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Full Length Research Paper

Antiproliferative activity of different extracts from *Daphne altaica* Pall. on selected cancer cells

Murat Kizaibek[1,3], Marzia Daniar[2], Lin Li[2] and Halmurat Upur[3]*

[1]Traditional Kazakh Medicine Research Institute of Ili Kazakh Autonomous Prefecture, 835000 Yining, Xinjiang, P. R. China.
[2]Department of Biochemistry and Molecular Biology, College of Basic Medicine, Xinjiang Medical University, 830011 Urumqi, Xinjiang, P. R. China.
[3]Faculty of Traditional Uighur Medicine, Xinjiang Medical University, 830011 Urumqi, Xinjiang, P. R. China.

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***Daphne altaica* Pall. (Thymelaeaceae) is a medicinal plant that has long been used to treat cancer and respiratory ailments in Traditional Kazakh Medicine. In order to systematically evaluate its potential anticancer activity, six extracts of different polarity, namely: aqueous, n-butanol, ethyl acetate, chloroform, petroleum ether and ethanol extracts were obtained from this plant and they were tested for their antiproliferative effects on four human cancer cell lines: esophageal squamous cell carcinoma, gastric carcinoma, hepatoma and cervical carcinoma cells. Results from the proliferation assay showed that all extracts, except for aqueous extract, exhibited a dose-dependent growth inhibitory effect on all cancer cell lines. Of these extracts, two fractions obtained from partitioning of ethanol extract, namely: chloroform extract and ethyl acetate extract, could be considered a potential source of anticancer compounds. Further studies are necessary for identification and chemical characterization of the active principles.**

Key words: *Daphne altaica*, traditional Kazakh medicine, antiproliferative.

INTRODUCTION

Cancer is the world's second biggest killer after cardiovascular disease. In 2005, 7.6 million people died from cancer, more than HIV/AIDS, malaria and tuberculosis combined. In addition, this number is expected to rise to 9 million in 2015 and increase further to 11.5 million in 2030 (WHO, 2007). Clearly, there is a great need to improve current cancer therapies and to search for new therapies. Throughout history, plants have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy and biology. Within the sphere of cancer, plants have played an important role as a source of effective anti-cancer agents and it is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Cragg et al., 2005). In Ili Kazakh Autonomous Prefecture of China, herbal remedies are frequently used by traditional Kazakh medicine (TKM) doctors to treat a large variety of diseases, for example hypertension, cancer, rheumatism and bone fracture. However, there is little information about their efficacy. *Daphne altaica* Pall., locally known as *uwsoyqi* or *qasqir jiydek*, is a deciduous herb of the Thymelaeaceae family. It is endemic to the north of Jungar Basin of Xinjiang, China (the Tacheng and Habahe areas), Altai, Manrak and Tarbagatai Mountains of Kazakhstan and Altai region of Russia as well as northwest Mongolia (Eastwood et al., 2009; Delectis et al., 1999). *D. altaica* have long been used in TKM to treat esophagus cancer, gastric cancer, tracheitis, common cold, soar throat, rheumatism, snakebite, and for their antitussive and diaphoretic properties (Xu et al., 2009). Its medicinal use was firstly recorded in a Kazakh medical classic *Shipagerlik Bayan*, which was written by Oteyboydak Tleukabyluly in 15th century (Tleukabyluly, 1994).

Although, many benefits of this plant have been

*Corresponding author. E-mail: halmurat@263.net. Tel: +86-991-4366176. Fax: +86-991-4366176.

claimed, no scientific datum is available so far about its biological properties, especially its anticancer activity. Therefore, this prompted us to investigate the antiproliferative activity of this plant on cancer cells. For these purposes, six extracts were prepared and tested for their potential antiproliferative properties with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay on different cancer cell lines. To our knowledge, this is the first time that the anticancer activity of *D. altaica* is evaluated.

MATERIALS AND METHODS

Plant material

The barks of *D. altaica* were collected from Altai Mountain, Xinjiang, China in July 2008. The plant was identified and authenticated by Bahargul Kongirkhan, a herbalist at the herbarium of Altay Institute for Drug Control, Xinjiang, China. A voucher specimen (No. 050036) was deposited at the same place.

Tested material

Dried barks of the plant (150 g) were chopped and extracted with 95% EtOH by maceration for 2 weeks in a dark place at a room temperature of 20 ± 2°C. The procedure was repeated twice. The extracts were combined, concentrated under reduced pressure and freeze-dried to yield the EtOH extract (DA-Et, 12.10 g). 0.5 g of this extract was kept for MTT assay and the rest was submitted to a sequential liquid-liquid extraction with solvents of increased polarity to yield petroleum ether (DA-Pt, 1.7064 g), chloroform (DA-Ch, 0.6915 g), ethyl acetate (DA-Ea, 1.8237 g), n-butanol (DA-Bu, 2.5242 g) and aqueous (DA-Aq, 2.5998 g) fractions.

Cell culture

The human esophageal squamous cell carcinoma (Eca-109), gastric carcinoma (AGS), hepatoma (SMMC-7721) and cervical carcinoma (HeLa) cells were purchased from the Cell Bank of Type Culture Collection of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained as exponentially growing cultures in RPMI 1640 cell culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) and 1% penicillin-streptomycin solution (10,000 units penicillin G and 10 mg streptomycin/ml) (Solarbio, Shanghai, China). All cell lines were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide.

Cell proliferation assay

All the samples were tested at 6.25, 12.5, 25, 50, 100 µg/ml concentrations. The samples were dissolved in dimethylsulfoxide (DMSO) and further diluted with cell culture medium. Cells incubated with the same concentration of DMSO were used as a control. The DMSO final concentration was adjusted to 1% of the total volume of medium in all treatments, including the control. For MTT assay, 1×10^5 cells/well were plated into 96-well plates (Nunclon, Denmark) and incubated for 24 h before the addition of drugs. After 48 h of incubation for all cells, 20 µl of MTT (Sigma, USA) reagent (5 mg/ml) in phosphate buffered saline (PBS) was added to each well. The plates were incubated at 37°C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (150 µl) was added to each well. The metabolized MTT product was quantified by reading the absorbance at 490 nm on a Beckman Coulter-AD340 (Beckman Coulter, Fullerton, CA, USA). Results were expressed as a percentage of cell viability (%), which was calculated by the following formula: percentage of cell viability (%) = (absorbance of treated sample at 490 nm/absorbance of untreated sample at 490 nm) × 100. All assays were performed in triplicate.

The median growth inhibitory concentration values (IC_{50}), defined as the amount of extract that induces 50% of cell death, were used to compare the antiproliferative activity of extracts on cancer cells.

Statistical analysis

The results of percentage of cell viability were presented as means ± SD. Statistical comparisons between treatment groups and the control group were performed using the one-way ANOVA followed by post hoc Tukey (in case of equal variance) or Dunnett's T3 (in case of unequal variance) test. These tests were performed using SPSS 13 for Windows. $p < 0.05$ was considered statistically significant. IC_{50} values and their 95% confidence interval (IC_{95}) were calculated using sigmoidal dose-response model with a variable slope in the Graphpad Prism software (version 4.03).

RESULTS AND DISCUSSION

Figures 1 to 4 shows the percentage of cell viability of Eca-109, AGS, SMMC-7721 and HeLa cells when treated by six extracts from the barks of *D. altaica*. All extracts, except for the aqueous one (DA-Aq), exhibited a dose-dependent growth inhibitory effect on all cell lines, but the data revealed that different cell lines have different sensitivities to the extracts. The IC_{50} values of extracts on these cell lines are shown in Table 1. Surprisingly, all doses of aqueous extract, together with low doses (6.25 and 12.5 µg/ml) of DA-Bu and DA-Ea, seemed to increase the viability of AGS gastric cancer cells, implying the possible presence of one or more high polar components with proproliferative activity in certain cancer cells (Figure 2).

This result also suggests that *D. altaica* must be carefully considered when used as a chemopreventive agent. However, for Eca-109, HeLa and SMMC-7721 cells, all doses of aqueous extract as well as the higher doses of DA-Bu and DA-Ea exhibited weak growth inhibition, while other extracts showed moderate to strong growth inhibition in two or more cell lines (Figures 1 to 4). Of these six extracts, only DA-Ch extract exhibited moderate to strong antiproliferative activity on all cell lines with lower IC_{50} values, while ethyl acetate extract showed very strong activity on Eca-109 cells with an IC_{50} value of 3.1 µg/ml (Table 1).

When compared with the crude extract DA-Et, its sub-fractions, namely DA-Ea, DA-Pt and DA-Ch, showed more significant antiproliferative activity on Eca-109, HeLa and all cell lines, respectively. These results indicated that antiproliferative principles of *D. altaica* bark have been effectively enriched in DA-Ea, DA-Pt and

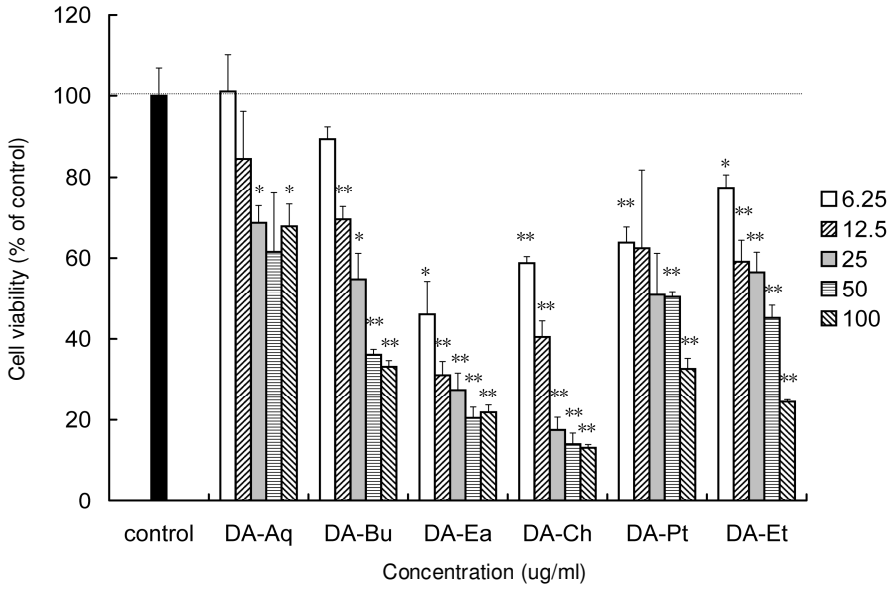

Figure 1. Antiproliferative activity of DA-Aq, DA-Bu, DA-Ea, DA-Ch, DA-Pt and DA-Et on esophageal squamous cell carcinoma (Eca-109) cells. All extracts were tested at 6.25, 12.5, 25, 50 and 100 µg/ml concentrations after 48 h of treatment. Results are means of three experiments ± standard deviation. ∗p < 0.05, ∗∗p < 0.01 versus control group (one-way ANOVA with post hoc Dunnett's T3 test).

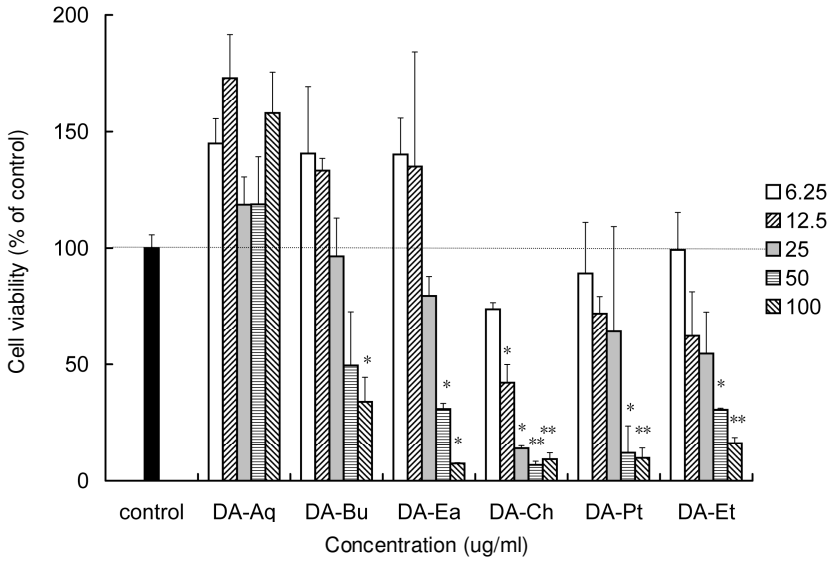

Figure 2. Antiproliferative activity of DA-Aq, DA-Bu, DA-Ea, DA-Ch, DA-Pt and DA-Et on gastric carcinoma (AGS) cells. All extracts were tested at 6.25, 12.5, 25, 50 and 100 µg/ml concentrations after 48 h of treatment. Results are means of three experiments ± standard deviation. ∗p < 0.05, ∗∗p < 0.01 versus control group (one-way ANOVA with post hoc Dunnett's T3 test).

DA-Ch fractions through liquid-liquid extraction. However, according to the criteria of the American National Cancer Institute, the IC_{50} limit to consider a crude extract promising for further purification is lower than 30 µg/ml (Suffness et al., 1990), indicating that only two fractions obtained from partitioning of the ethanol extract of *D. altaica*, DA-Ch and DA-Ea, could be considered a potential source of anticancer compounds.

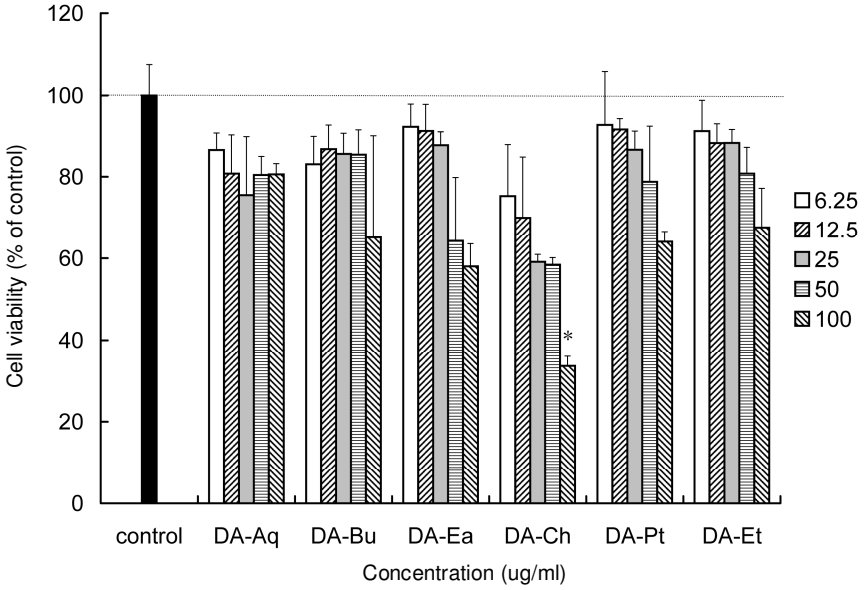

Figure 3. Antiproliferative activity of DA-Aq, DA-Bu, DA-Ea, DA-Ch, DA-Pt and DA-Et on hepatoma (SMMC-7721) cells. All extracts were tested at 6.25, 12.5, 25, 50 and 100 µg/ml concentrations after 48 h of treatment. Results are means of three experiments ± standard deviation. ∗p < 0.05 versus control group (one-way ANOVA with post hoc Dunnett's T3 test).

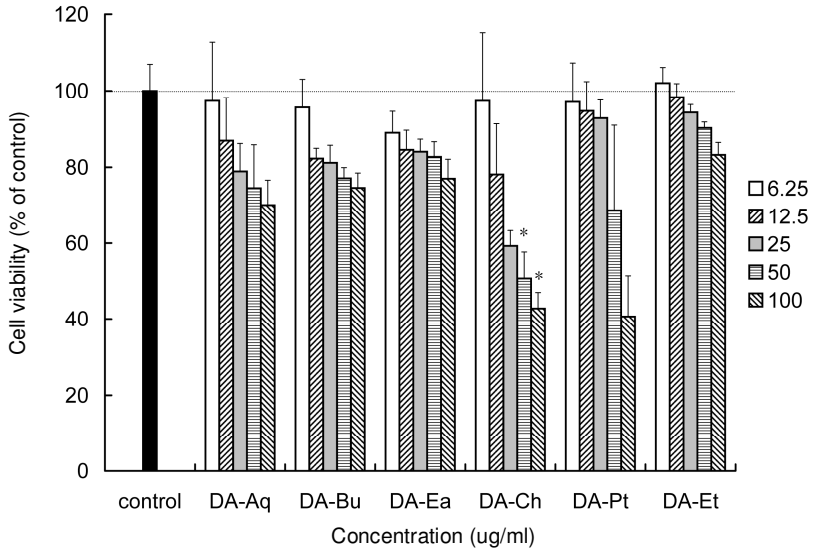

Figure 4. Antiproliferative activity of DA-Aq, DA-Bu, DA-Ea, DA-Ch, DA-Pt and DA-Et on cervical carcinoma (HeLa) cells. All extracts were tested at 6.25, 12.5, 25, 50 and 100 µg/ml concentrations after 48 h of treatment. Results are means of three experiments ± standard deviation. ∗p < 0.05 versus control group (one-way ANOVA with post hoc Dunnett's T3 test).

Future work in our laboratory will identify and characterize the active compounds present in these extracts, following fractionation using advanced techniques for fractioning and identification. Indeed, some species of genus *Daphne* were reported to have significant antitumor activity.

For example, it was described that extracts and compounds of *D. genkwa* (Zhan et al., 2005), *D. mucronata* (Mahdavi et al., 2007) and *D. odora* var. *marginata* (Zhan et al., 2006) markedly inhibits the

Table 1. Antiproliferative effect of six extracts from the barks of *D. altaica* on four different tumor cell lines. Data are presented as IC_{50} (µg/ml) values and their 95% confidence interval (IC_{95}) obtained by nonlinear regression.

Extracts	Cell lines			
	Eca-109	AGS	HeLa	SMMC-7721
DA-Aq	>100	U.C*	>100	>100
DA-Bu	33.9 (28.4-40.4)	57.5 (30.5-108.4)	>100	>100
DA-Ea	3.1 (1.6-6.4)	40.2 (25.5-63.2)	>100	>100
DA-Ch	8.5 (7.0-9.8)	10.6 (9.5-11.7)	56.1 (37.6-83.6)	49.9 (32.3-77.2)
DA-Pt	30.6 (18.2-51.5)	25.7 (16.9-39.3)	80.4 (62.7-103.0)	>100
DA-Et	29.5 (24.2-35.9)	26.7 (19.3-37.0)	>100	>100

*U.C: unable to be calculated due to enhanced cellular survival.

proliferation of several tumor cells *in vitro*. Crude extracts and isolated compounds from *D. tangutica* showed potent antitumor activity *in vivo* (Zhan et al., 2007).

From these species, different classes of natural compounds have been isolated, including coumarins, diterpenes, flavonoids, biflavonoids, lignans, simple phenylpropanoids and steroids.

Among these reported secondary metabolites, only daphnane type diterpene esters, biflavonoids, coumarins and lignans were reported to be the major bioactive compounds responsible for the antiproliferative activity of *Daphne* species (Zhan et al., 2005; Mahdavi et al., 2007; Zhang et al., 2006; Kasai et al., 1981; Yazdanparast et al., 2004; Zheng et al., 2007; Ma et al., 1994; Park et al., 2008).

Conclusion

The results of this study represent the first evidence that barks of *D. altaica* possess effective antiproliferative activities on human cancer cells. Future phytochemical investigations on this medicinal plant should be encouraged.

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