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Short Report

Anti-tumor Activity and Cell Cycle Arrest of a New Diterpene Ester from *Daphne mucronata* Using K562 Cells

Heibatollah Sadeghi and Razieh Yazdanparast*

Institute of Biochemistry and Biophysics, Tehran University, Tehran, Iran

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ABSTRACT

In our search for new anticancer medicinal plants, the antiproliferative activity of the methanol extract of *Daphne mucronata* (Thymelaeaceae) was evaluated using human myelogenous leukemia K562 cells. The cells responded to plant treatments in a dose dependent manner and the IC_{50} of the crude extract (equivalent to 1 g of plant leaves powder per ml) and the purified active component were found to be 42 µl and 1.3 µM, respectively. The antiproliferative activity of the plant was also evaluated using flow cytometry technique. The results indicated that the crude extract and the active purified component are capable of arresting the cells in G_1 phase of the cell progression cycle. This data may provide a mechanism for the antiproliferative action of the plant. *Iran. Biomed. J. 7 (3): 127-131, 2003*

Keywords: Cell cycle arrest, *Daphne mucronata*, Flow cytometry, G_1- phase

INTRODUCTION

Plant natural products are a valuable source of novel chemotherapeutic agents with wide range of mechanism of actions. The anti-tumor activity of gnidimacrin, isolated from *Stellera chamaejasme* L. [1], is mainly attributed to its effect(s) on protein kinase C (PKC) activity in various tumors, while the anti-proliferative effect of genkwadaphnin, purified from *Daphne genkwa* [2], is sought in its inhibitory effect on nucleic acids syntheses in transformed cells. Some of the anti-neoplastic agents exert their effect through the cell cycle progression machinery. For instance, the anti-tumor activity of artemisinin, and its derivatives [3], are mainly due to the arrest of the cell cycle in G_1 phase while paclitaxel, an anti-cancer agent from *Taxus brevifolia* L. [4] arrest the cell cycle in G_2/M phase transition.

The cell cycle coordinates a variety of cellular events to assure accurate replication of the genome and cytokinesis [5]. This coordination takes place mainly at G_1/S and G_2/M phase transitions by a series of checkpoints. The regulations of these checkpoints by specific factors allow the cells to respond properly to the proliferative signals [6]. In fact, it has been shown that the activity of many of these regulatory factors is lost or arrested during the process of tumorigenesis [7]. Some of those regulatory factors include cyclin-dependent kinases (e.g. CDK_4), cyclin partners (e.g. cyclin D and E), tumor suppressor proteins (e.g. p53) and CDK inhibitory proteins (e.g. p16, p21 and p27) [8-11]. It is generally believed that the restoration of altered regulatory checkpoints in defective systems will be an effective way of treating cancers.

Among the various suggested checkpoints in the cell cycle [8, 11], the G_1/S phase transition constitutes an important regulatory point. In G_1 phase, various complex signals interact to decide a cell's fate: proliferation, quiescence, differentiation, or apoptosis [12]. This phase is mainly characterized by gene expression and the syntheses of all proteins necessary for DNA replication in a cell. Therefore, this section of the cell cycle is very sensitive and responsive to various exogenous stimuli. In fact, several cystostatic agents such as sulfonamides [12] and histone deacetylase

*Corresponding Author; Tel. (98-21) 695 6976; Fax: (98-21) 649 8818; E-mail: yazdan@ibb.ut.ac.ir

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inhibitors [13], are currently under clinical investigations for treatment of cancer patients. In this investigation, we evaluated the effect(s) of *D. mucronata*, an anti-neoplastic plant [14] with unknown mechanism of action, and one of its active components, on the modulation of cell cycle machinery.

MATERIALS AND METHODS

Materials. The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). Cell lines were obtained from the Pasteur Institute of Iran (Tehran). Chloroform, diethyl ether and silica gel 60 for column chromatography were obtained from Merck (Germany). Silica gel G for thin layer chromatography (TLC) was obtained from Fluka (Sweden).

Plant material. Shoots of *Daphne mucronata* were collected from suburbs of Fars province at the end of spring. A voucher specimen was deposited in the Herbarium of Science Departments of Tehran University (Iran). The plant leaves were dried under shade and then powdered. The powder was kept in a closed container in a cold room.

Extraction and purification of the active component. The powdered plant material (500 g) was extracted three times with methanol-water (1:1, v/v). The accumulated alcoholic extract was concentrated under reduced pressure and the volume was adjusted to 500 ml. Some of the extracts were kept at -20˚C, in 1 ml aliquots, for cell treatments and the remaining were extracted five times with $CHCl_3$. The accumulated chloroform solution was concentrated under the reduced pressure to a final volume of 1 ml. The residue was fractionated on a silica gel column (40×1.5 cm) using diethyl ether: chloroform mixture (8:2, 6:4 and finally 4:6, v/v) as the eluting solvents, into three fractions. The active component was purified from the second fraction using TLC techniques. The molecular weight of the purified compound was 662 mass unit, using FAB/MS.The purity of the isolated compound has been confirmed by TLC, HPLC ^{1}HNMR and FAB/MS [15].

Cell culture. The human leukemia cell line (K562) was cultured using RPMI 1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg. ml^{-1}), penicillin (100 U. ml^{-1}) [1]. The cells (1×10^6) were seeded, in triplicate, into the culture dishes and incubated at 37°C with 5% CO_2 atmosphere for 24 hours. Then, the plant extract (42 µl per ml equivalent to 0.84 mg of plant powder) or the purified component (5×10^{-7} M, this dose identified as therapeutic effective dose) was added to the cells once a day and for two consecutive days. The cell viability was assessed by trypan blue exclusion test [16].

Cell cycle analysis. DNA content was analyzed on an EPICS II flow cytometer (Beckman counter, France) according to the established procedure [17]. Human leukemia cells (1×10^6) were seeded into

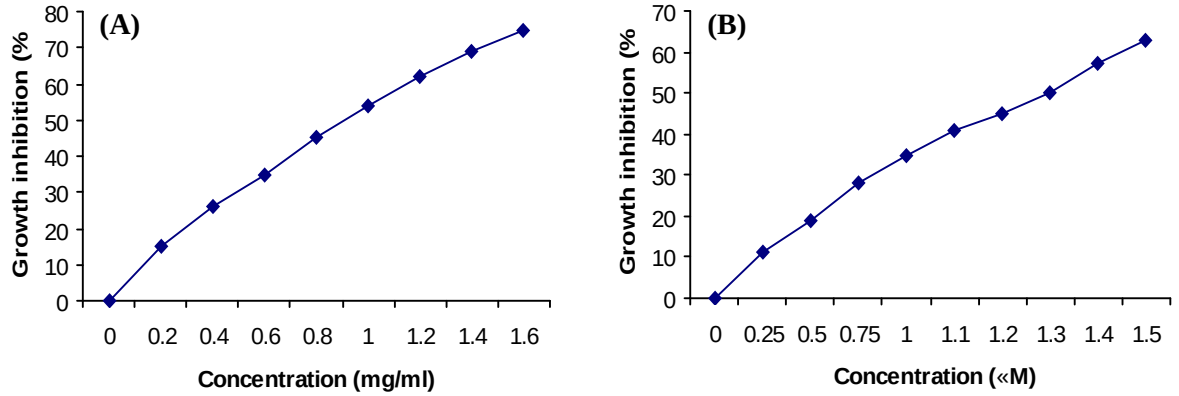


Fig. 1. Effects of plant extract **(A)** and the purified component **(B)** of *D. mucronata* on proliferation of K562 cells. The crude extract and the purified active component inhibited the cell proliferation in a dose dependent manner. The IC_{50} of the plant extract and the purified component are 0.84 mg/ml and 1.3 µM, respectively.

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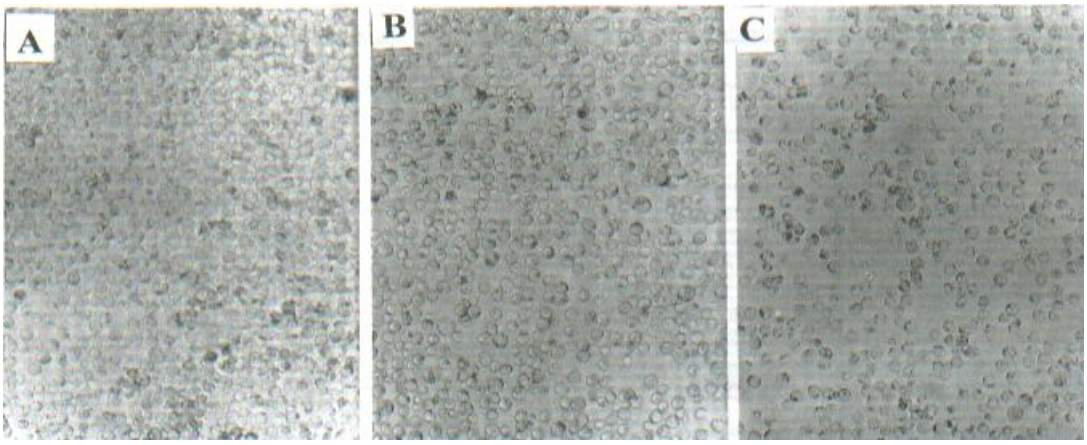


Fig. 2. Effects of *D. Mucronata* on K562 cells morphology. The cells were grown for 24 h in Petri dishes and then subjected for 48 h. to: no drug (**A,** control), the plant extract (**B,** 0.84 mg/ml) and the purified component (**C,** 1.3 µM).

culture dishes 24 hours prior to the treatments, according to the method describbed in cell culture section. The plant extract (28 µl/ml, corresponding to 0.56 mg of the plant leave powder) or the purified component (5×10^{-7} M) was given to the cells once a day and for two consecutive days [3].Cells were harvested, washed with PBS fixed in 70% ethanol, and kept at -20°C until analysis. Cells were then stained with 20 µg/ml propidium iodide containing 20 µg/ml RNase (DNase free) for 2 hours. The stained cells (1×10^6 cells/ml) were analysed by flow cytometry. The population of G_0/G_1, S, and G_2/M were quantitated using multicycle Cell Cycle Software. Results are expressed as percentage of the cells in each phase [18].

RESULTS AND DISCUSSION

The plant extract and the purified active component are capable of inhibiting the proliferation of the cancer cell lines used in this report. The results are expressed as $IC_{50,}$ the concentration required to reduce by 50% the number of the treated cells with respect to untreated samples. Figure 1 shows the dose responsive curves of K562 cells treated with crude extract (1a) and the purified component (1b). The data show that the purified component (MW of 662) is responsible for the action of the extract.

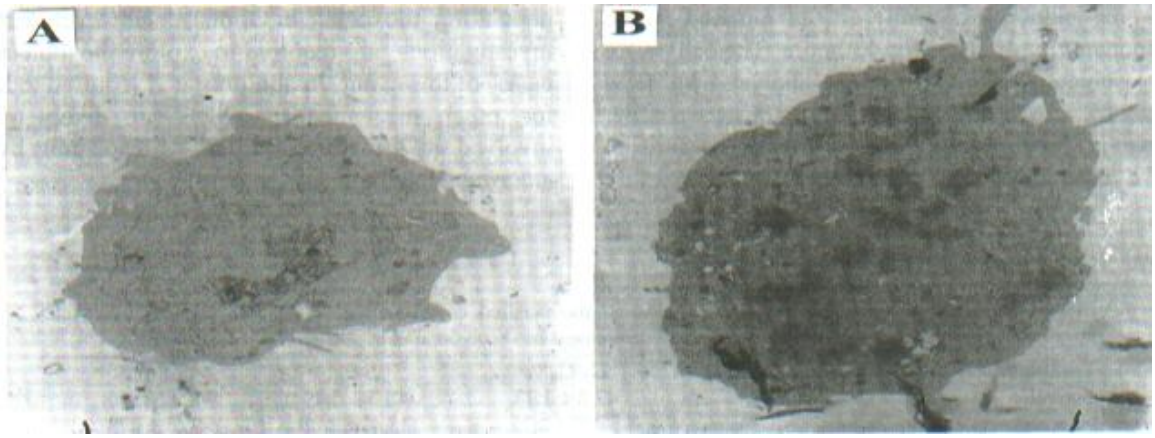


Fig. 3. Electronic micrographs of K562 cells before **(A)** and after **(B)** 48 hours of treatment with the purified active component of *D. mucronata* (5×10^{-7}M). Condensation of chromatin is markedly visible. (Electron micrograph, original magnification × 17,600).

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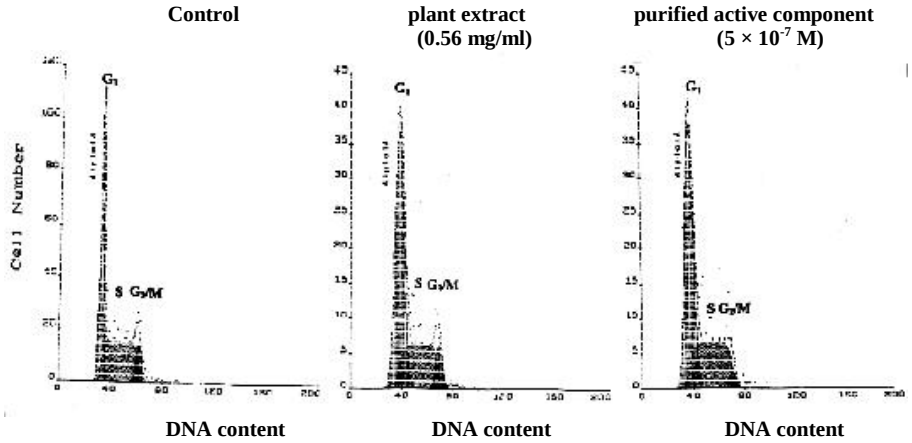


Fig. 4. Cell cycle analyses of K562 cells treated with the plant extract or the purified active component. Cells were treated for 48 hours, with the plant extract or its purified component at indicated concentrations. The cells were then washed with PBS and stained with propidium iodide, the DNA content was measured by flow cytometry and the cell distributions were determined using the multicycle Cell Cycle Software programme.

Using K562 cells, we found that the morphology of the cultured cells significantly changed upon treatment with the crude extract or the purified active component compared to the untreated control cells (Figs. 2 and 3).

Despite the fact that the K562 cells are resistant to induction of apoptosis by a variety of different agents, such as camptothecin, etoposide, and cytarabine [19], electron microscopic examination of the treated cells (Fig. 3b) indicates that the majority of the treated cells are in the early stages of apoptosis such as: condensation of chromatin, cytosolic vacuolization, cell shrinkage, and loss of microvilli.

To examine the effect of *D. mucronata* on cell cycle modulation, K562 cells were treated with two consecutive doses of the plant extract (equivalent to 0.56 mg plant powder/ml) or the purified active component (5×10^{-7}M) for 48 hours. The cells were then subjected to cell cycle analysis by flow cytometry. According to the flow cytometry profile, shown in Figure 4, the plant extract or the purified active component can effectively alter cell cycle distribution of the growing K562 cells. As shown in Table 1, the plant extract, after 48 hours of the treatment, has caused a marked reduction of the cell populations in S phase along with significant increase in cell numbers in G_1 phase. Similar results were also observed using the purified active component. For instance, at a dose of 5×10^{-7} M of the purified component, the G_1/G_0 ratio increased to 56.5% from 42.8% in the untreated cells. These results clearly suggest that *D. mucronata* suppresses the cell growth by inducing a specific block at G_1/S transition phase of the cell cycle. However, as it is evident from Table 1, the progression through G_2/M phase affected much less by *D. mucronata.*

The data presented in this report demonstrate that *D. mucronata* extract or the purified active component affected cellular proliferation, in part by populating the cells in G_1 phase. However, further investigations are required to explore the molecular basis of this event. In addition, it is required to look for other mechanisms, which are likely to be involved in growth inhibition of K562 cells by *D. mucronata.*

Table 1. Effect of the plant extract (28 µl) and the purified component (5×10^{-7}M) on K562 cell cycle progression compared to untreated cells. Each measurement has been done in triplicate.

Treatment	G_1/G_0 %	S %	G_2/M %
Control	42.8 ± 1.13	48.3 ± 0.49	8.9 ± 0.56
Plant extract	57.0[a] ± 2.75	35.4[a] ± 2.00	7.6[c] ± 0.78
Purified active component	56.5[a] ± 1.00	40.0[b] ± 2.83	3.3[a] ± 0.49

[a]significantly different from control (p<0.001); [b]significantly different from control (p<0.002); [c](p>0.05).

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