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# Anti-tumor Activity of *Dendrostellera lessertii* and the Inhibitory Effect of One of Its Purified Diterpene Ester on Wehi-164 cell Adhesion

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**Abstract** – In the course of our studies for new anticancer medicinal plants, we evaluated the effects of an alcohol-water (1:1, V/V) extract of *Dendrostellera lessertii* (Thymelaeaceae) leaves on the growth rates of breast tumors of rats. The breast tumors were induced in a group of rats by Dimethylbenz[a]anthracene (DMBA) injection. Our data showed that daily oral feeding of the crude extract to the rats, for 20 consecutive weeks, significantly repressed the growth rates of the breast tumors. In addition, the probable effect of *D. lessertii* crude extract or one of its purified active components on metastasis was evaluated using wehi-164 cells. Treatment of the cells with a single nontoxic dose of the purified active component for 48 hours inhibited the adhesion of the cells to the immobilized fibronectin molecules by almost 80% compared to the untreated control cells.
**Keywords** – adhesion, anti-tumor activity, breast cancer, *Dendrostellera lessertii*.

## Introduction

Tumor cell metastasis constitute a complex series of sequential events. Understanding the mechanism of this process in which malignant cells detach from the primary growth sites to form new secondary tumors in different organs, is certainly one of the challenging area of cancer research. Many steps of metastasis involve cellular interactions through cell surface molecules (Cavallare, 2001; Nicolson, 1984; Van Roy *et al.*, 1992). After detachment from the primary growth site, the circulating tumor cells either attach to each other (homotypic adhesion) or to the host cells such as platelets (heterotypic adhesion) through cell surface molecules to form cluster of cells named emboli. It is believed that these emboli play significant roles in tumor cell metastasis (Fidler, 1973; Glaves, 1983; Liotta *et al.*, 1976). Many of these cell surface molecules which mediate the cell-to-cell and the cell-to-extracellular matrix adhesion, have been characterized as cell surface glycoproteins (Danguy *et al.*, 2002; Humphries *et al.*, 1986; Springer *et al.*, 1991; Warren, 1999). It is now well documented that the chemical nature of carbohydrate moieties of these glycoproteins changes upon cell transformation (Bevilacqua *et al.*, 1987; Lin *et al.*, 2002; Yogeeswaran *et al.*, 1981). The oligosaccharide side chains of glycoproteins in tumor cells are usually larger in size, highly branched and well oversialylated at their chain termini (Humphries *et al.*, 1986; Lin *et al.*, 2002). On the other hand, drug induced modification of carbohydrate section of tumor cell glycoproteins, has altered their metastatic potential in experimental model systems (Hagmar *et al.*, 1973; Kijima-Suda *et al.*, 1986; Sinha *et al.*, 1974). Treatment with either swainsonine, an inhibitor of golgi α-mannosidase II, or tunicumycin, an inhibitor of synthesis of N-acetylglucosaminylpyrophosphatyl-polyisoprenol, an intermediate in the glycosylation process, has been reported to inhibit the experimental metastasis of $B_{16}$ melanoma cells (Elbein *et al.*, 1981; Humphries *et al.*, 1986; Humphries *et al.*, 1988; Olden *et al.*, 1991).

In this investigation, as part of our studies of plants for new anticancer agents with emphasis on Thymelaeaceae family, we examined the cytotoxicity and antitumor activity of an alcoholic extract of *D. lessertii* against breast tumors induced in rats by DMBA injection. After exploring the high antitumor activity of the plant extract, the anti-metastatic property of one of the purified active components of *D. lessertii* (Sadeghi *et al.*, 2002), was evaluated by measuring the extent of attachment of metabolically labeled and plant treated wehi-164 cells to fibronectin coated plates.

## Experimental

**Materials** – The cell culture medium (RPMI 1640), Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (life technologies, Scotland). The cell culture petri dishes were obtained from Nunc

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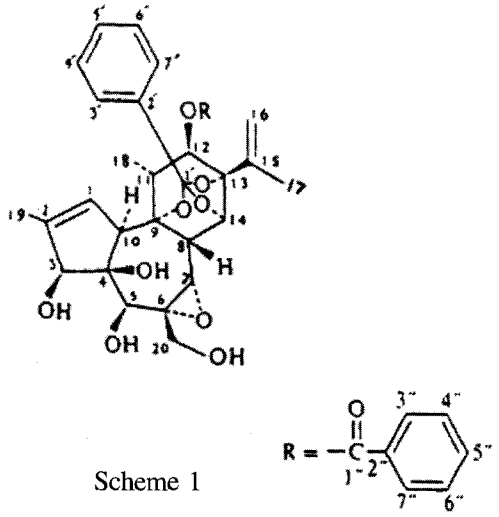
(Denmark). Fibronectin was purchased from Sigma Chemical Co. (USA). The [$^3$H]-Thymidine, L-[1-$^{14}$C] leucine and D-[2-$^3$H] mannose were purchased from Amersham (UK). Wehi-164 cell line (NCBI C200) was obtained from Iranian Pasteur institute (Iran). Organic solvents and silica gel 60 for column and tlc were obtained from Fluka (Sweden).

**Plant materials, Extraction and Purification** – The aerial sections of *D. lessertii* were collected from suburbs of Isfahan at the end of spring. The plant materials were dried, far from direct sunlight. The dried leaves were powdered and maintained in a closed container in the cold room. The plant powder (500g) was extracted three times with a mixture of methanol: water (1:1, v/v). The accumulated extract was concentrated under reduce pressure and then extracted with chloroform. The chloroform extract was concentrated to 2 ml under reduced pressure. The residue was fractionated on a silica gel column (40×1.5 cm) using diethyeter: chloroform mixture (8:2,6:4 and finally 4:6, v/v) as the eluting solvent. The most active component was localized in fraction no.2, using shrimp test (Sadeghi *et al.*, 2002). The structure of the purified active component was established to be a diterpene ester (scheme 1) With a molecular weight of 604 mu using different spectroscopic techniques. The structure elucidation results have been submitted elsewhere for publication.



Scheme 1

**$LC_{50}$ Estimation** – The biological potency of plant extract ($LC_{50}$) was obtained using *Artemia solina* test as described by McLaughline and coworkers (Meyer *et al.*, 1982) and as reported previousely (Sadeghi *et al.*, 2002).

**Cell culture** – The cells (K562, CCRF-CEM, HL-60, Wehi-164) were cultured in RPMI 1640 medium supplemented with FBS (10%, V/V), streptomycin (100 μg/ml), penicillin (100 U/ml) (yoshida *et al.*, 1996). The cells, $1\times10^6$, were seeded, in triplicate, into culture dishes incubated at 37°C in an incubator with 5% $CO_2$ atmosphere for 24 h prior to treatments. The plant extract or the purified component were given once, at various concentration, to the cells. The cell viability was assessed by trypan blue exclusion test (Gorman *et al.*, 1996), 24 h after treatments.

**Animal Treatments** – DMBA treatment: Female Sprague dawley rats (Hasarak, Iran) were fed high fat diet from age 21 days. The high fat diet was prepared by powdering ordinary diet and adding 20% corn oil to the powder followed by making pellets and drying in an oven at 37°C. The rats were housed in the temperature and light controlled animal house with free access to water and food. Mammary tumors were induced by i.g. administration of 5 mg DMBA (in 1 ml of corn oil). When the animals were 50 days old, the carcinogen was administered twice a week for four consecutive weeks. In order to minimized any possible effect of dietary fat on the carcinogen absorption from the intestine, rats were switched to the ordinary low fat diet 12 h before DMBA administration. The rats were returned to high fat diet 12 h after DMBA had been given (Clement, 1980). The control rats were given, in the same manner, equal amount of corn oil. Rats were palpated once a week, and tumors were identified by their location and their size were determined with a Vernier caliper in two perpendicular diameter.Rats with tumors were separated from other rats and divided into two groups of I and II. The plant extract (0.5 ml/ rat) was given i.g. to the rats of group II daily for 19 consecutive weeks. The rats of the control group (group I) received equal volume of distilled water by needle feeding. The changes in the length and width of each tumor was measured each week. The approximate volume of each tumor was calculated using the formula: $V=L.W^2/2$ in which V, L and W represent, respectively, the volume, length and the widths of each tumor (Hussy *et al.*, 1996).

**Radiolabeling of acid-insoluble and DNA materials in wehi-164 cells** – The wehi-164 cells ($3\times10^4$-$1\times10^5$ cells) were seeded into tissue culture wells using RPMI-1640 culture medium. After 48 h, the cells were treated with a single safe dose of plant extract (0.4 mg plant powder/ml) or the purified component (2 nM). The cells were incubated at 37°C for further 18 h. Then L-[$^{14}$C]leucine and D-[$^3$H] mannose or [$^3$H]-thymidine alone were added to a final radioactivity density of 0.2 μCi/ml, 5 μCi/ml and 0.2 μCi/ml, respectively.The cells were incubated for 20 min more at 37°C. Then the cell monolayers were washed twice with cold $PBS^-$.

**Cell attachment assay** – Attachment assays were conducted according to a method described by Nagata and colleaques (Nagata *et al.*, 1985). Briefly, the 24-well tissue culture plates were preincubated with various concentrations

of fibronectin solutions. The adhesion factor was diluted with $PBS^+$ and 300 µl aliquots were added to each well. The plates were incubated for 60 min at room temperature. The non-specific adsorption sites on the plastic surfaces were blocked by incubation with 300 µl heat-denatured BSA (10 mg/ml) for 30 min. The [$^3$H]-Thymidine labeled plant treated wehi-164 cells (from previous section) were washed with $PBS^-$, detached with 0.25% trypsin, containing 0.02% EDTA. After centrifugation for 5 min at 1000 rpm, the cells were resuspended to $3.3\times10^5$ cells/ml in RPMI-1640 medium and kept at 37°C for 10 min. Then 300 µl aliquots of this cell suspension were added to fibronectin coated wells and incubated for 20 min at 37°C. The unattached cells were removed and the attached cells were washed twice with $PBS^+$ and then solubilized using 300 µl of 0.1 N NaOH.The radioactivity content of each sample was determined by a β-scientillation counter.

**Cell Spreading assay** – The spreading assays were done according to Yamada's Method (Yamada *et al.*, 1984). The 96-well tissue culture plates were coated with 100 µl aliquots of fibronectin solution and then with 100 µl of heat-denatured BSA solution (10 mg/ml). On the other hand, the wehi-164 cells were treated with a single safe dose of the plant extract (equivalent to 0.4 mg plant powder/ml) or a single dose of the active component (2 nM) for 18 h. The treated cells, along with the untreated control cells, were detached using 0.25% trypsin in 0.02% EDTA, centrifuged, washed with $PBS^-$ and then resuspended to $3\times10^5$ cells/ml RPMI medium. The cells kept for 10 min at 37°C, centrifuged and resuspended in RPMI medium. Then 100 µl aliquots of the cell suspension were added to the fibronectin coated wells. The mixtures were incubated for 60 min at 37°C. The attached cells were then fixed with 3% formaldehyde containing 3% glutaraldehyde in PBS.The percentage of cells adapting a normal well-shaped morphology were estimated by counting 400 cells per well with randomized selected fields.

## Results and Discussion

**Bioassay** – The biological potency of the ethanol-water extract of *Dendrostelera lessertii* leaves after alcohol evaporation was evaluated using shrimp test (Meyer *et al.*, 1982). The $LD_{50}$ was determined to be equivalent to 0.062 mg of powdered plant leaves per ml of the bioassay solution. By the same approach the $LD_{50}$ of the powdered stem was determined to be equivalent to 0.3 mg powder per ml of the test solution. The entire investigation was therefore done using the more potent plant leaves extract. Using four different cancer cell lines, the $IC_{50}$ of the active component was established with respect to each cell line (Table 1).

**Table 1.** Inhibition of cell proliferation by *D. lessertii* and one of its purified active components. The cells were cultured in the presence of various concentrations of the crude extract or the purified component for 48 h. Each measurement is the average of three independent measurement

| Cell line | $IC_{50}$ | |
|---|---|---|
| | Plant extract (mg/ml) | Purified active component (nM) |
| K562 | 0.36 | 5 |
| CCRF-CEM | 0.42 | 12 |
| HL-60 | 0.40 | 8 |
| Wehi-164 | 0.42 | 12 |

**Antitumor Activity** – The effect of ethanol-water extract on the breast tumor was evaluated in rats. Breast tumors appeared in only 62% of the rats receiving DMBA injection and most of the tumor-bearing rats had 2-3 tumors. The tumors, as established by pathologists, were mostly adenocarcinoma and carcinomas. Our *in vivo* investigation showed that the ethanol-water extract of the *Dendrostelera lessertii* leaves had significant anti-tumor activity against rat breast tumors. As it is shown in Fig. 1, the tumor volume size of three selected tumors from different control rats increased up to $3\times10^{-3}$ $mm^3$ over a period of 20 weeks. In contrast, the volume sizes of breast tumors in rats treated daily with the plant extract in a dose of 0.5 ml per day (equivalent to 0.5 g of the powdered leaves) significantly declined over the same period of time. In some cases, as it is evident from Fig. 1, the tumor sizes were regressed totally so that no palpable tumors could be recorded after 15 weeks of daily treatment with the extract. Figure 2

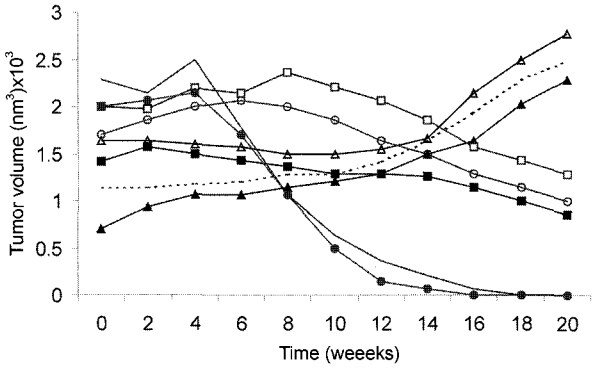



**Fig. 1.** The effects of oral administration of the plant extract on the breast-tumor volume in rats. After tumor induction with DMBA, the tumor-bearing rats were divided into two groups: The test rats (—, ○–○, ●–●, □–□ and ■–■) received by gastric intubation 0.5 ml of the plant extract daily for 20 consecutive weeks and the control rats (△–△, —, ▲–▲) received equal volume of water in the same manner and for the same period of time. The statistical significances of the observed differences in the treated rats, compared to the corresponding control rats, are: $P<0.001$.

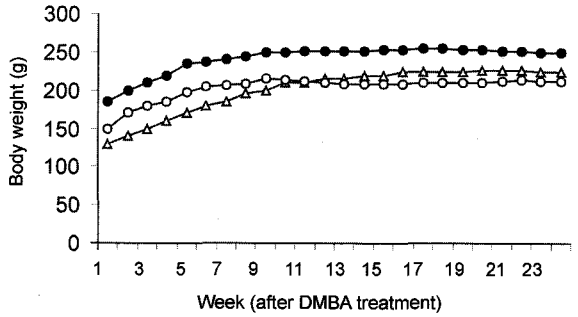



**Fig. 2.** Effects of ethanol-water plant extract on the body weight of rats pretreated with DMBA. Control (△−△), carcinogen treated (○−○) and carcinogen and plant extract treated (●−●). For experimental details see material and methods.

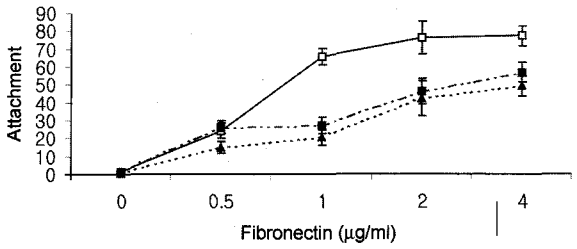



**Fig. 3.** Attachment of [$^3$H] thymidine-labeled control (□), crude extract (■) and the active component (▲) treated wehi-164 cells to plastic tissue culture wells coated with the indicated amount of fibronectin. Each measurement represents the mean of triplicate determination. ± SD.

indicates that the gain in body weight of all groups of rats: the healthy group, the control group (received only DMBA) and the test group (received DMBA followed by the plant extract) were similar and not affected by the consumption of the plant extract. Since the amount of daily water intake per rat was essentially the same in rats of all three groups, regression of the tumor growth rate by the plant extract can not be attributed to reduced food intake.

Since no toxicity was virtually observed during the long treatment period of tumor bearing rats with the plant extract and the inhibitory effects against the growth rate of mammary tumors were significant and reproducible, further research, concerning the mechanism of action of the plant extract was designed.

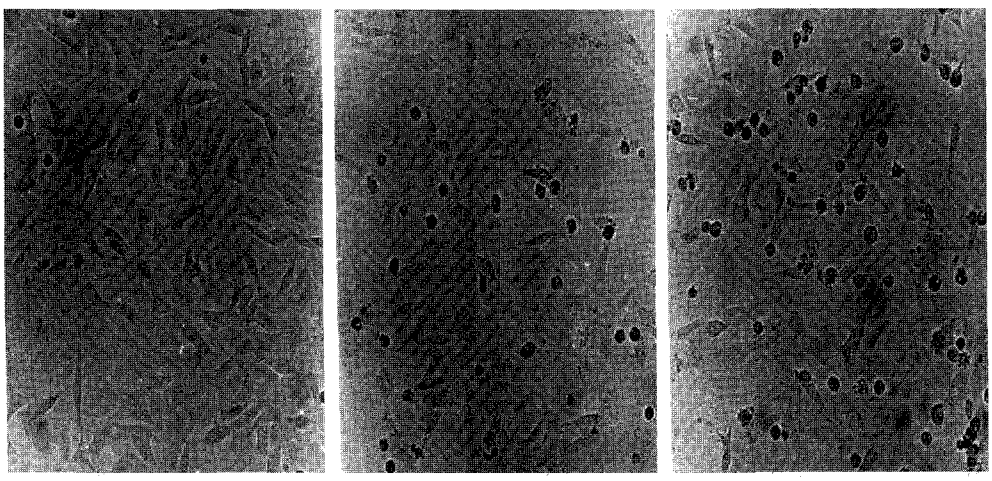
**Cell adhesion** – The effect of *D. lessertii* crude extract and the purified active component on the adhesive property of cells was evaluated using adherent wehi-164 cells. For this purpose the cells were treated with 20 μl of the crude extract (equivalent to 0.4 mg plant powder/ml) or with the active component (2 nM). The nontoxicity of these doses on wehi-164 cells were measured by comparing the extent of L-[$^{14}$C]-leucine and D-[$^3$H]-mannose incorporation into acid insoluble materials of the treated and untreated cell samples. As it is shown in Table 2, the net L-[$^{14}$C]-leucine and [$^3$H]-mannose incorporation into membrane proteins is not significantly affected by the treatment of cells with a single dose of the plant extract or a single dose (2 nM) of the purified component, compared to the untreated control samples.

**Attachment behavior of treated wehi-164 cells** – The ability of cells to attach to fibronectin macromolecules, immobilized on the surface of plastic tissue culture dishes, were measured and compared with the corresponding behavior of the untreated control cells. Fibronectin, as it is shown in Fig. 3, mediated concentration-dependent stimulation of attachment of untreated cells, with maximal level of approximately 80% attachment at a coating concentration of 1-3 μg/ml of adhesion factor. However, upon treatment of wehi-164 cells with a single dose (20 μl) of the crude extract (0.4 mg/ml) or the active component (2 nM), the extent of attachments to fibronectin coated wells (at a concentration of 2 μg/ml) were quenched by 20% and 30%, respectively (Fig. 3).

**Morphology of fibronectin-attached wehi-164 cells** – In an assay similar to that used to measure cellular attachment, the effect of treatment with *D. lessertii* crude extract or the active component on wehi-164 cell morphology and spreading capacity was examined after an extended 60 min incubation of cells in wells containing immobilized fibronectin. Untreated wehi-164 cells spread with characteristic polygonal morphology on fibronectin substance (Fig. 4,a). However, the crude extract treated (Fig. 4,b) or the active component treated (Fig. 4,c) cells showed poor adhesion, besides of exhibiting extensive rounding up of cell bodies. These data clearly showed the effects of *D. lessertii* crude extract and its purified active component on the molecular structure of tumor cell surface molecules which are involved in cell-to-cell and/or cell-to-matrix attachment. Further

**Table 2.** The L-[$^{14}$C]-leucine and D-[$^3$H]-mannose incorporation into the acid insoluble materials of wehi-164 cells under treatment with a single dose of *D. lessertii* crude extract (equivalent to 0.4 mg plant powder per ml of the cell culture medium) or 2 nM of the active component. Each measurement is the average of three independent measurements ±SD

| Treatment | Concentration | L-[$^{14}$C]Leucine incorporation (cpm/well) | [$^3$H]Mannose incorporation (cpm/well) |
|---|---|---|---|
| − | 0 | 132.87±14.67 | 147.13± 1.20 |
| + Crude extract | 0.4 mg/ml | 133.67± 6.12 | 140.67±12.70 |
| + Active component | 2nM | 130.02± 9.82 | 146.13±13.2 |



(a) (b) (c)

**Fig. 4.** Microscopic analysis of the morphology of untreated control wehi-164 cells (a), cells treated with the crude extract (0.4 mg/ml) (b), and cells treated with 2nM of the purified component (c) cultured in the fibronectin coated wells (3 μg/ml).

investigation, regarding the *in vivo* metastatic capacity of tumor cells under the influence of the crude extract or the purified active component, is going on in our laboratory and the results will be published soon.

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