

Basic Sciences in Medicine

CYTOTOXIC ACTIVITY OF *THYMUS VULGARIS*, *ACHILLEA MILLEFOLIUM* AND *THUJA ORIENTALIS* ON DIFFERENT GROWING CELL LINES

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ABSTRACT

The cytotoxic activity of ethanolic extracts of *Thymus vulgaris*, *Thuja orientalis* and *Achillea millefolium* was investigated on various growing tumor cell lines. MTT colorimetric assay was used for measuring the inhibition of cell proliferation. All of the three extracts showed a relatively dose-dependent inhibition of proliferation of human breast cancer (SK-Br-3, MDA-MB-435) and leukemia (U937 and K562) cell lines. The most sensitive cells to the effect of *Thuja orientalis* and *Achillea millefolium* extracts were K562 and SK-Br-3 and to *Thymus vulgaris* were U937 and MDA (50% inhibition at 10 µg/mL). Hela cell line was less sensitive than other tumor cell lines (% inhibition range -10 to 46). Despite this potential cytolytic activity against tumor cell lines, low concentrations of the extracts showed stimulatory effects on the growth of Vero, a non-malignant cell line. Proliferation of these cells was increased by 10 to 100 µg/mL of *Achillea millefolium* and *Thymus vulgaris* (%inhibition range -48.8 to -6.25) and suppressed by a concentration of 400 µg/mL (%inhibition 6.25 and 27.6, respectively). Various concentrations of *Thuja orientalis* showed stimulatory effects on this cell line (%inhibition range -34.00 to -21.4). Study of the effect of two low and high concentrations of the extracts on mitogen-induced human lymphocytes resulted in a slight increase at 50 µg/mL (Stimulation index, SI range 1.19 to 1.37, $p < 0.01$). In conclusion, although the extracts showed strong cytotoxicity for tumor cell lines, the proliferative lymphocytes and the non-malignant cell line used in this study were clearly less sensitive.

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Keywords: Cytotoxic activity, *Thymus vulgaris*, *Achillea millefolium*, *Thuja orientalis*, MTT assay.

INTRODUCTION

Medicinal plants have been used in the treatment of

malignant diseases for many years. Efforts have been applied to study the anti-tumor effects of these plants in order to identify the new potential compounds useful as anti-cancer drugs. During recent years, a variety of plant extracts have been investigated for their cytotoxic ability on cancerous cells including various cell-lines like HL60, human hepatoma cell line, HepG, U937 mono-

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cytic cell line, Hela cells, human lymphoid leukemia (MOLT-4B) cells, KB oral carcinoma, and K562 erythroleukemic cells. The agents found to have cytotoxic activity included extracts of plants like *Salvia miltiorrhiza*, a traditional Chinese herbal medicine, commonly used to treat liver diseases in China,¹ *Gynostemma pentaphyllum* mak,² *Anemarrhena asphodeloides* Bunge,³ mistletoe (*Viscum album* L)⁴ and *Semicarpus anacardium*.⁵ Isolated compounds like two diastereomeric saponins, julibrosides J1 and J9 obtained from the stem bark of *Albizia julibrissin* Durazz,⁶ Tanshinone II-A, a derivative of phenanthrene-quinone isolated from *Salvia miltiorrhiza* Bunge,⁷ bryonolic acid from *Trichosanthes kirilowii* var. *Japonica*,⁸ crocin from saffron⁹ and allicin from *Allium sativum*¹⁰ have also been found to cause growth inhibition and cell death. A microculture tetrazolium (MTT) assay in 21 tumor cell lines also revealed that d-Dicentrine, a naturally occurring aporphine type isoquinoline alkaloid, isolated from the root of *Lindera megaphylla* Hemsl. (Lauraceae) was most cytotoxic to esophageal carcinoma HCE-6, lymphoma cell lines, MOLT-4 and CESS, leukemia cell lines HL60 and K562, and hepatoma cell line MS-G2.¹¹

The hydroalcoholic extract of 14 species of Ayurvedic plants was also tested for cytotoxicity on COLO 320 tumor cells, using the MTT assay. The strongest cytotoxic effect causing 50% growth inhibition of the tumor cells was found for *Calotropis procera* (Ait). (Asclepiadaceae) and of the nuts of *Semecarpus anacardium* (Anacardiaceae). Even Nutraceuticals like soya bean,

some cytotoxic effects were found for crude extracts of three herbs including *Thuja orientalis*, *Thymus vulgaris* and *Achillea millefolium*. The present study was designed to evaluate the cytotoxic activity of these herbs against various tumor cell lines using MTT colorimetric assay. *Thuja orientalis*, *Thymus vulgaris* and *Achillea millefolium* belong to Cupressaceae, Lamiaceae and Compositae family of plants and popularly are known as sarv-e khomreii, avishan and boomadaran in Persian respectively. These plants are traditionally used for treatment of different disease such as cold, bronchitis and some chronic diseases. In several studies the biological activity and chemical composition of these herbs have been investigated and some anti-microbial and anti-inflammatory effects have been found.¹⁸⁻²⁶ No anti-tumor activity has been reported except for *Achillea millefolium*.²⁷ This herb has been found to be active against mouse P-388 leukemia cells.

MATERIAL AND METHODS

Preparation of culture medium with the extracts

Samples of fresh leaves and flower heads of *Thymus vulgaris* and *Achillea millefolium* in the summer and spring respectively and roots of *Thuja orientalis* during summer were collected from Fars province and authenticated by Mr. Iraj Mehregan from Shiraz School of Pharmacy. Voucher specimens were deposited in the Herbarium of the School of Pharmacy. Samples were washed, dried and then transferred into a percolator containing 70% ethanol. Extraction was done with a flow rate of 3 mL/min and then the extract solution was concentrated in a rotary evaporator (Heidolph, Germany). The yield (w/w) of extracts was 11.1%, 8.1% and 8% for each plant, respectively. Dried extracts were later dissolved in RPMI medium to obtain 20 mg/mL and mixed at 37°C for 20 minutes. This solution was centrifuged to remove insoluble ingredients, and then the supernatant was passed through 0.22 µm filters for sterilization. The solution was diluted with the medium and prepared at four concentrations (10, 50, 100, and 400 µg/mL).

Cell culture

Tumor cell lines including Hela (cervix epitheloid carcinoma), Ag8.653 (mouse myeloma), SK-Br-3 (breast cancer), K562 (myelogenous leukemia), MDA-MB-435 (breast cancer) and U937 (histiocytic lymphoma), and Vero (monkey kidney primary cells) purchased from Iranian cell bank and/or ATCC were used in this study. All the cell lines were kept in RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 10% fetal calf serum (Gibco, Germany) in culture flasks at 37°C in 5% humidified CO₂ incubator. The cells were fed until confluence (2×10⁶) and were expanded by trypsinization

Table 1. Effect of two concentrations of the extracts on the PHA-induced lymphocyte proliferation. SI (stimulation index) for PHA-treated cultures without the extract is 1. P values were all less than 0.01.

Extract	Concentration (µg/mL)	Mean SI±SD
<i>Thymus vulgaris</i>	50	1.27±0.08
	400	0.02±0.01
<i>Achillea millefolium</i>	50	1.37±0.07
	400	0.10±0.01
<i>Thuja orientalis</i>	50	1.19±0.01
	400	0.04±0.01

garlic, ginger, green tea, etc. have been suggested, in epidemiological studies, to reduce the incidence of cancer.^{5,13-16} Such studies have led to many classes of cytotoxic compounds including polyphenolic compounds, sesquiterpene lactones, lignans, quassinoids, triterpene glucosides, flavonoids, colchicine derivatives, and quinone derivatives.¹⁷

During the course of our study on different plants,

and subcultured at lower numbers in new culture flasks. Viability of cells was determined by trypan blue dye exclusion test.

MTT colorimetric assay

A colorimetric assay using 3 (4,5-dimethylthiazoyl-2, 5-diphenyltetrazolium bromide) (MTT) was performed as described.²⁸ Briefly, cells were added onto the flat-bottomed micro-culture plates in the presence or absence of the various concentrations of the extracts (in triplicate) and incubated at 37°C in a 5% humidified CO₂ incubator for 48 hours. Then, 10 µL of MTT (5 mg/mL, Gibco, Germany) was added to each well and incubation was continued for a further 4 hours at 37°C. 100 µL/well of solubilization solution containing isopropanol and 10% SDS in 0.01 M HCl was added into each well. After complete solubilization of the dye, plates were read at 570 nm on an ELISA reader. The reference wavelength was 690 nm. The mean optical density (OD)±SD for each group of replicates was calculated. Percent inhibition of cells exposed to various treatment was obtained as follows:

$$\% \text{Inhibition} = 100 - [(\text{Test OD} / \text{Non-treated OD}) \times 100]$$

Mitogen-induced lymphocyte proliferation

Human peripheral blood lymphocytes (PBL) were separated by gradient centrifugation with Ficoll-hypaque. After washing with RPMI medium, cells were resuspended in the same medium at a concentration of 1×10^6 cells/mL. 100 µL of the cells were added into each well of microtissue culture plate (in triplicate). Then an appropriate volume of phytohemagglutinin (PHA, Bahar-afshan Co., Iran) in a suboptimal dose (10 µg/mL) and extracts in the final concentrations of 50 and 400 µg/mL were added. The plates were incubated in a CO₂ incubator for 3 days. [³H]-Thymidine (0.5 µCi/well) was then added to the wells and after 18 hours harvesting was done. [³H]-Thymidine incorporation was measured using a beta-counter (Pharmacia, Sweden). Stimulation index (SI) was obtained by dividing (the mean CPM of treated cultures - the mean CPM of untreated cultures) to (the mean CPM of cultures treated with PHA alone - the mean CPM of untreated cultures).

Data were analyzed using SPSS software and Duncan test.

RESULTS

Thymus vulgaris extract showed a relatively dose-dependent inhibitory effect on the proliferation of all tested cell lines except Vero cells. According to 50% inhibition of cell proliferation, the order of sensitivity

of the cell lines to this extract was U937>MDA>K562>SK-Br>Ag8. Treatment of MDA cells with 400 µg/mL of the extract resulted in 100% inhibition. The extract showed a dose-dependent inhibition of Hela cells, but this inhibitory effect didn't reach 50%. In the culture of Vero cells, this extract showed stimulatory effects at concentrations less than 400 µg/mL (%inhibition range -46.8 to -14.3). The highest stimulatory effect was observed at 50 µg/mL. At 400 µg/mL cell proliferation was markedly decreased (%inhibition

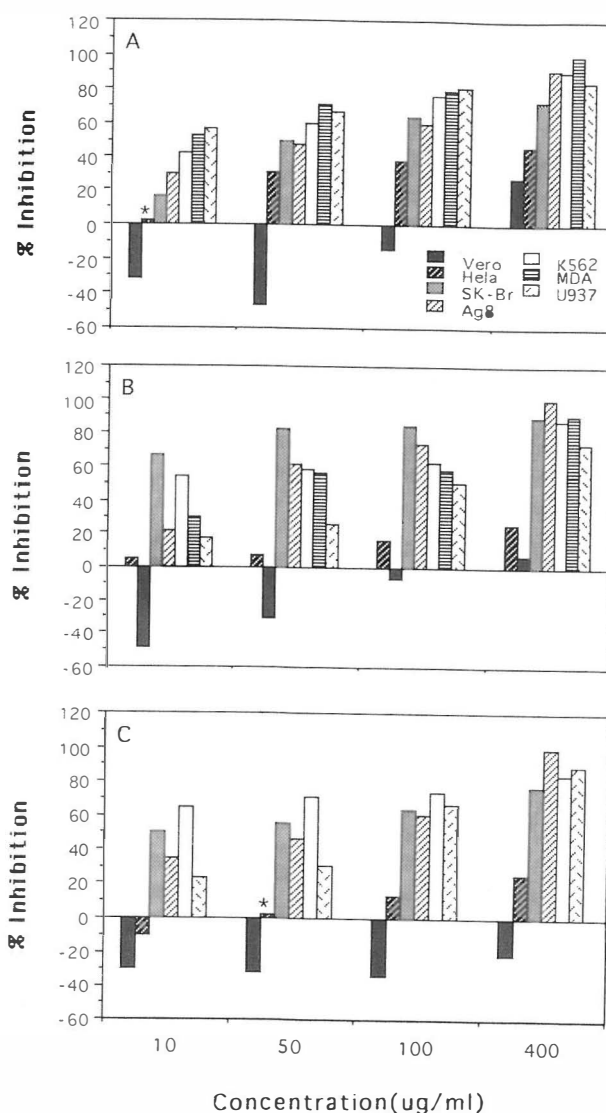


Fig. 1. Effect of various concentrations of the extracts obtained from A) *Thymus vulgaris* B) *Achillea millefolium* and C) *Thuja orientalis* on a 48 hour culture of different cell lines. Data represents the mean of triplicate determinations. All values except those shown with a star are significant ($p < 0.05$). (SD ranged 1-8).

27.6) (Fig. 1-A).

As shown in Figure 1-B, in the culture of tumor cell lines an inhibitory effect due to *Achillea millefolium* was observed. This extract at a concentration of 10 µg/mL caused 50% inhibition of SK-Br and K562 cells growth. The proliferation of these cells was strongly decreased at 400 µg/mL (%inhibition>87). Addition of various concentrations of the Achillea to the Vero cell culture increased the proliferation activity of these cells (%inhibition range -48.8 to -6.25). The highest activity was observed at 10 µg/mL. At 400 µg/mL a 6.25% inhibition of cell proliferation was detected.

More than 50% inhibition of K562, U937, SK-Br and Ag8 cells was observed at a concentration of 100 µg/mL of *Thuja orientalis*. The most sensitive cells to Thuja were K562 and SK-Br cell lines (50% inhibition at 10 µg/mL).

Treatment of Hela cells with 10 µg/mL of the Thuja extract slightly increased the cell proliferation but in concentrations of 50 µg/mL and higher, the proliferation activity decreased to reach 26.3% inhibition. In culture of Vero cells, the cell proliferation was increased by concentration of 10 to 400 µg/mL (%inhibition -34.00 to -21.4) (Figure 1-C).

To demonstrate the effect of extracts on normal human lymphocytes two concentrations of the extracts were examined on the proliferation of mitogen induced lymphocytes. As indicated in Table I, although all the extracts showed strong cytotoxic activity at 400 µg/mL, at a lower concentration (50 µg/mL) not only did the survival of cells remain unaffected but also a slight increase in the proliferation of treated lymphocytes compared to non-treated cells was observed.

DISCUSSION

In order to demonstrate a cytotoxic effect, nothing can replace the observation of animal models, but as they are expensive and often difficult to interpret, simpler tests are used. These tests require less effort and also make possible a better understanding of the mechanisms of action of substances being tested. MTT colorimetric assay is one of these tests which is easy and sensitive for measuring agent cytotoxicity. In the present study this method was used to assess the *in vitro* cytotoxicity of *Thymus vulgaris*, *Achillea millefolium* and *Thuja orientalis* against different tumor cell lines. Generally, all the tumor cell lines responded to the extracts in a very similar fashion, perhaps indicating a common mechanism of action of the extracts in all the cell lines. The anti-tumor activity of *Achillea millefolium* at a concentration as low as 10 µg/mL was mainly against a breast cancer cell line, SK-Br, and a leukemic cell line, K562. Similar inhibitory effects of the other two extracts on proliferation of

the breast and leukemic cell lines indicated the strong anti-proliferative activity of the extracts against these types of tumor cell lines. As our study showed, Hela cells which originate from epitheloid cervix carcinoma, were less affected by the extracts. The stronger cytotoxic activity of the extracts against breast cancer and leukemic cell lines and weaker activity against Hela cells demonstrated the dependency of the effect of the extracts on the origin and nature of the tumor cell lines.

A quite different behavior was observed in Vero cells. In contrary to tumor cell lines, the proliferation of this non-malignant fibroblast-like cell line was increased by low concentrations of all the extracts. At higher concentrations, in case of *Thuja orientalis* the stimulatory effect was still observed, whereas in the cases of *Achillea* and *Thymus* the proliferation activity was inhibited. One possible explanation for these different responses might be the presence of a complex of stimulatory and inhibitory components in the extracts. At low concentrations the stimulatory component increased the proliferation of Vero cells. As the concentration of the inhibitory molecules was increased the cell proliferation decreased. The results of our study indicated that the extracts at a concentration of 50 µg/mL had no cytotoxic activity but conversely had slight stimulatory effect on the mitogen-induced human lymphocytes. This observation, in addition to no cytotoxic activity of the extracts against Vero cells, showed that these extracts might be good targets for more anti-cancer studies. Previous studies on these herbs indicated that the Thyme extract could elevate the number of normal leukocytes *in vivo*.²⁹ This elevation of leukocytes is of importance for various possibilities of immunostimulation. *Thymus vulgaris* has also been introduced as a source of natural antioxidants and thus could play an important role in the chemoprevention of diseases resulting from lipid peroxidation.³⁰ In a study on the *Labiatae* family, 26 active compounds from *Rosmarinus officinalis*, *Thymus vulgaris*, *Origanum vulgare* and *O. majorana* have been determined.³¹ A biphenyl compound, and a flavonoid, eriodicytol, were also isolated as antioxidative components from the leaves of *Thymus vulgaris* by bioassay-directed fractionation.³² Moreover, dietary supplementation of thyme essential oil during the lifetime of rats showed its effects on the antioxidant status in liver, kidney and heart tissues.³³ In another study, four acetophenone glycosides have been isolated from the butanol-soluble fraction of thyme extracts. Among these, two new compounds were determined, one of which showed weak cytotoxicity and inhibited the DNA synthesis of human leukemia cells.³⁴

In terms of *Thuja orientalis* no anti-tumor activity or immunostimulatory effect has been reported. A polysaccharide fraction from another species of the *Cupressaceae* family, *Thuja occidentalis* has been

shown to be an inducer of CD4+ T cells as well as cytokine production.³⁵⁻³⁷

Regarding the inhibitory activity of *Thuja orientalis* on various tumor cell lines, study of the induction of T cells and production of cytokines due to this herb will be an interesting subject. In our study *Achillea millefolium* was found to be slightly more toxic than other herbs. Three flavones from this herb have been isolated and identified.³⁸ Tozyo et al. have reported the anti-tumor activity of sesquiterpenoids from *Achillea millefolium*.²⁷ This structure has been found to be active against mouse P-388 leukemia cells *in vivo*. Our results are further evidence that strengthen the possible anti-cancer usefulness of this plant.

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