

## STUDY OF CYTOTOXIC ACTIVITY OF *DAPHNE MUCRONATA* ROYLE GROWN IN IRAN

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### ABSTRACT

**Background:** Plants are a proven source of anti-tumor compounds and it is reasonable to assume that many such substances remain to be discovered. In Iranian folk medicine, ethanolic extract of the aerial parts of *Daphne mucronata* Royle is used against various skin disorders.

**Objective:** In order to evaluate anti-neoplastic plants, we initiated a cytotoxic study using *Daphne mucronata* that is indigenous to Iran.

**Methods:** The cytotoxic activity of two hydroalcoholic and chloroformic extracts of *Daphne mucronata* Royle were examined on seven different cell lines by MTT assay. Cell lines used in this study included SK-Br-3 and MDA-MB-435 (breast cancer), Hela (cervical epitheloid carcinoma), K562 (myelogenous leukemia), U937 (monoblastic leukemia), Ag.8 (mouse myeloma) and Vero (primary monkey kidney).

**Results:** The highest cytotoxic activity of the hydroalcoholic extract of *D. mucronata* was found on breast cancer cell lines. 50 µg/ml of the extract inhibited proliferation of 24-hour cultures of MDA-MB-435 and SK-Br-3 cell lines (73% and 34% inhibition, respectively). The extract showed anti-leukemic activity particularly against the U937 cell line. A 50% inhibition of cell proliferation due to 100 µg/ml of the extract in 24-hour culture of U937 and 48-hour culture of Ag.8 cell lines was observed. Despite the result of MTT assay showing a reduction of Hela cell line viability after 24-hour exposure to 10-50 µg/ml of the extract, a significant stimulatory activity at concentrations more than 400 µg/ml was noted. No significant cytotoxic effect was detected in relation to the Vero cell line. The chloroformic extract showed weak cytotoxic effect on MDA-MB-435, SK-Br-3 and U937 cells but had no significant effect on the other cell lines.

**Conclusion:** Hydroalcoholic extract of *D. mucronata* showed anti-tumour activity particularly against breast and leukemia cell lines.

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**Key Words** • Cytotoxicity • MTT assay • cell line • *Daphne mucronata*

### Introduction

Plants are a valuable source of useful anti-tumor compounds, and it is reasonable to assume that additional existing substances remain to be discovered.<sup>1,2</sup> However, at a

conservative estimate, there are 250,000 species of plants worldwide. When this is coupled with the fact that there is no definitive method of selecting the proper starting material, screening for appropriate biological activity are necessary.<sup>3-5</sup>

In Iranian folk medicine, ethanolic extract of aerial parts of *Daphne mucronata* Royle (Thymelaeaceae) indigenous to Iran and Turkey is used against various skin disorders such as vitilligo.<sup>6,7</sup> The aerial parts of the

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*Daphne* species (e.g. mezerium) have been used in traditional medicine as a remedy for rheumatism and as a purgative and abortifacient.<sup>8</sup> On the other hand, literature reviews show that the different species of *Daphne* such as *D. mezereum*, *D. genkawa*, *D. olidoies* and *D. odora* have good cytotoxic effects. In earlier reports active compounds have been isolated and identified from this *Daphne* genera.<sup>9-11</sup> Mezerine obtained from *D. mezereum* has been shown to possess anti-leukemic activity. Odoricin is a new nematocidal compound derived from *D. odora* and daphnetin-8-glucoside from *D. mucronata* (accuminata) possesses cardiotoxic activity.<sup>12-14</sup>

In order to find an anti-cancer plant, we initiated a cytotoxic study on *D. mucronata* that grows in Iran.

## Materials and Methods

### Plant materials:

The plant was collected from Abadeh in Fars Province (south central part of Iran). The plant was identified by the Department of Botany and voucher specimens were deposited in the Herbarium of the Faculty of Pharmacology, Tehran University of Medical Sciences (Herbarium no. 6528). Aerial parts were dried in shade and chopped into small pieces. The powder was divided into two parts; the first part was extracted with chloroform in a Soxhlet's apparatus for 8 hours. The solvent was then removed under reduced pressure (yield=8% w/w). The second part was extracted by percolation in methanol/water (70:30) for 24 hours and the solvent was evaporated and dried under reduced pressure (yield=6.8% w/w).

### Preparation of culture medium with *Daphne mucronata* extract:

RPMI 1640 medium supplemented with 5% fetal calf serum (Gibco, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin was used as culture medium. Dried extract was

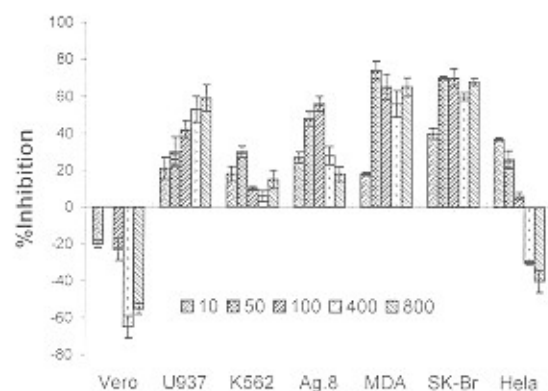
dissolved in this medium to the concentration of 20 mg/ml and was mixed at 37 °C for 20 min. This solution was centrifuged to remove insoluble ingredients, and the supernatant was passed through 0.22 µm filters for sterilization. The solution was diluted with the medium and five concentrations (10, 50, 100, 400, 800, µg/ml) were prepared.

### Cell culture and the cytotoxicity assay:

Cell lines including Vero, Hela, K562, SK-Br-3, Ag.8, U937 and MDA-MB-453 were maintained in medium. Cytotoxicity assay was carried out in 96-well tissue culture plates using an MTT proliferation kit (Gibco, Germany). Briefly, cells were cultured in triplicate at a concentration of 10<sup>5</sup> cells/well in 100 µl culture medium containing various concentrations of *D. mucronata* extracts. Treated cultures were incubated in a CO<sub>2</sub> incubator (37 °C, 5% CO<sub>2</sub>) for 24 to 72 hours. After the incubation period, 10 µl of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] in final concentration of 5 mg/ml was added and then the plates were incubated for 4 hours in the incubator. The formation of the blue formazan was assessed by addition of 100 µl of solubilization solution. Plates were allowed to stand overnight in the incubator. The absorbance of the samples was measured at 570 and 630 nm with an ELISA reader (Pharmacia, Sweden). The mean optical density (OD)±SD for each group of replicates was calculated. Inhibition percentage of cell proliferation was obtained as follows: %Inhibition=100-[(test OD/nontreated OD) x 100].<sup>15-18</sup> A concentration of 10 µM doxorubicin was used as positive control without adding the extract.

### Statistical analysis:

Data were analyzed using SPSS software, one-way ANOVA and Duncan test. A p-value of ≤ 0.05 was considered significant.



**Figure 1:** Exposure of tumor cell line to various concentrations (10-800  $\mu\text{g/ml}$ ) of hydroalcoholic extract of *Daphne mucronata* in a 48-hour culture. Data represents the mean value of triplicates with bars indicating SD (SD ranged 1-8). All values are significant.

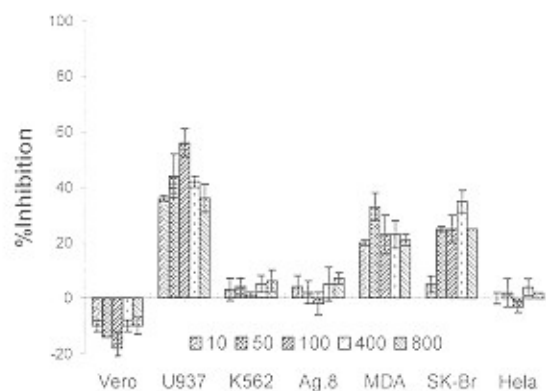
## Results

The cytotoxic activity of two hydroalcoholic and chloroformic extracts of *D. mucronata* was examined on seven different cell lines. Results of the cytotoxic activity of hydroalcoholic extract are shown in Figure 1 and are as follows:

**U937:** The extract showed a dose-dependent inhibitory effect on the proliferation of this cell line after 24 hours (%inhibition range, 24-56%). After 48 hours, the inhibitory effect was continuously dose-dependent and increased up to 60% at 800  $\mu\text{g/ml}$ . Finally this effect reached 75% in the 72-hour culture.

**K562:** The extract showed no marked cytotoxic activity on this cell line after 24 hours. Weak cytotoxic activity was observed on 48-hour culture at all concentrations (%inhibition range, 6%-30%). This cytotoxic activity reached up to the 38% in the 72-hour culture.

**MDA-MB-453:** The extract showed a significant cytotoxic activity in all concentrations after 24 hours (%inhibition range, 23-73%). The highest activity was at 50



**Figure 2:** Exposure of tumor cell line to various concentrations (10-800  $\mu\text{g/ml}$ ) of chloroformic extract of *Daphne mucronata* in a 48-hour culture. Data represents the mean value of triplicates with bars indicating SD (SD ranged 1-8). All values are significant.

$\mu\text{g/ml}$  (73%). Almost, the same results were obtained after 48 and 72 hours (%inhibition 18-74%).

**Ag.8:** Proliferation was inhibited in all concentrations. The highest percent inhibition after 48 hours was found at a concentration of 100  $\mu\text{g/ml}$  of the extract (56%).

**SK-Br-3:** Anti-proliferative activity after 24 hours was found at all concentrations of the extract (%inhibition range, 14-47%). This activity was strongly increased after 48 hours (%inhibition range, 40-70%) and 72 hours (%inhibition range, 45%-75%). The best result was obtained at 100  $\mu\text{g/ml}$  after 72 hours (%inhibition, 75%).

**HeLa:** The extract showed no marked effect on the 24-hour culture of this cell line. Mild cytotoxic activity at 10-50  $\mu\text{g/ml}$  of the extract (%inhibition range, 26%-37%) and stimulatory activity at concentrations more than 400  $\mu\text{g/ml}$  after 48 hours were observed. A mild increase in the stimulatory activity was seen in the 72-hour culture (%inhibition -48%)

**Vero:** No cytotoxic activity but rather stimulatory effect was detected after 48 hours (%inhibition range, 0% to -65%). Due to an overload of cells, the stimulatory activity after

72 hours was not measurable. Treatment of cell lines with different concentrations of the chloroformic extract reduced the cell viability of MDA-MB-453, SK-Br-3 and U937 lines after 48 hours (%inhibition range 20-33%, 5-35% and 36-56%, respectively). Slight stimulatory activity was observed for the Vero cell line (%inhibition, -10% to -18%). Other cell lines were almost not affected (%inhibition < 7%) (Fig. 2). Doxorubicin as the positive control killed the cell lines (100% inhibition).

## Discussion

The development of the effect of cytotoxic agents with therapeutic application remains an important need in clinical oncology. In recent years, the cytotoxicity and chemical constituents of different medicinal plants have been evaluated. *Typhonium flagelliforme* (Araceae) commonly known as the "rodent tuber" in Malaysia is one of these plants, its hexane extract has shown cytotoxic activity against P388 murine leukaemia cells.<sup>19,20</sup> *Albizia julibrissin*, another herbal plant has also shown good inhibitory action against the KB cancer cell line *in vitro*.<sup>21</sup> Aqueous extract of *Embllica officinalis* has been found to be cytotoxic to L 929 cells in culture in a dose dependent manner.<sup>22</sup> And the extract of *Eupatorium perfoliatumb* has shown potent cytotoxicity with EC<sub>50</sub> values (12-14 µg/mL) comparable to a standard cytotoxic agent, chlorambucil.<sup>23</sup>

The possible anti-proliferative effect of the hydroalcoholic and chloroformic extracts of *D. mucronata* is the subject of this study. This effect was determined in four groups of tumor cell lines including 3 leukemic cell lines, 2 breast cancer, one cervix cancer and one non-malignant cell line. The most anti-proliferate activity of alcoholic extract of *D. mucronata* was observed on breast cancer cell lines. This

extract, at a concentration of 50 µg/ml, showed 73% and 34% inhibition in 24-hour culture of MDA-MB-453 and SK-Br-3 cell lines, respectively. The inhibitory effect of the extract on the latter cell line increased to 70% after 48 hours which is indicative of the strong anti-tumor activity of the extract on this type of cell line. This extract also showed cytotoxic activity on leukemic cell lines. Among these lines, U937 and Ag.8 were more sensitive than K562. A 50% inhibition of cell proliferation due to 100 µg/ml of the extract was observed in 24-hour culture of U937 and 48-hour culture of Ag.8 cell lines whereas the highest % inhibition of K562 after 48 hours was 30%. In comparison to the hydroalcoholic extract, the chloroformic extract showed a weaker cytotoxic activity and the effect was only on MDA-MB-453, SK-Br-3 and U937 cell lines. Taking into consideration that these lines were the most affected cells after exposure to hydroalcoholic extract, it is concluded that the major components with cytotoxic property in *D. mucronata* might be the polar agents. In previous studies, the anti-leukemic activity of *D. genkawa*, another species of thymelaeaceous genus has been reported.<sup>13</sup> In that study, Liou et al. showed that the two natural products isolated from *D. genkawa* including Genkwadaphnin and Yuanhuscine were able to inhibit DNA and protein synthesis in P-338 leukemic cells. The major effects of these diterpene esters were the blockage of the elongation process and an interference with peptidyl transferase reaction.<sup>13</sup> Anti-leukemic activity has also been reported for mezerein, a potent anti-tumor agent isolated from *D. mezereum*.<sup>12</sup> It is interesting to note that mezerein structurally resembles phorbol esters which are tumor promoting agents.<sup>24</sup> In our study, despite the weak cytotoxic activity of *Daphne* extract on Hela cells at low concentrations, a stimulatory activity at concentrations more than 400 µg/ml was observed. This result shows the difference in mode of action of the extract on different cell

lines. In addition, it is possible that the stimulatory activity may in part be due to the presence of some similar weak tumor promoting or mitogenic agents in the extract. *D. mucronata* also displays a stimulatory activity on Vero, which is a non-malignant fibroblast-like cell. This finding suggests that this plant deserves further study as an anti-neoplastic therapeutic agent. Indeed, further studies on *D. mucronata* extract and identification of its effective compounds will help to obtain more information with regard to its anti-tumor activity.

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