Assessment of selective growth inhibitory effects of HESA-A on some human neoplastic cell lines

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Background: During the past few years, HESA-A, a herbal-marine mixture, has been used to treat cancer as an alternative medicine in Iran. However, weight of the evidence is not sufficient to accept or refuse the use of this compound as a cytotoxic drug. We investigated the selective anticancer effects of HESA-A on breast, prostate, colon, and glioblastoma multiforme (GBM) neoplastic cell lines.

Methods: MTT-based cytotoxicity assay was performed on HCT-116 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), PC-3 (prostate adenocarcinoma), U-87MG (GBM), and HDF-1 (normal dermal fibroblast) cell lines using different concentrations of HESA-A (0, 1, 3.3, 10, 33 and 100 µg/ml) and doxorubicin as positive control (10 µM). If there was seen an inhibitory response, median inhibitory concentration (IC₅₀) was determined. We defined the cytotoxicity as the extrapolated IC₅₀ became equal to or lower than 50 µg/ml.

Results: HESA-A at the highest concentration (100 µg/mL) significantly inhibited the growth of HCT-116 cell line (P = 0.003; compared to control). Percentage growth inhibition of HESA-A at this concentration was determined as 40.13%. IC₅₀ of this compound on HCT-116 cell line was extrapolated 117.28 µg/mL. There were no statistically significant differences between the mean absorbance measures of HESA-A treated groups in other cell lines.

Conclusion: This study showed that HESA-A doesn’t fulfill the predetermined criterion of cytotoxic agents. More preclinical investigations are needed to assess the efficacy of HESA-A in cancer.

Keywords: HESA-A; cytotoxicity; MTT assay; neoplastic cell lines.
Introduction

Cancer is considered as a major public health problem throughout the world. The global burden of this disease continues to increase in developing countries.\(^1,2\) Although progress has been made in reducing mortality rate and improving survival, chemotherapy, as the primary means of treatment, is only effective in approximately one-fourth of those treated.\(^3\) This failure in treatment has led to the increasing attitudes toward complementary and alternative medicine, especially in the Middle East.\(^4\)

HESA-A is a herbal-marine mixture, including king prawn (*Penaeus latisulcatus*), caraway (*Carum carvi*), and celery (*Apium graveolens*).\(^5\) During the past few years, this compound has been used to treat cancer as an alternative medicine in Iran. Data derived from an in vitro study suggest that HESA-A has selective anticancer properties on some neoplastic cell lines of breast, cervix and liver.\(^6\) But, an important limitation of this work is that the cells were treated with suprapharmacological concentrations of the compound. It has been also claimed that this compound can improve the quality of life in patients with metastatic colon cancer.\(^7\) Published toxicological data indicated to the safety of HESA-A.\(^8\)

Although literature support exists for the anticancer effects of HESA-A,\(^7,8\) but weight of the evidence is not sufficient to accept or refuse the use of this compound in cancer treatment. On the other hand, if there is an anticancer property, cytotoxic or cytostatic nature of HESA-A has not been directed in the published articles. Since rational for the design of a clinical trials depends on what is preclinically known about the agent,\(^9\) it seems necessary to accurately study the anticancer effects of HESA-A according to standard methodologies at the preclinical level. In the present study, we aimed to determine whether this compound meets the criteria of an anticancer drug at in vitro level. This study performed to investigate the anticancer effects of HESA-A in breast, prostate, colon, and GBM neoplastic cell lines.

Methods

Materials and cell lines

Dulbecco’s modified eagle medium (DMEM), fetal bovine serum, phosphate-buffered saline (PBS), trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA), amphotericin B and penicillin-streptomycin solutions were obtained from Invitrogen (Carlsbad, CA, USA). All cell culture vessels were purchased from BD Biosciences (Franklin Lake, NJ, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) glycine and doxorubicin hydrochloride were obtained from Sigma-Aldrich (Steinheim, Germany). MCF-7 (human breast adenocarcinoma), HCT-116 (human colon cancer), PC-3 (human prostate cancer) and U-87MG (human GBM) cell lines were purchased from National Cell Bank of Iran (Tehran, Iran). Human normal dermal fibroblast cell line (HDF-1) was obtained from Iranian Biological Resource Center (Tehran, Iran). HESA-A powder was gifted by Osveh Pharmaceutical Company (Tehran, Iran) and prepared according to the criteria of National Cancer Institute (NCI).\(^{10}\)

MTT-based cytotoxicity assay

MTT-based cytotoxicity assay was carried out in accordance with the protocol previously described by Plumb and her colleagues.\(^{11}\) Based on population doubling time of cell lines, cytotoxicity test was designed as short-form and long-form assays. Since the doubling time of HCT-116 is shorter than 24 hours, this cell line was included in the short-form assay. But the cytotoxicity of HESA-A on MCF-7, PC-3, U-87MG and HDF-1 cell lines with the doubling times longer than 24 hours was examined with the long-form assay.

In the short-form assay, HCT-116 cells were conveyed to 96-well microtitration plates with a seeding density of 5,000 cells per well in 200 µL DMEM medium containing 10% FBS and 2mM L-glutamine. The plates were incubated in humidified air containing 5% CO2 at 37°C. The next day, when the cells were entered to the logarithmic phase of growth, exposure period was started by adding the drugs. In this period, the cells were exposed to the five concentrations of HESA-A including 1 µg/mL, 3.3 µg/mL, 10 µg/mL, 33 µg/mL, and 100 µg/mL. Doxorubicin was used with the concentration of 10 µM as positive control. Each treatment was run triplicate. 24 hours later, the drugs were removed from the wells. In order to demonstrate retention of regenerative capacity of the exposed survived cells, a 48 hours recovery period were
In the long-form assay, cells were seeded in microtiteration plates with the density of 1,000 cells per well. Drugs were added 3 days later with the same concentrations mentioned for short-form assay. In the long-form assay, the durations of exposure and recovery periods were considered as 72 and 96 hours, respectively.

During the recovery period, the plates were fed daily with fresh medium. At the end of recovery period, 50 µL of MTT (5 mg/mL) solution was added to each well and then the plates were further incubated for 4 hours. All remaining supernatant were removed and 200 µL of DMSO was added to dissolve the formed insoluble formazan crystals. 25 µL of glycine buffer was added to each well to adjust the final pH. Then, absorbance was immediately recorded at 570 nm using microtitration plate reader (BioTek®, USA). The absolute values of the absorbance converted to survival fraction data.

If there was seen an inhibitory response, median inhibitory concentration ($IC_{50}$) was determined as the drug concentration that is required to reduce the absorbance to half that of the control.

According to the criteria of NCI, we have defined a compound as a cytotoxic agent if it could significantly inhibit the growth of the cells (50% $\square$) at relatively low concentrations (50 µg/mL) 10.

### Statistical analysis

Data were represented as mean ± standard error of the mean (SE). Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Bonferroni test. Linear regression analysis was used to calculate $IC_{50}$. Level of significance was set at $P < 0.05$. The statistical analyses were carried out using BioStat 2008 software.

### Results

The absorbance measures are summarized in Table 1. There was shown a statistically significant difference between the mean absorbance measures of HESA-A treated groups in HCT-116 cell line, as determined by one-way ANOVA ($P = 0.022$). HESA-A at the highest concentration (100 µg/mL) significantly inhibited the growth of HCT-116 cell line ($P = 0.003$; compared to placebo-treated group). Percentage growth inhibition of HESA-A at this concentration was determined as 40.13%. This compound did not show any significant effects at lower concentrations on the cell line of colon adenocarcinoma.

As shown in Figure 1, there was seen a significant nega-

| Table 1. MTT-based cytotoxicity assay of HESA-A on cell lines of colon adenocarcinoma (HCT-116), breast adenocarcinoma (MCF-7), prostate cancer (PC-3), glioblastomamultiforme (U-87MG) and normal skin fibroblast (HDF-1). Measures were determined by MTT assay at 570 nm. Data are represented as mean ± standard error. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | HESA-A          | Doxorubicin     |
|                 | 1 µg/mL         | 3.3 µg/mL       | 10 µg/mL        | 33 µg/mL        | 100 µg/mL       | 10 µM           |
| HCT-116         | 1.52 ± 0.07     | 1.45 ± 0.16     | 1.43 ± 0.02     | 1.33 ± 0.16     | 1.09 ± 0.15     | 0.91 ± 0.07$^*$ |
| MCF-7           | 0.86 ± 0.04     | 0.94 ± 0.03     | 0.90 ± 0.01     | 0.97 ± 0.10     | 0.94 ± 0.09     | 0.96 ± 0.05     |
| PC-3            | 2.90 ± 0.59     | 1.55 ± 0.29     | 1.24 ± 0.12     | 1.57 ± 0.58     | 1.93 ± 0.40     | 1.41 ± 0.44     |
| U-87MG          | 0.80 ± 0.14     | 0.91 ± 0.17     | 0.95 ± 0.07     | 1.27 ± 0.18     | 0.73 ± 0.08     | 0.78 ± 0.20     |
| HDF-1           | 1.00 ± 0.03     | 0.96 ± 0.02     | 1.08 ± 0.07     | 0.98 ± 0.07     | 0.84 ± 0.02     | 0.88 ± 0.09     |

Asterisk: $P < 0.05$; compared to control  
Double-asterisk: $P < 0.01$; compared to control  
Stagger: $P < 0.001$; compared to control
The present study aimed to assess the selective cytotoxicity of HESA-A on some neoplastic and normal cell lines. To accomplish this objective, MTT-based cytotoxicity assay was performed using neoplastic cell lines of colon (HCT-116), breast (MCF-7), prostate (PC-3) and GBM (U-87MG), and a normal fibroblastic cell line (HDF-1). This work has shown that HESA-A can inhibit the growth of HCT-116 cell line in a concentration-dependant manner. Our results showed that the IC_{50} of this compound for this cell line is 117.28 µg/mL, which is about 2.4 times higher than predetermined threshold for cytotoxic agents. The results of this study indicate that HESA-A has not growth inhibitory effects on other neoplastic and normal cell lines included in this work.

Previously, Sadeghi-Aliabadi and Ahmadi have studied the selective cytotoxicity of HESA-A in neoplastic cell lines of breast (MDA-MB-468), cervix (Hela) and liver (Hep-2). They reported that this drug inhibited the growth of neoplastic cells at therapeutic concentrations. In another study, Ahmadi and his colleague evaluated anticancer effects of HESA-A in patients with metastatic colon cancer. They reported that this drug could improve the quality of life in metastatic setting of colon adenocarcinoma. These researchers suggested HESA-A as an effective anticancer drug.

Our results demonstrate that HESA-A inhibits the growth of HCT-116 cell line to some degree. This finding can be rooted in the ingredients of this compound. A compositional analysis of HESA-A performed by X ray fluorescence assay, showed the presence of elements such as zinc, selenium, strontium, vanadium, gallium and manganese in it. The role of some of these trace elements has been widely studied in prevention or treatment of cancer. Zinc can increase chemosensitivity of neoplastic cells to anthracyclines. Several human studies have shown a potentially protective effect of selenium on prostate cancer. Several studies over the past few decades have shown the protective effects of vanadium against initiation, promotion and progression of cancer at preclinical levels. Gallium-containing complexes inhibit the proliferation of neoplastic cells in vitro and in vivo and have shown activity against some cancers in clinical trials. It has been shown that some manganese complexes exert anti-cancer properties. Sadeghi-Aliabadi and Ahmadi reported that the organic fraction of HESA-A had no effects on neoplastic cells. So, based on the literature about the importance of trace elements on cancer, it can be proposed that the observed inhibitory effects of HESA-A may be partly due to the existence of such elements and, or their different complexes.

Despite the observed inhibitory effects of HESA-A on HCT-116 cell line, our results showed that the IC_{50} of this compound for this cell line is about 2.4 times higher than the maximal IC50 for typical cytotoxic agents as defined by the NCI. Since the calculated IC_{50} is higher than 50 µg/mL, HESA-A cannot fulfill the predetermined criterion of cytotoxic agents in HCT-116 cell line. It is noteworthy...
that Sadeghi-Aliabadi and Ahmadi calculated the IC$_{50}$ of HESA-A for MDA-MB-468, Hela and Hep-2 as 400 µg/ml, 400 µg/ml and 800 µg/ml, respectively. So it seems that these inhibitory effects can be observed at concentrations which cannot be attained clinically by conventional dosages.

Another finding of this study is that HESA-A has no growth inhibitory effects on all neoplastic cell lines as like as normal cells. Previously, it has been indicated that this compound has no striking cytotoxic effects on the normal cells.

One limitation of the present study is that the growth inhibitory properties of HESA-A were evaluated only on four cell lines from four different cancer types. According to the guidelines of the NCI, the anticancer properties of each substance must be tested in a 60-human cell line panel from 9 common tumor histotypes. So, to determine the complete growth inhibitory profile of HESA-A, further comprehensive evaluation of this compound in the 60-human cell line panel of the NCI is suggested. Such a study will help to identify the potentially sensitive cancer histotypes.

Further research is needed to identify the active substances of HESA-A which are responsible to the anticancer effects of this compound. This requires bioassay guided purification of this compound by repeated fractionation, as described by the NCI.

It merits emphasis that the assessment of synergistic effects of HESA-A with conventional chemotherapeutic agents in future researches, especially in the colon adenocarcinoma can be of great value.

**Conclusion**

We can conclude that HESA-A inhibits the growth of HCT-116 cell line to some degree at high concentrations. But, it seems that this inhibitory effect can be observed at concentrations which cannot be attained clinically by conventional dosages. Also it doesn’t fulfill the predetermined criterion of cytotoxic agents in this cell line. The evidence is not sufficient to continue prescription of HESA-A to the cancer patients. More preclinical investigations are needed to evaluate the efficacy of HESA-A or its active components in cancer therapy.

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