ANTINEOPLASTIC EFFECTS OF *DAPHNE MUCRONATA*: INHIBITION OF DNA AND RNA SYNTHESIS

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Abstract- Cytotoxicity evaluation of *Daphne mucronata* (*Thymelaeaceae*) extract and one of its active purified components, using seven different cancerous cell lines, revealed the strong antiproliferative activity of the plant. Using flow cytometry technique, it was found that treatment of the most responsive cells (K562) with the plant extract or the active component inhibited the progression of cells through G_1 phase by almost 15% compared to the untreated cells. Based on the extent of [^{3}H]-thymidine and [^{3}H]-uridine incorporation into DNA and RNA, respectively, the major metabolic effects of *D. mucronata* were found to be mainly on DNA and to a less extent on RNA synthesis. These data strongly support the flow cytometry observation and provide a mechanism for the antiproliferative activity of *D. mucronata*.

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Key Words: Cell cycle arrest, *daphne mucronata*, nucleic acid synthesis

INTRODUCTION

Plant-drived chemotherapeutical agents exert their action through wide range of mechanisms. Some of the antineoplastic agents exert their effects through cell cycle progression machinery. For instance, the antitumor activity of artemisinin and its derivatives, are mainly due to arresting the cells in G_1 phase of their progression cycle (1) while paclitaxel, an anticancer agent from *Taxus brevifolia L*., arrest the cells in M stage of their cycle (2). The cell cycle coordinates a variety of cellular events in order to assure accurate replication of genome (3). These coordinations take place at different checkpoints. The regulation of these checkpoints by specific factors allow the cells to respond properly to the proliferative signals (4). Some of those regulatory factors include cyclin-dependent kinases (e.g. CDK4), cyclin partners (e.g. cyclin D and cyclin E), CDK inhibitory proteins (e.g. p16, p21and p27) and tumor suppressor proteins (e.g. p53) (5-8). Among the various suggested checkpoints in the cell cycle, 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. This phase is mainly characterized by gene expression and the synthesis of all the proteins needed for DNA replication in a cell. Therefore, this section of cell cycle is very sensitive and responsive to various exogenous stimuli (9). In the current study, chemosensitivity of three different human cancer cell lines and a mouse BALB/C fibrosarcoma cell line with respect to the crude extract of *Daphe. mucronata* and one of its purified active components, was assessed by nucleic acids synthesis inhibition assay which measures the extent of [^{3}H]-thymidine and [^{3}H]-uridine incorporations into DNA and RNA, respectively.

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MATERIALS AND METHODS

The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (life technologies, Paisley, Scotland). The cell culture petri dishes and 96 well flat-bottom microtiter plates were obtained from Nunc (Denmark). Cancer cell lines were obtained from Pasteur Institute of Iran (Tehran, Iran). Chloroform, diethylether, ethanol, and silica gel 60 were obtained from Merck (Germany). Silica gel G for TLC was obtained from Fluka (Sweden).

Plant material

Aerial parts of *D. mucronata* were collected from the suburbs of Kermanshah Province at the end of spring. A voucher specimen was deposited in the herbarium of Science Department of Tehran University. The plant leaves were dried far from direct light, 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 ethanol–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 extract was kept at -20°C, in 1ml aliquots, for cell treatments and the remaining (450 ml) were extracted five times with chloroform ($CHCl_3$). The accumulated chloroform solution was concentrated under the reduced pressure to a final volume of 1ml. The residue was fractionated on a silica gel column (40×1.5 cm) using diethylether: 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 structure elucidation of the active component is under our present investigation.

In vitro anti-tumor activity

The antitumor activity of *D. mucronata* extract and the purified component against human tumor cell lines were determined by an MTT-based chemosensitivity assay. Several leukemia cell lines (e.g., K562, HL-60, and MOLT-4) and a mouse BALB/C fibrosarcoma cell line were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells (5×10^4 cell/ml) were plated in a 96-well microplate to a final volume of 180µl. The cell cultures were maintained at 37°C in an atmosphere of 5% CO_2. After 24 hr, twenty microliters of various concentrations of *D. mucronata* extract or the purified component were given to the cells once a day and for two consecutive days. Fifty microliters of MTT (2 mg/ml) in Ca^{2+} and Mg^{2+}- free PBS [PBS (-)] was added to each well and the plates were incubated for 4 hours. The plates were then centrifuged (820g, 15 min) prior to removal of the medium and MTT, and then MTT-formazan crystals were dissolved in DMSO (200 µl/well). Glycine buffer (25µl/well, 0.1M, pH 10.5) was added to each well and the absorbance was measured at 570 nm (10).

Cell cycle analysis

DNA content was analyzed on an EPICS II flow cytometer according to the established procedure (11). Human leukemia cell line, K562, was cultured in RPMI 1640 medium supplemented with FBS (10%,v/v), streptomycin (100 $\mu g.ml^{-1}$), and penicillin (100 $U.ml^{-1}$). The cultured cells were incubated in a CO_2 incubator (5% CO_2) at 37°C. The cells (1×10^6 cells) were triplicately seeded into culture dishes 24 hours prior to treatments. The plant extract (28 µl/ml, corresponding to 0.56 mg of the plant leave powder) or the active component (5×10^{-7} M) was given to the cells once a day and for two consecutive days. Cells were harvested, washed with PBS, fixed in 70% ethanol, and kept at -20°C until analysis. Cells were then stained with 20µg/ml propidium iodide containing 20 µg/ml RNase (DNase free) for 2 hours. The stained cells (1×10^6 cells/ml) were analyzed by flow cytometry. The population of G_0/G_1, S, and G_2/M were determined using Multicycle Cell Cycle Software. Results are expressed as percentage of the cells in each Phase (1,12).

Inhibition of nucleic acids biosynthesis

DNA

Three different human cancer cells lines (K562, HL-60 and MOLT-4 leukemia cell lines) and WEHI-164, a mouse BALB/C fibrosarcoma cell line, at a cell concentration of 5×10^4/0.2 ml/well were seeded into 96 well flat-bottom microtiter plates 24 hours prior to the treatments. The plant extract (28 µl/ml, corresponding to 0.56mg of plant leaves powder) or the active component (5×10^{-7} M) was given to the cells once a day and for two consecutive days. A total of 0.5 µCi of [^{3}H]-thymidine was added to each well, 16 hours before cell harvest. The cells were then harvested onto glass fiber filters (Labo Science Tokyo, Japan) with a semiautomatic harvester (Titertek). After drying at room temperature, the glass fiber filters were placed into counting vials with 5 ml of the scintillation fluid and their radioactivity measured in a liquid scintillation counter. All experiments were performed in triplicate and mean values were used to calculate the percent inhibition of radioactivity incorporation. The extent of inhibition of DNA synthesis was determined by following equation: %inhibition = (1- test (cpm)/control (cpm)) × 100 (13,14).

RNA

[^{3}H]-uridine incorporation into RNA was achieved similar to the incorporation of [^{3}H]-thymidine into DNA except that 1 hour before cells harvesting, 1µCi of [^{3}H]-uridine had been added to each well (15).

Statistics

The Student's t-test has been used to evaluate the significance of data. A P-value less than 0.05 was considered statistically significant.

RESULTS

Among the seven different cancer cell lines used to assay the cytotoxicity of the crude leave extract of *D. mucronata* and one of its purified active components, K562 cells showed the smallest IC_{50} (Table 1). Light microscopic examination of K562 cells indicated that while the untreated cells were smaller in size and morphologically in the process of mitosis, the majority of the treated cells had round morphology and were relatively larger in size (data not shown).

To examine the probable effects of *D. mucronata* on cell progression cycle, K562 cells were treated with 0.28 µl/ml (equivalent to 0.56 mg of plant leaves powder) of the plant extract or the purified active component (5×10^{-7}M) once a day and for two consecutive days. The cells were then subjected to cell cycle analysis by flow cytometry. According to data, the plant extract and the purified active component can effectively halt the progression of the cell cycle at some stages. For example, after treatment with gnidilatimonoin, the population of cells in G_1 phase increased from 42.8% to 56.5% and that of S phase decreased from 48.3% to 35.4%. However, the progression of cells through G_2/M phases were affected much less by *D. mucronata* (Table 2).

Despite the fact that K562 cells are resistant to induction of apoptosis by a variety of agents (e.g. camptothecin, etoposide and cytarabine) (16), light and electron microscopic examinations of the treated cells indicate that the majority of these cells are undergoing apoptosis (Fig. 1): chromatin condensation, apoptotic bodies formation, and loss of microvilli are markedly visible in the Figure.

Table 1. Inhibition of cell proliferation by *D. mucronata*. Cells were cultured in the presence of various concentrations of the crude extract or the active component. The plant extract or the active component was given to the cells once a day and for two consecutive days. Cell proliferation was measured by MTT assay. Each measurement has been done in triplicate

	IC_{50}	
Cell type	Plant extract (mg/ml)	Active component (µM)
K562	0.84	1.3
HL-60	0.90	1.38
MOLT-4	0.94	1.4
WEHI-164	0.90	1.4

Table 2. Effects of the plant extract (28 µl/ml, corresponding to 0.56 mg of the plant leaf powder) 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	48.3	8.9
Plant extract	57	35.4	7.6
Purified component	56.5	40	3.5

The effect of *D. mucronata* on nucleic acid synthesis was also evaluated in whole and homogenized cells (K562, HL-60, MOLT-4 leukemia cell lines and WEHI-164 cell line). The cells were treated with two consecutive doses of the plant extract or the purified active component for 48 hours, the effect was followed by the extent of [^{3}H]-thymidine incorporation into DNA.

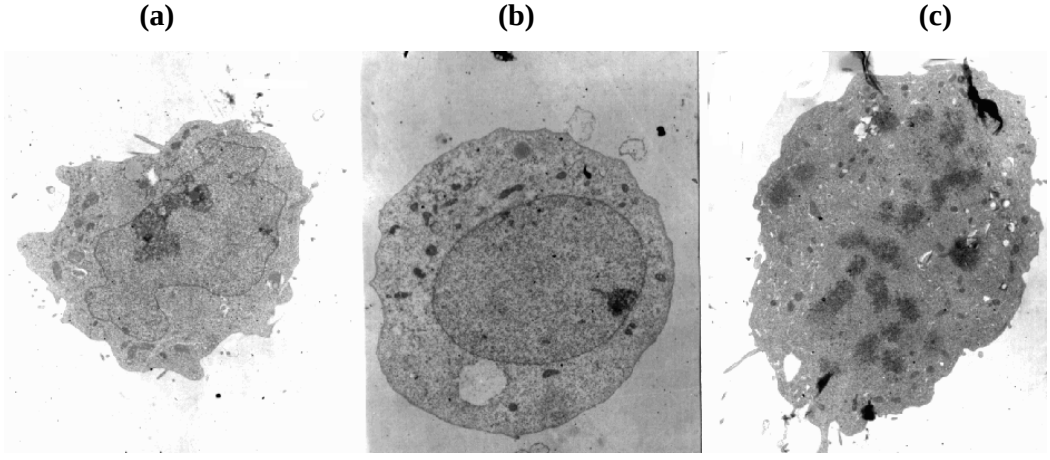

Fig. 1. Electronic micrographs of K562 cells before (a) and after (b and c) 48 hours of treatment with the purified active component (5×10^{-7}M). Condensation of chromatin is markedly visible in b and c (electron micrograph, original magnification × 17,600)

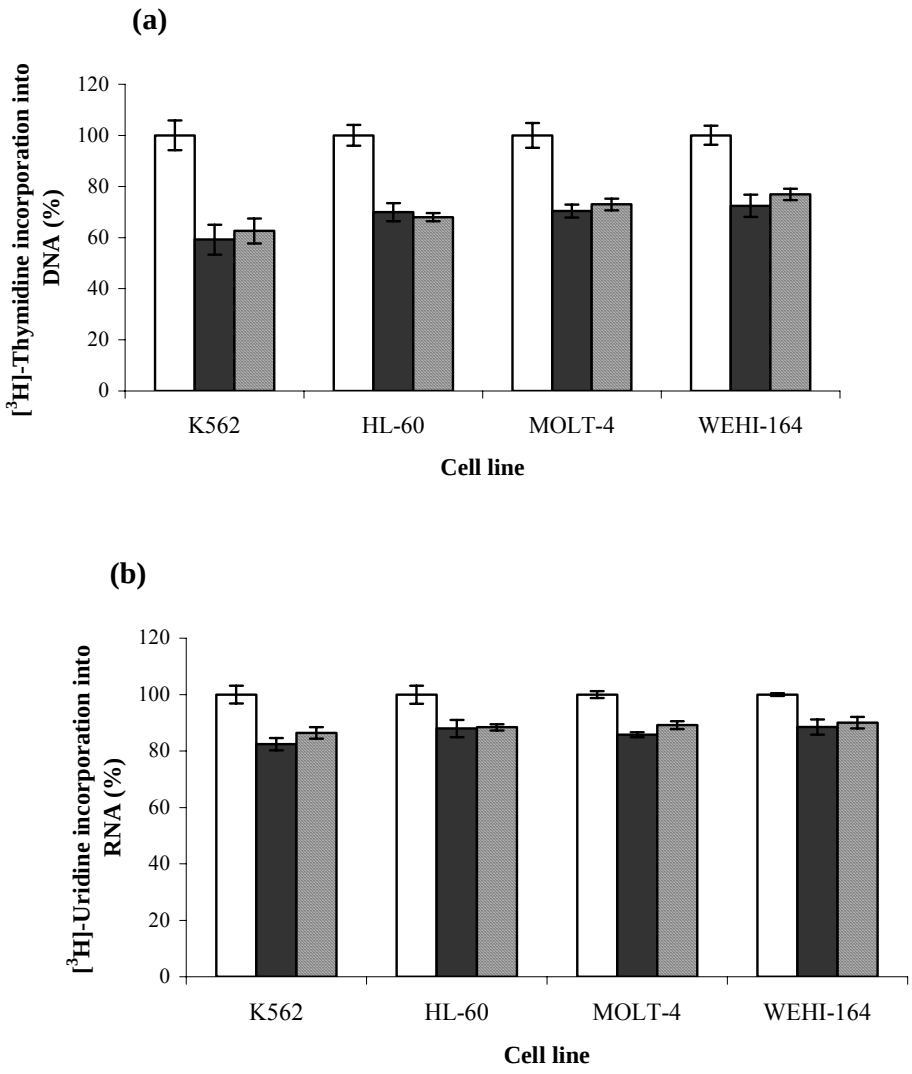

Fig. 2. Effects of plant extract (0.56 mg/ml of plant leaves powder, ■) and the active component (5×10^{-7} M, ▧) on the nucleic acids biosynthesis (a: DNA; b: RNA) in the cultured cell lines (K562, HL-60, MOLT-4 and WEHI-164) with respect to the untreated cells (□). Results are the mean ± SD of measurements

Fig. 2a shows that the plant crude extract caused a 40.79% reduction in [^{3}H]-thymidine incorporation into DNA. The reduction of DNA synthesis in other cells (HL-60, MOLT-4 and WEHI-164) under the effect of plant crude extract were 30.05%, 29.62% and 27.6%, respectively. Similarly, the purified active component inhibited DNA synthesis in K562, HL-60, MOLT-4 and WEHI-164 cell lines by 37.4%, 25.49%, 21.87% and 23.1%, respectively. According to Fig. 2b, the RNA synthesis, measured by [^{3}H]-uridine incorporation, was marginally quenched by *D. mucronata*. Despite this significant quenching effect on DNA synthesis, there was no detectable molecular interaction between DNA or RNA molecules and the purified active compound using band shift assay technique (data not shown). Based on these data, it may be concluded that *D. mucronata* exert its biological action mainly through inhibition of DNA synthesis.

DISCUSSION

The active component of *D. mucronata* appeared to have significant effects on cellular metabolism which probably would explain its high antineoplastic activity against several human cancer cell lines, mainly K562 cells. The major effect is the suppression of DNA synthesis, which was measured by the extent of [^{3}H]-thymidine incorporation into DNA using whole and homogenized cells. In addition, flow cytometry evaluation of the cells under the effect of

plant crude extract or the active component, clearly indicated that the cells have been arrested in the G_1 phase of their growth progression cycle meaning that they are not duplicating their DNA content.

Despite significant inhibition of DNA synthesis by the plant, no direct molecular interactions between DNA and the active component were observed using electrophoretic techniques. These observations suggest a different regulatory role for the active component possibly at the genomic level. Further investigations are required to explore the mode of action of the active component on DNA synthesis.

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