Tehranolide Could Shift the Immune Response towards Th1 and Modulate the Intra-Tumor Infiltrated T Regulatory Cells

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ABSTRACT

Background: *Artemisia diffusa* contains a new type of sesquiterpene lactone with an endoperoxide group (Tehranolide). **Objective:** Due to the existing similarity between the structures of Tehranolide and Artemisinin, it was hypothesized that Tehranolide would have similar effects as Artemisinin. In this study, the immunotherapeutic effectiveness of Tehranolide was investigated by direct intra-tumoral injection. **Methods:** Tehranolide was purified from *Artemisia diffusa*, and its effect on the tumor volume was investigated. The splenocyte proliferation, shifting of cytokine profile, and the presence of naturally-occurring CD4+CD25+Foxp3+ Treg cells were assessed to describe the anti-tumor immune response. **Results:** Analysis of immune response showed that, intra-tumoral injection of Tehranolide decreased the rate of tumor growth compared to control group. Furthermore, the proliferative response of mice treated with Tehranolide was enhanced. In comparison with the control group, production of both IL-4 and IFN-γ was induced ($p < 0.05$). The results indicated a decrease in tumor CD4+CD25+Foxp3+ T lymphocytes in the Tehranolide-treated group compared to the control group. **Conclusion:** Treatment of tumors with Tehranolide attenuated CD4+CD25+Foxp3+ Treg cell-mediated immune suppression and elicited a persistent anti-tumor immunity against cancer.

Keywords: Tehranolide, T Regulatory Cell, IFN-γ, IL-4

INTRODUCTION

Artemisinin, as the active component of *Artemisia annua* L, was isolated in 1972 (1) and its structure, being unique among anti-malarial drugs, was determined in 1979 (2). Artemisinin is a sesquiterpene lactone containing an endoperoxide bridge essential for its activity. Deoxyartemisinin is by no means toxic, indicating that the endoperoxide bridge contributes significantly to cytotoxicity for artemisinin (3). Because of its profound anti-malarial activity, this compound has been of special biological interest. Moreover,

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its anti-tumor activity has been reported recently (4). T helper cells are generally grouped into Th1 and Th2 cells; the former produce interferon, while the latter are characterized by IL-4, IL-5, IL-10 and IL-13 production (5).

Immune deviation towards Th1 responses results in tumor rejection as Th1 pathways typically lead to the activation of cytotoxic T-cell lymphocytes (CTL), natural killer (NK) cells, macrophages and monocytes, generally defending the body against tumors. Almost all malignancies are associated with the suppression of cell mediated immunity (CMI) (6). In screening for cancer recurrence and also for cancer monitoring, assessment of Th1/Th2 balance in cancer patients may be beneficial (7). Destruction of virus-infected cells, or malignant cells as is aimed in cancer immunotherapy, has been shown to be often but not always Th1-mediated (8). Treg cells are developed in the thymus and migrate to peripheral lymphoid tissues. The Foxp3 transcription factor is specifically expressed in natural Treg cells, and is a master gene governing the development of these cells [9]. The CD4+CD25+Foxp3+ regulatory T (Treg) cells which can exert a broad suppressive effect on anti-tumor immunity (10,11) are present in breast cancer patients (12,13). In a number of malignancies, Tregs are elevated in the peripheral blood and tumor tissues, and such an increase correlates with disease progression and worsens prognosis (14). The dramatic enhancement in anti-tumor immunity can be simply induced by depleting Treg cells (15).

The insight on Treg cell biology as well as the available molecular tools will be critical in cancer immunotherapy (16). In the present paper, we attempted to provide evidence indicating that the intra-tumoral injection of Tehranolide can greatly enhance its antitumor immunotherapeutic potency in an aggressive tumor model in which no therapy alone results in a significant anti-tumor effect.

MATERIALS AND METHODS

Isolation and Purification of Tehranolide. One thousand grams of *Artemisia diffusa* was collected from North of Iran. All parts of the dry plant was extracted by a mixture of n- hexane/ ethyl acetate / methanol (1:1:1) and was kept at room temperature for 24 h. The extract was then passed through Whatman filter paper. Subsequently, the filtrate was concentrated by a vacuum rotary evaporator at 45°C for 2 h, and kept at -20°C. The concentrated filtrate was then run through a silica gel column at different solvent polarities starting from a non-polar solvent (n-hexane) to a medium polarity mixture (n-hexane/ ethyl acetate) and ending with ethyl acetate alone. Afterwards, mixtures of different concentrations of n-hexane/ ethyl acetate / methanol were used with increasing concentrations of methanol to produce higher polarities. Fractions were collected and the purity of components in each fraction was evaluated by thin layer chromatography (TLC). Fractions 35, 36, 37, 38, 39, 40, 41, and 42 exhibited a single band on TLC. Tehranolide was then identified by the 500 MHz ^{13}C-NMR spectra, using $CDCl_3$ as a solvent.

Mice. Six-to-eight-week-old inbred female Balb/c mice were obtained from Pasteur Institute of Iran. Food and water were provided *ad libitum* and the mice were maintained under standard conditions for one week prior to experimentation. All the experiments were done according to the Animal Care and Use Protocol of Tarbiat Modares University (Tehran, Iran).

Spontaneous Mouse Mammary Tumor (SMMT). SMMT, an invasive ductal carcinoma, was developed spontaneously in female Balb/c mice (17). Tumor tissue was separated from the breast-cancer-bearing Balb/c mice and was cut by forceps and scalpel into pieces less than 0.5 cm^3. Each piece was then transplanted subcutaneously into syngenic female Balb/c mice. After tumor tissue appearance, the mice were divided into two groups of five mice each. About 17 days later, when the size of tumors reached 1500 mm^3, intra-tumoral injection of Tehranolide was started and its optimum does selected according to the results obtained from Delayed Type Hypersensitivity (DTH) test. The test group was intra-tumorally inoculated with 5.64 µg/mouse/day of Tehranolide for 6 days. PBS was injected to the control group. The tumor volume was measured in the two groups by a digital caliper. Tumor size was calculated by means of the following formula:

$$V = 1/2 \times LW^2$$

where *V* represents volume, *L* the length, and *W* the width.

Mononuclear Cell Separation. The animals from both test and control groups were killed by neck dislocation and the solid tumors were cut into small pieces and minced by forceps and scalpel. The pieces were rinsed twice with phosphate buffered saline (PBS) and passed through a 150 µm mesh stainless steel filter. The mononuclear cells (MNCs) were isolated from the suspension by density centrifugation (700×*g*, 15 min, and 20°C) using Ficol hypaque (Baharafshan, Iran). The cell layer was then removed and washed twice with PBS for 10 min by centrifugation at 360×*g* and 4°C. The precipitated cells were re-suspended in PBS containing 2% fetal calf serum (Gibco, England) and stained with Trypan Blue. The resultant viability was 90%. Ten thousand cells were poured into each of the flow-cytometric tubes and labeled with monoclonal antibodies.

Measurement of Cytokine Profiles by ELISA. To evaluate the effect of Tehranolide on cytokine production of the MNCs, spleen MNCs were separated. Spleens were removed under sterile conditions, and single cell suspensions were prepared in RPMI 1640 medium (GIBCO, UK). RBCs were osmotically lysed using 0.75% NH_4Cl in Tris buffer (0.02%, pH 7.2). The MNCs were subsequently isolated by density centrifugation (700×*g*, 15 min, and 20°C) using Ficol Hypaque (Baharafshan, Iran). Then, the cell layer was removed and washed twice by centrifugation at 360×*g* and 4°C with PBS for 10 min. The precipitated cells were re-suspended in RPMI 1640 (GIBCO, UK) medium containing 10% fetal calf serum (GIBCO, UK). After Trypan Blue staining and counting, cell viability was more than 90%. Then, 4×10^5 of the cells were poured into each of the wells of 96-well micro-plates and lysate antigen, at the final concentration of 5 µg/ml, was then added with the purpose of stimulating the cells. The resulting mixture was incubated for 72 h at 37°C and 5% CO_2. Finally, the supernatants were collected and kept at −70°C until analysed for cytokines. ELISA kit (R&D systems, Country, USA) was used to measure IFN-γ and IL-4. Briefly, subsequent to washing the wells with the buffer, the standard samples and biotin conjugates were added to each well. The mixtures were incubated for 2 h. The micro-plates were then washed three times with the washing buffer and Stereptavidin-HRP was added. The plates were further incubated for 1 hr at 37°C, and again washed with the washing buffer. The TMB substrate solution was added after the wash and after 15 min was followed by the addition of the stop solution. The results were read by an ELISA reader at 450 nm.

Flow-cytometric Analysis of T- lymphocyte Subpopulation in the Tumors. The animals were sacrificed, their tumors removed and separately cut into small pieces, rinsed twice with PBS and minced by forceps and scalpel. The suspensions were passed through a 100 μm mesh stainless steel filter. The cells were then washed twice and labeled with monoclonal antibodies. The freshly-prepared cells were analyzed by direct immuno-fluorescence staining. The antibodies and reagents used for staining were: FITC-conjugated anti-CD4, PE-CY5 conjugated anti-CD25 and PE conjugated anti-Foxp3. All the stainings were performed in a washing buffer consisting of PBS supplemented with 1% heat-inactivated fetal bovine serum (FBS, GIBCO, UK), 0.1% sodium azide (Sigma, USA), and 2 mM EDTA (Sigma, USA). After determining the viability of the cells by Trypan Blue exclusion, the cells were washed twice in the washing buffer. Each sample was immuno-stained with antibodies for 45 min at 4°C. The cells were then washed again in washing buffer and fixed with 2% paraformaldehyde. Flow-cytometric analysis was carried out using an EPICS flow-cytometer (Beckman Coulter, UK). The analysis was focused on the lymphoid areas of the forward and side scatters. Double-stained cells were analyzed using Coulter software.

Antigen Preparation. Tumors were allowed to attain a size of approximately 3000 mm^3. Afterward, the tumor cells were extracted from the breast-cancer-bearing Balb/c mice and the tumor suspension was prepared by three rounds of freezing and thawing. It was then passed through a stainless steel 150 μm filter and sonicated with a power of 4 Watts for 30 seconds followed by a 20-second incubation period for three alternative times. Finally, the extract was filtered. In order to inhibit serine proteases, 1 mM Phenylmethylsulfonyl fluoride (PMSF) was added to the cell lysates. Protein concentration of the extract was determined using Bradford method (18) and the resulting extract was stored at −20°C until used.

Proliferation Index. Treated and untreated animals were sacrificed and their spleens were removed. Splenocytes were isolated by needle perfusion method using sterile RPMI-1640. Erythrocytes were lysed at room temperature using ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3 and 0.1 mM Na2EDTA). The cells were washed and resuspended in RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin (100 μg/ml streptomycin and 100 IU/ml penicillin). Cell density was measured using hemocytometer with light microscopy and viability test was performed as before using Trypan Blue dye exclusion method. The splenocytes at a concentration of 5×10^5 cells/well were cultured in 96-well plates in triplicates in the presence of 4 μg/well of prepared antigen (T-test group) or 6 μg/ml PHA (P-positive control) or nothing (N-negative control), in a total volume of 200 μl. RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 25 mM HEPES was used without phenol red indicator. The plates were incubated for 36 h at 37°C in a humidified 5% CO$_2$ atmosphere. Cell proliferation was determined by Bromodeoxyuridine (BrdU) labeling solution. The uptake of BrdU was estimated using cell proliferation BrdU kit (Roche Diagnostic GmbH, Mannheim, Germany), and expressed as stimulation index (S.I.) by the following formula:

$$S.I. = [(T-N)/(P-N)] \times 100.$$

Statistical Analysis. The results are expressed as the mean ± standard deviations of triplicate determinations. Statistical analysis was performed using one-way ANOVA

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and two-tailed Student's *t* test. A value of p<0.05 was considered to be statistically significant.

RESULTS

Characterization of the isolated Molecules using ^{13}C NMR Spectroscopy. The purified material was characterized by ^{13}C NMR spectroscopy, as shown in Figure 1.
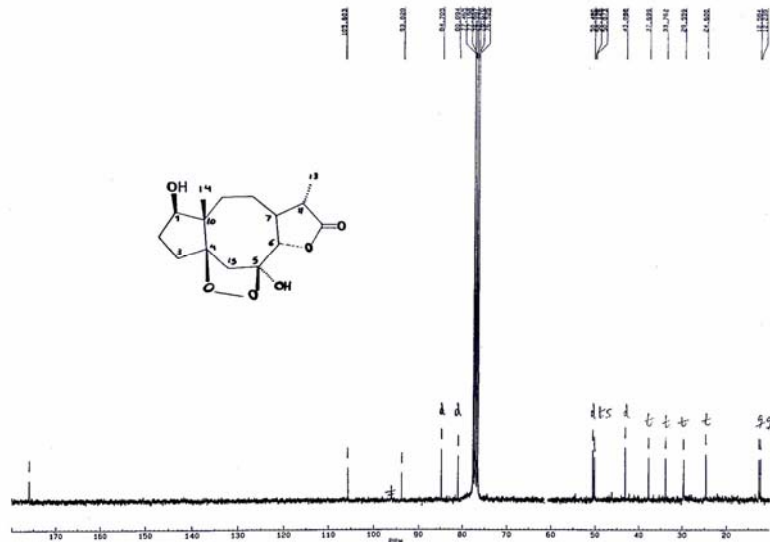

Figure 1. ^{13}C NMR spectroscopy of Tehranolide.

Effect of Tehranolide intra-tumoral Injection on Tumor Volume. In order to evaluate the tumor volume in the tumor-bearing animals, ten Balb/c mice were used and the protocol in Figure 2 was applied. Tehranolide was intra-tumorally injected every day and the tumor size was measured daily. The results indicated that Tehranolide (5.64 µg/mouse/day) decreased the rate of tumor growth significantly (p<0.05) in comparison with the control group (Figure 2).
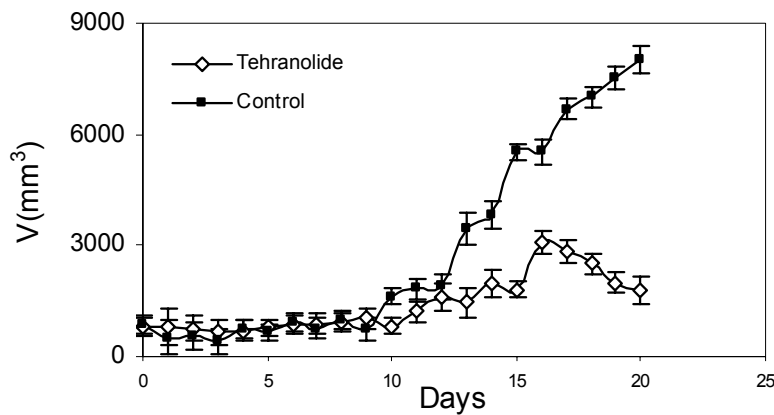

Figure 2. Tumor volume increase in the experimental and control groups. A significant difference was observed (p<0.001) when comparing the Tehranolide group with the control.

Effect of intra-tumoral injection of Tehranolide on Lymphocytes Proliferation Index. The effect of intra-tumoral injection of Tehranolide was evaluated using proliferation test. Spleen cells were collected 6 days after intra-tumoral injection and re-stimulated with the lysate antigen. The proliferative response of mice treated with Tehranolide was enhanced (p<0.001). Moreover, an increase in the stimulation index in the Tehranolide-treated animals was observed as depicted in Figure 3.
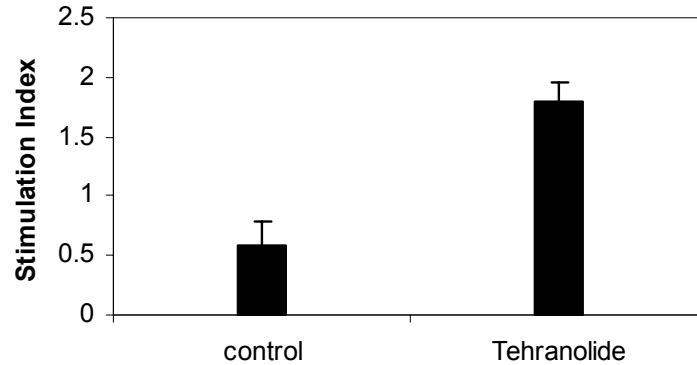

Figure 3. Lymphocyte proliferation shown as the Stimulation Index for the intra-tumorally injected mice. Splenocytes were obtained from intra-tumorally injected mice and re-stimulated with lysate antigen. Cell proliferation assay was performed using Brdu.

Effect of intra-tumoral injection of Tehranolide on the level of IFN-γ and IL-4. The splenocytes obtained from intra-tumorally injected mice were analyzed for the production of IFN-γ and IL-4. The splenocytes of Tehranolide group, which were re-stimulated *in vitro* with lysate antigen, showed a significant (p<0.05) increase in the level of IFN-γ compared to the control group. In addition, a significant decrease (p<0.05) was noticed in the level of IL-4 production in the Tehranolide group compared with the control group as shown in Figure 4.
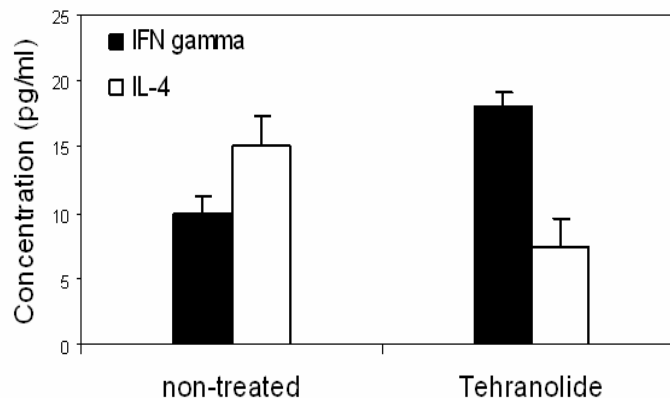

Figure 4. IFN-γ and IL-4 responses in intra-tumorally Tehranolide injected mice.

Effect of intra-tumoral injection of Tehranolide on the Level of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. The effect of the administration of intra-tumoral Tehranolide injection on the tumor CD4+CD25+Foxp3+ regulatory T lymphocytes was measured by

flow cytometry. The results in Figure 5 indicate a significant ($p<0.05$) decrease in tumor infiltrated CD4+CD25+Foxp3+ T lymphocytes in the Tehranolide-treated group compared to the control group.
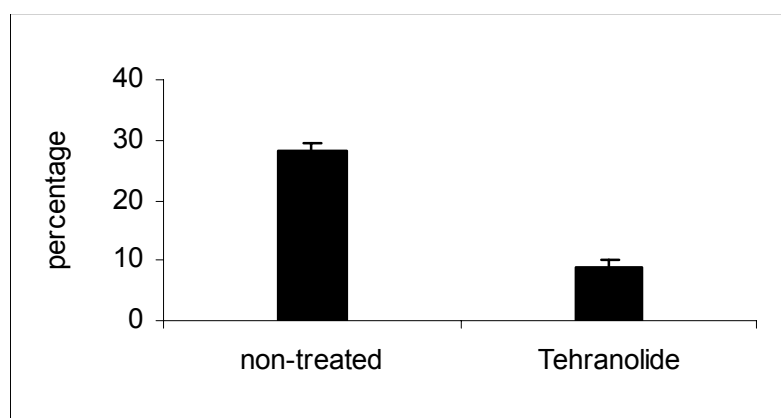

Figure 5. Effect of intra-tumoral injections of Tehranolide on the level of CD4+CD25+Foxp3+ regulatory T cells. A significant decrease ($p<0.001$) was noted in the Tehranolide treated group compared to the control group.

DISCUSSION

Artemisinin is a sesquiterpene lactone isolated from the plant *Artemisia annua* L. The compound and its analogs are being used as anti-malarial agents and their pharmacology and pharmacokinetics have been well studied (19). Artemisinin contains an endoperoxide that can react with an iron atom to form a carbon-based free radical. Such a free radical, when formed intra-cellularly, can cause macromolecular damage that leads to cell death. Since cancer cells uptake a large amount of iron compared to normal cells, they are more vulnerable to the cytotoxic effect of Artemisinin than the normal cells. *In vitro* experiments (20) have shown that, Molt-4 cells, a human leukemia cell line, and human breast cancer cells are more susceptible to the cytotoxic effect of Artemisinin than their normal counterparts; i.e. human lymphocytes and normal breast cells, respectively. Concerning the controversial effects of Artemisinin on the immune responses, i.e. its immuno-stimulative or immuno-suppressive effects, we hypothesized in this regard that Artemisinin excerts its effect in a dose-dependent manner by possessing two efficient opposite effects (biphasic). Our previous study confirmed that Artemisinin behaves differently in different doses (21). For instance, at high doses it acts as an immuno-suppressive agent (22) while it has immuno-stimulative effects at low doses [present study]. Also, we reported that Artemisinin's mechanism of inhibition may be interacting with intracellular calmodulin in high doses (23).

Due to similar structures of Tehranolide and Artemisinin, we devoted our present study to investigate the effects of Tehranolide on tumors. Tehranolide was purified from *Artemisia diff*usa. The data initially provided direct evidence that intra-tumoral injection of Tehranolide caused a shift in Th1/Th2 balance toward Th1-dominant immunity. Tehranolide shifted Th2 response towards Th1 and increased the production of IFN-γ and decreased that of IL-4 in mice, as can be seen from Figure 4. Th1-biased immune responses, often sought in current experimental cancer immunotherapy, are thought to be

more effective in malignant cell killing than Th2-biased responses. The shift to the Th1 response induced by Tehranolide can be used as an attempt to increase effectiveness of cancer immunotherapy in both human malignancy and animal models. Our findings showed a significant reduction in tumor size, as demonstrated in Figure 2. In addition, a significant increase was noticed in the proliferation of lymphocytes in the Tehranolide group in comparison with the PBS group (Figure 3). Treg cells protect the host from autoimmune diseases by suppressing the self-reactive cells. As such, Treg cells may also block anti-tumor immune responses. Treg cell frequencies and functions are important because their increased number might favor tumor development or growth, which will influence the course of the disease. Woo *et al.* were the first to report increased percentages of CD4+CD25+Foxp3+ Treg cells in TILs in non–small cell lung cancer and the ovarian cancer. These Treg cells were shown to secrete TGF-ß, providing first evidence that Treg cells contribute to immune dysfunction in patients with cancer. More importantly, Treg cells mediate potent inhibition of T cell proliferation (24). Supporting this initial report, a larger study concluded that prevalence of CD4+CD25+Foxp3+ Treg cells is increased not only in the tumor micro-environment of patients with invasive breast or pancreas cancers but also in PB (peripheral blood), suggesting that the enhancement of Treg cells is a generalized phenomenon (25). Immunotherapy has been proposed as an excellent approach to prevent tumor recurrence by destroying tumor cells while saving the normal-surrounding healthy cells (26). In recent years, several immune-based therapeutic strategies have been demonstrated to be effective against experimental tumor models in laboratory animals. Moreover, scientists have investigated whether depletion of regulatory T lymphocytes (Tregs) can augment immunotherapies against cancer.

Tregs are a subpopulation of CD4+CD25+Foxp3+ T lymphocytes constitutively expressing the transcription factor Foxp3, the high affinity IL2 receptor CD25 and the B7 ligand CTLA4 (27). Tregs are required for the maintenance of tolerance throughout the lifetime of the organism (28). In this regard, our finding that Tehranolide significantly ($p<0.05$) decreases the tumor infiltrating CD4+CD25+Foxp3+ lymphocytes compared to the control group (Figure 5) is important. It was also demonstrated that Tehranolide could cause tumor size reduction and as such, this compound may be a chemotherapeutic and immunotherapeutic agent. In conclusion, Tehranolide has a great potency as an antitumor agent.

In summary, our results indicate that a significant decrease in the proportion of functional CD4+CD25+Foxp3+ Treg cells were present following the intra-tumoral injection of Tehranolide to tumor-bearing mice. Inclusion of Tehranolide in the treatment group attenuated CD4+CD25+Foxp3+ Treg cell-mediated immune suppression, and elicited a robust and persistent anti-tumor immunity against cancer. Therefore, a possible approach for developing clinically-applicable chemotherapeutic and immunotherapeutic agents against cancer is to screen traditional medicinal plants which have been used for thousands of years for their anti-cancer activities with few side effects.

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