NUTRIENT COMPOSITION COMPRISING GREEN TEA POLYPHENOLS FOR TREATING OSTEOSARCOMA

The present invention relates to the use of a composition comprising an ascorbic acid compound, a L-lysine compound, a L-proline compound, and a polyphenol compound for the preparation of a pharmaceutical composition for treating osteosarcoma. Moreover, the invention further relates to a method of treatment wherein said composition is administered to a subject suffering from osteosarcoma.

Osteosarcoma, a primary malignant tumor of bone or soft parts that arises from bone-forming mesenchymal cells, primarily develops in the distal femur, the proximal tibia, the proximal humerus and the distal radius. Classic osteosarcoma demonstrates aggressive, rapid growth with a high risk of local, "skip" metastases and early, pulmonary metastasis. It is the most common bone cancer and the sixth most common cancer in children, and is more frequent in males than females. Most osteosarcomas arise from non-inherited errors in the DNA of growing bone cells. Because these errors occur randomly and unpredictably, there is currently no effective way to prevent this type of cancer [Miller, Dowshen et al.(2002)].

For decades, standard treatment for osteosarcoma has consisted of surgery (amputation or limb salvage surgery) and chemotherapy, which focus on cancer cell destruction, but do not address metastasis.

Radiation and chemotherapy have not only been ineffective in providing a cure, but also indiscriminately attack all cells – causing cellular damage and destruction of the body's connective tissue, and thus facilitate cancer metastasis. For example, of 31 patients studied with localized osteosarcoma [Jaffe, Carrasco et al. (2002)] and treated with conventional chemo-

therapy (high-dose methotrexate and leucovarin rescue in 3 patients and intra-arterial cis-platin in 28 patients) at the Anderson Cancer Center, only 3 patients did not experience local recurrence or pulmonary metastases during the follow-up period of 225+ months. Side effects of chemotherapy include: anemia, abnormal bleeding, increased risk of infection due to destruction of bone marrow, liver and kidney damage, heart problems, and hearing loss. Approximately 20% of children diagnosed with osteosarcoma have an advanced stage of osteosarcoma that has metastasized to the lungs, brain and other bones [Miller, Dowshen et al. (2002)]. Even resection of the primary tumor has been reported to potentiate distant metastasis in osteosarcoma [Tsunemi, Nagoya et al. (2003)].

Several nutrient compounds have been reported to exert anticancer activity. Ascorbic acid has been reported to have cytotoxic and antimetastatic actions on malignant cell lines [Koh, Lee et al. (1998), Roomi, House et al. (1998), Naidu, Karl et al.(2003)]; in addition, low levels of ascorbic acid have been reported in cancer patients [Anthony and Schorah (1982), Nunez, Ortiz de Apodaca et al. (1995), Kurbacher, Wagner et al. (1996)]. ECGC is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines [Valcic, Timmerman et. al. (1996), Mukhtar and Ahmed (2000), Yang, Liao et al. (1998)]. The observed effects were, however, rather weak and, thus, not be a suitable basis for a therapeutic approach for a rapid growing and aggressive cancer. Other studies suggested that the synergistic anticancer effect of ascorbic acid and EGCG together with other compounds on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients [Netke, Roomi et al.(2003)].

Clearly, there is a need for safe and effective therapeutic approaches to control the process of cancer metastasis, in particular for aggressive cancers such as osteosarcoma.

The technical problem underlying the present invention, thus, must be seen as the provision of means and methods to comply with the aforementioned needs.
This problem is solved by the embodiments characterized in the claims and herein below.

Accordingly, the present invention relates to the use of a composition comprising

(a) an ascorbic acid compound,

(b) a L-lysine compound,

(c) a L-proline compound, and

(d) a polyphenol compound

for the preparation of a pharmaceutical composition for treating osteosarcoma.

The term "composition" encompasses liquid and solid preparations of the nutritional compounds referred to above as well as gels thereof. The solid preparations may be manufactured in a suitable form including tablets, capsules, powders, granules, tea preparations or the like. Its well known in the art how to manufacture said liquid, gel-like and solid preparations referred to herein. The composition referred to herein may be provided in accordance with the uses of the invention as mixture of the compounds or by means of a kit including the ingredients separately. The ingredients may be packaged in said kit in separate vials. The composition is also suitable for human or animal use. Preferably, said animal is mammal, most preferably a dog, cat or horse.

The amino acids "proline" or "lysine" referred to in accordance with the present invention are preferably the L-amino acids. "Proline" or "Lysine" also encompasses its hydroxyl derivatives hydroxyproline and hydroxylysine as well as salts thereof.

The term "ascorbic acid" preferably refers to ascorbate, ascorbic acid and salts thereof. Sometimes ascorbate compounds may be referred to as vitamin C.

The term "polyphenol compound" as used in accordance with the present invention, preferably, refers to a preparation of green tea plants comprising the polyphenolic compounds that are present in green tea. Polyphenolic compounds may be present as up to 30% dry weight in green tea. They include bioflavinoids such as flavanols, flavandiols, flavonoids, and phenolic acids. Flavanols represent the most abundant polyphenols in green tea and are commonly known as catechins. Most preferably, said catechins are EGCG, EG, ECG or EC. EGCG refers to (-)-epigallocatechin-3-gallate, EC refers to epicatechin which refers to (-)-epicatechin, ECG refers to eipcatechin-3-gallate which refers to (-)-epicatechin-3-gallate, EGC refers to epigallocatechin which refers to (-)-epigallocatechin. It is well known in the art how such preparations may be obtained. Preferably, polyphenols are to be administered in an amount of 200 mg to 5000 mg per day and subject.

The compounds referred to in accordance with the uses of the present invention may be admixed in any suitable ratios or amounts. Whether such a ratio or amount is suitable can be

determined by the skilled person by using the assays specified in the accompanied Examples referred to below. Most preferably, the composition referred to in accordance with the present invention provides a daily dosage of 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 (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1mg.

The pharmaceutical composition to be administered in accordance with the present invention may include a pharmaceutically acceptable carrier, diluent, or excipient. The composition to be used in accordance with the present invention can be prepared by procedures known in the art. Respective ingredients may be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. xamples of excipients, diluents, and carriers include: i) fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; ii) binding agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; iii) moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; iv) resorption accelerators such as quaternary ammonium compounds; v) surface active agents such as acetyl alcohol, and glycerol monostearate; v) adsorptive carriers such as kaolin and bentonite; and vi) lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols. The compositions may also be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for example, by intramuscular, subcutaneous or intravenous routes. Ideally the formulation is in the form of a pill, tablet, capsule, lozenge, liquid or similar dosage form as referred to aove. The compositions may well be suited to formulation as sustained release dosage forms and the like.

As used herein, the term "treating" is used to mean reducing, inhibiting, attenuating or treating the syndromes accompanied with the pathological conditions referred to in accordance with the present invention. Treating becomes apparent for the clinician by monitoring the symptoms accompanied with the said pathological conditions. The symptoms are described in detail in standard text books such as Stedman or Pschyrembel. Treatment preferably refers to significant reduction, inhibition, attenuation or treatment. The significance can be determined by standard methods of statistics, e.g., Student´s t-test, chi square test an others.

The term "ostosarcoma" refers to cancer types of the bone. The symptoms accompanied with said diseases or disorders are well known in the art and described in detail in medical text books such as Stedman or Pschyrembel. Accordingly, the clinician can determine without further ado whether a patient suffers from osteosarcoma. The term "prevention" means said the nutritional composition may also be administered in order to avoid the development of osteosarcoma.

The results of the study underlying the present invention, surprisingly, demonstrated significant suppression of osteosarcoma tumor growth in immune impaired (athymic) male nude mice by supplementation with 0.5% of the nutrient mixture (which contains ascorbic acid, lysine, proline, and epigallocatechin gallate). Furthermore nutrient supplementation resulted in decreased mitotic index when contrasted with the control mice, see accompanied Examples. Current treatment of osteosarcoma is associated with poor prognosis, especially due to the increased risk of developing other cancers with chemotherapy. Therefore, new safe effective treatment strategies are needed. In accordance with the present invention the synergistic effect of a nutrient mixture (NS) of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on the growth of human osteosarcoma xenografts in athymic nude mice has been investigated. Moreover, the effect of NS on human osteosarcoma cell line MNNG-HOS in vitro was investigated by measuring: cytotoxicity, modulation of MMP-2 and -9, cancer cell invasive potential, and angiogenesis. After one-week of isolation, 5-6 week old athymic male nude mice (n=12) were inoculated with $3x10^6$ osteosarcoma cells MNNG-HOS. After injection, the mice were randomly divided into two subgroups; group A was fed a regular diet and group B was fed a regular diet supplemented with 0.5% of the nutrient mixture. Four weeks later, the mice were sacrificed, and their tumors were excised, weighed, and processed for histology. Cell proliferation was evaluated by MTT assay, MMP expression by gelatinase zymography, and invasion through Matrigel. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) to study enhanced MMP expression. Results showed that the nutrient mixture (NS) inhibited the growth and reduced the size of tumors in nude mice. Furthermore, the mitotic index was decreased in the supplemented group (4-5) in contrast to the control group (12-15). The invasion of osteosarcoma MNNG-HOS cells through Matrigel was significantly reduced in a dose-dependent fashion, with 100% inhibition of invasion of MNNG cells at 50 μg/ml concentration of the nutrient mixture. No significant anti-proliferative effect by NS was seen with MNNG cells. Zymography showed dose-dependent

inhibition of MMP secretion by MNNG in the presence of NS. Nutrient synergy strongly suppressed the growth of tumors without any adverse effects in nude mice, suggesting the nutrient combination has potential as an anticancer agent. In vitro studies demonstrated inhibition of cancer cell invasion and secretion of MMPs - critical parameters for cancer control and prevention.
Thanks to the present invention osteosarcoma can be treated without harmful side effects of the conventional therapies such as surgery and/or radiation therapy.

The definitions and explanations of the terms made hereinabove apply mutatis mutandis for the other embodiments of the present invention disclosed hereinafter.

In a preferred embodiment of the use of the present invention said ascorbic acid compound is selected from the group consisting of ascorbic acid, a pharmaceutically acceptable ascorbate salt, an ascorbate ester, more preferably ascorbyl palmitate, and/or mixtures of the aforementioned compounds. More preferably, said pharmaceutical acceptable ascorbate salt is selected from the group consisting of calcium ascorbate salts and magnesium ascorbate salts.
Suitable amounts of the compounds to be used for the preparation of the composition referred to in accordance with the present invention are disclosed in WO 03/057201 A2, which is hereby incorporated by reference, in detail. Preferably, the ascorbic acid compound is ascorbic acid in an amount of 25 mg to 5000 mg and/or calcium ascorbate and/or magnesium ascorbate and/or ascorbyl palmitate in the same amounts. All amounts are calculated per day and subject.

In a further preferred embodiment of the use of the present invention said L-lysine compound is selected from the group consisting of L-lysine hydrochloride, L-lysine, and pharmaceutically acceptable L-lysine salts.
Suitable amounts of the compounds to be used for the preparation of the composition referred to in accordance with the present invention are disclosed in WO 03/057201 A2, which is hereby incorporated by reference, in detail. The L-lysine compound, preferably, may be also a mixture of at least two of the chemicals of the aforementioned group. Preferably, the L-lysine compound is L-lysine in an amount of 50 mg to 5000 mg per day and subject.

In another preferred embodiment of the use of the present invention said L-proline compound is selected from the group consisting of L- proline hydrochloride, L- proline, and pharmaceutically acceptable L- proline salts.
Suitable amounts of the compounds to be used for the preparation of the composition referred to in accordance with the present invention are disclosed in WO 03/057201 A2, which is hereby incorporated by reference, in detail. The L-proline compound, preferably, may be also a mixture of at least two of the chemicals of the aforementioned group. Preferably, the L-proline compound is L-proline in an amount of 25 mg to 3000 mg per day and subject.

In yet another preferred embodiment of the use of the present invention said composition further comprises a trace element selected from the group consisting of selenium, manganese, magnesium, calcium, and copper.
Suitable amounts of the compounds to be used for the preparation of the composition referred to in accordance with the present invention are disclosed in WO 03/057201 A2, which is hereby incorporated by reference, in detail. The composition, preferably, comprises at least two, at least three or more of the trace elements of the aforementioned group. Preferably, the trace elements may be used together, separately or in any comination in the following amounts per day and subject: selenium: 1 µg to 200 µg; cooper: 20 µg to 9000 µg; manganese: 50 µg to 10000 µg; calcium: 300 mg to 600 mg; magnesium: 300 mg to 600 mg.

In a further preferred embodiment of the use of the present invention said composition further comprises an L-arginine compound. More preferably, said L-arginine compound is selected from the group consisting of L- arginine hydrochloride, L- arginine, and pharmaceutically acceptable L- arginine salts.
Suitable amounts of the compounds to be used for the preparation of the composition referred to in accordance with the present invention are disclosed in WO 03/057201 A2, which is hereby incorporated by reference, in detail. The L-arginine compound, preferably, may be also a mixture of at least two of the chemicals of the aforementioned group. Preferably, the L-arginine compound is L-arginine in an amount of 50 mg to 3000 mg per day and subject.

In another preferred embodiment of the use of the invention, the composition may further comprise N-acetyl cysteine.
"N-acetyl-cysteine" as used herein comprises cysteine or cystine (dimer of cysteine) and cysteine salts thereof. Suitable amounts of the compounds to be used for the preparation of the composition referred to in accordance with the present invention are disclosed in WO 03/057201 A2, which is hereby incorporated by reference, in detail. Preferably, the N-acetyl cystein is administered in an amount of 10 mg to 1500 mg per day and subject.

In a preferred embodiment of the use of the present invention said composition is to be administered in oral, parenteral, subcutaneous, intraarterial, or intravenous form.

The invention also relates to a method for treating osteosarcoma in a subject comprising the step of administering to a subject suffering from osteosarcoma a composition as defined hereinabove in a therapeutically efficient amount.
It is well known for the person skilled in the art how such compositions can be administered to a subject. Particularly preferred techniques are describe in detail in WO 03/057201 A2 which is hereby incorporated by reference. Moreover, all embodiments of the use of the present invention apply mutatis mutandis for the aforementioned method for treating osteosarcoma.

In a preferred embodiment of the method said subject is a human.

In a further preferred embodiment of the method the composition is administered orally, parentarally, subcutaneously, intraarterially, or intravenously.

Full bibliographic information on the references cited above and below will be found below. The disclosure content of these references is hereby incorporated by reference.

References

1. H.M. Anthony, C.J. Schorah (1982). Severe hypovitaminosis C in lung-cancer patients: The utilization of vitamin C in surgical repair and lymphocyte related host resistance. *Br J Cancer.* 46: 354-367

2. N. Jaffe, H. Carrasco, K. Raymond, A. Ayala, F. Eftekhari (2002). Can cure in patients with osteosarcoma be achieved exclusively with chemotherapy and abrogation of surgery? *Cancer.* 10: 2202-10.

3. W.S. Koh, S. J. Lee, H. Lee, C. Park, M.H. Park, W.S. Kim, S.S. Yoon, K. Park, S.I. Hong, M.H. Chung, C.H. Park (1998). Differential effects and transport kinetics of ascorbate derivatives in leukemic cell lines. *Anticancer Res* 8: 2487-2493.

4. C.M. Kurbacher, U. Wagner, B. Kolster, P.E. Andreotti, D. Krebs, H.W. Bruckner (1996). Ascorbic acid (vitamin C) improves the antineoplastic activity doxorubicin, cisplatin and paclitaxel in human breast carcinoma cells in vitro. *Cancer Lett.* 103(2): 183-189.

5. J.T. Masman (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J Immunol Methods* 65: 55-57

6. R. Miller, S. Dowshen, and M. Trigg, Childhood Cancer: Osteosarcoma (May 2002) *The Nemours Foundation: Kids Health* http://kidshealth.org/parent/medical/cancer/cancer_osteosarcoma_p1-3.html

7. H. Mukhtar and N. Ahmed (2000). Tea polypheonols: prevention of cancer and optimizing health. *Am J Clin Nutr.* 71: 1698S-1720S.

8. K.A. Naidu, R.C. Karl, K.A. Naidu, D. Coppola D (2003). Antiproliferative and proapoptotic effect of ascorbyl stearate in human pancreatic cancer cells: association with decreased expression of insulin-like growth factor 1 receptor. *Dig Dis Sci.* 48(1): 230-7.

9. S.P. Netke, M.W. Roomi, V. Ivanov, A. Niedzwiecki, and M. Rath. A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits prolif-

eration and extracellular matrix invasion of various human cancer cell lines. *Research Communications in Pharmacology and Toxicology: Emerging Drugs.* 2, 37-50.

10. C. Nunez, Y. Ortiz de Apodaca, A. Ruiz (1995). 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.*10: 68-372.

11. M. Rath and L. Pauling (1992). Plasmin-induced proteolysis and the role of apoprotein(a), lysine and synthetic analogs. *Orthomolecular Medicine.*7:17-23.

12. M.W. Roomi, D. House, M. Eckert-Maksic, Z.B. Maksic, C.S. Tsao (1998). Growth suppression of malignant leukemia cell line in vitro by ascorbic acid (vitamin C) and its derivatives. *Cancer Letters* 122: 93-99.

13. T. Tsunemi, S. Nagoya, M. Kaya, S. Kawaguchi, T. Wada, T. Yamashita, S. Ishii (2003) Postoperative progression of pulmonary metastasis in osteosarcoma. *Clin Orthop.* 407:159-66.

14. S. Valcic, B.N. Timmerman, D.S. Alberts, G.A. Wachter, M. Krutzch, J. Wymer, J.M. Guillen (1996). Inhibitory effects of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. *Anticancer Drugs.* 7: 461-468.

15. G.Y. Yang, J. Liao, K. Kim, E.J. Yurkow, C.S. Yang (1998). Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis.* 19:611-616.

The figure show:

Figure 1: (A) Effect of NS on total weight of osteosarcoma MNNG xenografts in male nude mice; (B) Histology of tumor tissue in supplemented (Suppl) and contol mice, (C) Photographs of tumors from control and supplemented nude mice.

Figure 2: (A) Effect of the nutrient mixture (NS) and PMA on human osteosarcoma cells MNNG-HOS proliferation; (B) Effect of exposure to nutrient composition (NS) on MMP-2 expression by human osteosarcoma MNNG HOS cells.

Figure 3: Effect of the nutrient mixture (NS) on Matrigel invasion and migration by human osteosarcoma cells MNNG-HOS.

The invention will be illustrated by the following Examples. However, the Examples shall not be used to limit the scope of the invention.

Example 1: Treatment of osteosarcoma in nude mice

Human osteosarcoma cells MNNG-HOS obtained from ATCC (American Type Culture Collection, Rockville, MD) were maintained in MEM culture, 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 $3x10^6$ cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA) for inoculation. Male athymic nude mice (NCr-nu/nu), approximately six 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 $3x10^6$ human osteosarcoma MNNG-HOS 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, mice from Group A were fed a regular diet and those in Group B were fed a regular diet supplemented with 0.5% NS. After four weeks, mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin and processed for histology. Results showed that the nutrient supplemented nude mice developed significantly smaller tumors (by 53%, $p = 0.0001$) and less vascular ones than did the control group of nude mice (Figure 1A). Furthermore, histological examination revealed that mitotic figures in the control group averaged 12-15 per high power field; in contrast, the nutrient supplemented rats developed tumors with a decreased mitotic figure (4-5 per high-power field) (Figure 1B). The tumors, located in the subcutaneous layer, were expansile, with evidence of peripheral invasion. The neoplasm was composed of spindle shaped or irregularly round cells with large, irregularly round to oval hyperchromatic nuclei and scant cytoplasm with indistinct borders. Irregular areas of tumor necrosis involved about 70% of the tumor mass.

Stock solution of the nutrient mixture (total weight 4.4 Gm) for the experiments 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 (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1mg.

The results were expressed as means ± SD for the groups. Data were analyzed by independent sample "t" test.

Example 2: Effects of the nutrient composition on isolated MNNG-HOS cells from nude mice in culture

Human osteosarcoma cells MNNG-HOS obtained from ATCC (American Type Culture Collection, Rockville, MD) were maintained in MEM culture, 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 $3x10^6$ cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA) for inoculation.

Male athymic nude mice (NCr-nu/nu), approximately six 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 $3x10^6$ human osteosarcoma MNNG-HOS 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, mice from Group A were fed a regular diet and those in Group B were fed a regular diet supplemented with 0.5% NS. After four weeks, mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin and processed for histology.

Human osteosarcoma cells were grown in MEM in 24-well tissue culture plates (Costar, Cambridge, MA). Cell cultures were supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated with 1 ml of media at 37^0 C in a tissue culture incubator equilibrated with 95% air and 5% CO_2.

At near confluence, the cells were treated with the nutrient mixture (NS) dissolved in media and tested at 0, 10, 100, 500, and 1000 μg/ml in triplicate at each dose. A group of cells were also treated with PMA 200 ng/ml. The plates were then returned to the incubator. Cell proliferation was evaluated 24 hrs following incubation with test reagents.

Cell proliferation was evaluated based on the MTT assay. The MTT assay [Masman JT, (1983)] is 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. After MTT addition (0.5mg/ml) the plates were covered and returned to the 37^0C 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 Bio Spec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

MMP expression in condition media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel (Invitrogen Corporation) in the presence of 0.1% gelatin. Culture media (20μl) was loaded and SDS-PAGE was performed with a tris-glycine SDS buffer. After electrophoresis, the gels were washed with 5% Triton X-100 for 30 minutes. The gels were then incubated for 24 hours at 37^0C in the presence of 50mM Tris-HCl, 5mM $CaCl_2$, 5μM $ZnCl_2$, PH 7.5 and stained with Coomassie Blue R 0.5% for 30 minutes and destained. Protein standards were run concurrently and approximate molecular weights were determined.

Invasion studies were conducted using Matrigel™ (Becton Dickinson) inserts in 24-well plates. Suspended in medium, osteosarcoma cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with Hematoxylin and Eosin and visually counted under the microscope.

Stock solution of the nutrient mixture was as described above. The results were expressed as means $\pm$ SD for the groups. Data were analyzed by independent sample "t" test.

Claims

1. Use of a composition comprising
   (a) an ascorbic acid compound,
   (b) a L-lysine compound,
   (c) a L-proline compound, and
   (d) a polyphenol compound
   for the preparation of a pharmaceutical composition for treating osteosarcoma.

2. The use of claim 1, wherein said ascorbic acid compound is selected from the group consisting of ascorbic acid, a pharmaceutically acceptable ascorbate salt, an ascorbate ester and/or mixtures of the aforementioned compounds.

3. The use of claim 2, wherein said pharmaceutical acceptable ascorbate salt is selected from the group consisting of calcium ascorbate salts and magnesium ascorbate salts.

4. The use of claim 1, wherein said ascorbate compound is an ascorbate ester.

5. The use of claim 4, wherein said ascorbate ester is ascorbyl palmitate.

6. The use of any one of claims 1 to 5, wherein said L-lysine compound is selected from the group consisting of L-lysine hydrochloride, L-lysine, and pharmaceutically acceptable L-lysine salts.

7. The use of any one of claims 1 to 6, wherein said L-proline compound is selected from the group consisting of L- proline hydrochloride, L- proline, and pharmaceutically acceptable L- proline salts.

8. The use of any one of claims 1 to 7, wherein said composition further comprises a trace element selected from the group consisting of selenium, manganese, magnesium, calcium, and copper.

9. The use of any one of claims 1 to 8, wherein said composition further comprises an L-arginine compound.

10. The use of claim 9, wherein said L-arginine compound is selected from the group consisting of L- arginine hydrochloride, L- arginine, and pharmaceutically acceptable L- arginine salts.

11. The use of any one of claims 1 to 10, wherein said composition further comprises N-acetyl cysteine.

12. The use of any one of claims 1 to 11, wherein said composition is to be administered in oral, parenteral, subcutaneous, intraarterial, or intravenous form.

13. A method for treating osteosarcoma in a subject comprising the step of administering to a subject suffering from osteosarcoma a composition as defined in any one of claims 1 to 12 in a therapeutically efficient amount.

14. The method of claim 13, wherein said subject is a human.

15. The method of claim 13 or 14, wherein the composition is administered orally, parentarally, subcutaneously, intraarterially, or intravenously.