Original Article

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Comparison of the Effect of Different Concentrations of Aqueous and Hydroalcoholic Extracts of *Zophobas Morio* (Coleoptera: Tenebrionidae) Larvae on Breast Cancer Cells and Human Umbilical Vein Endothelial Cells

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ABSTRACT

BACKGROUND AND OBJECTIVE: Cancer is one of the challenges of the medical world. Recently, in the search for new compounds with anti-cancer properties, attention to natural compounds has increased. The aim of this experimental study was to investigate the anti-cancer properties of various concentrations of aqueous and hydroalcoholic extracts of *Zophobas morio* (Coleoptera: Tenebrionidae) larvae, as a mass-rearing and available insect, on breast cancer cells.

METHODS: In this experimental study, the insect was reared at a temperature of 25 °C and a relative humidity of 60% on wheat bran. At the final larval stage, the insects were dried and pulverized. Breast cancer cells (MCF-7) and normal cells (HUVEC) purchased from Pasteur Institute, were treated for 24 and 72 hours by different concentrations of aqueous and hydroalcoholic extracts (zero, 25, 100, 400 and 1200 µg/ml). Water was used as a control. Cell viability was measured by MTT assay. In five treatment groups, 10,000 cells of each extract were cultured in three replicates.

FINDINGS: Adding high concentration of hydroalcoholic extract (39.42±4.48 in 24 hours, 23.52±1.82 in 72 hours) compared to aqueous extract (56.02±9.43 in 24 hours, 36.52±4.07 at 72 hours) had a better effect on inhibiting the proliferation of cancer cells ($p<0.05$). The effect of these extracts on normal cells was observed only in high concentrations and its amount was lower than cancer cells (aqueous and hydroalcoholic extracts [61.77±3.44 and 57.21±1.10 in 24 hours] and [39.25±6.53 and 27.14±1.38 in 72 hours], respectively) ($p<0.05$).

CONCLUSION: The results showed that the high concentration of hydroalcoholic extract of *Z. morio* larvae played an effective role in inhibiting breast cancer cells, without having a significant effect on normal cells.

KEY WORDS: *Darkling Beetles, Breast Cancer Cells, Cytotoxicity, Aqueous and Hydroalcoholic Extracts*.

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Introduction

Cancer reports are increasing every year, with 18.1 million new cases and about 9.6 million deaths in 2018. The most common types of cancer in women are breast, cervical, colorectal, lung and thyroid cancers (1). Conventional treatments for cancer include radiation therapy, chemotherapy, or surgery to remove the damaged parts (2). Conventional chemical treatments have various side effects (3). Novel therapies can be effective in controlling the disease, especially when conventional chemotherapy is not effective. Therefore, it is necessary to adopt new cancer treatment strategies to control the mortality rate of the disease. Insects are one of the most successful organisms in the entire planet with about 55% biodiversity and are a source of antimicrobial and anticancer compounds (4).

Among the characteristics of insects, we can mention their success in the process of evolution and their dominance over their parasitic competitors (5, 6). In traditional Chinese medicine and other parts of the world, some ointments are made from roasted insects and the secretions of bees and parasitoids (7-9). Honey is used to treat wounds, infections (tuberculosis, flu and colds) and cancer. In Chinese medicine, cantharidin, derived from blister beetles and other insect-protective secretions, is used to treat cancer. Extracts of the body of many insects such as bees, predatory and parasitoid bees, beetles, etc. have antiviral, antibacterial and anti-cancer effects. The larvae of flesh flies and houseflies are used to treat wounds and reduce infections, and the saliva of blood-eating insects is used to treat blood problems (10).

Darkling beetles of the family Tenebrionidae feed on broken grains, flour, bran and similar crops in protected and dark areas and are among the storage pests. In addition to their economic importance as pests, these insects are used for biophysiological studies due to their ease of reproduction in vitro. They are also resistant to high and low temperatures and hunger as they can survive 6 to 8 months without food (11).

Due to their large size, high quality proteins and fatty acids in the body, chitin in their skin and high conversion ratio of food in their body, these insects are used in the food of livestock, poultry, aquatic animals and pets. Their body powder and oil are nutritious and are used in human food in some countries (12). Superworms *Zophobas morio* (Fabricius) are one of the insects of this family, which are known for their large size. They are not native to Iran, but today they are reared in Iran to feed reptiles, birds and domestic animals. The full size of the larvae of these beetles is 50 to 60 mm and the end of their body is brown, unlike mealworms. Antibacterial, antioxidant, anti-allergic, anti-cancer and probiotic properties have been reported for the extract of this insect (13).

There are few studies on the role of compounds extracted from *Z. morio* larvae on cancer cells. In only one study, the effect of different concentrations of ethanolic and isopropanolic extracts of the larvae of this insect on breast cancer cells was observed and evaluated (14). In investigating the role of aqueous and alcoholic extracts of *T. molitor* (a similar insect of the same family), Darbemamieh et al. reported the inhibitory role of high concentrations of insect extracts on breast cancer cells (15). The aim of this study was to evaluate the cytotoxicity of different concentrations of aqueous and hydroalcoholic extracts (0, 25, 100, 400 and 1200 µg/ml) of *Z. morio* larvae on MCF-7 breast cancer cells and endothelial cells of the inner wall of HUVEC umbilical vein as normal cells after 24 and 72 h.

Methods

Materials: In this experimental study, after approval by the ethics committee of Razi University, Kermanshah with the code IR.RAZI.REC.1399.019, the materials were purchased from Sigma Company.

Breeding of *Zophobas morio* larvae: Insect larvae were raised from a series of similar eggs in a useful insect breeding center in the Faculty of Agriculture of Razi University at a temperature of 25 °C and a humidity of 60% (Figure 1). Wheat bran was used to feed them. Potato slices were placed in storage containers every three days as a source of water (12). To get the extract, adult larvae were starved in separate containers for 24 hours to empty their intestinal contents. Due to their cannibalistic behavior, they feed on each other if kept together without food. The larvae were placed in the freezer for half an hour to reduce motility, after which they were washed twice with distilled water and once with 70% ethanol to remove their external contaminants. They were then dried with a paper towel to remove the ethanol. The larvae were then placed in a freezer at -20 °C for one hour to be killed. Then they were dried in an oven at 60 °C. Dried larvae were ground for extraction (14).

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Figure 1. Larvae of *Zophobas morio*

Sample size: 10,000 cells per well and three wells were used as replicates per treatment. The 5 treatment groups were in concentrations of zero (control), 25, 100, 400 and 1200 µg/ml of each aqueous or alcoholic extract and were used on two types of cells (normal and cancer) in 24 and 72 hours. Distilled water was used for control treatments.

Preparation of aqueous and hydroalcoholic extracts:

Aqueous extract: 10 g of superworm dry larval powder was transferred to sterile distilled water, boiled for 10 minutes and then centrifuged. The supernatant was passed through Whatman No. 1 filter paper and the aqueous extract was dried in an oven. The powder was separated and stored in the freezer for later use (16).

Hydroalcoholic extract: 10 g of dry *Z. morio* larval powder was transferred to 100 ml of 70% alcohol, stirred for 72 hours at room temperature and then centrifuged. The supernatant was removed via Whatman No. 1 filter paper . The aqueous phase was separated from the alcohol by rotary, then dried and stored in the freezer for later use (16).

Evaluation of cytotoxicity by MTT assay: MCF-7 and HUVEC cells were purchased by Kermanshah University of Medical Sciences from Pasteur Institute of Iran. These cell lines were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic (Pen/Strep) in an incubator with 5% carbon dioxide, 95% humidity and 37 °C, and after reaching cell density of about 80%, were isolated from the bottom of the flask using Trypsin-EDTA and used for experiments (17). normal and cancer cells with a concentration of 10,000 cells were added to each 96-well plate well with at least 3 replicates. After 24 hours of culture and adhesion of cells to the bottom of the plate, the supernatant was removed and a new medium was added with different concentrations of aqueous and alcoholic extracts of *Z. morio* (zero, 25, 100, 400 and 1200 µg/ml). At the end of the period, about 20 µl of MTT solution was added and kept in the incubator for 4 hours. Finally, the supernatant was removed and 100 µl of DMSO solution was added to each well. The reading was performed by ELISA reader at 570 nm (18) and the percentage of living cells was calculated.

Survival rate with fluorescent microscope: After culture, the supernatant of MCF-7 and HUVEC cells treated with different concentrations of aqueous and hydroalcoholic extracts of *Z. morio* larvae was removed. The cells were then fixed with 4% paraform-aldehyde and stained with acridine orange-ethidium bromide in a 1: 1 ratio. Appotosis was assessed using a fluorescent microscope at 400x magnification (19).

Statistical analysis of data: The data obtained in this experiment were analyzed with SPSS software version 16. To analyze the data, the statistical test of completely randomized factorial design was used. The means were compared with Duncan's test. At least three replicates were used for the experiments. Data were expressed as Mean±SD and $p<0.05$ was considered significant.

Results

The results of toxicity test after 24 hours showed that by adding high concentrations of two aqueous and hydroalcoholic extracts of Superworm larvae (1200 µg/ml), a decrease in cell proliferation was observed in HUVEC cells (61.77±3.44 and 57.21±1.10, respectively) (Table 1). Although this reduction trend was lower compared to cancer cells, but the difference with the control group was significant ($p<0.05$) (Table 1).

Adding the high concentration of aqueous extract of Superworm larvae (1200 µg/ml) (56.02±9.43%) caused a significant reduction in cancer cell proliferation compared to the control group and other concentrations of the same extract and other concentrations of hydroalcoholic extract of Superworm larvae, except for 400 and 1200 µg/ml concentrations of hydroalcoholic extract ($p<0.05$). Addition of 1200 µg/ml hydroalcoholic extract of Superworm larvae significantly reduced the proliferation of cancer cells (39.42±4.48%) compared to the control and all concentrations of aqueous and hydroalcoholic extract of Superworm larvae ($p<0.05$) (Table 1).

Addition of 1200 µg/ml hydroalcoholic and aqueous extracts of Superworm larvae significantly reduced the proliferation of normal cells (27.14±1.38

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and 39.25±6.53%, respectively) compared to all treatment and control groups ($p<0.05$) (Table 2). This decrease was due to the addition of hydroalcoholic extract (1200 µg/ml) more than aqueous extract and this difference was also significant ($p<0.05$). Addition of 1200 µg/ml of hydroalcoholic extract of Superworm larvae (23.52±1.82) reduced the proliferation of cancer cells, which showed a significant difference compared to the control group and other treatment groups ($p<0.05$) (Table 2). Fluorescent microscopy images show the intensity of cell apoptosis at a concentration of 1200 µg/ml for both types of extracts for both cell types. With increasing the concentration of the extract, cell apoptosis intensified (Figure 2). This effect was less in the aqueous extract, but was noticeable. Such a concentration-dependent increasing trend was observed more strongly after 72 hours and, of course, was more visible in the hydroalcoholic extract than in the aqueous extract (Figure 3). The extent and severity of cell apoptosis and cell necrosis in human umbilical vein endothelial cells used as a normal cell, non-cancerous cell in this experiment were significantly lower than breast cancer cells and were not seriously damaged at low and moderate concentrations.

Table 1. Evaluation of cytotoxicity of different concentrations of aqueous and alcoholic extracts of Superworm-based on MTT test (24 hours)

Treatment	Percentage of Huvec live cells after MTT test (24 hours)	Percentage of MCF-7 live cells after MTT test (24 hours)
Control	100^{b}	100^{ed}
Aqueous extract of superworm (25 µg/ml)	$122.36±12.07^{c}$	$88.71±9.96^{cd}$
Aqueous extract of superworm (100 µg/ml)	$133.32±8.15^{d}$	$96.82±9.80^{cde}$
Aqueous extract of superworm (400 µg/ml)	$120.49±4.78^{c}$	$106.43±8.55^{e}$
Aqueous extract of superworm (1200 µg/ml)	$61.77±3.44^{a}$	$56.02±9.43^{b}$
Alcoholic extract of superworm (25 µg/ml)	$107.11±3.48^{b}$	$84.27±4.91^{c}$
Alcoholic extract of superworm (100 µg/ml)	$97.76±5.19^{b}$	$83.29±9.11^{c}$
Alcoholic extract of superworm (400 µg/ml)	$96.44±4.76^{b}$	$61.27±7.17^{b}$
Alcoholic extract of superworm (1200 µg/ml)	$57.21±1.10^{a}$	$39.42±4.48^{a}$

The means with different letters in each column are significantly different. Differences at 5% level have been investigated and the means with similar letters in each column indicate no significant differences between the treatment groups.

Table 2. Evaluation of cytotoxicity of different concentrations of aqueous and alcoholic extracts of Superworm-based on MTT test (72 hours)

Treatment	Percentage of Huvec live cells after MTT test (72 hours)	Percentage of MCF-7 live cells after MTT test (72 hours)
Control	100^{c}	100^{d}
Aqueous extract of superworm (25 µg/ml)	$124.32±8.64^{d}$	$121.19±14.09^{e}$
Aqueous extract of superworm (100 µg/ml)	$143.86±3.60^{e}$	$124.64±10.25^{e}$
Aqueous extract of superworm (400 µg/ml)	$157.73±6.22^{f}$	$113.74±9.29^{de}$
Aqueous extract of superworm (1200 µg/ml)	$39.25±6.53^{b}$	$36.52±4.07^{b}$
Alcoholic extract of superworm (25 µg/ml)	$106.72±0.79^{c}$	$84.02±9.98^{c}$
Alcoholic extract of superworm (100 µg/ml)	$99.28±8.20^{c}$	$74.27±7.96^{c}$
Alcoholic extract of superworm (400 µg/ml)	$100.20±8.86^{c}$	$51.80±4.64^{b}$
Alcoholic extract of superworm (1200 µg/ml)	$27.14±1.38^{a}$	$23.52±1.82^{a}$

The means with different letters in each column are significantly different. Differences at 5% level have been investigated and the means with similar letters in each column indicate no significant differences between the treatment groups.

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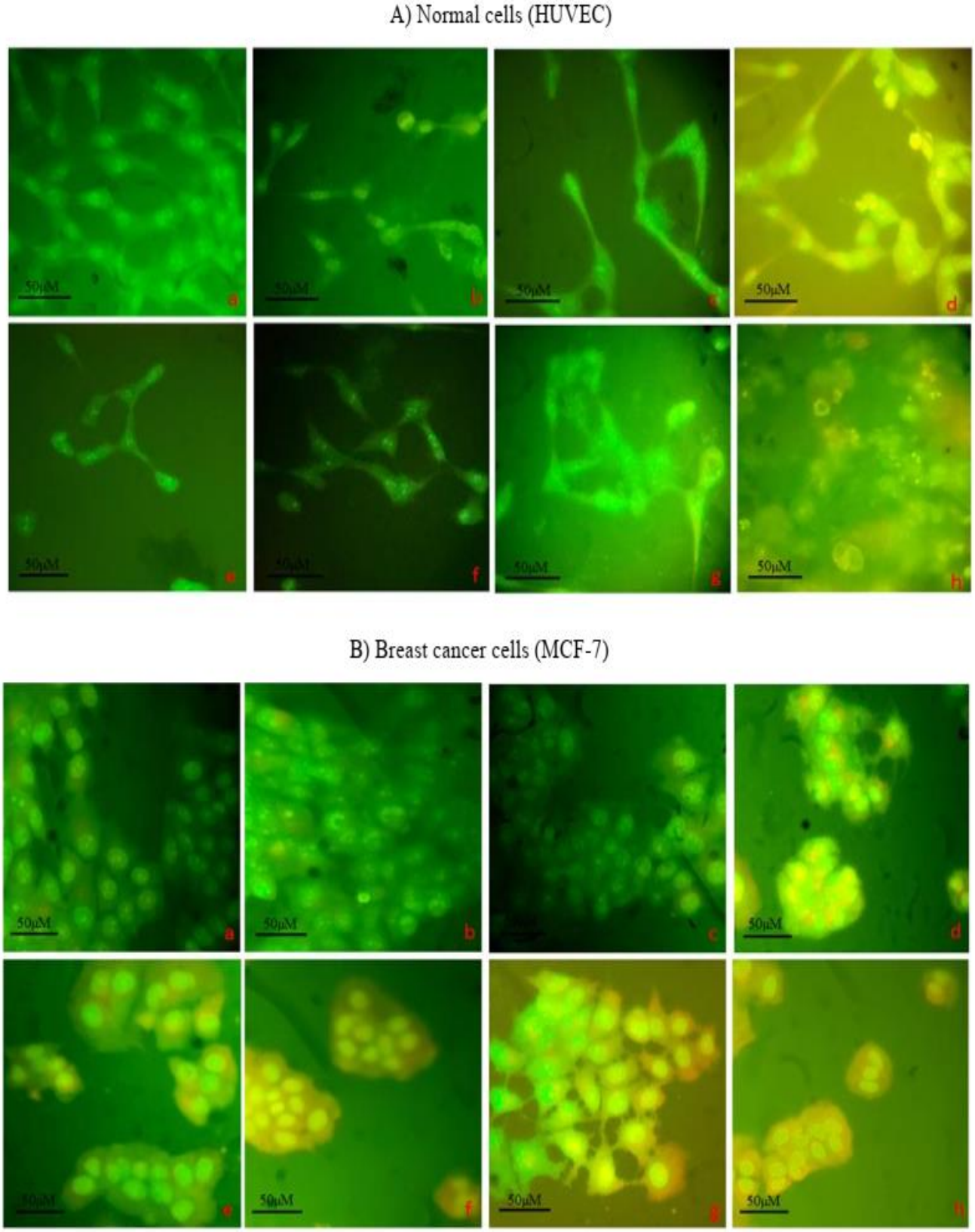

Figure 2. Acridine orange/ethidium bromide (AO/EB) staining of normal cells (a) and cancer cells (b) treated with different concentrations of aqueous and hydroalcoholic extracts of Superworm larvae after 24 hours. a-d) 25, 100, 400 and 1200 µg/ml of aqueous extract, respectively, e-h) 25, 100, 400 and 1200 µg/ml of hydroalcoholic extract, respectively.

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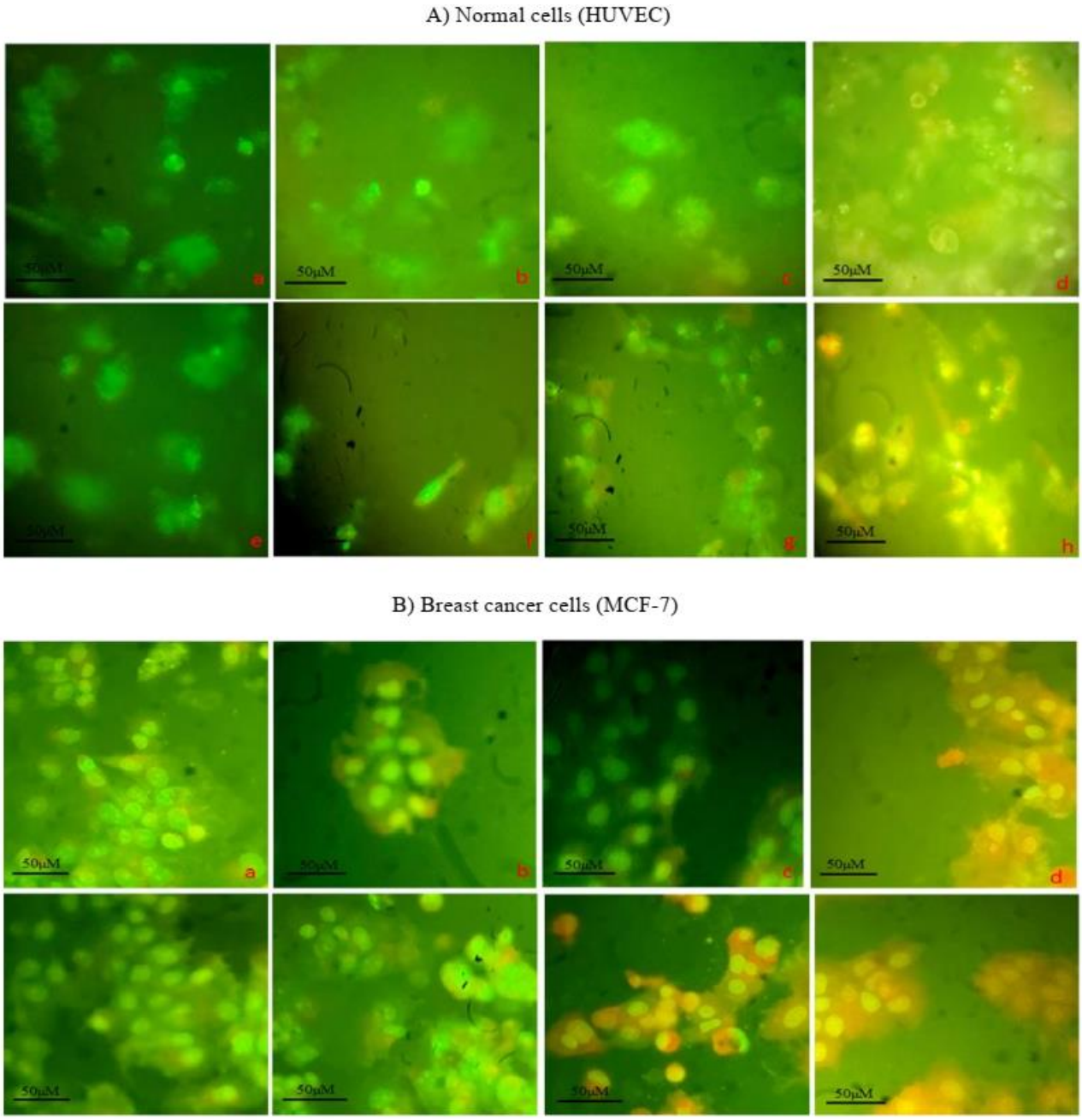

Figure 3. Acridine orange/ethidium bromide (AO/EB) staining of normal cells (a) and cancer cells (b) treated with different concentrations of aqueous and hydroalcoholic extracts of Superworm larvae after 72 hours. a-d) 25, 100, 400 and 1200 µg/ml of aqueous extract, respectively, e-h) 25, 100, 400 and 1200 µg/ml of hydroalcoholic extract, respectively.

Discussion

The results of this study showed that in their high concentration, both types of extracts induced death and inhibited proliferation in these breast cancer cells. Hydroalcoholic extract at concentrations of 25, 100 and 400 µg/ml also showed a decrease in proliferation of cancer cells, unlike aqueous extract. Few studies have investigated the role of extracts and compounds extracted from these larvae. Similar to the present study, the anti-cancer activity of ethanolic and isopropanolic extracts of *Z. morio* larvae on breast cancer cells indicates their direct effect. This inhibitory effect on isopropanol extract was much stronger than ethanolic extract (14). In the study by Hou et al., the extracts of *Musca domestica* L. larvae were used against colon

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cancer cells. This extract had no adverse effects on normal cells, while inhibiting the proliferation of cancer cells (20). Hasaballah et al. investigated the anticancer effects of different concentrations of extracts of the larvae of *M. domestica*, *Lucilia sericata* and *Chrysomya albiceps* against human colon cancer cells. The most effective against colon cancer cells were related to methanolic extracts of *L. albiceps* and *M. domestica* (6).

A peptide extracted from *Calliphora vicina* called alloferon has been shown to have toxic effects on cancer cells (21). The anticancer effects of ethanolic extract, and hexane and ethyl acetate fragments of *T. molitor* larvae (very similar to Superworm) against prostate, cervical, liver, colon, lung, breast and ovarian cancer cells were investigated and inhibitory effects were observed (22). In investigating the role of aqueous and alcoholic extracts of *T. molitor* on breast cancer cells, Darbemamieh et al. observed the relative effect of high-concentration hydroalcoholic extract on breast cancer cells (15).

Based on the results of this study, hydroalcoholic extract had a better effect on the inhibition of cancer cells compared to aqueous extract, especially at its high concentration. More information about the effective compounds of these larvae and the isolation and use of these compounds in laboratory and in vivo studies is needed to provide the conditions for the development of new drugs effective in breast cancer and other cancers based on the findings of this study. The anticancer properties of this insect extract on other types of cancer cells also need to be investigated.

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