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Original Article

HESA-A Exerts Its Cytoprotective Effects through Scavenging of Free Radicals: An in Vitro Study

Mehryar Habibi Roudkenar[1], Parisa Bahmani[1], Raheleh Halabian[1], Amaneh Mohammadi Roushandeh[2], Ali Jahanian Najafabadi[3], Mohammad Ali Shokrgozar[4]

Abstract

Background: Natural medicines have been recently considered more reasonable for human use most notably due to their safety and tolerance. HESA-A is a marine-originated herbal medicine with a variety of healing effects. However, its exact biological mechanism is not clear. The present study aimed at the evaluation of the HESA-A antioxidant effect.

Methods: Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) cells were treated with different concentrations of HESA-A and H_2O_2 followed by cell proliferation assays. The antioxidant effect of the HESA-A preparations was evaluated by an antioxidant assay kit.

Results: The viability of CHO and HEK293T cells were about 89% following their incubation with 100 and 200 ng/ml HESA-A, respectively for 1.5 hrs. However, when the cells were incubated with concentrations of 300 ng/ml or more, the cell viability significantly decreased to 48% compare to the control cells. The cytotoxic effects of H_2O_2 were observed after 2 hrs of incubation of the HEK293T or CHO cells with 10 mM or 16 mM H_2O_2, respectively, while in the presence of HESA-A the cytotoxicity was significantly decreased. Antioxidant assay revealed that HESA-A scavenges free radicals.

Conclusion: The findings indicate that HESA-A had cytoprotective effects in vitro, and that such an effect might be due to antioxidant properties.
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Keywords ● HESA-A ● reactive oxygen species ● hydrogen peroxide

[1]Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran,
[2]Department of Anatomy, Faculty of Medicine, Medical University of Hamadan, Hamadan, Iran.
[3]Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran.
[4]National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran.

Correspondence:
Mohammad Ali Shokrgozar PhD,
National Cell Bank of Iran,
Pasteur Institute of Iran,
Pasteur street,
Pasteur Square,
Tehran, Iran.
Tel/Fax: +98 21 66492595
Email: mashokrgozar@pasteur.ac.ir
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Introduction

History of medicine reveals that about 60% of anticancer and 75% of anti-infective drugs, which were approved from 1981-2002, could be traced to natural origins, which are cheaper and perhaps more productive than chemical compounds.[1] Most natural compounds are part of routinely-used traditional medicine, therefore the tolerance and safety of them are almost better known than those of chemical entities, which are new for human use.[2] In addition, a large number of the naturally derived medicinal compounds is originated from micro-organisms and marine organisms that contain remedies against tuberculosis, malaria, cancer, HIV and other diseases.[1]

HESA-A is a drug of herbal-marine origin (Wild celery, Cumin and King Prawn) which is obtained based on anecdotal

evidence from Persian folk and traditional medicine. HESA-A showed hepatoprotective and anti tumor properties, and have been patented under Iranian authority.[3,4] It is composed of organic constituents, mineral elements such as CaO (43.787%), P_2O_5 (6.63%), Na_2O (3.689%), MgO (2.897%), SO^3 (2.193%), K_2O (1.988%), SiO_2 (1.09%), Fe_2O_3 (0.375%), Al_2O_3 (0.354%), and trace elements which are known to possess anti-oxidant and potential anti-cancer properties such as vanadium (V), nickel (Ni), titanium (Ti), zinc (Zn), strontium (Sr) and selenium (Se).[4-6] This compound appears to be an effective and safe anticancer remedy that may increase survival of end–stage patients, and can be used in some patients.[3,7,8] Protective effects of HESA-A such as decrease of morbidity and increase of the life span of cancer patients already has been shown.[9] HESA-A inhibits the growth of cancer cells in a concentration dependent and selective manner.[9] It increases the appetite, and can be considered as an immune-modulator, anti-inflammatory, and anti-viral agent.

Although HESA-A shows some clinical effects, its precise biological mechanisms remain unclear. It has been shown that in rabbits under oxidative stress challenges, treatment with HESA-A led to an increase in total antioxidant and antierythrocytelysis.[10] In addition, the hepatoprotective effect of HESA-A against hepatic damage in rabbits was demonstrated in previous study.[8] Since HESA-A revealed considerable effects in the treatment of psoriasis vulgaris, a T cell-mediated inflammatory disorder, it seems that it has immunomodulator characteristics too.[5] Such findings indicate that HESA-A may have antioxidant activity. Furthermore, the finding that HESA-A is rich in trace elements such as Cu, Mn, Se and Zn, which are involved in enzymatic detoxification and antioxidant systems,[10] suggests that HESA-A may possess antioxidative effects.

Given such evidences, the present study aimed to test whether or not HESA-A has antioxidant properties. To answer such a question, the cytotoxic effects of hydrogen peroxide (H_2O_2) on Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) cell lines were determined following exposure of the two cell lines to HESA-A by cell proliferation and antioxidant assays.

Materials and Methods

Cell Culture

The CHO andHEK293T cell lines were obtained from national cell bank of Iran (Pasteur Institute of Iran). The cells were grown in RPMI 1640 medium (Gibco-BRL, Germany) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Germany) and 1% penicillin streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were cultured in 25 cm^2 cell culture flasks. For experimental purposes, cells were seeded at a density of 2×10^4 cells/well in 96-well plates 24 h before any treatment. Then, they were treated with 5-20 mM concentrations of H_2O_2 for different time lengths. HESA-A compound was diluted in phosphate buffered saline (PBS) and the cells were separately treated with 100-1000 ng/ml of HESA-A for different durations. The CHO and HEK293T cells were also exposed to various concentrations of HESA-A (100, 200, 300, 500 ng/ml), followed by treatment with different concentrations of H_2O_2. Controls were maintained under normal condition.

Cell Proliferation Assays

Cytotoxic effects of HESA-A and H_2O_2 on CHO and HEK293T cell lines were assessed by 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT). The MTT assay was performed as described previously by Roudkenar et al.[11] Briefly, at the end of any treatment, the cells were incubated with a 1:10 dilution of the 5 mg/ml 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Dusseldorf, Germany) stock for four h at 37°C in a cell culture incubator. Subsequently, the cells were washed with PBS followed by the addition of acidic isopropanol (0.04 M HCl in absolute isopropanol).Then the plates were shacked for one min and the absorbance was recorded at 570 nm using a microplate reader system.

Determination of Total Antioxidant Activity of HESA-A

The activity of HESA-A against oxidative stresses was measured with an antioxidant assay kit (Sigma Aldrich, USA). The kit provides for an efficient measurement of the total antioxidant activity. For the evaluation of the antioxidant property, different concentrations of HESA-A (20-100 µg/ml) were added to 96 well plates, and the antioxidant capacity was evaluated according to the kit manufacture's protocol. The CHO and HEK293T cells were grown in 96 well plates. Then, various concentrations of HESA-A (100-800 ng/ml) were added to the culture medium one hour before H_2O_2 treatment. Afterwards, CHO cells and HEK293T cells were treated with 16 and 10 mM H_2O_2, respectively. Finally, the culture medium was

collected and antioxidant capacity of HESA-A was measured according to the supplier protocol. Trolox, a water-soluble vitamin E analog, was provided by the kit and was used as a positive control of antioxidant activity. Absorbance was monitored at 405 nm using a ELx800 Absorbance Microplate Reader.

Statistical Analysis

The results are expressed as mean ± SD of three independent experiments. Differences between groups were compared using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparison Test. A probability of committing type one error of ≤0.05 was considered statistically significant.

Results

Cytotoxic Effect of HESA-A on CHO and HEK293T Cell Lines

Different concentrations of HESA-A were used to clarify direct effects of HESA-A on the viability of CHO and HEK293T cells. At first, the cells were exposed to HESA-A (100-1000 ng/ml). As determined by MTT assay, the viability of the cells incubated with the concentrations of 100 and 200 ng/ml of HESA-A was shown to be about 89% after 90 min, while in the presence of 300 ng/ml or higher concentrations of the HESA-A the viability was decreased down to 48% comparing to the controls (figure 1a). This indicates that cytotoxicity of HESA-A is dose dependent. Next, for optimization of the treatment duration the cells were exposed to 100 and 200 ng/ml HESA-A for one h, 1:30 h, two h and 2:30 h. Then the cytotoxicity was determined by MTT assay. Compared to the control, no changes were observed in the viability of the cells In the presence of 100 and 200 ng/ml of HESA-A. However, the cytotoxic effects of HESA-A at 200 ng/ml were shown to be time dependent (figure 1b). Therefore, the minimal toxic doses of HESA-A were determined.

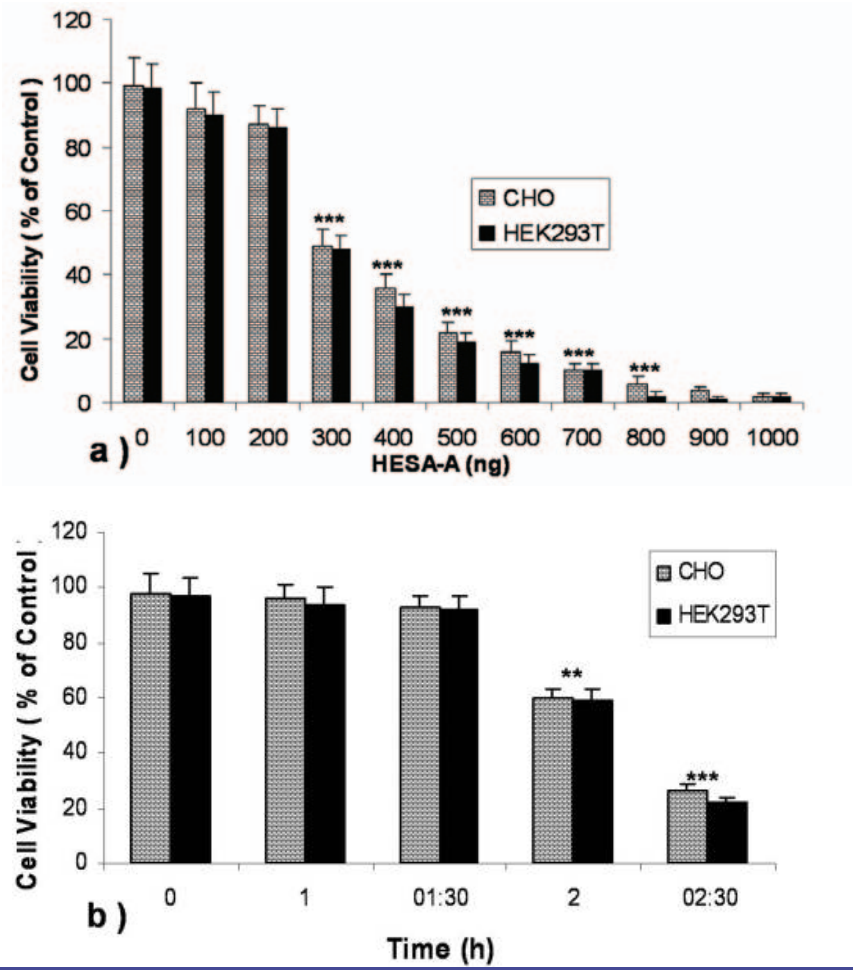

Figure 1: The effects (mean±SD, three replicates) of HESA-A on viability of CHO and HEK293T cells. a) The cells were treated with different concentrations of HESA-A for 1.5 hrs. The signs of cytotoxicity were first observed at the concentration of 300 ng/ml of HESA-A. b) The cells were treated with HESA-A (200 ng/ml) for different time courses.
** indicates significant difference (P<0.01) from the control; *** indicates significant difference (P<0.001) from the control

HESA-A Reduced H_2O_2 Toxicity on CHO and HEK293T Cell Lines

The cytoprotectivity of HESA-A against oxidative stress was examined by the treatment of CHO and HEK293Tcells with H_2O_2. First, the toxicity of H_2O_2 was determined by treatment of the cells with different concentrations (6-20 mM) of H_2O_2. The cytotoxicity effects were observed 2 hrs following incubation of HEK293T and CHO cells with 10 and 16 mM H_2O_2, respectively (data not shown). But the addition of 100, 200 and 300 ng/ml of HESA-A considerably reduced cytotoxic effects of H_2O_2 (figures 2a, 2b). Compared to the control, H_2O_2 at 10 mM in case of HEK293T cells and 16 mM in case of CHO cells was not toxic to the cells in the presence of 100 to 300 ng/ml HESA-A. The results suggest that HESA-A could scavenge reactive oxygen species (ROS) in vitro. It is noteworthy that HESA-A was toxic to the cells at the concentrations of 300 ng/ml and more (figure 1a), but unexpectedly, in the presence of H_2O_2, the toxic effects of HESA-A was decreased suggesting that HESA-A and H_2O_2 might have neutralized each other slightly.

Total Antioxidant Activity of HESA-A

The above-mentioned results showed that HESA-A could protect cells against H_2O_2 toxicity, but the mechanisms underlying this effect was not clear. First, the antioxidant activity of HESA-A were considered, therefore, total anti-

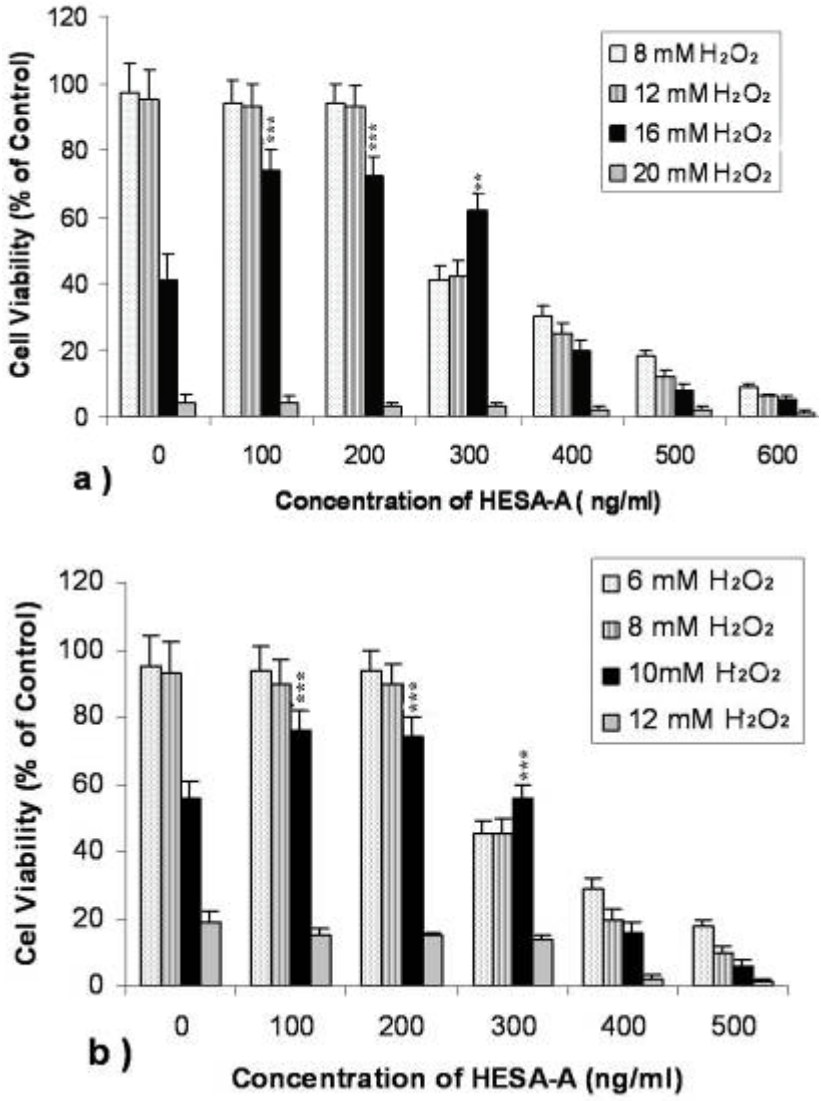

Figure 2: The effects (mean±SD, 3 replicates) of HESA-A on hydrogen peroxide (H_2O_2)-induced toxicity in a) CHO and b) HEK293T cells. The cells were treated with different concentrations of HESA-A and H_2O_2. a) HESA-A at 100 and 200 ng/ml protected CHO cells against H_2O_2-induced toxicity. b) HEK293T cells
** indicates significant difference (p<01) from the control (without HESA-A treatment); *** indicates significant difference (P<0.001) from the control (without HESA-A treatment)

oxidant activity of HESA-A was determined. As figure 3 shows, HESA-A scavenges free radicals like Torolox, which was used as positive control. It should be noted that when a compound contains antioxidant property, the number of free radicals is lower; therefore, the absorbance is lesser. Next, we hypothesized that HESA-A exerted its cytoprotective effects on the cells through same mechanisms i.e. antioxidant activity. To examine this, the cells were exposed to H_2O_2 in the presence of HESA-A followed by antioxidant capacity assay of cell culture medium. As shown in figure 4 the antioxidant capacity of the cell culture medium was highest at the concentrations of 100, 200 and 300 ng/ml of HESA-A. This indicates that HESA-A protect cells against H_2O_2 induced cytotoxicity.

Discussion

The current study was designed to determine whether HESA-A could act as an antioxidant compound. First we incubated CHO and HEK293Tcells with HESA-A to test whether this compound has any toxic effect on normal cells. Although low concentrations, HESA-A did not change cell viability compared to untreated cells, but at higher concentrations it caused significant decrease in cell viability. This suggests that HESA-A at high concentrations was toxic to the cells; however, in most of the concentrations below the lethal doses no changes in cells' survival were observed. These findings show that that HESA-A is a safe compound; however, it should be noted

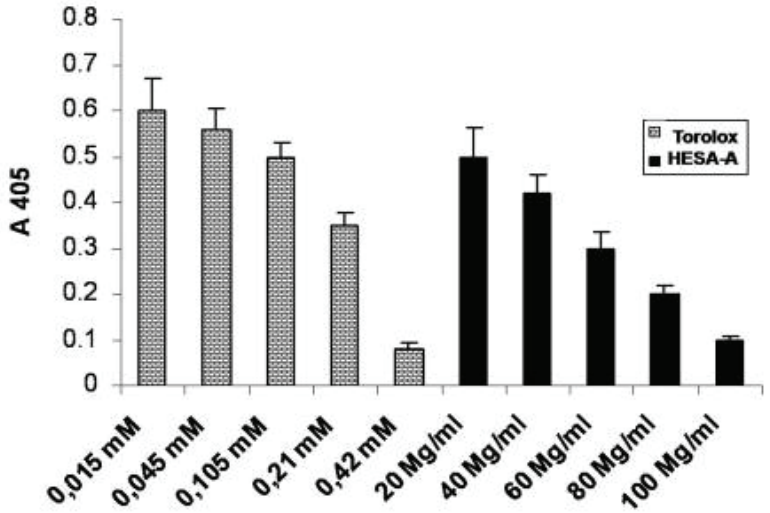

Figure 3: The effects (mean±SD, number of replicates, 3) of various concentrations of torolox and HESA-A on A 405, which is a measure of total antioxidant capacity. The antioxidant activity of both compounds increases with increasing concentration.

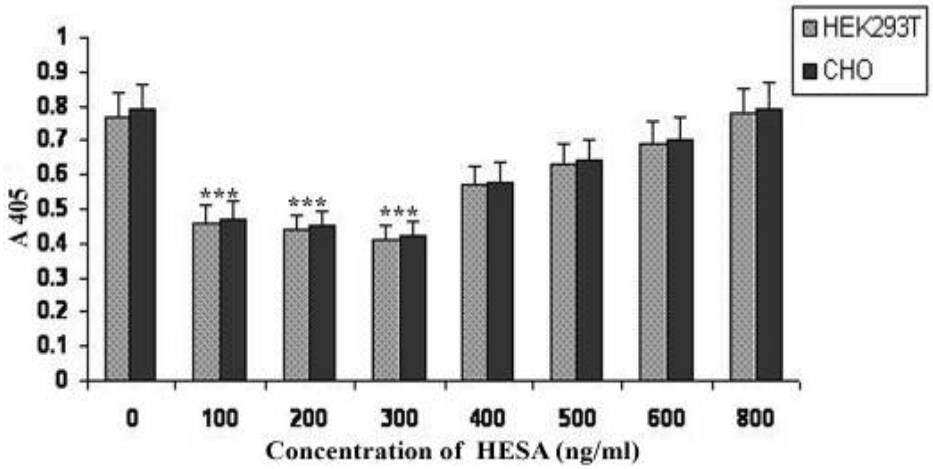

Figure 4: The effects (mean±SD, number of replicates, 3).of various concentrations of HESA-A on A 405 in Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) cell lines. A 405 is a measure of total antioxidant activity. The cells were exposed to H_2O_2 in the presence of HESA-A followed by the measurement of antioxidant capacity assay of cell culture medium. In concentrations of 200 and 300 ng/ml of HESA-A, the antioxidant capacity of the cell culture mediums were the highest indicating that HESA-A inhibited the toxicity of H_2O_2 to the cells. Chinese hamster ovary cells were treated with 16 mM H_2O_2 and HEK293T cells were treated with 10 mM H_2O_2.
*** indicates significant difference ($P<0.001$) from the control (without HESA-A treatment)

that despite the successful use of HESA-A as a therapeutic agent, it must be used with care to minimize or to avoid its deleterious effects.

The cytoprotective effects of HESA-A against ROS toxicity was examined by treating CHO and HEK293Tcells with H_2O_2. For this purpose, CHO and HEK293T cells were treated with H_2O_2, and proliferation of cells in the presence of HESA-A was compared with that of control cells. While the addition of H_2O_2 to HEK293T and CHO cells caused cell toxicity, treatment of these cells with HESA-A ameliorated the H_2O_2-induced cytotoxicity. These results showed that this compound could protect cells against ROS produced by H_2O_2. This was the first finding that HESA-A had antioxidant properties. We also examined our hypothesis by the evaluation of total antioxidant capacity of HESA-A with an antioxidant assay kit.

HESA-A demonstrated a concentration-dependent antioxidant activity with an antioxidant capacity at 200 ng/ml. This indicates that HESA-A prevents oxidation and suggests that the medical benefits associated with HESA-A might be due to its antioxidant capacity. This idea is further supported by the observation that the administration of HESA-A supplements in rabbits improved their antioxidant status by decreasing erythrocyte lysis and lipid peroxidation following exogenous oxidative stress challenges.[10] It has been clearly documented that tumor cells are under persistent oxidative stresses,[12] and it has been suggested that, because of antioxidant properties, HESA-A is effective in curbing the growth of cancer cells.[8] Thus, it seems that antioxidant properties of HESA-A is responsible for its anticarcinogenic effects by scavenging cancer-promoting oxidants. Such a conclusion is also supported by the constituents of HESA-A. For example, celery, the vegetable part of HESA-A, shows a minor antioxidant activity and is an effective scavenger of ROS.[13,14] Additional support for cytoprotective activity of HESA-A comes from X-ray studies that revealed the presence of certain trace elements (Cu, Mn, Se, V and Zn) in HESA-A.[4] It has been known that one of the important biological functions of trace elements is antioxidant effect. The activity of antioxidant enzymes depends on a sufficient supply of the trace elements.[15] These elements function at the active site of metal ion-dependent enzymes such as superoxide dismutase, catalase or glutathione peroxidase.[16] For example Cu acts as a reductant in the enzymes superoxide dismutase, cytochrome oxidase, lysil oxidase, dopamine hydroxylase, and several other oxidases that reduce molecular oxygen.[17] In addition, Zn has been shown to have an antioxidant role (s) in organelle-based systems and isolated cell-based systems in vitro.[18] In support of this notion, it was shown that the administration of pharmacological doses of Zn in vivo had a protective effect against general and liver-specific prooxidants.[18] Moreover vanadium, one of HESA-A constituents, exerts an antitumor effects through inhibition of cellular tyrosine phosphatases and/or activation of tyrosine phosphorylases, which result in the activation of signal transduction pathways leading to apoptosis and/or activation of tumor suppressor genes.[9] Other studies suggest that carcinogenic metals such as chromium and vanadium can cause cell death through DNA damage, protein modifications, or lipid peroxidation.[19] Selenium, another trace element of HESA-A, is an essential constituent of a number of enzymes, the antioxidant property of which have been reported.[20] Selenium may cause DNA fragmentation and decreased DNA synthesis, and provides the catalytic centers for a number of seleno-enzymes.[21]

Given the above discussion about the role of trace elements and the findings of the present study, it may be possible to suggest that antioxidant properties of HESA-A may be due to its trace elements. It has been known that high supplementation of many trace elements can help in the reductive activation of H_2O_2. For example, interaction of H_2O_2 with Cu generates more reactive species, such as hydroxyl radicals.[22,23] Therefore, our findings suggest that cytotoxic effects of HESA-A in higher concentration may be due, at least in part, to the catalysis of oxidative stress by Cu. Importantly, the present study shows that although HESA-A resulted in cell death at concentrations of 300 ng/ml and more, this effect may be blocked by H_2O_2. This finding provokes the fascinating possibility that HESA-A may be consumed for scavenging reactions, which could result in neutralization of HESA-A and banishment of its cytotoxic effects.

The finding of this study also suggest that the antioxidant capacity of HESA-A can be the result of its ability to counteract oxidative stress. Altogether, based on the findings of this study, we propose that HESA-A can dramatically scavenge ROS in vitro. Therefore, further investigation should be performed to evaluate the possibility of using HESA-A for the treatment of various diseases including cancer and hypertension by modulating the generation of ROS.

Conclusion

The findings of this study show that HESA-A can scavenge free radicals, and contains antioxidant

property. This finding highlights potential application of HESA-A to treat various diseases in which ROS generation is a major problem. However, precise functions of HESA-A warrants further investigation.

Conflict of Interest: None declared

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