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ORIGINAL ARTICLE

GE132+Natural: Novel promising dietetic supplement with antiproliferative influence on prostate, colon, and breast cancer cells

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Summary

Purpose: *Natural products have been investigated for promising new leads in pharmaceutical development. The purpose of this study was to analyze the biological effect of GE132+Natural, a novel supplement consisting of 5 compounds: Resveratrol, Ganoderma lucidum, Sulforaphane, Lycopene and Royal jelly.*

Methods: *The antiproliferative activity of GE132+Natural was tested on 3 different human cancer cell lines: MCF7 (breast cancer cells), PC3 (prostate cancer cells), and SW480 (colon cancer cells), as well as on EA.hy 926 (normal human endothelial cell line). In addition, the cytotoxicity of GE132+-Natural on the proliferation of primary human mesenchymal stem cells isolated from dental pulp (DP-MSC), along with its in vitro impact on different peripheral blood parameters, was determined.*

Results: *The results revealed high antiproliferative activity of GE132+Natural on all tested cancer cell lines (PC3, MCF7 and SW480), as well as on the EA.hy 926 endothelial cell line in a dose-dependent manner. However, applied in a wide range of concentrations GE132+Natural did not affect both the proliferation of primary mesenchymal stem cells and the peripheral blood cells counts.*

Conclusion: *The data obtained demonstrated that GE132+Natural is effective in inhibiting cancer cell proliferation, indicating its potential beneficial health effects. In addition, the results pointed that adult mesenchymal stem cells might be valuable as a test system for evaluating the toxicity and efficacy of new medicines or chemicals.*

Key words: *antiproliferative effect, breast cancer, colon cancer, dietary supplement GE132+Natural, prostate cancer*

Introduction

Epidemiological data suggest that ingestion of some compounds from fruits and vegetables is associated with lower risk of cancer. In the present study we estimated possible cytotoxic effects and antitumor activity of the dietetic supplement GE132+Natural, available in the form of capsules containing combination of 5 very powerful antioxidants: *Ganoderma lucidum* (Reishi mushroom), Royal jelly, Resveratrol, Lycopene and Sulforaphane. *Ganoderma lucidum* (Polyporaceae family) is a medicinal mushroom whose fruiting bodies have been used for their medicinal characteristics in traditional Chinese medicine for over 2000 years [1]. Resveratrol is a phytoalexin, or plant antibiotic, mostly known as a constituent of red wine detected in more than 70 plant species, including grapes, berries, plums, peanuts, and pines [2]. Regarding lycopene, literature data claim that this is the carotenoid responsible for tomato's cancer preventive effects [3]. For the next component, Royal jelly, excreted by the mandibular and hypopharyngeal glands of worker bees of the genus *Apis mellifera*, its unique and major components (medium

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chain fatty acids: 10- hydroxy-2-decenoic, 3,10 dihydroxydecanoic and sebacic acids) are shown to be responsible for its modulation of estrogen receptor function *in vitro* and *in vivo* [4], knowing that estrogen is implicated in the development or progression of numerous diseases, which include various types of cancer (breast, ovarian, colorectal, prostate and so forth) [5]. The anticarcinogenic effect of sulforaphane isothiocyanate, found in cruciferous vegetables (watercress, broccoli, cabbage, and so forth) has also been previously reported [6]. Epidemiological studies suggest that increased consumption of all 5 components of the tested GE132+ Natural is related to improving the prognosis for various cancers.

To investigate the GE132+Natural antiproliferative effects we tested its activity on 3 human tumor cell lines: PC-3, MCF7, and SW-480, as well as on EA.hy 926 cell line. In addition, we estimated its potential cytotoxicity on primary human cells by analysing the effects of this product on the proliferation of MSC, isolated from the pulp of deciduous teeth (DP-MSC), as well as its *in vitro* impact on different peripheral blood parameters.

Methods

Preparation of dietary supplement GE132+Natural extract

GE132+Natural product was purchased from the International Health, Belgrade. Due to its incomplete solubility in many solvents [7-11], stock solutions of the investigated compound were prepared as follows: the content of one capsule (500 mg) was mixed with 10ml of absolute ethanol and incubated at 37°C for 24 h with occasional mixing. Following incubation, the solution above the sediment was collected and filtered through 0.2µm filter in order to obtain clear and sterile solution for further work with cell cultures. This solution was entitled as 'fraction 1' and arbitrary marked as 1:10 dilution of the GE132+Natural supplement (50 mg/ml). For further examinations this 'fraction 1' of the GE132+Natural extract was diluted with growth culture medium up an arbitrary concentration of 0.5 µg/ml.

Cell cultures

Human breast carcinoma cell line (MCF7,ATCC/HTB-22) and human colon adenocarcinoma cell line (SW-480, ATCC/CCL-228) were maintained in Ham's F12: DMEM (1:1) (Sigma Chemicals Co, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA GmbH, Austria). Human prostate cancer cell line PC-3 and human endothelial cell line EA.hy 926 were maintained in DMEM culture media supplemented with 10% FBS. All cell lines were purchased from The American Type Culture Collection (ATCC), MD, USA. Cell and all culture media were supplemented with 100U/ml penicillin/streptomycin (PAA GmbH, Austria), HEPES (10 mM, PAA GmbH, Austria) and L-glutamine (2 mM, PAA GmbH, Austria). DP-MSCs were isolated from deciduous teeth and their multi-lineage mesenchymal differentiation ability, as well as positive expression of MSC markers were evidenced as previously described [12]. DP-MSCs were maintained in DMEM with 10% FBS and 200 µM L-ascorbic acid-2-phosphate (Sigma, St Louis, MO, USA). All cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air and maintained at 70–90% confluence with medium replacement 3 times a week. After washing in phosphate buffered saline (PBS, PAA GmbH, Austria), cells were dislodged for both passaging and harvesting by a brief incubation in 0.25% trypsin-0.2% EDTA, resuspended in DMEM supplemented with 10% FBS, stained with trypan blue stain (Gibco BRL, Life Technologies) and counted using a hematocytometer.

Cell proliferation assay

The effect of the investigated GE132+Natural product on the cell proliferation was determined using MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide; Sigma-Aldrich, St. Louis, MO, USA), based on the ability of viable cells to convert soluble MTT into an insoluble formazan. All tested cells were seeded in 96-well cell culture plates in appropriate culture medium ($1x10^4$cells/100µl/well) and grown over a period of 24 h. Then, increasing concentrations of GE132+Natural extract solution (serial dilutions of the 'fraction 1' in growth culture medium) were added to the cell cultures in triplicate and cells were exposed to each concentration at 37°C, 5% of CO_2 for 24 and 48 h. Cell cultures grown in culture medium alone, as well as cultures incubated in medium containing only the corresponding volume of solvent absolute ethanol, were used as controls. After the incubation period, 10 µL of MTT solution (5 mg/mL in PBS, pH 7.2) were added to each cell culture and incubated for a subsequent 3 h at 37 °C, with 5% CO_2. To dissolve formazan crystals in the cell cultures, the media were discarded and formazan crystals were dissolved with 0.1 N HCl in isopropanol. Optical density at 540nm was measured using an automatic reader for microtiter plates (Labsystems Multiskan PLUS, Finland). Tests were performed at least 3 times for each cell line.

Analyses of peripheral blood parameters

The cytotoxic influence of the investigated GE132+Natural product was determined by analyzing its influence on different peripheral blood parameters, including the total number of white blood cells (WBC), erythrocytes and platelets, the differential count of nucleated cells, and hematocrit. To this end, peripheral blood of healthy donors was collected using heparin as

anticoagulant. To every 1 ml of tested blood, 0.5 ml of increasing concentrations of the tested GE132+ Natural extract or the control solution (saline, growth medium alone or growth medium with appropriate volume of absolute ethanol) was added. Samples were incubated for 1 or 4 h at 37oC in humidified atmosphere with 5% CO2. The total number of WBC, as well as erythrocyte and platelets counts, were determined using a hemocytometer. Differential counts of nucleated cells were made on 100 counted cells on blood smears stained by May-Grunwald-Giemsa procedure. Hematocrit was determined by the microhematocrit method.

Statistics

Results were presented as mean ± standard deviation (SD) of 3 independent experiments. Student's *t*-test, using the Origin PC Program, was performed to evaluate the probability of significant differences among the samples with $p<0.05$ considered significant.

Results

The results obtained revealed significant antiproliferative effect of GE132+Natural on all tested tumor cell lines in a dose-dependent manner. Namely, although the low doses of GE132+Natural extract of up to 125 μg/ml or 250 μg/ml (400-fold or 200-fold diluted 'fraction 1', respectively) were either not or slightly effective, higher doses significantly inhibited the growth of all cancer cell lines tested.

As for the human PC3 prostate cancer cell line, statistically significant inhibition of cell growth, for approximately 30%, was detected at an arbitrary concentration of 500 μg/ml GE132+Natural extract (Figure 1), while at higher concentrations, ranging from 750 to 2000 μg/ml, GE132+Natural inhibited the growth of PC3 cells by 40 - 50%.

The cell growth suppression induced by GE132+Natural extract on the MCF7 breast cancer cell line, was significantly more potent than the effects observed on the other cancer cell lines (Figure 2). The highest concentrations used (750-2000 μg/ml) led to 60% inhibition of MCF7 cell proliferation, while the treatment of the cells with concentrations 250 μg/ml and 500 μg/ml resulted in lower, although still significant (20 – 40%) inhibitory effect.

Regarding the SW-480 human colorectal adenocarcinoma cell line, the results demonstrated that GE132+Natural supplement had weaker, but statistically significant inhibitory effect on cell growth (Figure 3). Namely, while GE132+Natural extract was ineffective at doses ranging from 4 to 125 μg/ml, concentrations of 250 μg/ml and higher inhibited the growth of SW480 cells for approximatelly 30-40%.

Human endothelial cell line EA.hy 926 was used as one control, since this is a "hybrid" cell line made by fusion of HUVEC cells, primary cells of human umbilical vein, with A549 cells of human lung adenocarcinoma. When the antiproliferative effect of GE132+Natural extract on EA.hy 926 cells was evaluated, the results obtained showed that GE132+Natural inhibited the proliferation of these cells by 40–50%, at concentrations ranging from 500 to 2000 μg/ml (Figure 4).

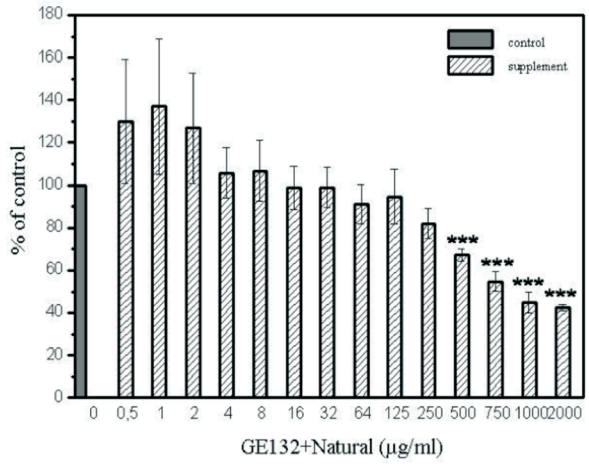

Figure 1. Antiproliferative effects of GE132+Natural on PC-3 tumor cell line. Inhibition of proliferation of PC3 tumor cell line treated with different concentration of GE132+Natural extract, expressed as percentage of control-100%. Results are determined by MTT test after 24h of incubation and shown as mean value ± standard deviation for 3 experiments. Statistically significant difference in relation to control: ***p<0.001.

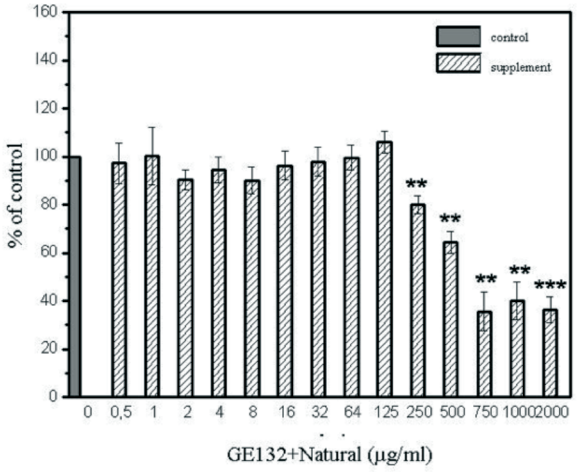

Figure 2. Antiproliferative effects of GE132+Natural on MCF7 tumor cell line. Inhibition of proliferation of MCF7 tumor cell line treated with different concentration of GE132+Natural extract, expressed as percentage of control–100%.Results are determined by MTT test after 24h of incubation and shown as mean value ± standard deviation for 3 experiments. Statistically significant difference in relation to control: **p<0.01, ***p<0.001.

To distinguish the antitumor activity from the cytotoxicity caused by the GE132+Natural, we further evaluated the antiproliferative effect of this supplement on normal human cells, choosing mesenchymal stem cells as primary human cells with high self renewing and proliferative capacities. As shown in Figure 5 GE132+Natural did not exert any significant effect on the growth of DP-MSCs, since applied in a wide range of concentrations did not affect the proliferation of these cells.

To confirm the absence of cytotoxic effects of GE132+Natural on normal human cells, we additionally tested the effects of GE132+Natural on different peripheral blood parameters, by *in vitro* subjecting peripheral blood of healthy donors to increasing concentrations of GE132+Natural extract. Results of these experiments revealed that during incubation for either 1 h or 4 h GE132+-Natural, in very broad range of concentrations, did not cause significant changes of blood cell counts. Namely, the obtained values of white blood cells, erythrocytes, and platelets counts, as well as hematocrit, in peripheral blood cultures exposed to GE132+Natural were similar to those found in control experimental groups treated with saline, growth medium alone or medium containing absolute ethanol (data not shown). Furthermore, GE132+Natural supplement did not affect the peripheral blood differential count (data not shown).

Discussion

Natural products of strong medicinal values are gaining importance in cancer therapy and prevention, especially in the light of the serious side effects posed by chemically produced medicinal derivatives. Hence, it becomes pivotal to investigate other strategies to control tumor proliferation. In the present study, we evaluated the effects of GE132+-Natural dietary supplement containing 5 components (Ganoderma lucidum, Royal jelly, Resveratrol, Lycopene and Sulforaphane) on the proliferation of 3 different human tumor cell lines including PC-3, MCF7, and SW480, along with the effects on human endothelial EA.hy26 cell line and human primary cells (DP-MSCs). The results showed significant antiproliferative activity of GE132+Natural on all 3 tested tumor cell lines, as well as on the endothelial cell line used. However, as no changes were observed in the proliferation of DP-MSC, as well as in different peripheral blood parameters after *in vitro* incubation of these cells with GE132+Natural, the results indicated that this supplement might exhibit beneficial health effects.

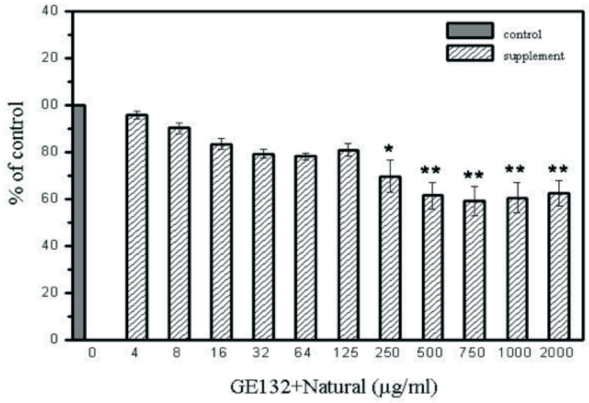

Figure 3. Antiproliferative effects of GE132+Natural on SW480 tumor cell line. Inhibition of proliferation of SW480 tumor cell line treated with different concentration of GE132+Natural extract, expressed as percentage of control–100%.Results are determined by MTT test after 24h of incubation and shown as mean value ± standard deviation for 3 experiments. Statistically significant difference in relation to control: *p<0.05,**p<0.01.

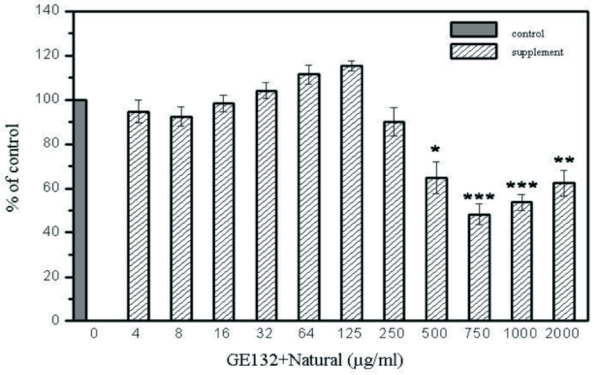

Figure 4. Antiproliferative effects of GE132+Natural on EA.hy 926 cell line. Inhibition of proliferation of EA.hy926 cell line treated with different concentration of GE132+Natural extract, expressed as percentage of control–100%.Results are determined by MTT test after 24h of incubation and shown as mean value ± standard deviation for 3 experiments. Statistically significant difference in relation to control: *p<0.05,**p<0.01, *** p<0.001

The antitumor activity of the GE132+Natural confirmed by the significant growth suppression of all cancer cell lines tested is in agreement with previous studies demonstrating the antitumorigenic effects of all components of the GE132+Natural supplement themselves [13,14]. Resveratrol, a dietary polyphenol derived from grapes, berries, peanuts, and other plant sources, is one of the GE132+Natural components with well documented anticancer properties. Namely, resveratrol was shown to inhibit the proliferation of a variety of human tumor cells *in vitro*, including B cell lymphoma, myeloid leukemia, pancreatic, gastric, liver and lung cancer through

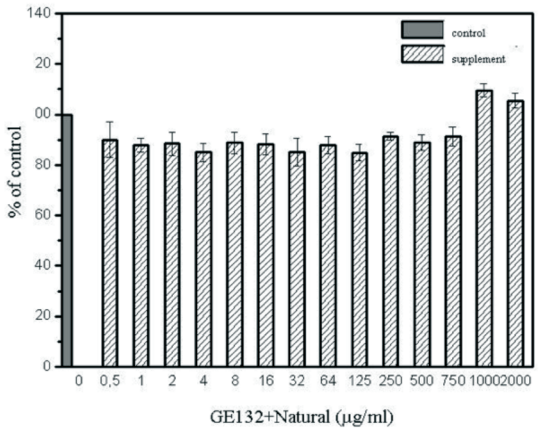

Figure 5. Effects of GE132+Natural on DP-MSCs growth. Proliferation of DP-MSC cell line treated with different concentration of GE132+Natural extract was determined by MTT test after 24h of incubation. Results are shown as mean value ± standard deviation for 3 experiments and presented as percentage of control-100%.

cell-cycle arrest and activation of caspases [15,16]. Moreover, several reports documented its *in vitro* antioxidant and proapoptotic activity on prostate cancer cells [17], as well as breast (MCF7, MCF10F and MDAMB231) and colon cancer cell lines [18]. However, resveratrol appears to impact the proliferation not only of tumor cells but also of normal primary cells as it suppressed the proliferation of keratinocytes, endothelial and smooth muscle cells [18], although the proliferation of normal human peripheral blood mononuclear cells (PBMC) was unaffected [18]. According to numerous literature data demonstrating the inhibitory effect of resveratrol on colon, breast and prostate cancer cell growth, one can suppose that this component of GE132+Natural dietary supplement contributed to growth reduction of SW480, MCF7 and PC3 cells observed in our experiments.

Yet another component of GE132+Natural dietary supplement, Ganoderma lucidum, an oriental fungus, has been widely used as a medical drug in China and other Asian countries. Previous reports showed antiproliferative and apoptotic effect of *Ganoderma lucidum* on MCF7 cells proliferation in both dose- and time-dependent manner, mediated by the modulation of estrogen receptor (ER) and NF-kB signaling and subsequent down-regulation of c-myc expression [19]. In addition, it was demonstrated that *Ganoderma lucidum* inhibits the prostate cancer PC3 cells proliferation in a dose- and time-dependent manner by cell cycle arrest at G2/M phase through regulation of cyclin B and Cdc2 expression and up-regulation of p21 expression, as well as the apoptosis induction through decrease in the of NF-kB-regulated Bcl-2 and Bcl-xl expression [20]. Concerning colorectal cancer, one of the most common cancers worldwide, suppressive effects of *Ganoderma lucidum* on the proliferation of different colorectal cell lines, including HCT-116, HT-29 and SW480 cells, were also shown before [21,22]. Having in mind all these findings, one can assume that *Ganoderma lucidum* within GE132+Natural also contributed to the observed reduction of SW480, MCF7 and PC3 cancer cells growth.

Another component of GE132+Natural, lycopene, the major carotenoid and active compound in tomatoes, was also proved to be involved in cancer prevention [3]. Moreover, it was shown that carotenoids may influence processes such as proliferation of cancer cells, independently of their role as antioxidants. Namely, higher cytostatic and cytotoxic potential of lycopene, as compared to other carotenoids, was previously demonstrated in prostate cancer PC3 cells *in vitro* [23]. Regarding MCF7 human breast cancer cells, it has previously been published that carotenoids inhibit the growth of both ER(-) and ER(+) breast cancer cell lines, indicating that ER status is not substantial factor for the reaction of breast cancer cells to carotenoid treatments [24], while other studies revealed mechanisms underlying antiproliferative effect of lycopene on MCF7 cells growth based on bcl-2 down-regulation and cell cycle inhibition [25]. In case of colon cancer cells there are some assumptions that lycopene could modulate PI3-K/Akt signaling pathways to suppress growth of HT-29 colon cancer cells [26]. However, further studies should confirm whether and through which mechanisms lycopene contributes to cancers cells' growth suppression observed in this study.

Considering the next component of GE132 supplement, Royal jelly, literature is poor regarding its effect on tumor cell lines. Since MCF7 cells are expressing ER, and there have been proofs that Royal Jelly increases the proliferation in ER(+) cells [27], further studies should be performed to determine the exact influence of this component on this breast cancer cell line. The anticancer effectiveness of sulforaphane (SFN), a natural compound derived from broccoli, has been determined in various cancers. Regarding breast cancer MCF7 cells, it has been demonstrated that SFN induces mitotic arrest and microtubule disruption by stimulating G2/M accumulation through elevation of cyclin B1 protein and cdc2 kinase activation [28]. In case of PC3 cells, there is evidence that SFN is highly effective in suppressing proliferation of these cells in culture through targeting cancer cells by multiple chemopreventive mechanisms [29], even more by target-

ing benign hyperplasia cells and cancerous prostate cells while leaving the normal prostate cells unaffected. Further on, although there is little evidence in the literature, especially for SW480 colon cancer cells, results concerning human colon carcinoma HT29 cells revealed that SFN in these cells induces cell cycle arrest, followed by apoptosis [30,31]. It is very likely that SFN is another component of GE132+Natural that has high inhibitory effect on the proliferation of cancer cells tested in this study. However, further studies are needed to confirm this assumption.

When the antiproliferative effect of GE132+-Natural extract on EA.hy 926 cells was evaluated, the results obtained showed that GE132+Natural inhibited the proliferation of these cells up to 50%, at concentrations ranging from 500 to 2000 μg/ml. Assuming that "hybrid" cell lines are often designated as normal cell lines and are used as control cells to prove the non-cytotoxic effects of various chemotherapeutic agents, the growth inhibition pattern obtained was, in a way, unexpected. However, immortalized cells usually show anomalous behavior and phenotype, which do not reflect the mechanisms observed in their normal homologous cells. Therefore, since EA.hy 926 cells were immortalized by fusion of HUVEC cells, primary cells of human umbilical vein, with A549 cells of human lung adenocarcinoma, the possibility that the compounds present in GE132+Natural affected the cancer-like mechanisms in these cells could not be excluded. On the other hand, since the vascular endothelial cell proliferation is a key initial step in angiogenesis, the effect of GE132+Natural could be related to the involvement of the supplement components in this process. Namely, there are data pointing that *Ganoderma lucidum* polysaccharides peptides (GLPP) directly inhibit HUVEC primary endothelial cell proliferation *in vitro* and induce endothelial cell apoptosis [32]. In addition, there are reports demonstrating that Resveratrol, Royal jelly and SLF suppress HUVEC cell proliferation by inducing cell apoptosis [18,33,34]. Therefore, the antiproliferative effect of GE132+Natural on EA.hy 926 cells could be related to the specific anti-angiogenic effect of Ganoderma lucidum, Resveratrol, Royal jelly and/or SLF components. Further detailed studies of GE132+Natural activity on the primary endothelial cells' growth should be performed in order to confirm its effect on angiogenesis, since it is well known that tumor growth and metastasis are angiogenesis-dependent processes, and angiogenesis inhibitors preventing the proliferation of vascular endothelial cells are thought to have potential for treatment applicable to many types of cancer.

To determine the cytotoxic effect of GE132+-Natural, we next evaluated its effects on primary human cells, which are often considered a better option as model systems for predicting toxicological behavior of various substances. The toxicity of chemotherapeutic agents is usually detected by measuring the effect on mature blood cells, erythrocytes, leukocytes, and platelets, and our results indicated that GE132+Natural does not cause significant changes in various peripheral blood cell counts *in vitro*. However, more important and valuable are the data obtained with the human MSCs, in which GE132+Natural, in a wide range of concentrations, did not affect their proliferation and viability. The unique properties of MSCs, such as unlimited proliferation ability and plasticity to generate other cell types, along with various readily available sources of primary human cells, clearly identify their potential benefits in toxicology, although data demonstrating that these cells can confidently be used to perform *in vitro* acute toxicity tests are just starting to be reported [35]. Our results support the approach that human adult stem cells provide a more accurate model for *in vitro* studies, replacing the transformed cell lines with anomalous behavior, as well as the mature primary cells that are limited in quantity, express batch-to-batch variation and need to be isolate freshly for each study. Another advantage is the ability to derive stem cells from each individual human subject, offering the opportunity to analyze the genetic background that might affect the susceptibility to toxicity.

In conclusion, our results demonstrated that GE132+Natural is beneficial in inhibiting cancer cell proliferation and indicated its potential antiangiogenic effects. Additionally, our data pointed that adult mesenchymal stem cells might be valuable as a test system for evaluating the toxicity and efficacy of new medicines or chemicals.

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