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G1 Phase Arrest and Apoptosis Induction in Human Thyroid Cancer Cell Line Thr.C1.PI33 by 3-Hydrogenkwadaphnin Isolated from *Dendrostellera lessertii*

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ABSTRACT

Background: *Dendrostellera lessertii* (*Thymelaeaceae*) is a toxic plant that grows in parts of Iran. The anti-proliferative properties of its crude methanol extract and one of its active components, 3-hydrogenkwadaphnin (3-HK), have been established using several cancer cell lines. **Methods:** In a further attempt to determine the mode of action, two groups of synchronously growing cells were treated with a single dose of 3-HK (3.5 nM) and/or a single dose of the crude extract (equivalent to 0.36 mg plant powder). Every 8 hours, the percentages of cells within G1, S, and G2-M phases were determined by flow cytometric (FCM) analysis; electron microscopic pictures were taken after fixation with 2% glutaraldehyde. **Results:** Twelve hours after treatments, apoptotic cell death was confirmed by the observation of marked morphological changes of the plasma membrane as microvillar disappearance and the appearance of apoptotic bodies in the treated cells. FCM analyses revealed that the G1 phase arrest was under the influence of the pure substance. **Conclusion:** The results confirmed the previously drawn conclusion that the raw material and the pure substance from *D. lessertii* exert their anti-tumor effects through cell cycle arrest at G1 phase and diversion of cell fate toward programmed cell death. *Iran. Biomed. J. 11 (4): 215-221, 2007*

Keywords: Thyroid cancer, Apoptosis, *Dendrostellera lessertii*, Flow cytometry, Transmission electron microscopy

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INTRODUCTION

Medicinal plants have been used for millennia as remedies for a variety of disease conditions [1]. It is estimated that plant-derived chemicals constitute up to 50% of all modern drugs [2, 3]. However, as most species of higher plants have never been examined, more devoted research is required in order to extend and enhance the usefulness of plants in prevention and treatment of various illnesses, including cancer, and to understand the mechanism of action of their biologically active components [4]. Some clinically useful anti-cancer agents have been derived from highly toxic plants [5, 6]. *Daphne mezereum* is a plant native of Europe, Caucasus, Turkey and Siberia. The toxic nature and its anti-leukemic effect were initially described in 1962 and 1975, respectively, and to date, several of its active ingredients have been isolated and purified [7-12]. *Dendrostellera lessertii* is another member of the *Thymelaeaceae* family that grows in parts of Iran. Its strong anti-tumor activity in animal models and in cultures has been established using its crude methanol extract as well as one of its purified substances, 3-hydrogenwadaphnin (3-HK), a daphnane-type diterpene ester [13-19]. The present study is an illustration of the ability of *D. lessertii* crude methanol extract and 3-HK to cause apoptotic body formation and G1-phase cell cycle arrest in thyroid cancer cells in culture.

MATERIALS AND METHODS

Reagents. The cell culture medium (RPMI 1640), and penicillin-streptomycin were purchased from Gibco BRL (Life technologies, Paisley, Scotland). Fetal bovine serum was purchased from School of Veterinary Medicine of the University of Tehran

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(Iran). The cell culture Petri dishes and flasks were obtained from Nunc (Roskilde, Denmark). Crude alcoholic extract of *D. lessertii* (1:1 methanol-water) and the purified compound, 3-HK were prepared as reported previously [13, 16, 18].

Cell culture. Human thyroid cancer cell line Thr.C1.PI33 was purchased from Pasteur Institute of Iran (Tehran) where it was developed from a 55-year-old Iranian female patient. The cells were maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO_2. The adherent cells were dislodged by EDTA/BSA solution every 3-5 days, made into suspension and used for seeding.

Drug treatment and cell viability. Cells were plated, in triplicate, into Petri dishes at a density of 1×10^5 cells/ml. After overnight growth, the cells were exposed to a single dose of crude extract (equivalent to 0.36 mg of plant powder). The cultures were then incubated for 18 h, 24 h, and 48 h and live cells were counted manually by hemacytometer and trypan blue exclusion test [20]. Photographs were taken by light microscopy (Olympus, Japan).

Flow cytometric (FCM) analysis. Cells were seeded in 14 (7 controls and 7 tests) Petri dishes at a concentration of 1×10^5 and allowed to adhere for 24 h. Synchronization was achieved by depriving the cells from their medium and replacing it by medium without fetal bovine serum for 72 h, after that complete medium in the case of controls, and the same containing 3.5 nM 3-HK in the case of tests, were added. According to Park *et al.* [21], at time zero and every 8 h afterwards for the first 16 hours and every 4 h for the rest of the time, control and drug-treated cells were dislodged using EDTA/BSA solution and rinsed with PBS. The cell suspensions were centrifuged at 84 ×g for 3 min, and the supernatants were discarded. Pellets were suspended in PBS, fixed with 75% ethanol at room temperature for 30 min, and then averted to -20°C. Cells were digested by 1% RNase A (ribonuclease A) at 3°C for 30 min, stained by propidium iodide fluorescent staining at 4°C, protected from light, filtered twice and then DNA content was analyzed on an EPICS II flow cytometer (Epics Division of Coulter Corporation, Florida, USA).

Transmission electron microscopy (TEM). Cells were seeded into 4 culture flasks at a density of 1×10^6/ml and allowed to reach 75-80% confluency. The cells in two of the flasks were then treated with the crude extract for 12 hours. The control and treated cells were dislodged by EDTA/BSA solution, made into suspension and washed 2 times with PBS. The cell fixation was proceeded as described by Maunsbach *et al.* [22]. Briefly, each cell suspension was mixed rapidly with an equal volume of 2% glutaraldehyde solution in 0.1 M cacodylate buffer and fixed for two hours. The pellet of cells, after centrifugation at 1,000 ×g for 5 minutes, was resuspended twice in an excess of 0.1 M cacodylate buffer with a 15-min interval. Cells were resuspended in 1% osmium tetroxide in 0.1 M cacodylate buffer and fixed for 30 minutes and then centrifuged at 1,000 ×g for 5 min. Then, 0.1 M cacodylate buffer was added and this step was repeated. To the pellet, an equal volume of 2% agar in 0.1 M sodium cacodylate buffer (heated to 40°C) was added and then the agar and cells were mixed rapidly. After cooling, the pellet was cut with razor blade into 2-mm cubic pieces and stored in 2% glutaraldehyde solution at 4°C until further processing and viewing by Zeiss Electron Microscope EM109 (Germany).

RESULTS

The effect of crude methanol extract of *D. lessertii* on cell survival has been shown in Figure 1. Compared to the control samples (untreated), the treated cells were characterized by shrinkage, and longer distances between cells. The number of live cells decreased about 40% after treatment with the crude extract for 24 hours.

The results from the single trial FCM analysis (Fig. 2) suggest that pure 3-HK at a concentration of 3.5 nM caused an accumulation of 35% of the cells in G1 phase after about 20 hours; which was accompanied by a 25% reciprocal decrease of cell population in the S phase. The peak level of S phase was 20% less than that of controls presumably due to gradual apoptotic elimination of cells. The cell population in G2-M phase was not affected although the treated cells progressed through G2-M phase at a slower rate than controls.

Figure 3 illustrates the electron microscopic evaluation of untreated and treated cells; apoptotic bodies containing nuclear fragments are visible; also the disappearance of microvilli in the treated cells is visible. Microvilli are actin filled membranous extensions common to epithelial cells. They perform

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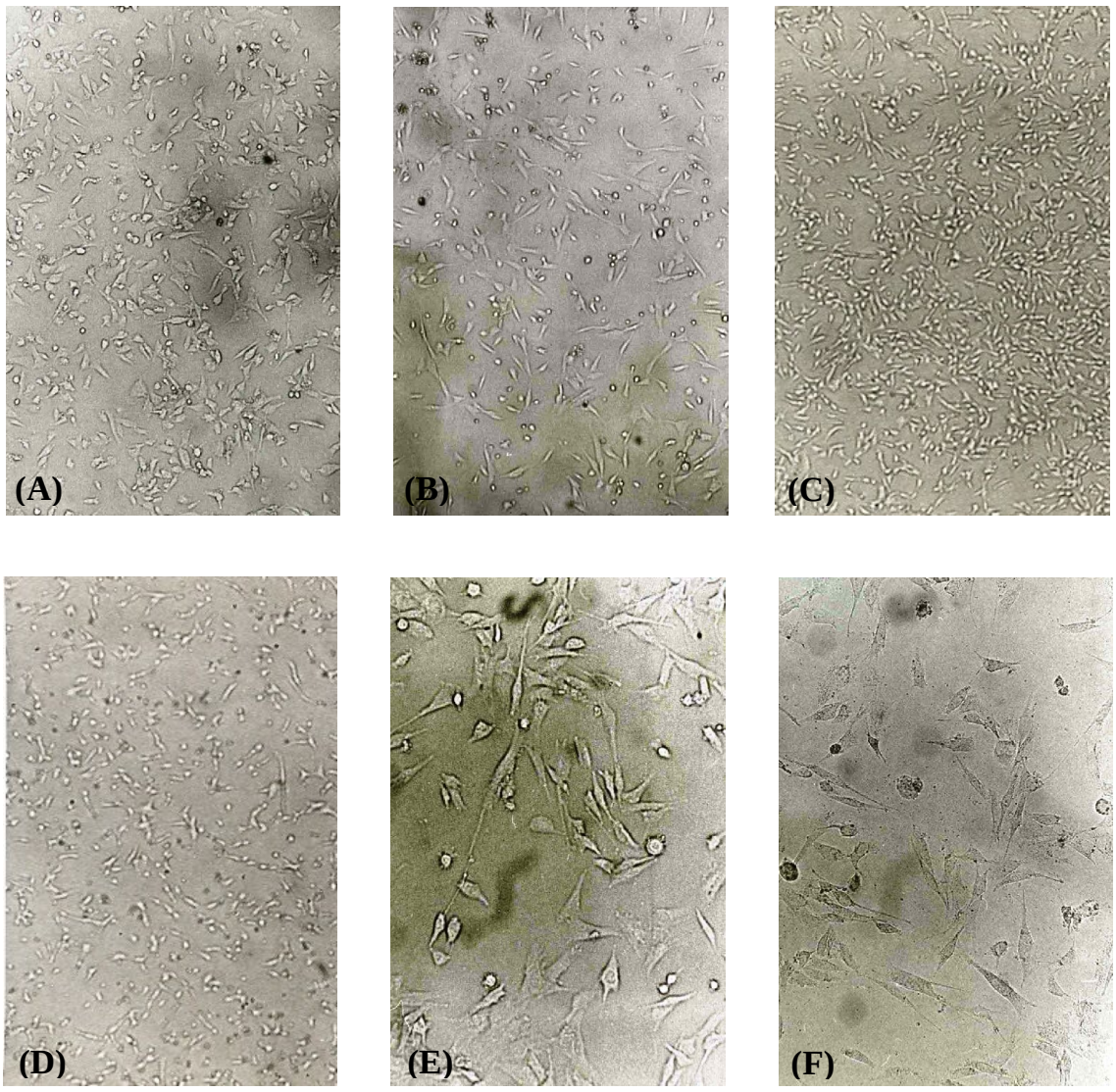


Fig. 1. The effect of *D. lessertii* crude extract on human thyroid cancer cell line Thr.C1.PI33. **(A)** Untreated and **(B)** treated cancer cells after 18 h (Magnification ×10); **(C)** untreated and **(D)** treated cells for 24 h (magnification ×4); **(E)** untreated and **(F)** treated cells for 48 h (magnification ×20).

as a grouping site for a variety of surface signaling and adhesion molecules such as CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) [23].

DISCUSSION

Many anti-tumor agents derived from plant sources function by targeting G1 phase of cell cycle and inducing apoptosis [24-26]. It has been reported that in thyroid carcinoma, interleukin-1α regulates G1 cell cycle progression and arrest [25] while the apoptotic effect and the cell cycle arrest of *D. lessertii* pure extract on a variety of cancer cell lines have been suggested to result from perturbation of GTP metabolism [14].

After exposure to the crude extract and the pure active compound of *D. lessertii*, Thr.C1.PI33 thyroid cancer cells, manifested the morphological features of apoptotic cells that can be recognized by light and electron microscopy, including cell shrinkage, loss of surface contact as the cells separated from their neighbors, loss of specialized

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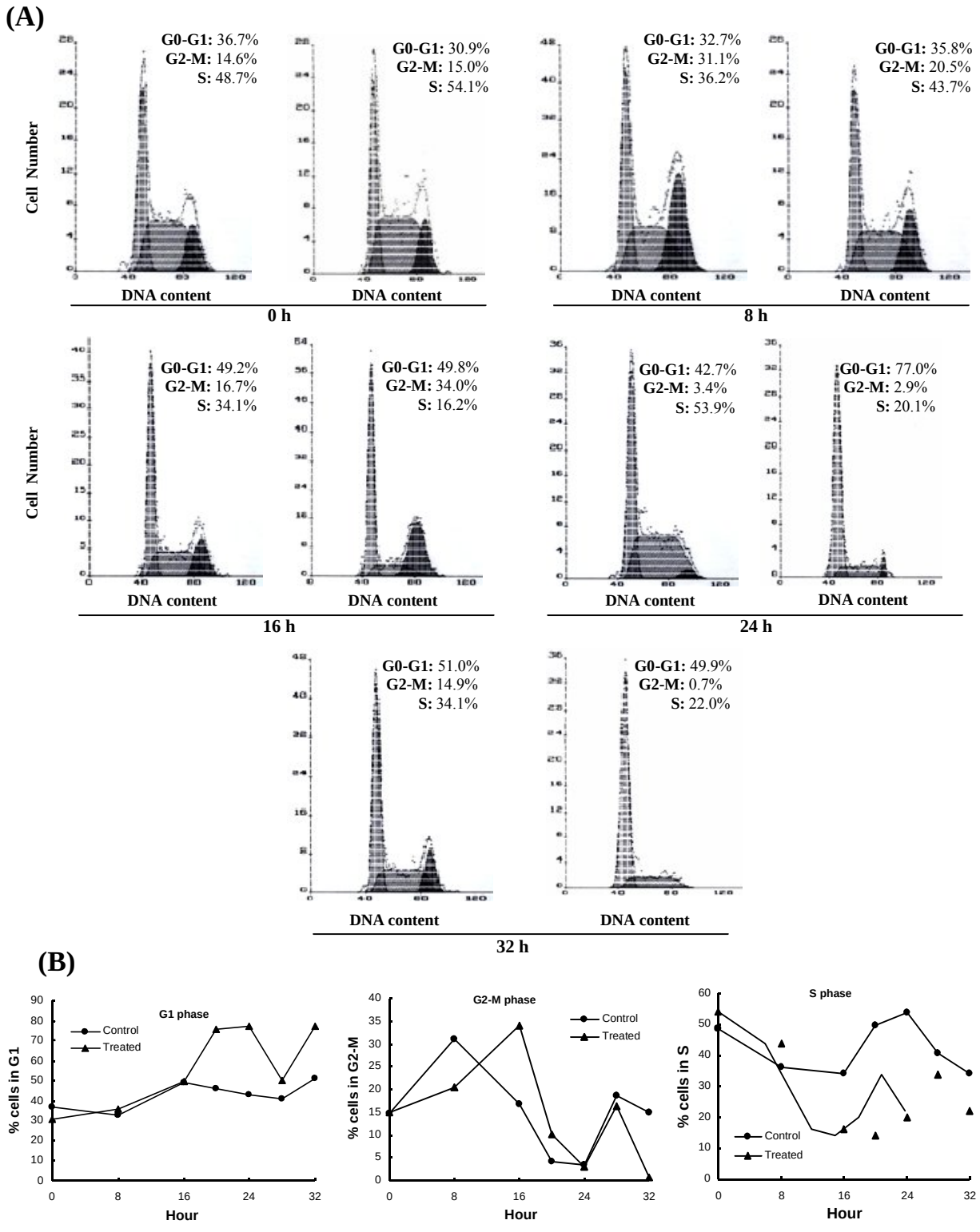


Fig. 2. Inhibition of cell cycle progress in Thr.C1.PI33 cell line by treatment with 3-hydrogenkwadaphnine (3-HK). **(A)** Distribution of the cells in the cell cycle. Cells were synchronized and replaced by a medium containing 5% FBS. Every 4h cells were collected fixed and stained with propidium iodide. DNA content was analyzed by flow cytometry. The percentage of cells within the G1, S, and G2-M phases of the cell cycle was determined. Every graph is the result of a single trial. **(B)** Graphic representation of the percentage of cells in G1 phase, G2-M phase and S phase at every 4 h for 32 hours. Note the accumulation of treated cells in G1, and slow and fewer number of cells progressing through G2-M phase and S phase under the effect of 3-HK.

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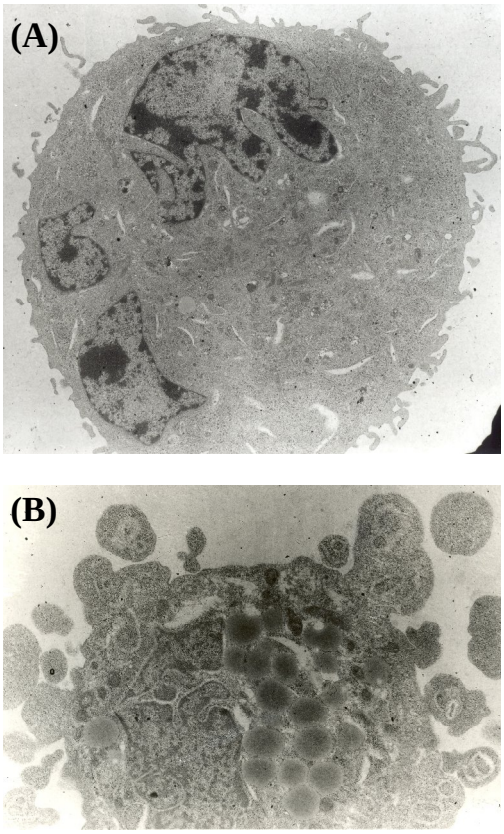


Fig. 3. Human thyroid cancer cell line Thr.C1.PI33 before and after treatment with *D. lessertii* crude extract visualized by transmission electron microscopy. **(A)** A normal cell with intact nucleus and mitochondria and with a number of microvilli (Magnification ×3000). **(B)** An apoptotic cell with fragmented nucleus, apoptotic bodies containing DNA material, and smooth cell surface without microvilli (Magnification ×7000).

membrane structures such as microvilli, and cytoplasmic membrane blebbing and formation of apoptotic bodies containing cytoplasm, organelles and nuclear fragments.

Microvilli are believed to participate in the mobilization of thyroid hormones. In thyroid as well as other cell types microvilli function to increase surface area for important secretory, transport and enzymatic activity. Microvilli also serve as grouping sites for surface molecules that mediate cell-cell adhesion and signaling, movement, and extravasation of lymphocytes [27].

The abundance of microvilli is found to be the major differences among different types of thyroid carcinoma [28]. Although the density of microvilli decrease in neoplastic compared to normal states, the TEM pictures of the individual cells in this study show that the cancerous cells still possess some of their microvilli which they lose after treatment with lessertii extract (Fig. 3). There is evidence that microvilli may serve as a facilitating mechanism for metastasizing cancer cells enabling them to invade distant parts of the body. Metastasizing cancer cells have perhaps developed a control mechanism over organization of microvillar morphology and structure. Also, the timing of their induction and differentiation disassembling them when they need to lose contact with the original cancer tissue and assembling in time of extravasation and adherence. The dynamic nature of the microvilli renders them susceptible to a wide variety of drugs that like *lessertii* extracts can eliminate them or like low doses of lovastatin induce them as reported by Wang *et al.* [28]. Whether the elimination of microvilli from thyroid cancer cells by the lessertii crude extract and 3-HK is permanent or temporary could not be commented at this stage, but the ablation of these structures permanently or for a long time to permanently prevent their adherence merit further investigation. This is a way to avoid cancer cell invasiveness by developing anti-metastatic agents. It may be worth to mention that the disappearance of microvilli as one of the early events in apoptosis may be under the control of Rho protein. The Rho family of proteins is a GTPase able to suppress apoptosis while metastasizing (i.e. anoikis) by interacting with a wide variety of proteins that mediate cytoskeletal remodeling [29, 30].

All parts of the *Dendrostellera lessertii* plant are highly toxic, causing swelling of lips and tongue, thirst, nausea and vomiting, internal bleeding, weakness, coma and even death if eaten; it causes irritation and permanent darkening of the skin. The liver is the site of detoxification in the body, therefore, it may be worth to mention that low concentrations of the crude extract of this plant enhanced cell proliferation and had pronounced effects on the morphology of liver cancer cells, causing detachment and loss of anchorage without cell death (the IC_{50} of the crude extract for HepG2 cells was found to be around 5 mg/ml, which is about 10 times that of Thr.C1.PI33 cells, 0.45 mg/ml) while the pure compound did not show such immediately visible effects which may suggest the safety of pure substance (3-HK) compared to the raw material in terms of possible liver damage (unpublished data). While the traditional herbal medicines are often useful in their wholeness and/or mixed with other herbal preparations, this may not apply to toxic plants that may contain potentially valuable anti-disease chemicals.

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