (human imatinib-resistant) and the Ba/F3-(T315I/E279K/Y253H) (mouse BCR/ABL point mutation-transfected cells). The results revealed that AVM inhibited the phosphorylation of BCR/ABL and their subsequent molecular signals including AKT and MAPK activation. AVM induced the release of cleaved PARP and cleaved caspase-3 caused apoptosis and inhibited the viability of these cells. Interestingly, AVM appeared to be more sensitive to imatinib-resistant (T315I, Y253H, and E279K) than wild-type BCR/ABL cells, indicating its potential to overcome imatinib-resistant severe issues in CML. Moreover, the effects of various sub-fractions of AVM were then investigated in order to determine the optimal solvent for the identification of anticancer bioactive compounds. The results demonstrated that the ethyl acetate and chloroform fractions were more effective than the n-hexane and water fractions. It is thus concluded that AVM inhibits the activity of BCR/ABL and their subsequent molecular signals, including AKT and MAPK, resulting in cytotoxicity via apoptosis.

Introduction

Chronic myeloid leukemia (CML) is a malignancy of the blood and bone marrow that affects children and adults. An abnormal chromosomal translocation known as t(9;22) results in the establishment of the Philadelphia chromosome, which contains the BCR-ABL gene, culminating in the development of this syndrome (1). Due to the introduction of imatinib, a tyrosine kinase inhibitor (TKI), CML patients now benefit from treatment (2). However, imatinib resistance has developed as a significant issue. At least four generations of TKIs have been produced and demonstrated to have therapeutic efficacy (3). However, not all TKI resistance issues have been resolved.

The mechanism of resistance has been the subject of numerous studies. Alterations in the *BCR-ABL* sequences are one of the most common explanations (3). Additionally, several resistance mechanisms, such as *BCR-ABL* genomic amplification, are considered (4). Focusing on preventing BCR-ABL activation is quite efficient, as imatinib has demonstrated, but may not be the optimal option. Combining reagents with distinct mechanisms of action may be the solution to the problem of drug resistance (5).

Traditional medicine, which focuses on plants that have medicinal effects, has attracted criticism from a wide range of scientists. Herbal medicine has been shown to be effective against cancer in a vast number of trials, however the specific mechanism by which they work remains a mystery to scientists to date. It could be a combination of acts with varying degrees of variance (6). However, due to their long history of use in the community, most herbal medicine is safe for humans, with a few exceptions (7). As such, it represents a potential source of effective therapeutic reagents for human use.

Artemisia vulgaris (*A. vulgaris*) is a significant medicinal plant species belonging to the genus *Artemisia*. It is most well-known for its volatile oils. Due of the chemical and biological richness of the genus *Artemisia*, it has garnered considerable interest. The discovery and isolation of the promising antimalarial medication artemisinin is one of the beneficial applications of *A. vulgaris* (8,9). *A. vulgaris* has a long history of being used to cure human illness. This medicinal plant is anti-malarial, anti-inflammatory, anti-hypertensive, antioxidant, anticancer, immunomodulatory, liver-protective, antispasmodic, and anti-infection (9).

In previous research, the authors demonstrated that the methanol extract of *A. vulgaris* (AVM) has the ability to inhibit the viability of CML cells (10). The aim of present study was to determine and clarify the mechanism of action of AVM. BCR/ABL activation is present in >90% of CML cases (11). Thus, cells expressing different forms of BCR/ABL were recruited for the present study, including K562 [human

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wild-type (WT)] or TCCY-T315I [human imatinib-resistant (IR)] and the Ba/F3-(T315I/E279K/Y253H) (mouse BCR/ABL point mutation-tranfected cells).

Materials and methods

Plant materials and standard extraction preparation. *A. vulgaris* was gathered in the southern region of Vietnam (Bay Nui-An Giang). As mentioned in a previous study, *A. vulgaris* was identified by herbalists at the Traditional Medical Center in Tinh-Bien, An-Giang, Vietnam (voucher no. BNAG-2017-0102) (10).

Drying of the samples was carried out in a dry oven at a temperature of 40°C until they were completely dry. Using a blender, dry samples were ground to a fine powder. The samples were then dissolved in methanol [1:10 (w/v)]. The powder and methanol mixture was continuously swirled at room temperature for four days before being filtered through a Whatman filter paper. To get crude methanol extracts, filtrates were evaporated at 40°C in a vacuum. The powder was then weighed and diluted in methanol yielding stock solutions of plant extracts (200 mg/ml). Subsequently, the solution was divided into aliquots and stored at 20°C until needed. The crude methanol extract was saturated in water and then partially fractionated with n-hexane (to generate the n-hexane fraction), chloroform (to generate the chloroform fraction), ethyl acetate solvent (to generate the ethyl acetate fraction), and finally distilled water to reach the aqueous fraction.

Cell lines, culture conditions. Professor Yuko Sato (University of Tokyo, Tokyo, Japan) provided the human leukemia cell lines TCCY-T315I, K562, and Mus musculus (B cells) Ba/F3 cells; the Ba/F3 cells with E279K, Y253H and T315I were created by the authors as previously described (12). A humidified incubator (5% CO_2 at 37°C) was used to grow Vero cells (ATCC CCL-81™; American Type Culture Collection) in DMEM and other cells (TCCY-T315I, K562, Ba/F3-E279K/Y253H/T315I) in RPMI-1640 medium (both from Sigma-Aldrich; Merck KGaA) supplied with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.) and 100 IU/ml penicillin and 0.1 mg/ml streptomycin.

Western blot analysis. At a density of $1x10^5$ cells/ml, the cells (K562 and TCCY-T315I) were plated onto a 10-cm dish with varied AVM concentrations (25, 50 or 100 μg/ml). Cells were collected and washed twice with PBS (-) after the specified incubation durations (8, 16 or 24 h). On ice for 30 min, the cells were dissolved in a protein lysis buffer ($Na_2H_2P_2O_7$ 10 mM, NaF 50 mM, EDTA 5 mM, Na_3VO_4 1 mM, HEPES 5 mM, phenylmethylsulfonyl fluoride 1 mM, Triton X-100 0.01%, NaCl 150 mM, and aprotinin 75 μg/ml). Total protein cell lysates were then obtained after centrifugation at 15,000 x g for 10 min at and 4°C. A total of 20 μg of total protein samples (determined by BCA protein assay kit) were placed into wells and separated through polyacrylamide gel electrophoresis (12.5%) and electroblotting onto a Hypond-P membrane (Amersham; Cytiva). Subsequently, 5% skim milk buffer was used to block the membrane for 1 h at room temperature. Antibodies were employed to probe the membrane after washing, and antibody binding was detected using enhanced

Table I. Effect of AVM on the cell proliferation of leukemia cell lines, determined using the IC_{50} value and SI.

Leukemia cell lines	IC_{50} (μg/ml)	SI
K562	44.27±1.42	3.46
TCCY-T315I	29.81±1.61	5.14
Ba/F3-T315I	4.78±0.19	31.98
Ba/F3-Y253H	26.34±0.94	5.81
Ba/F3-E279K	18.89±0.70	8.11

AVM, methanol extract of *Artemisia vulgaris*; SI, selectivity index.
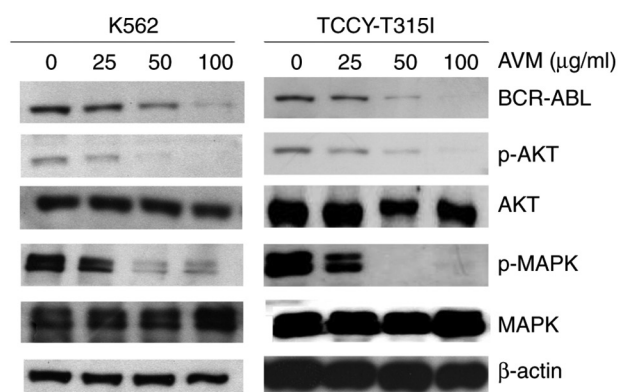

Figure 1. AVM has the ability to inhibit BCR-ABL expression. K562 and TCCY-T315I cells were treated with the specified concentrations of AVM or DMSO alone as a control. Western blot analysis with the indicated antibodies was performed on whole cell lysates. AVM, methanol extract of *Artemisia vulgaris*.

chemiluminescence ECL (Amersham; Cytiva). The following primary antibodies were used: anti-c-Abl (cat. no. sc-23; dilution 1:500; Santa Cruz Biotechnology, Inc.), anti-actin (cat. no. A2066; 1:1,000; Sigma-Aldrich; Merck KGaA), caspase-3 (1:1,000; cat. no. 9662), phosphorylated (p)-p44/42 MAPK (Thr202/Tyr204; 1:1,000; cat. no. 9101S), and AKT (1:1,000; cat. no. 9272; all from Cell Signaling Technology, Inc.), and anti-PARP (1:1,000; cat. no. 016-16831; FUJIFILM Wako Pure Chemical Corporation). The primary antibodies were incubated for 1 h at room temperature or overnight at 4°C. Subsequently, the membranes were washed for 15 min, twice, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody [1:1,000 anti-mouse IgG HRP (cat. no. sc-2031) or anti-rabbit IgG HRP (cat. no. sc-2317)] provided by Santa Cruz Biotechnology, Inc. for 1 h at room temperature.

Cell viability. Cell viability was assessed using the trypan blue dye exclusion test on suspension cells, as previously described (13). The half maximal inhibitory concentration (IC_{50}) was determined by plotting a graph between the various concentrations of AVM (6.25, 12.5, 25, 50 and 100 μg/ml) and the percentage of inhibition in cell viability. The ratio of the IC_{50} value for Vero cells to the IC_{50} value for cancer cell lines (K562, TCCY-T315I, Ba/F3-T315I/Y253H/E279K) was used to construct the selectivity index (SI). The SI values imply
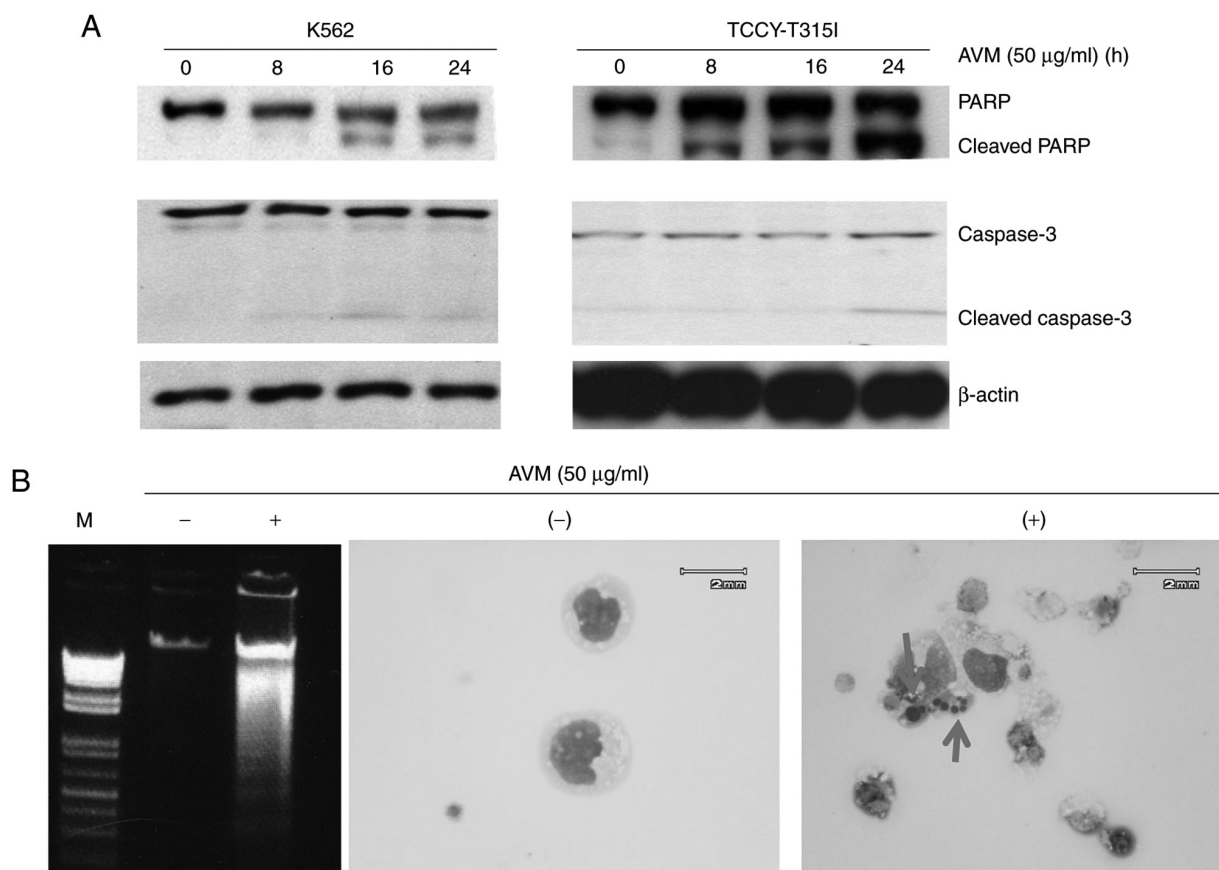
Figure 2. AVM induces apoptosis in cells. (A) K562 and TCCY-T315I cells were treated with 50 μg/ml AVM. Western blot analysis was performed on cell lysates using caspase-3 and PARP antibodies. The results indicated that AVM promoted the release of cleaved caspase-3 and cleaved PARP. (B) TCCY-T315I cells were treated with 50 μg/ml AVM for 24 h. The results revealed that the DNA was fragmented (left panel). In addition, the nucleus was not whole, and the arrows indicate that there were apoptotic bodies (right panel). AVM, methanol extract of *Artemisia vulgaris*.

that the plant extracts kill leukemia cells preferentially, rather than being non-selective cytotoxic extracts. SI values >3 were considered to be highly selective for cancer cells (14).

Morphological changes in AVM-treated cells. Cells (K562, TCCY-T315I, Ba/F3-T315I/Y253H/E279K) were seeded at a density of $1x10^5$ cells/well in six-well culture plates, overnight. Subsequently, the cells were treated with various concentrations of AVM (50 and 100 μg/ml) and maintained at 37°C with 5% CO_2 for 72 h. The cells that were not treated acted as a control. A 10-fold magnification-inverted light microscope was used to detect the morphological changes in the cells.

Detection of DNA fragmentation. TCCY-T315I cells were treated with or without 50 μg/ml AVM for 3 days. Subsequently, the cells were harvested and total genomic DNA was extracted using a standard procedure. A total of 10 μg genomic DNA from each sample was blotted and electrophoresed on a 1.2% agarose gel for the DNA fragmentation experiment. UV light was used to detect DNA fragmentation.

Statistical analysis. Data were compiled from three independent experiments and were presented as the mean ± SEM. Data were compared using unpaired Student's t-test or one-way ANOVA with Tukey's post hoc test. GraphPad Prism version 8.3.0 (GraphPad Software, Inc.) was used to perform

statistical analysis. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

AVM suppresses the expression of BCR-ABL. BCR/ABL activation is present in >90% of CML cases (15). Thus, it was investigated whether treatment with AVM had any effect on BCR-ABL expression. For 24 h, K562 and TCCY-T315I cells were treated with varying concentrations of the AVM extract. BCR-ABL1 is ~210 kDa in size (11). As illustrated in the western blot results of Fig. 1, BCR-ABL expression was dose-dependently decreased. Additionally, downstream signaling pathways of BCR/ABL, including as AKT and MAPK, were impacted, consistent with BCR-ABL inhibition (Fig. 1). These results indicated that the antileukemic activity of AVM may be mediated by disruption of the BCR/ABL signaling cascade.

Cell apoptosis is induced by AVM. The purpose of the following experiments was to determine whether treatment of cells with the AVM extract induces apoptotic biomarkers such as caspase activation, DNA fragmentation, and an intact nucleus in cells. K562 and TCCY-T315I cells were co-cultured with 50 μg/ml of AVM for 24 h. Subsequently, western blotting was performed to determine the effect of AVM on molecule
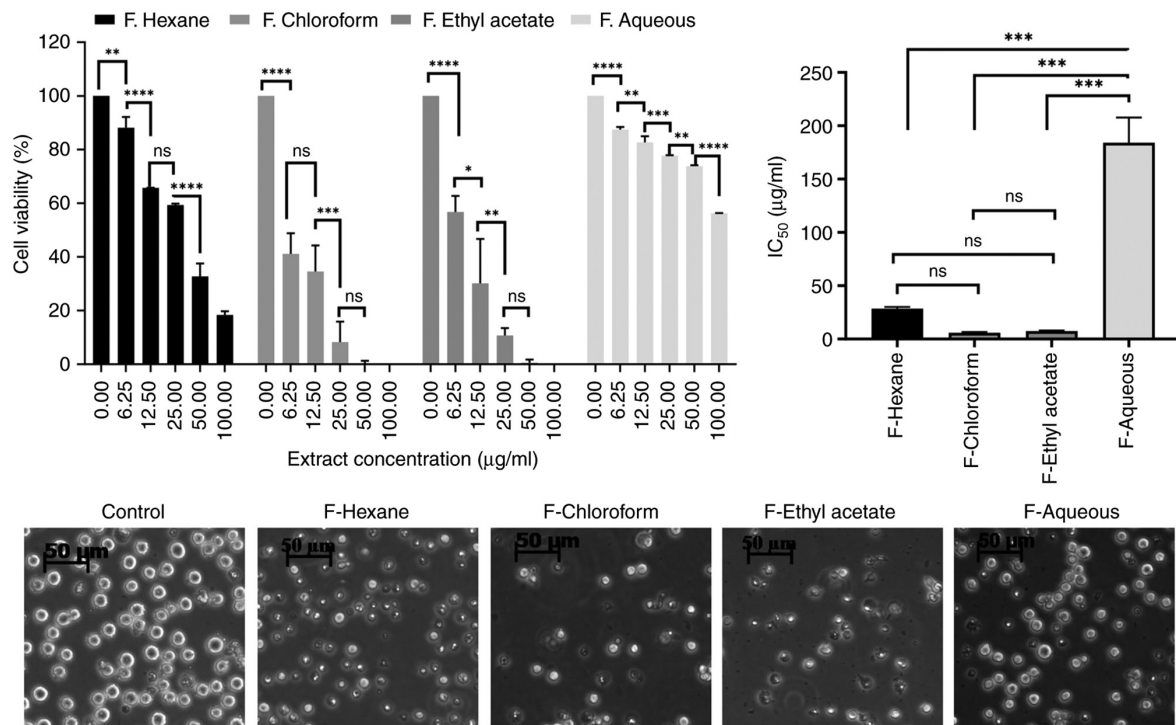
Figure 3. Chloroform and ethyl acetate fractions of the AVM are responsible for the majority of the antileukemia activity. Different concentrations of n-hexane, chloroform, ethyl acetate, and the aqueous fraction of the AVM were co-cultured with K562 cells and cell viability was determined as indicated in Materials and methods, followed by observation under phase contrast inverted microscope. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. AVM, methanol extract of *Artemisia vulgaris*; ns, not significant.
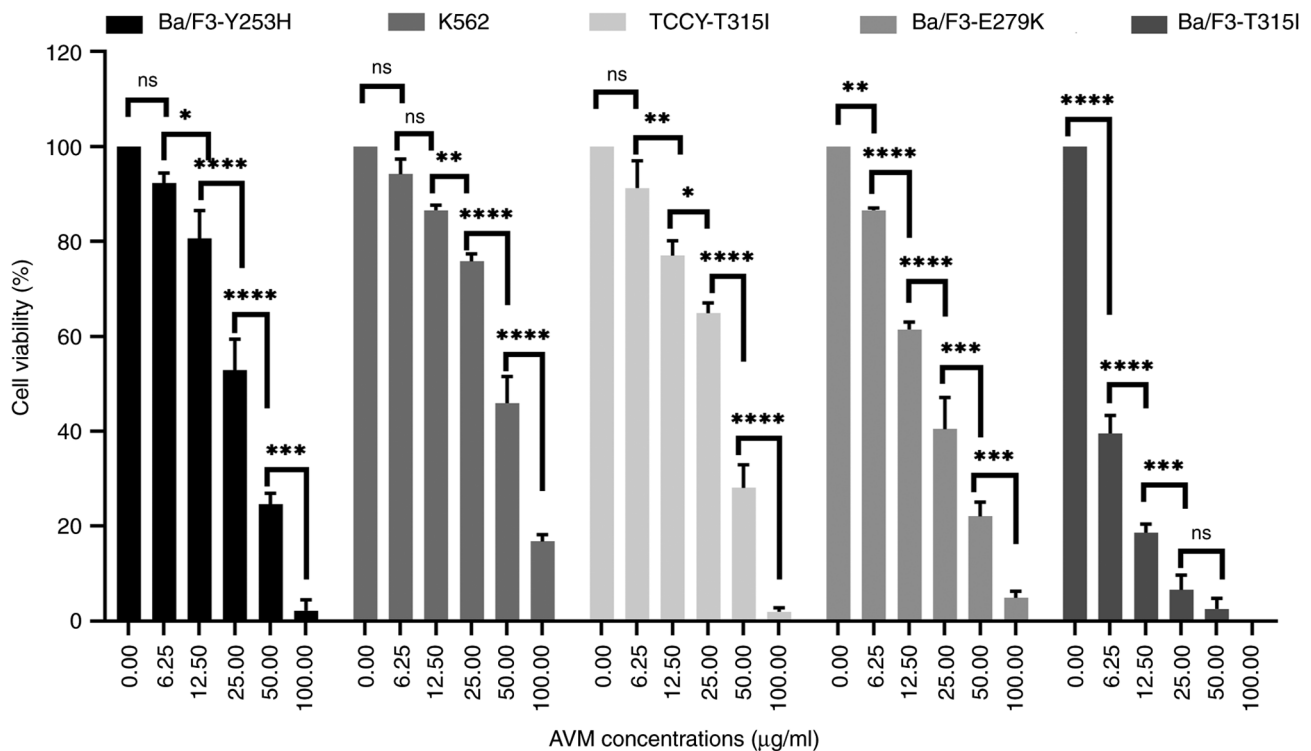

Figure 4. Cell viability is inhibited by treatment with AVM in a dose-dependent manner. AVM was added to Ba/F3-(Y253H/E279K/T315I), TCCY-T315I as well as K562 cells for three days at various concentrations (6.25-100 µg/ml), and the viability of cells was assessedby Trypan blue test. Three independent tests were carried out. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. AVM, methanol extract of *Artemisia vulgaris*; ns, not significant.

activation of the caspase pathway. It was observed that PARP and caspase-3 molecules were cleaved (Fig. 2A), indicating that apoptosis occurred in leukemia cells following AVM treatment. Moreover, the total DNA was extracted and run
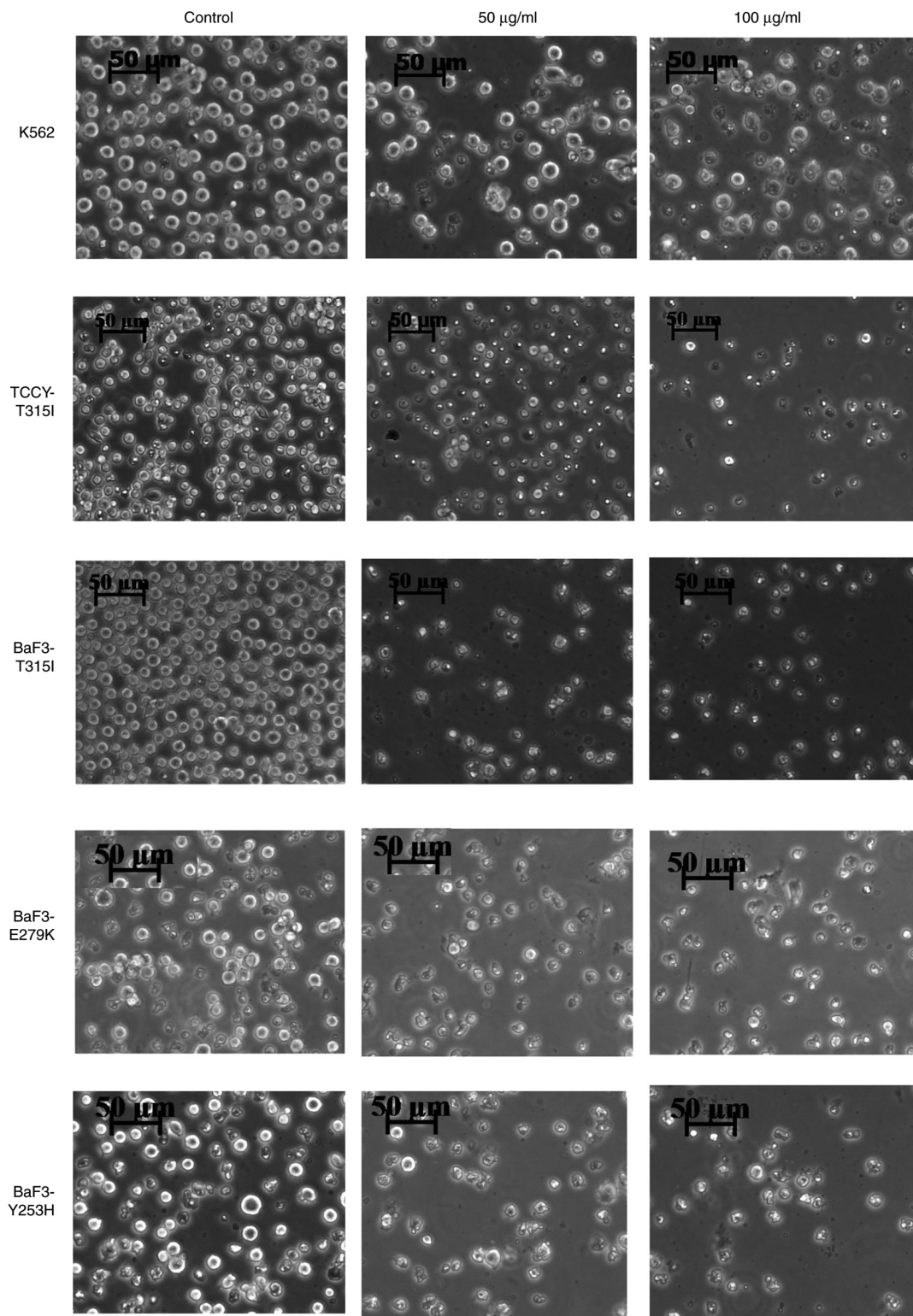
Figure 5. Morphological changes in AVM-treated cells. The Ba/F3-(T315I/E279K/Y253H), TCCY-T315I as well as K562 cells were treated with 50 or 100 μg/ml of AVM for 24 h and then viewed under phase contrast inverted microscope. AVM, methanol extract of *Artemisia vulgaris*.

through electrophoresis, and the results showed the fragmentation of DNA (Fig. 2B, left panel). To further demonstrate apoptosis, the nucleus of cells was stained; as revealed in Fig. 2B (right panel), the nucleus was broken in cells treated with AVM. In comparison, control cells retained their nucleus (untreated with AVM). Taken together, apoptosis was the form of cell death produced by the AVM extract.

Chloroform and ethyl acetate fractions of the AVM extract cause the main anti-leukemic effect. Since the AVM crude extract contains numerous different components, it was dissolved into solvents ranging from non-polar (n-hexane) to low polarity (chloroform), medium polarity (ethyl acetate), and strong polarity (water), and generated fractions, which were then assessed for toxicity to K562 cell viablity. Interestingly, it
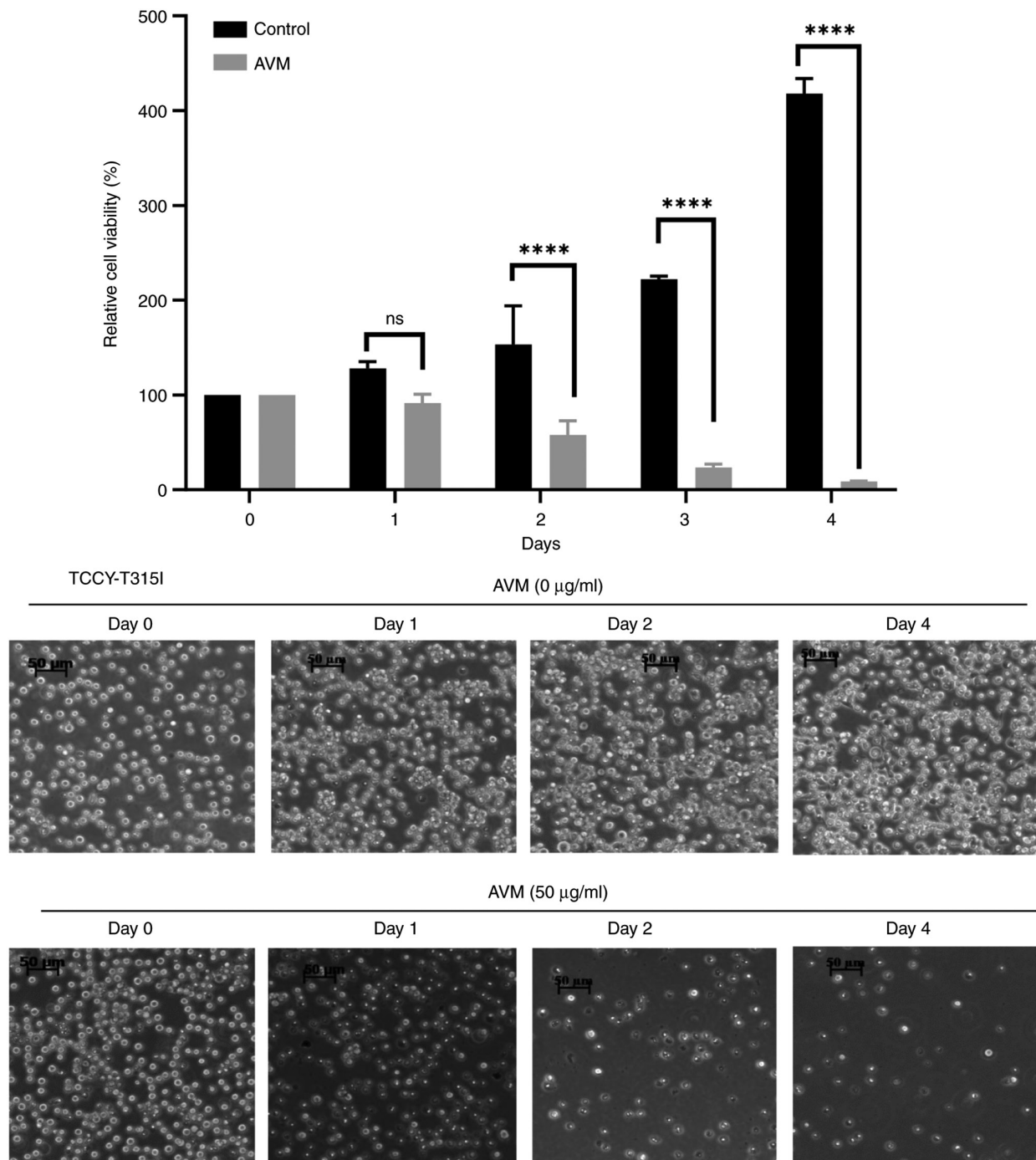
Figure 6. Time-dependent effect of AVM. Phase contrast inverted microscopy was used to observe the TCCY-T315I cells cultured with AVM (50 μg/ml) for varied time-points (0 to 4 days) and cell viability was assessed as indicated in Materials and methods. Cell viability was calculated relative to day 0. ****P<0.0001. AVM, methanol extract of *Artemisia vulgaris*; ns, not significant.

was determined that the chloroform and ethyl acetate fractions suppressed the viability of K562 cells very effectively, whereas the aqueous fraction did not (Fig. 3).

Potential of AVM to overcome imatinib-resistance. Despite the marked success of imatinib in improving survival rates (16), resistance development continues to be a challenge. The mechanism of resistance most frequently described is point mutations in the BCR-ABL1 gene. Numerous studies indicate that BCR-ABL point mutations are detected at a rate of 12-63% in imatinib-resistant patients with CML. Over 90

different mutations have been found, but ~2/3 of mutated cases have amino acid substitutions in T315, Y253, E255, M351, G250, F359, and H396 (17,18). In this next experiment, cells with BCR/ABL point mutations including human TCCY-T315I and stable murine-transfected Ba/F3 cells with BCR/ABL point mutations including T315I, Y253H, and E279K, were used.

It was first verified whether AVM affected the viability of imatinib-resistant cells. The viability rate of the cells was determined using a trypan blue test. The results demonstrated that the AVM extract inhibited the viability of K562 and TCCY-T315I
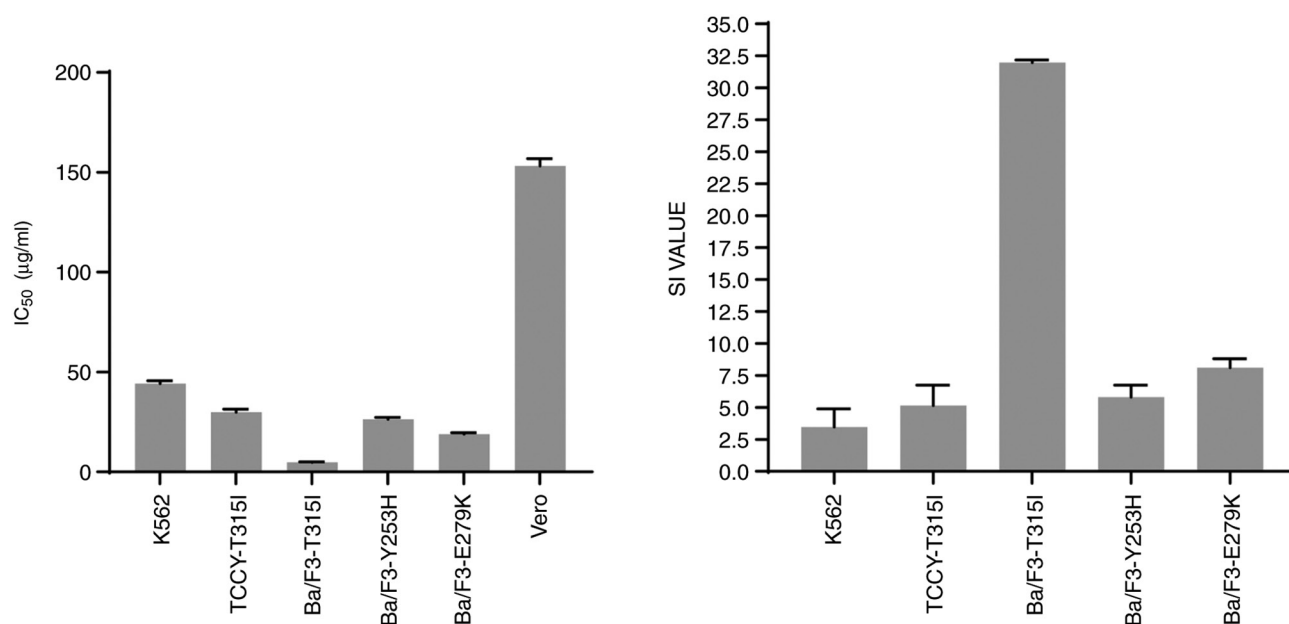
Figure 7. IC_{50} and SI values of AVM. The IC_{50} value of AVM in Ba/F3-(T315I/Y253H/E279K), TCCY-T315I, K562 and Vero cells (left). The SI value of AVM was derived by dividing the IC_{50} against Vero cells by the IC_{50} against leukemia cells (right). AVM, methanol extract of *Artemisia vulgaris*; SI, selectivity index.

cells (Fig. 4), indicating that the AVM extract could suppress the viability of human leukemic cells. Similar results were obtained with the murine Ba/F3-(T315I/Y253H/E279K). This finding is significant because it suggests that AVM may be able to overcome imatinib resistance, allowing it to be used in the future as a new tyrosine kinase inhibitor.

The morphological changes of these cells were observed using a phase contrast inverted microscope following treatment with AVM at concentrations of 50 as well as 100 μg/ml for 24 h. Cells appeared to have shrunken in size (Fig. 5). Interestingly, this finding corresponds to the results presented in Figs. 2 and 4.

Effect of AVM on the viability of TCCY-T315I cells in a time-dependent manner. To determine whether AVM has a time-dependent effect on cells, a time-dependent experiment with TCCY-T315I cells was performed. The results indicated that cells treated with control (methanol alone) continued to develop well up to the 4th day. However, cells treated with 50 μg/ml AVM extract did not grow and were almost completely attenuated by day 4 of culture (Fig. 6). This study demonstrated, once again, that AVM may decrease cell viability in a dose- and time-dependent manner, as previously demonstrated.

Because the SI value reflects the differential behavior of the extract, the higher the SI value, the more selective the extract. An SI number >3 units reflects the general toxicity of the pure chemical (14). The SI value in this study was calculated as the difference between the IC_{50} values of the Vero cells and cancer cell lines. It was clear from the results obtained that the Vero cells were the least sensitive to the action of AVM extract compared with the other cell-lines (left panel, Fig. 7). The AVM extract had SI values >3 (Table I and Fig. 7, right panel), indicating that it had a significant cytotoxic action and strong selectivity against BCR-ABL leukemia cells.

Discussion

Cancer is a leading cause of death worldwide, owing to factors such as late diagnosis, poor prognosis, and resistance to chemotherapy and radiation. A significant increase in cancer-related mortality is projected in the following several decades (19). Although treatment with imatinib, for CML with BCR-ABL, or erlotinib, for non-small cell lung cancer have been found to be effective, treatment resistance continues to be a challenge that must be resolved in order to provide improved healthcare (20,21). Reagents for evaluating possible candidates for therapeutic use can be found in natural chemicals generated from plants. In fact, a significant number of cancer-fighting drugs are natural compounds or are derived from natural substances (22). *Artemisia santolina, Artemisia kulbadica, Artemisia diffusa, Artemisia turanica* and *Artemisia sieberi* extracts demonstrated cytotoxic activity against a variety of cancer cell lines (23). Recently, it was demonstrated that AVM significantly decreases the viability of a hepatocellular cancer cell line (24) Additionally, *A. vulgaris* essential oil has been demonstrated to cause apoptosis in leukemic cell line, HL-60 (25). In the present study, it was determined that AVM inhibited the development of leukemia cells and induced death in CML cells. The present study is consistent with a previous study (10). In a previous study, it was discovered that AVM contains a significant amount of courmarin and flavonoid (10). As a result, it is reasonable to assert that these secondary metabolite groups contributed to the anti-leukemic action of AVM.

Previous research has demonstrated that the IC_{50} values of AVM vary greatly among cancer cell lines, such as 50 ng/ml for human HCT-15 colon cancer (24), 100 mg/ml for hepatocellular carcinoma (HepG2) (24), 190 μg/ml for MCF7, 778 μg/ml for A549, 284 μg/ml for HeLa, 317 μg/ml for A7R5, and 317 μg/ml for 293T cells (26). Compared with the preceding findings, leukemia cells appear to be significantly

more sensitive. Further study in an *in vivo* model is required to further elucidate the toxicity of AVM.

As was revealed, AVM may also alter the levels of BCR-ABL, which in turn affects the stimulation of MAPK and AKT (Fig. 1). The oncoprotein BCR-ABL was revealed to play a role in the development of leukemia. BCR-ABL inhibitors have been developed and successfully used in clinical treatment based on earlier findings (27). Additional therapeutic strategies, however, have aimed to decrease BCR-ABL expression in order to inhibit BCR-ABL signals. In the present study it was demonstrated that AVM treatment inhibits BCR-ABL. Subsequently, the downstream signals of the BCR-ABL cascade may be impacted as well. This finding may shed light on the mechanism by which AVM affects leukemia cells.

Several important post-translational modifications, such as ubiquitination, SUMOylation, phosphorylation, neddylation, and acetylation, control how active and stable the BCR-ABL protein is (28). It has been shown that the ubiquitin-proteasome (UPP) pathway can be used to break down the BCR-ABL protein (29). Protein ubiquitination is a process that uses a bioactive enzyme called the ubiquitin-conjugate enzyme and a protein called the ubiquitin ligase (30). It has been reported that the ubiquitin ligases CHIP11, c-CBL, and SH2-U-box12 cause BCR-ABL to multiply and then break down. However, adding ubiquitin to a protein is a dynamic process, and a certain enzyme called deubiquitinase can remove ubiquitin molecules that have already been attached (Dub) (31-33). Several Dubs, such as USP25 (31), HAUSP (USP7) (32), and USP9x (33), have been found to be linked to BCR-ABL1. Recent research has revealed that Dub USP7 keeps BCR-ABL and USP7/BCR-ABL from becoming unstable (32). USP7 interacts with the Y-kinase domain of BCR-ABL. Due to this, USP7 and BCR-ABL can form a positive feedback loop in which USP7 stabilizes BCR-ABL and BCR-ABL phosphorylates and activates USP7, which further promotes the pathophysiology of CML (32). Thus, drugs that inhibit the function of USP7 could also be used to treat CML.

Artesunate is a semisynthetic version of the active ingredient in the Chinese herb *Artemisia annua*, which is called artemisinin (34). It has been shown that artesunate inhibited the interaction between USP7 and BCR-ABL. This led to more polyubiquitination of BCR-ABL, which accelerated its breakdown. Notably, artesunate functioned well with imatinib, which is the main drug used to treat CML and is a specific inhibitor of BCR-ABL kinase, to cause CML cells to commit suicide (apoptosis) (32). Artemisinin is the primary bioactive compound in *A. vulgaris*. (8). Thus, it is considered that it is possible that AVM slows the viability of CML cells through a mechanism that affects how USP7 and BCR-ABL work together. However, this hypothesis needs to be assessed in more detail to be confirmed.

It is important to note that AVM has a negligible effect on Vero cells when compared to leukemia cells in the laboratory (Fig. 7). In addition, in the present study it was determined that AVM had a high selective effect on aberrant leukemia cells or Ba/F3 cells that had been transfected with the oncogene BCR-ABL (Fig. 7 and Table I). This finding suggests that AVM may have potential in treatment for leukemia. However, additional research is necessary before AVM can be applied in practice.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HTC designed the study and performed the experiments. BTKL analyzed the data and wrote the manuscript. BTKL and HTC confirm the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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