

# In Vitro and In Vivo Anti-tumor Effect of a Nutrient Mixture Containing Ascorbic Acid, Lysine, Proline and Green Tea Extract on Human Synovial Sarcoma Cancer Cells

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## ABSTRACT

Synovial sarcoma, a soft tissue cancer that most often occurs around leg or arm joints, has a 50% rate of metastasis. Structural changes in the extracellular matrix (ECM) are necessary for cancer cell migration and invasion. This prompted our team to investigate the effect of a nutrient mixture containing lysine, proline, ascorbic acid and green tea extract (NM) on the growth of human synovial sarcoma cell Hs 701.T xenografts in athymic nude mice, and on the invasive potential of human synovial sarcoma cells SW 982, in vitro. Male nude mice, 5-6 weeks of age, were inoculated with  $3 \times 10^6$  synovial sarcoma Hs 701.T cells, divided into two groups, and fed either a regular diet or a diet supplemented with 0.5% NM. After four weeks, tumors were excised, weighed and processed for histology. In addition, the in vitro effect of NM on human synovial sarcoma cells SW 982 was evaluated: cell proliferation by MTT assay, MMP secretion by gelatinase zymography, and invasion through Matrigel. NM reduced the size of synovial sarcoma tumors in nude mice by 44%. In vitro, NM inhibited the secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500  $\mu\text{g/ml}$  and MMP-9 at 50  $\mu\text{g/ml}$  concentration and blocked the invasion of

human synovial sarcoma cells through Matrigel at 1000  $\mu\text{g/ml}$  NM ( $p < 0.0001$ ). Our results suggest that NM is potentially an excellent candidate for therapeutic use in the treatment of synovial sarcoma, by inhibiting tumor growth and critical steps in cancer development, such as inhibiting MMP secretion and invasion.

**Keywords:** synovial sarcoma, nude mice, xenograft, nutrients, ascorbic acid, green tea extract, anti-tumor, MMP, invasion.

## INTRODUCTION

Synovial sarcoma is a rare soft tissue cancer (accounting for less than 1% of all new cancer cases each year) that most often occurs around leg or arm joints (50%), but can also occur in the trunk (40%) and head/neck (10%). This cancer is found more often in adolescents and young adults than in older people, and slightly more often in men than women. Due to the elasticity of this soft tissue cancer, tumors can grow rather large before they are noticed. Initially, synovial sarcoma presents as a painless lump, which becomes more painful as the tumor grows and presses against nearby nerves and muscles. The only reliable method of determining whether the tumor is malignant is by biopsy. Treatment depends upon location and stage of the disease, most commonly surgery (removing the entire tumor and nearby muscle and tissue), sometimes in conjunction with radiation and chemotherapy. Synovial sarcoma tends to recur locally, involving regional lymph nodes, and distant metastasis occurs in about 50% of the cases.<sup>1</sup>

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Cancer cells form tumors and spread by degrading the extracellular matrix (ECM) through various matrix metalloproteinases (MMPs). The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. Rath postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, have the potential to inhibit tumor growth and expansion.<sup>2</sup> These nutrients can exercise their anti-tumor effect through the inhibition of MMPs and in addition, strengthen connective tissue surrounding cancer cells through their effect on collagen synthesis. These two processes are essential for a tumor encapsulating effect.

We investigated the effects of a nutrient formulation containing lysine, proline, arginine, ascorbic acid and green tea extract (NM) on the growth of human synovial sarcoma Hs 701.T xenografts in athymic nude mice and on the in vitro anti-proliferative and anti-invasive potential of NM on human synovial sarcoma cells SW 982.

## MATERIALS AND METHODS

### IN VIVO STUDY

#### Cell Culture

Synovial sarcoma Hs 701.T cells from ATCC (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY. At near confluence, the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to a concentration of  $3 \times 10^6$  cells in 0.2 ml phosphate buffered saline (PBS) and 0.1 ml Matrigel (BD Bioscience, Bedford, MA) for inoculation.

#### Animals

Male athymic nude mice (NCr-nu/nu), approximately 6 weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA, and maintained in microinsulator cages under pathogen-free conditions on a 12-hour light/12-hour dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals. After housing for a week, the mice were inoculated with  $3 \times 10^6$  synovial sarcoma Hs 701.T cells in 0.2 ml of PBS and 0.1 ml of Matrigel. After injection, the mice were randomly divided into two groups, A and B. Six mice were allocated to each group. From day one, Group A mice were fed a regular diet and Group B mice were fed a regular diet supplemented with 0.5% of the nutrient formulation (NM). The growth of tumors was monitored by measuring the length and width of the tumors with a caliper every three days. After four weeks, mice were sacrificed and tumors were excised, weighed and fixed in 10% (v/v) buffered formalin, and processed for histology.

### IN VITRO STUDY

#### Cell culture

Human synovial sarcoma cells SW982 were obtained from ATCC and grown in modified Eagle medium, supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/ml), streptomycin (100 µg/ml), and amphotericin (0.25 µg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO<sub>2</sub>. At near confluence, the cells were treated with the nutrient mixture dissolved in media and tested in triplicate at 0 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 500 µg/ml and 1000 µg/ml. The plates were then returned to the incubator. The cells were washed with PBS and 500 µl of MTT (Sigma, St. Louis, MO) 0.5 mg/ml in media was added to each well. Cell proliferation was evaluated 24 hrs. following incubation with test reagents. Culture media components were purchased from Gibco (Grand Island, NY). All other chemicals used were purchased from Sigma.

#### MTT ASSAY

Cell proliferation was evaluated by MTT assay,<sup>3</sup> a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and cell viability. After MTT addition (0.5mg/ml), the plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells; the formazan product was dissolved in 1 ml DMSO and absorbance was measured at 570 nm in a Bio Spec 1601, Shimadzu spectrometer. The OD<sub>570</sub> of the DMSO solution in each well was considered to be proportional to the number of cells. The OD<sub>570</sub> of the control (treatment without supplement) was considered to be 100%.

#### GELATINASE ZYMOGRAPHY

MMP secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20 µl) mixed with sample buffer was loaded, and SDS-PAGE was performed with tris glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM

Tris-HCl and 10 mM CaCl<sub>2</sub> at pH 8.0, and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

## MATRIGEL INVASION STUDIES

Invasion studies were conducted using Matrigel™ (Becton Dickinson, Franklin Lakes, NJ) matrix-coated 9-mm cell culture inserts (pore size, 8 μm) set in 24-well plates using a modified Boyden Chamber method as described by Albini et al.<sup>4</sup> 200 μl of cell suspension (3x10<sup>4</sup> cells) supplemented with nutrients, as specified in the design of the experiment, in triplicate, were seeded on the insert in the well. The lower chambers also contained 5% fetal bovine serum as a chemoattractant. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO<sub>2</sub> for 24 hours. After incubation, the remaining cells in the upper layer of the insert were carefully swabbed with cotton. The penetrating cells in the lower layer were fixed with cold methanol and stained with hematoxylin and eosin. The cells that invaded the lower side of the filter were counted using optical microscope.

### Nutrient Mixture (NM) Composition

The stock solution of the nutrient mixture (total weight 4.2 gm) used for testing was composed of the following: vitamin C (as ascorbic acid and as Mg, Ca and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Lab). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, EGCG 35%, caffeine 1.0%, selenium 30 μg, copper 2 mg, and manganese 1mg.

### Statistical analysis

The results were expressed as means ± SD for the groups. Data was analyzed by independent sample “t” test. Correlation coefficients were determined for dose-dependent studies using MedCalc Software (Mariakerke, Belgium).

## RESULTS

### IN VIVO STUDY

#### Tumor Growth

Nude mice receiving the nutrient supplemented diet developed significantly smaller tumors (by 44%, p=0.0099) than the mice receiving the control diet, as shown in Figure 1.

#### Histopathology: H&E

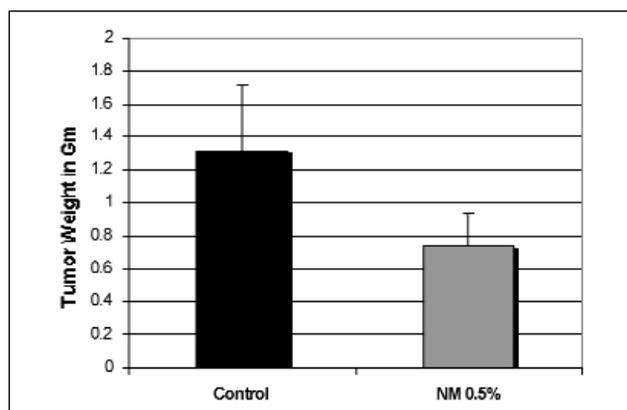
In the control group, the ulcerated tumor masses (Figures 2A,B) revealed dense sheets of cells with irregu-

larly round to oval vesiculated nuclei and scant, ill-defined cytoplasm. Extensive necrosis was present in the central area of the mass involving 50% to 60% of the neoplasm. Severe neutrophilic infiltration was associated with the necrotic areas bordering the area of ulceration. Mitotic figures averaged five per high-power field. Tumor sections from the supplemented mice (Figures 2C,D) showed morphological similarity to those from the control group. Mitotic figures in tissue sections from supplemented mice averaged four per high-power field.

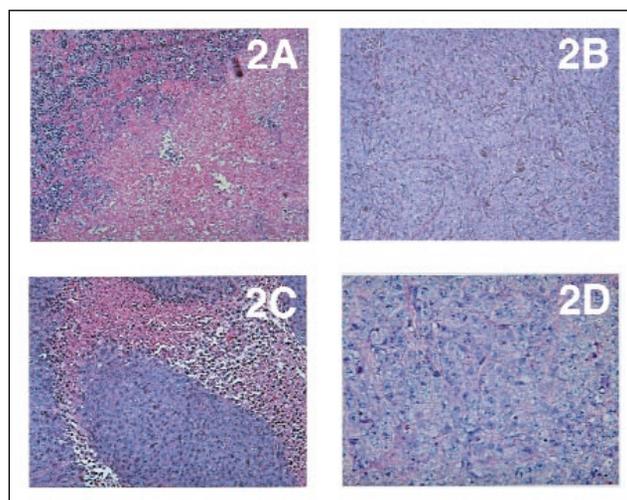
### IN VITRO STUDY

#### Proliferation study

NM did not show any significant effect on synovial sarcoma cell growth up to a concentration of 500 μg/ml (Figure 3). Antiproliferative effect of NM at 500 μg/ml was 20% (p<0.003) and at 1000 μg/ml was 28% (p<0.006).



**Figure 1.** Effect of the nutrient supplemented (NM 0.5%) on synovial sarcoma Hs 701.T xenograft tumor growth in nude mice. Tumor growth was reduced by 44% (p=0.0099) with supplementation.



**Figure 2.** Tumor histopathology from control and supplemented mice: 2A – Control 20x, 2B – Control 40x, 2C – Supplemented 20x, 2D – Supplemented 40x.

### Gelatinase zymography study

As shown in Figure 4A, zymography demonstrated secretion of MMP-2 and a very faint band of MMP-9 by uninduced human synovial sarcoma cells. PMA (200 ng/ml) treated cells showed enhanced MMP-9 expression, but at much lower degree than is found with other cell lines (Figure 4B). The nutrient mixture inhibited the secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500 µg/ml and MMP-9 at 50 µg/ml NM.

### Invasion study

The invasion of human synovial sarcoma cells through Matrigel was significantly reduced in a dose-dependent manner ( $r=0.9415$ ,  $p=0.0050$ ) with 79% inhibition at 500 µg/ml and total block at 1000 µg/ml NM, as shown in Figures 5 and 6.

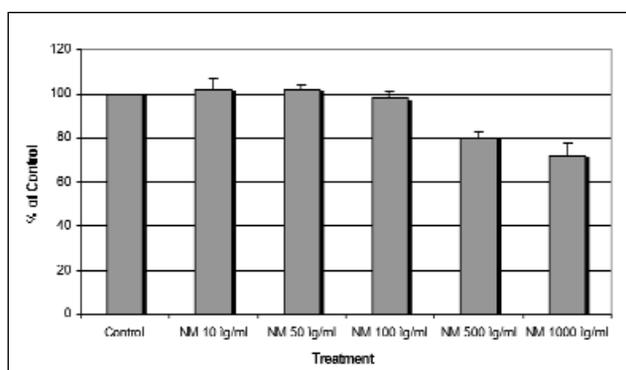
### DISCUSSION

The results of this study demonstrated significant inhibition of tumor growth of synovial sarcoma Hs 701.T xenografts in nude mice supplemented with 0.5% NM. The in vitro studies demonstrated profound anti-invasive effects of NM on human synovial sarcoma SW 982. Matrigel invasion and MMP-2 and MMP-9 secretion by synovial sarcoma cells decreased in a dose-dependent fashion in the presence of NM, with complete inhibition of invasion at 1000 mg/ml, MMP-2 secretion at 500 µg/ml and MMP-9 at 50 µg/ml concentration.

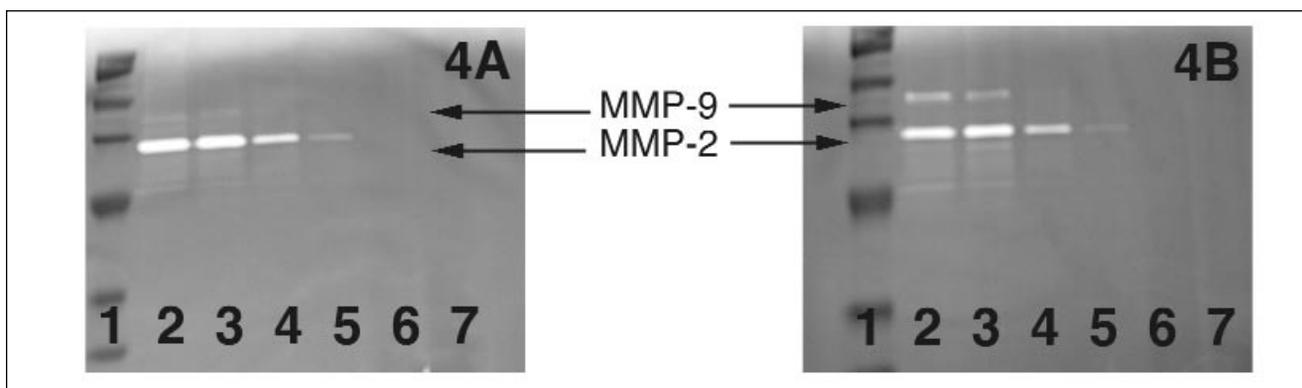
Matrix invasion can be controlled by inhibition of MMP activity, as well as by increasing connective tissue strength and stability, contributing to the “encapsulation” of the tumor. In this study, the increased inhibitory effect from the combined effects of the nutrients on MMP-9 and MMP-2 secretion by synovial sarcoma cells was consistent with its inhibition of matrix invasion. In addition, matrix invasion was modulated by enhanced stability and strength of the connective tissue secondary to the activity of the mixture of

nutrients provided. Optimization of synthesis and structure of collagen fibrils depends upon hydroxylation of proline and lysine residues in collagen fibers. It is known that ascorbic acid is essential for the hydroxylation of these amino acids. Since it is not produced in the human body, however, sub-optimal levels are common. Additionally, low levels of ascorbic acid have been reported in cancer patients.<sup>5-7</sup>

The inhibitory effects of the individual nutrients composing the test nutrient mixture have been reported in both clinical and experimental studies. In an in vitro study, human fibrosarcoma cells were treated with ASC-2-0-phosphate-6-O-palmitate (a lipophilic and auto-oxidation-resistant derivative of ascorbic acid), and in as little as 30 minutes, tumoral invasion was inhibited by 50%, with 80% inhibition achieved in 90 minutes, without cytotoxic effect. Additionally, zymography and Western blots showed significant inhibition of MMP-9 and MMP-2 expression, suggesting powerful chemopreventative and anti-metastatic ability via potent antioxidant activity.<sup>8</sup> Other studies<sup>9-11</sup> on the effect of tea polyphenols on MMP activity and invasion in human



**Figure 3.** Effect of the nutrient mixture (NM) on proliferation of synovial sarcoma cell SW 982: MTT assay. NM showed no significant effect on synovial sarcoma cell growth up to 500 µg/ml NM, and some antiproliferative effect at 500 µg/ml (20%,  $p<0.003$ ) and at 1000 µg/ml (28%,  $p<0.006$ ).



**Figure 4.** Effect of the nutrient mixture (NM) on MMP secretion of untreated (4A) and PMA 200 ng/ml induced (4B) synovial sarcoma cell SW 982; Legend: 1 – Markers, 2-Control, 3-7 NM 10, 50, 100, 500, and 1000 µg/ml. Zymography demonstrated MMP-2 and MMP-9 with induction of MMP-9 with PMA treatment. NM inhibited secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500 µg/ml and MMP-9 at 50 µg/ml NM.

fibrosarcoma, which show that addition of EGCG to the cells significantly decreased invasion and suppressed gelatin degradation by MMP-2 and MMP-9, support our findings.

However, individual nutrients are not as powerful as nutrient synergy. The results of this study showed significant anti-invasive action on the synovial sarcoma SW 982 cells tested in the presence of the nutrient mixture. In a previous study,<sup>12</sup> we demonstrated that the synergistic anti-cancer effect of ascorbic acid, proline, lysine and EGCG on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients.

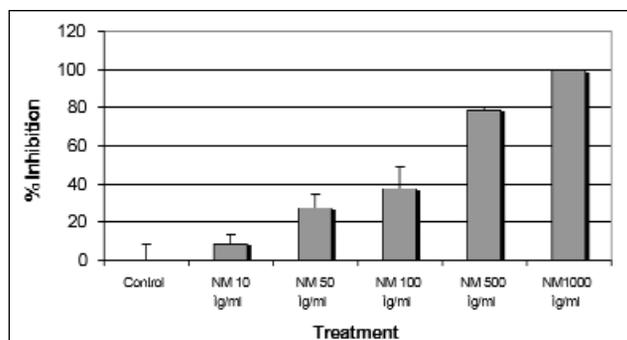
While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer prevention and treatment, the results of these studies suggest that the formulation of green tea extract, lysine, proline and ascorbic acid, is an excellent candidate for adjunctive therapeutic use in the treatment of highly metastatic synovial sarcoma by inhibiting MMP secretion and invasion without toxic effects.

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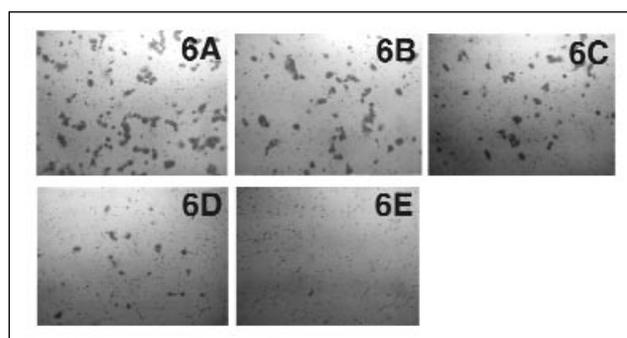
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#### REFERENCES

1. Childhood Soft Tissue Sarcoma (PDQ®): Treatment." National Cancer Institute. <http://www.nci.nih.gov/cancerinfo/pdq/treatment/child-soft-tissue-sarcoma/patient>. Accessed February 2004.
2. Rath M, Pauling L. Plasmin-induced proteolysis and the role of apoprotein (a), lysine and synthetic analogs. *Orthomolecular Medicine*. 1992;7:7-23.
3. Masman TJ. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Methods*. 1983;65:55-57.
4. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SR, Kozlowski JM, McEwan ER. A rapid in vitro assay for quantitating the invasion potential of tumor cells. *Cancer Res*. 1987;47:3239-3245.
5. Anthony HM, Schorah CJ. Severe hypovitaminosis C in lung-cancer patients: the utilization of vitamin C in surgical repair and lymphocyte related host resistance. *Br J Cancer*. 1982;46:354-367.
6. Nunez C, Ortiz de Apodaca Y, A Ruiz. Ascorbic acid in the plasma and blood cells of women with breast cancer. The effect of consumption of food with an elevated content of this vitamin. *Nutr Hosp*. 1995;10:68-372.
7. Kurbacher CM, Wagner U, Kolster BK, Andreotti PE, Krebs W, Bruckner HW. Ascorbic acid (vitamin C) improves the antineoplastic activity doxorubicin, cisplatin and paclitaxel in human breast carcinoma cells in vitro. *Cancer Letters*. 1996;103(2):183-189.



**Figure 5.** Effect of the nutrient mixture (NM) on Matrigel invasion of human synovial sarcoma cell line SW 982. The invasion of human synovial sarcoma cells through Matrigel was significantly reduced in a dose-dependent manner ( $r=0.9415$ ,  $p=0.0050$ ) with 79% inhibition at 500 µg/ml and total block at 1000 µg/ml NM.



**Figure 6.** A-E – Invasion photomicrographs: 6A – Control, 6B – NM 50 µg/ml, 6C – NM 100 µg/ml, 6D – NM 500 µg/ml, 6E – NM 1000 µg/ml.

8. Liu JW, Nagao N, Kageyama K, Miwa N. Antimetastatic and anti-invasive ability of phospho-ascorbyl palmitate through intracellular ascorbate enrichment and the resultant antioxidant action. *Oncol Res*. 1999;11(10):479-87.
9. Maeda-Yamamoto M, Kawahara H, Tahara N, Tsuji K, Hara Y, Isemura M. Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells. *J Agric Food Chem*. 1999; 47(6):2350-4.
10. Maeda-Yamamoto M, Suzuki N, Sawai Y, Miyase T, Sano N, Hashimoto-Ohta A, Isemura M. Association of suppression of extracellular signal-regulated kinase phosphorylation by epigallocatechin gallate with the reduction of matrix metalloproteinase activities in human fibrosarcoma HT1080 cells. *J Agric Food Chem*. 2003;51(7):1858-63.
11. Dell'Aica M, Dona L, Sartor E, Pezzato-Garbis S. (-) Epigallocatechin-3-gallate directly inhibits MT1-MMP activity, leading to accumulation of nonactivated MMP-2 at the cell surface. *Lab Invest*. 2002;82(12):1685-93.
12. Netke SP, Roomi MW, Ivanov V, Niedzwiecki A, Rath M. A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits proliferation and extracellular matrix invasion of various human cancer cell lines. *Research Communications in Pharmacology and Toxicology: Emerging Drugs*. 2003; 2:37-50.