

## ORIGINAL ARTICLE

# Screening of medicinal herbs for cytotoxic activity to leukemia cells

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## Summary

**Purpose:** This study aimed to find a new source of anti-leukemia agents from Vietnamese medicinal plants.

**Methods:** The human leukemia cell lines TCCY, KU-812, TCC-S, KOPB-26, and HL60 were used. The crude ethanol extracts of 17 medicinal plants were collected and evaluated for their cytotoxicity against these leukemia cell lines by the trypan blue dye exclusion test. Morphological changes of cells were observed under phase-contrast inverted microscope. Bioactive compounds were evaluated using the method described by Ciulei. 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was carried out for evaluating the antioxidant effect.

**Results:** Among the tested samples, *Artemisia vulgaris* (*A.vulgaris*) crude ethanol extract effectively inhibited the viability of leukemia cell in both dose and time-dependent

manner. The  $IC_{50}$  value was different for cell lines and ranged from  $18.07 \pm 1.64 \mu\text{g/ml}$  to  $45.87 \pm 3.49 \mu\text{g/ml}$ . Moreover, the phytoconstituents analysis results showed coumarin, flavonoid, anthocyanin, cardiac glycoside, tannins, reduced sugar compounds were present in the *A.vulgaris* extract. The total polyphenol and flavonoid contents of the dry extract were calculated as 3.81 mg GAE/g dry weight and 11.64 mg RUE/g dry weight of *A.vulgaris*. *A.vulgaris* exhibited antioxidant activity with  $IC_{50}$  is  $145.10 \pm 6.34 \mu\text{g/ml}$ .

**Conclusions:** Among the 17 Vietnamese plants used to treat a variety of cancer-related diseases, *A.vulgaris* has been able to suppress the growth of leukemia cells.

**Key words:** *A.vulgaris*, antioxidant, leukemia, Vietnamese, herbal plant

## Introduction

In 2018, registered were about 18.1 million new cases and 9.6 million cancer deaths worldwide [1]. Cancer is one of the most prevalent, dangerous diseases and the first leading cause of death before the age of 70 years in Vietnam [1]. The total number of new cases was 164,671 (approximately 0.17%) and the number of deaths was 114,871 (approximately 0.12%) of the Vietnamese population. Leukemia ranks 7<sup>th</sup> and accounted for 3.73% of new cancer cases in 2018 and but ranks 5<sup>th</sup> among the

leading causes of total deaths [1]. And like other low-middle income countries, Vietnam only received about 5% of the global cancer financial resources [2]. Therefore, in Vietnam cancer is a disease of the day and an economic burden for the nation.

Thanks to therapies such as radiation, chemotherapy, immunotherapy, stem cell therapy, etc., cancer patients today have a good chance to live. Chemotherapy is perhaps the most widely utilized.

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Received: 14/04/2020; Accepted: 19/05/2019

Drugs used in chemotherapy are also chemically or semi-synthetically synthesized. The side effects of these medications remain, however, an issue that needs to be tackled. More focus is slowly paid to research on the screening of natural compounds with anticancer activity. The available plant resources are vast, and their potential is limitless, so the continuation of such a research is necessary.

Plants still remain a primary source of drugs for cancer treatment and can lead to the development of novel anticancer agents. Evidence from ancient medicinal practices shows that phytochemicals have their inherent potential to cure diseases [3,4]. In addition, certain cancer chemotherapeutic

drugs obtained from natural sources have shown excellent impact [5]. In Vietnam, the roles of traditional medicine have been established and gradually contribute to support the health care system as an alternative treatment. There are over 12,000 plants and no less than 2,500 species have been used in ethnomedicine [6,7], but few Vietnamese plants have been investigated for anticancer activities [8-10].

The objective of this research was to screen 17 herbal medicinal plants for their antileukemia properties. The results indicated that *A. vulgaris* crude extract has been able to inhibit the growth of leukemia cells.

**Table 1.** The seventeen different plant species tested in the bioassays of this screening study

Botanical name	Common name	Family	Part tested	Place	Voucher number
<i>Asparagus cochinchinensis</i>	Chinese Asparagus (Thiên môn)	Asparagaceae	Roots and rhizome	An Giang	BNAG-2017-0101
<i>Artemisia vulgaris</i>	Mugwort (Ngải cứu)	Asteraceae	Aerial parts	An Giang	BNAG-2017-0102
<i>Boerhavia diffusa</i> L.	Punarnava (Sâm đất)	Nyctaginaceae	Roots	An Giang	BNAG-2017-0103
<i>Eurycoma longifolia</i>	Tongkat ali (Mật nhân)	Simaroubaceae		An Giang	BNAG-2017-0104
<i>Houttuynia cordata</i>	Fish mint (Diếp cá)	Saururaceae	Aerial parts	An Giang	BNAG-2017-0105
<i>Usticia gendarussa</i>	Willow-leaved justicia (Thanh táo)	Acanthaceae	Leaf	Ho Chi Minh	HCM-2017-0101
<i>Wedelia chinensis</i>	Chinese Wedelia (Sài đất)	Asteraceae	Aerial parts	An Giang	BNAG-2017-0107
<i>Dialium cochinchinensis</i>	Velvet Tamarind (Xay nhung)	Dialium	Seeds	Kon Tum	KT-2017-0101
<i>Plantago major</i>	Broadleaf plantain (Mã đề)	Plantaginaceae	Aerial parts	An Giang	BNAG-2017-0106
<i>Eclipta prostrata</i>	False daisy (Cò mực)	Asteraceae	Leaf and stem	An Giang	BNAG-2017-0107
<i>Acanthopanax senticosus</i> (Rupr. et Maxim.) Harms	Eleuthero (Ngũ gia bì gai)	Araliaceae	Stem	An Giang	BNAG-2017-0108
<i>Gynura procumbens</i> (Lour) Merr.	longevity spinach (Kim thất tai)	Asteraceae	Aerial parts	Da Lat	DL-2017-0101
<i>Lantana camara</i>	Umbelanterna (Ngũ sắc)	Verbenaceae	Leaf and stem	Ho Chi Minh	HCM-2017-0102
<i>Scoparia dulcis</i>	Goatweed (cam thảo)	Plantaginaceae	All parts	An Giang	BNAG-2017-0109
<i>Curcuma aromatica</i>	Wild turmeric (Nghệ trắng)	Zingiberaceae	Rhizome	An Giang	BNAG-2017-0110
<i>Lonicera japonica</i> Thunb	golden-and-silver honeysuckle (Kim ngân hoa)	Caprifoliaceae	Flowers	An Giang	BNAG-2017-0111
<i>Citrus hystrix</i>	Kaffir lime (Lá chúc)	Rutaceae	Leaf	An Giang	BNAG-2017-0112
<i>Plectranthus amboinicus</i>	Country Borage (Rau Tần dày lá)	Lamiaceae	Leaf	An Giang	BNAG-2017-0113

## Methods

### Plant materials and standard extraction preparation

Seventeen Vietnam medicinal plants were collected from distinctive areas within the south of Vietnam (Bay Nui-An Giang) in 2017 (Table 1). The different plants were identified by Dr. My Van Dang, a herbalist of the Traditional Medicine Centre, Tinh Bien, An Giang province. The voucher specimens (Table 1) have been deposited in this department's herbarium.

After collection, the samples were washed and dried in a dry oven until they were completely dry at 40°C. Dry samples were ground into fine powder form using a blender and they were mixed with methanol (1:10 (w/v)). The mixture of powder and methanol was swirled continually at room temperature for 4 days before being filtered using Whatman filter paper. Filtrates evaporated under vacuum at 40°C to produce crude methanol extracts. A total of 17 herbal extracts were obtained. By weighing the powder and dissolving it in methanol, stock solutions of plant extracts (200 mg/mL) were ready. The solution was divided into aliquots and kept at -20°C until used.

### Cell lines and culture conditions

The human leukemia cell lines TCCY, HL60, KU-812, TCC-S and KOPB-26 received from Prof. Yuko Sato (Tokyo, Japan) and the African green monkey kidney (Vero) cell lineage (ATCC CCL-81™) were used in this study [11,12]. Vero cells were grown in Dulbecco's modified

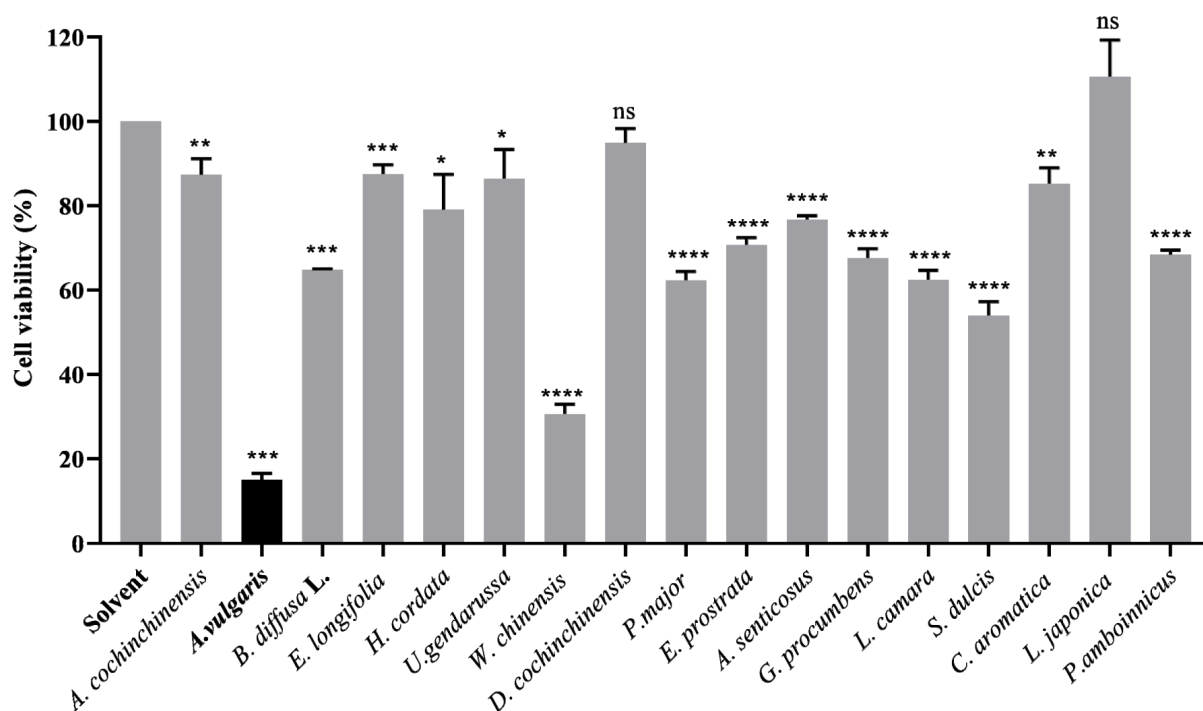
Eagle's medium (DMEM, Sigma- Aldrich, Ho Chi Minh City, Vietnam) and other cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Sigma-Aldrich, TBR Technology Corporation, Hochiminh City, Vietnam) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ThermoFisher Scientific, Hochiminh City, Vietnam), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (P4333, Sigma-Aldrich, Hochiminh City, Vietnam) in a humidified incubator of 5% CO<sub>2</sub> at 37°C.

### Cell viability

Cell proliferation was conducted on suspension cells and was determined by the trypan blue dye exclusion test as described previously [13]. The selectivity index (SI) was expressed as IC<sub>50</sub> value of Vero cell/IC<sub>50</sub> value of cancer cell line. The SI values indicate the plant extracts selectively kill leukemia cells and not just non-selective cytotoxic extracts. Samples with an SI value >3 were considered highly selective for cancer cells [14].

### Morphological changes of cells by phase-contrast microscope

TCCY cells were seeded in 6-well plates at a concentration of 10<sup>5</sup> cells per well and incubated overnight. Then, the cells were treated with different concentrations of plant extracts (0, 50 and 100 µg/ml) followed by 72-h incubation at 37°C with 5% CO<sub>2</sub>. The untreated cells served as control. The morphological changes of the cells were observed using an inverted light microscope at 10x magnification.



**Figure 1.** Cytotoxic effect of 17 whole plant extracts against TCCY cells. Plant extracts were added at 100 µg/ml to TCCY cells for 72h and cell viability assays were performed as described in Methods. The results are displayed as percentage of dead cells induced by each plant extract as compared to untreated cells (treated with ethanol at the same concentration as the plant extracts). Data are shown as mean ± SD of 3 independent experiments (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).

### Phytochemical screening

Bioactive compounds were evaluated using the method described by Ciulei [15].

### Determination of total content polyphenols and flavonoids

The total polyphenols contents (TPC) of the plant extracts was determined using Folin-Ciocalteu reagent [16] following a slightly modified method of Nunzia et al [17]. A volume of 200  $\mu$ L extract in a concentration of 1000  $\mu$ g/mL was mixed with 200  $\mu$ L Folin-Ciocalteu reagent 100%. The mixture was incubated at room temperature for 5 min, after which 1600  $\mu$ L of sodium carbonate solution 5% (w/v) was added. The reaction mixture was incubated at 40°C for 20 min before putting into 96-well plates and measured the absorbance at 765 nm with a VersaMax™ ELISA microplate reader. Zero to 500  $\mu$ g/mL of gallic acid was used for the linear equation of a standard curve. The content of TPC was expressed as mg/g gallic acid equivalent (GAE) of dry extract.

The total flavonoids contents (TFC) of the plant extracts was determined by using Aluminium Chloride Colorimetric Method [18]. The range of 0 to 500  $\mu$ g/mL of Rutin was used for the standard curve. 500  $\mu$ L of Rutin solution were added to 2000  $\mu$ L distilled water and 150  $\mu$ L  $\text{NaNO}_2$  5%. After incubating at room temperature for 6 min, 150  $\mu$ L  $\text{AlCl}_3$  10% were added and then incubated for 6 min at room temperature. A volume 2000  $\mu$ L of  $\text{NaOH}$  4% and 200  $\mu$ L distilled water were added to the mixture and incubated for 15 more min at room temperature before putting into 96-well plates and measured the absorbance at 510 nm with a microplate reader. The extract with a concentration of 1000  $\mu$ g/mL was determined to contain TFC as mg/g Rutin equivalent (RUE) of dry extract.

### Antioxidant properties

DPPH radical scavenging activity was carried out according to the method of Ghatak et al with slight modification [19]. The solution of DPPH 0.3 mM was prepared to react with the plant extract at a ratio of 1:1. The mixture's absorbance was measured at 517 nm after incubation at 37°C for 30 min. Vitamin C was used as positive control for this assay. The ability to scavenge DPPH radical scavenging activity was calculated using following formula:

$$\% \text{ DPPH radical scavenging} = \frac{[\text{sample} - \text{negative control}]}{[\text{negative control}]} \times 100$$

**Table 2.** The  $\text{IC}_{50}$  value and selectivity index (SI) of *A.vulgaris* extract on the growth of leukemia cell lines

Leukemia cell lines	<i>A.vulgaris</i>	
	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ )	SI
TCCY	$45.87 \pm 3.49$	3.34
TCC-S	$22.89 \pm 0.68$	6.68
HL60	$29.36 \pm 2.26$	5.21
KU-812	$18.07 \pm 1.64$	8.47
KOPB-26	$22.00 \pm 1.33$	6.96

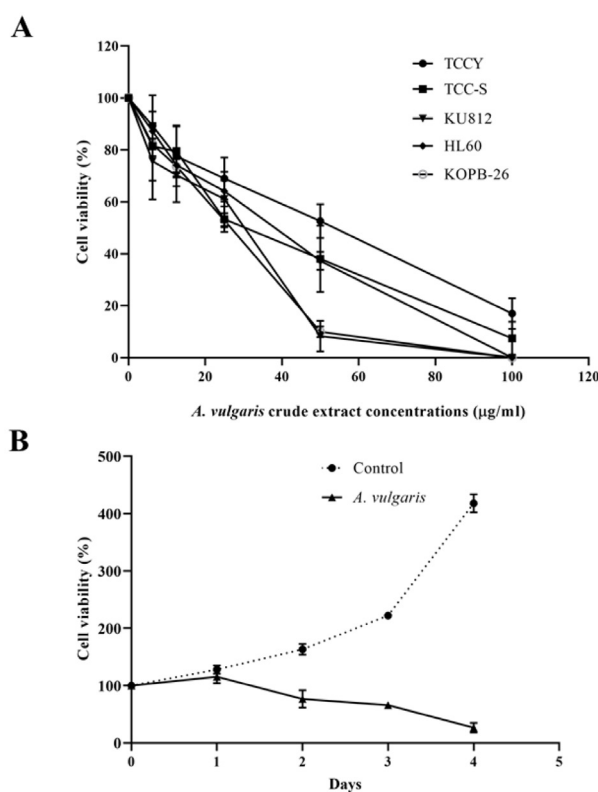
### Statistics

The data were analyzed using the unpaired Student's t-test between the control and compounds. A p value <0.05 was considered statistically significant. Data were compiled from three independent experiments and values were expressed as mean $\pm$ SD. For data calculations GraphPad prism software, version 8.3.0, was used.

## Results

### Screening plants for their inhibition activity on leukemia

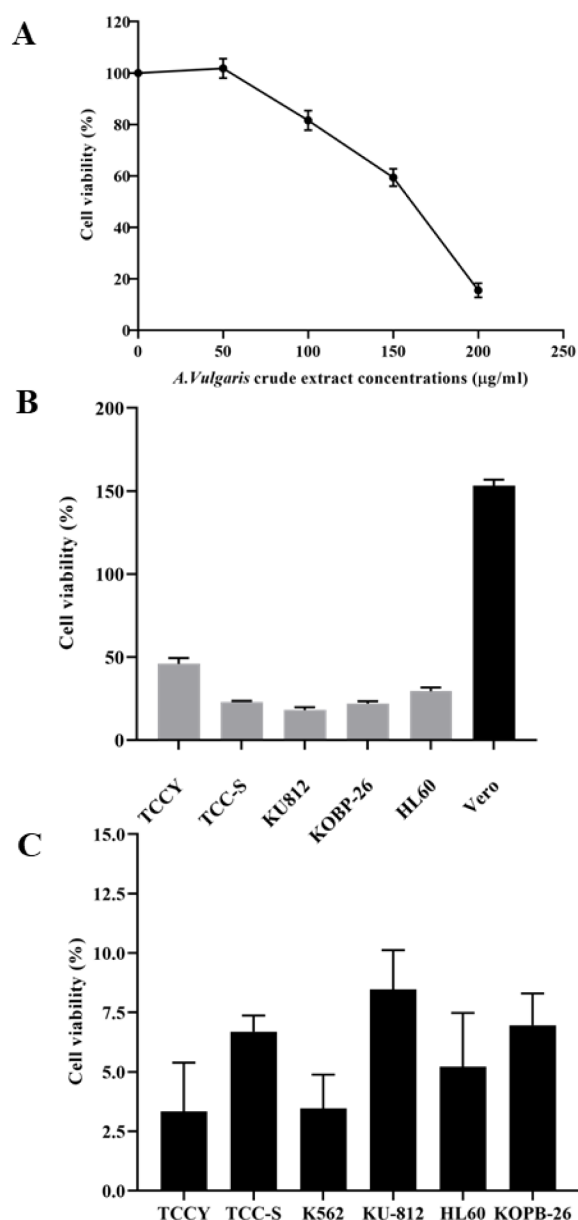
For searching potential anti-leukemia reagents, we used the TCCY cell line as the screening model for the primary screen. Seventeen selective plants were collected in different places in Vietnam, based on their known anticancer activity. All plant etha-



**Figure 2.** Cell growth inhibition induced by *A.vulgaris* ethanol extracts in dose- and time-dependent manner. **A:** Effect of *A.vulgaris* extract is dose-dependent. Plant extracts were added at different concentrations (6.25 to 100  $\mu\text{g/ml}$ ) to TCCY, TCC-S, HL60, KU-812, and KOPB-26 cells for 72h and cell viability assays were performed as described in Methods. The results are presented as a percentage of death from each plant extract in comparison with non-treated cells (handled with ethanol at the same concentration as plant extracts). The experiments were performed in triplicate. P values <0.0001 for all tested cells. **B:** Effect *A.vulgaris* extract is time-dependent. Plant extracts (50  $\mu\text{g/ml}$ ) were added to TCCY cells for various time periods (0 to 4 days) and cell viability assay was determined as described in Methods (p<0.0001).



nol extracts have been prepared and analyzed for their potential antitumor. We tested the effect of all plant extracts at the concentration of 100 µg/ml in TCCY cells. Results are presented in Figure 1. In all tested samples, *A.vulgaris* extract showed the strongest inhibition on the growth of cells. Thus, *A.vulgaris* extract was subjected to further test for phytochemicals as a potential candidate.

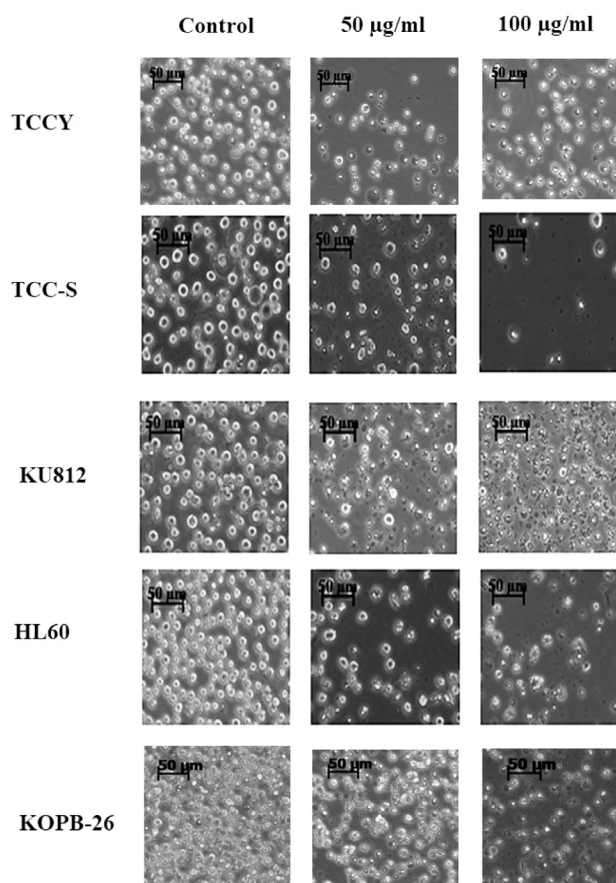


**Figure 3.** The  $IC_{50}$  and SI values of *A.vulgaris* ethanol extracts on cells. **A:** Plant extracts were added at different concentrations to Vero cells for 72h and cell viability assays were performed as described in Methods. **B:** The  $IC_{50}$  value of *A.vulgaris* ethanol extracts on TCCY, KU812, TCC-S, HL-60, KOPB-26, and Vero cell ( $p > 0.05$ ). **C:** The SI value of *A.vulgaris* ethanol extracts on TCCY, KU812, TCC-S, HL-60, and KOPB-26 cells was calculated by dividing  $IC_{50}$  against Vero cells to  $IC_{50}$  against leukemia cells. SI values of *A.vulgaris* were higher than 3 indicating that the *A.vulgaris* has potent cytotoxic activity and good selectivity against leukemia cells.

*A.vulgaris* inhibited the growth of leukemia cells in a dose and time-dependent manner

The most effective plant extracts *A.vulgaris* was first tested for their effect on different leukemia cell lines at different lower concentrations. As shown in Figure 2A, the two plant extracts inhibited cell growth also at lower concentrations, with varying efficacy depending on the specific plant extract and the tested cancer cell line, and the  $IC_{50}$  were recorded in Table 2 and were illustrated in Figure 3B. Inhibition of cell growth was also time-dependent, as demonstrated in Figure 2B.

Since the SI value indicates the differential behavior of the extract, the more selective it is, the higher the SI value. SI value greater than 3 units indicates the general toxicity of the pure compound [14]. In this study, the SI value was expressed as  $IC_{50}$  value of Vero cell (Figure 3A)/ $IC_{50}$  value of cancer cell line. The results showed that the SI values of *A.vulgaris* were higher than 3 (Table 2 and Figure



**Figure 4.** Morphological changes of cells by phase contrast microscope. TCCY, HL-60, KU812, KOPB-26 and TCC-S cells were treated with 0, 50 or 100 µg/ml of *A.vulgaris* ethanol extracts for 72h and then observed under phase contrast inverted microscope. Compared to untreated cells, treated-leukemia cells showed characteristics of apoptosis in a dose-dependent manner.

3C), indicating that the *A.vulgaris* extract had potent cytotoxic activity and good selectivity against leukemia cells.

#### Cell morphological changes using phase contrast inverted microscope

Apoptosis was based on certain morphological changes of cell death including cell shrinkage, chromatin condensation and fragmentation, and membrane blebbing [20,21]. In this test, the morphological properties of the TCCY cells were determined by Phase Contrast Inverted Microscope after exposure to different concentration of the plant extracts (50-100 µg/ml) and compared to untreated cell morphology. Differences in cell morphology have been identified in both treated and untreated cells after 3 days of culture. As shown in Figure 4, compared to untreated cells, treated-leukemia cells showed characteristics of apoptosis such as cell shrinkage and membrane blebbing in a dose-dependent manner.

#### Phytochemical screening

The results of phytochemical analysis (Table 3) show that the coumarin, flavonoid, anthocyanin, cardiac glycoside, tannins, reduced sugar compounds were present in the *A.vulgaris* extract.

**Table 3.** Secondary metabolites groups existing in *A.vulgaris*

Natural products	<i>A.vulgaris</i> fractions		
	Diethyl ether	Ethanol	Water
Alkaloid	±	±	±
Coumarin	++	+++	
Flavonoid		++	+
Cardiac glycoside		+	±
Saponin		-	-
Anthocyanin		+	+
LAC/PAC		±	±
Tanin		±	+
Reduced sugar		+	+
Organic acid		-	-
Polyuronid			-

+: weak intensity reaction. ++: Medium intensity reaction. +++: Strong intensity reaction. -: No reaction.

**Table 4.** Total phenols contents and total flavonoids contents in *A.vulgaris* extract

	<i>A.vulgaris</i>
% Yield	5.51
TPC (mgGAE/g dry wt)	3.81
TFC (mgRUE/g wt)	11.64

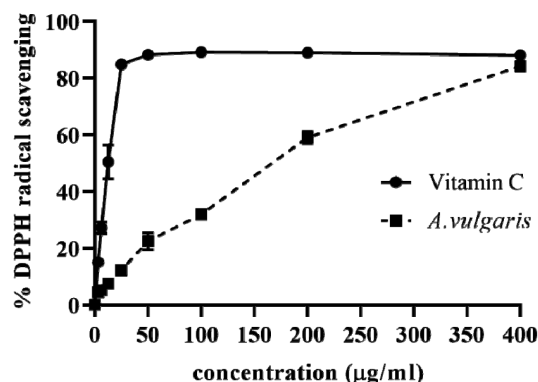
The TPC of *A.vulgaris* extracts were calculated as 3.81 mg GAE/g dry weight, respectively (Table 4). Meanwhile, TFC results indicated that these extracts were rich in flavonoid with a total flavonoid content of 11.64 mg RUE/g dry weight of *A.vulgaris* extract.

#### Antioxidant properties

*A.vulgaris* antioxidant properties as calculated by DPPH and DPPH- scavenging are shown by changing color from purple to yellow when the radicals come into contact with the antioxidants [22]. *A.vulgaris* extract scavenging DPPH activity was performed and compared with vitamin C. The findings shown in Figure 5 suggested that the antioxidant effect of *A.vulgaris* extract is not so high compared to positive control (vitamin C). The IC<sub>50</sub> value of DPPH assay was inferred by nonlinear regression analysis. As usual, vitamin C antioxidant had very low scavenging activity (IC<sub>50</sub> = 11.36±0.78 µg/ml) and the IC<sub>50</sub> value of *A.vulgaris* extract was quite high IC<sub>50</sub>, 145.10±6.34 µg/ml).

#### Discussion

Ethnopharmacological knowledge of traditional herbal remedies is a promising source for finding potential bioactive plant material. Plants that are used as traditional medicines represent an appropriate tool for the selection of plant candidates that may have anticancer properties [3]. In all 17 tested samples, *A.vulgaris* extract showed the strongest inhibition to the growth of cells (p=0.0003). Thus, *A.vulgaris* extract was subjected to further tests for phytochemicals as a potential candidate.



**Figure 5.** Antioxidant activity of the *A.vulgaris* ethanol extract. Determination of antioxidant activity of the *A.vulgaris* ethanol extracts by the DPPH method, using different concentrations, expressed as a percentage of antioxidant activity. The results showed that the antioxidant effect of *A.vulgaris* was not so high compared to positive control (vitamin C, p<0.0001). The experiments were performed in triplicate.

*A.vulgaris* is commonly used as a medicinal herb in traditional medicine in many countries including India [23], China [24], Nepal [25] and Vietnam. *A.vulgaris* is used in the Indian system of medicine as an emmenagogue, antihelmintic, antiseptic, antispasmodic, stomachic and in the treatment of respiratory and nervous diseases [23]. However, there is less scientific data on the anticancer inducing effect of *A.vulgaris*. In 2013, the methanolic extract of the leaves of *A.vulgaris* was shown to inhibit the growth of hepatocellular carcinoma (HepG2) cells *in vitro* with  $IC_{50}$  calculated around 100 µg/ml [26]. Recently, the methanolic extract of *A.vulgaris* has been shown to exhibit significant anticancer activity against colon cancer (HCT-15 cell line with an  $IC_{50}$  is 50 µg/ml) [24]. The methanol extract activity of flower, leaf, stem and root extracts of *A.vulgaris* could suppressed the proliferation of MCF-7 and HEK293 cell lines at very high concentration ( $IC_{50}$  value > 500 µg/ml) [27]. Another research by Saleh et al showed *A.vulgaris* essential oil mediated dose- and time-dependent apoptosis in human acute myelogenous leukemia (HL-60) cells via mitochondrial and caspase-dependent mechanisms [28]. Thus, compared to previous studies, the results of our study showed that *A.vulgaris* is more likely to selective toxicity to leukemia cells than other cancer cell lines with  $IC_{50}$  ranging from 18.07±1.64 (KU-812) to 45.87±3.49 (TCCY) µg/ml (Table 2).

Cell shrinkage as one of the foremost predominant morphological properties in nearly all of apoptotic cell death is caused by the extraordinary change in intracellular water. Despite the fact that necrotic cells assimilate the water resulting in enlarging their size and at long last burst, apoptotic cells lose water caused to cell shrinkage and lesser in the measure, taken after arrangement of the blebs at the cell surface due to the partition of the plasma membrane from the cytoskeleton [20]. The observational experiment displayed that the higher plant extracts concentration exposed to the leukemia cells, the more the destructive changes in the cell morphology compared to control cells.

These results indicated that *A.vulgaris* extracts exhibited time- and dose-dependent anticancer activity.

The traditional medicinal herbs are used for the therapy of diseases because of pharmacological actions which could be attributed to the presence of natural products such as alkaloids, flavonoids, tannins, etc. Some of them have potential to reduce the risk and progression of acute and chronic diseases like cancer, heart-related disease and strokes due to antioxidant effect [29]. The results presented in Table 3 showed that coumarin, flavonoid, anthocyanin, cardiac glycoside and tannins reduced sugar compounds present in the *A.vulgaris* extract. In plants, phenols and phenolic compounds play a major role in the plant's antioxidant function. It was known that there is a strong correlation between the amount of phenols and antioxidant activity, because the hydrogen groups of phenol compounds are excellent donors of hydrogen and have a high capacity for scavenging [30]. However, the findings shown in Figure 5 suggested that the antioxidant effect of *A.vulgaris* extract is not so high compared to positive control (vitamin C). A previous paper in Nepal has shown that the  $IC_{50}$  value of methanol extract of *A.vulgaris* was 48.77±0.11 µg/ml by Pandey et al [25]. Compared to this result, the scavenging properties of *A.vulgaris* in our study were less active, probably due to differences in the chemical composition of these extract, which require further studies to be clarified.

## Conclusions

Among the 17 Vietnamese plants used to treat a variety of cancer-related diseases, *A.vulgaris* has been able to suppress the growth of leukemia cells.

## Acknowledgements

We thank Prof. Yuko Sato (Tokyo, Japan), Dr. Hoang Thanh Chi (Ho Chi Minh City, Vietnam) for providing the cell lines utilized in those studies and Dr. My Van Dang for his help of plant collecting and identification.

## Conflict of interests

The authors declare no conflict of interests.

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