Original Article

The effect of hydro-alcoholic extracts of Nigella sativa, Carum carvi, Taraxacum officinale, and royal jelly on the survival of HDF cell line under H2O2-induced oxidative stress compared to metformin

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Abstract

Background and aims: Highly active oxygen causes DNA or protein damage. This study aimed to determine the effect of the extract of three medicinal plants, royal jelly, and metformin on the survival of human dermal fibroblast (HDF) cells under oxidative stress induced by H_2O_2.
Methods: Hydroalcoholic extracts were prepared from *Nigella sativa, Carum carvi* seeds, and *Taraxacum officinale* flower. The growth curve of HDF cells was plotted in Dulbecco's modified eagle *medium* (DMEM). The cells were exposed to H_2O_2 stress and treated with different concentrations of the extracts, royal jelly, and metformin. The cells viability was then calorimetrically analyzed during different times in 69-well plates.
Results: The exponential growth phase of HDF cells was observed during 24–72-hour growth. High cell survivals were seen during 72 hours of exposure to 25 mg/mL royal jelly ($99.13 \pm 3.22\%$), 15 mg/mL *C. carvi* extracts and *N. sativa* ($97.87 \pm 3.25\%$ and $96.22 \pm 5.47\%$, respectively), 10 mg/mL royal jelly ($96.14 \pm 4.72\%$), 16 mg/mL metformin ($91.23 \pm 5.34\%$), and 8 mg/mL *T. officinale* ($86.31 \pm 7.44\%$), respectively. These results were significantly higher than the results of 48 and 24 hours of exposure to 15 mg/mL *N. sativa* ($P=0.013$), 15 mg/mL *C. carvi* ($P=0.019$), and 16 mg/mL metformin ($P=0.027$). Moreover, royal jelly (25 mg/mL) significantly indicated greater effect on the cell survival compared with the highest concentrations of the extracts and metformin at different exposure times ($P=0.016$-0.032).
Conclusion: Royal jelly, *N. sativa*, and *T. officinale* extracts revealed remarkable effects on the stressed HDF cells; therefore, they can be proposed for treatment of wounds and burns after clinical analysis.
Keywords: Plant extracts, Royal jelly, Metformin, Oxidative stress, HDF cell line

Introduction

Free radicals are produced during several natural processes in the body, including breathing, food digesting, alcohol and drug metabolizing, and converting fats into energy. Natural antioxidant systems usually destroy free radicals in the body. Nevertheless, if these systems do not work well, free radicals can involve in some reactions that result in the cell membrane and DNA destructions or inhibition of crucial cell activities such as enzymatic reactions and cellular processes involved in cell division and energy generation (1). These injuries lead to oxidative stress associated with several metabolic and chronic disorders including cancers (2- 4).

Nigella sativa is a medicinal plant in the Ranunculaceae family. The plant's seeds and oil are known as a traditional food or drug in Unani Tibb, Ayurveda Siddha, and Islamic medicines. *N. sativa* has been widely used or investigated for its therapeutic properties such as antihypertensive, antibacterial, anti-inflammatory, anticancer, antioxidant, diuretic, liver tonic, respiratory and gastrointestinal, analgesic, spasmolytic, and immunomodulatory effects along with the treatment of skin diseases. The therapeutic effects of this plant are attributed to the presence of thymoquinone, the major bioactive component found in its essential oil (5).

Carum carvi is mainly grown in West Asia, North Africa, and Europe. Whole fruits or their vapors have been described in traditional medicines, especially Unani medicine, because of their lung and stomach tonic, astringent, coagulant, eupeptic, analgesic, antispasmodic, antidiarrheal, diuretic, and galactagogue effects. Major effective compounds in *C. carvi* are carvacrol, carvone, α-pinene, limonene, γ-terpinene, carvenone, linalool, and *p*-cymene (6).

Taraxacum officinale in the *Compositae* family, closely related to chicory, obtained its name from Greek and Persian-Arabic botanical origins. *Taraxacum* spec has been traditionally used for centuries as a medicinal plant to treat several diseases such as cancer, acne, liver disorders, and digestive tract dysfunctions. Sesquiterpenes, saponins, phenolics, and flavonoids are the most frequent effective compounds in the plant organs including roots, leaves, and the whole plant. In addition, the plant has nutritious consumption such as food and beverage (7).

Royal jelly is a crucial food for queen honeybees and is mainly produced in the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*). The most frequent compounds in royal jelly are proteins (12%-15%), sugars (10%-16%), lipids (3%-6%), vitamins, free amino acids, and minerals (8). Investigations carried out on experimental animals exhibited many pharmacological functions of royal jelly including antibacterial (8), anti-oxidant (9), antitumor (10), anti-inflammatory (8), anti-allergic (11), anti-aging (12), wound healing (10), and antihypertensive functions (13). In addition, the improved lipoprotein metabolism, reduced serum total cholesterol, and low-density lipoprotein levels were also observed following the consumption of royal jelly by humans (14). Royal jelly contains active compounds including 1.28 µg/mg total flavonoids and 23.3 µg/mg phenolics (8).

Human dermal fibroblasts (HDFs) are extracellular matrix forming cells that produce skin connective tissues. These cells play an essential role in wound healing and have been used as excellent model systems in different studies conducted on many aspects of cell physiology, especially on skin biology and reprogramming induced pluripotency (15). The cells were also used in the studies on the effects of anticancer agents on cell growth (16). Some studies have reported the cytotoxic effects of metformin on cancerous cells such as cervix cells. These effects are proposed as a result of apoptosis induction which leads to the necrosis of the cancer cells. Normal cells have not been affected by metformin. Metformin also has protective antioxidant effects on nerve cells and improver effects on blood factors such as malondialdehyde, superoxide dismutase, and glutathione peroxidase following H_2O_2-induced oxidative stress. Therefore, metformin can be considered an antioxidant agent that combats the free radicals (17).

The present study aimed to evaluate the effects of *N. sativa*, *C. carvi*, and *T. officinale* hydro-alcoholic extracts as well as royal jelly on the survival of HDF cells involved in H_2O_2-induced oxidative stress.

Materials and Methods
Plant extract preparation
The studied plants including the seeds of *N. sativa* and *C. carvi* and the flowers of *T. officinale* were obtained from Isfahan Agricultural and Natural Resources, research center, Iran, and approved by the center's herbarium. First, the flowers and seeds were washed with sterile distilled water to remove the dust, and then, they were thoroughly dried in the dark and ground. Next, to prepare the hydroalcoholic extract, 50 grams of each plant powder were separately transferred to an Erlenmeyer flask containing 500 mL methanol 80% and placed on a shaker at 120 rpm for 72 hours in darkness. The resulting solution was then filtered, poured into a sterile plate, and dried in an oven at 50 °C. Finally, the extracts were placed in closed vials in darkness at 4 °C (18).

Cell line preparation
The used HDF cell line was prepared in a flask from Pasteur Institute, Tehran, Iran. For the cultivation of the cells, first DMEM culture medium (HiMedia, India) was prepared in supplementation with 1% of a 10X streptomycin and penicillin solution (10 000 U penicillin and 10 mg streptomycin per mL in 0.9% normal saline) and 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA). The pH of the medium was adjusted to 7.3-7.6 by NaOH and HCl (19). Then, the culture medium was added to the cells, vortexed, and transferred to a filtered flask. The flask was incubated at 37°C in 5% CO_2. After 48 hours, the cells had grown as a single layer in the bottom of the flask. All cell cultivation steps were performed aseptically under an equipped laminar air flue (20). For cell re-culturing, the liquid medium was discarded and 3 to 5 mL of trypsin was added to the cells adhering to the bottom of the flask. The flask was then incubated at 37°C for 5-10 minutes. After that, the exfoliation of all cells was checked under a light microscope. If some cells were still attached to the bottom of the flask, they were detached by a few vertical blows. Then 5 to 7 mL of fresh culture medium was added to the flask to dilute the existing trypsin. The flask contents were then transferred to a falcon tube and centrifuged at 1300 rpm for 5 minutes, and the obtained supernatant was discarded (21). Finally, the precipitated cells were washed with phosphate buffered saline (PBS) and incubated using the abovementioned method. For long preservation, the cells were suspended in the solution containing 10% dimethyl sulfoxide (DMSO) and 90% FBS. After that, they were kept at -20°C for 4 hours and then at -80°C for a week and finally transferred to a liquid nitrogen tank (19).

Cell counting
In this phase, 100 µL cell suspension was mixed with 300 µL culture medium and 100 µL trypan blue in an Eppendorf vial. Then, 20 µL of the suspension was poured on a hemocytometer slide and counted by a light microscope at × 10 magnification, and the unstained cells were counted as the living cells (19).

Cellular standard curve plotting
First, a suspension of HDF cells with the concentration of 50×10^4 cells per mL was prepared in a Dulbecco's modified eagle *medium* (DMEM). Then, 200 µL of the uninoculated culture medium and 200 µL of the prepared cultured cells were poured into the first row with 8 wells

(A1-H1) and the second row with 8 wells (A2-H2) of a 96 well-plate, respectively. In the third row (A3-H3), 180 μL of cell suspension was poured. Reducing 20 μL of culture medium totaled 11 rows. Live cells with active metabolism converted MTT to purple formazan, which absorb light at 530 to 600 nm, while dead cells lost the ability to convert MTT to formazan. Therefore, in the next step, 20 μL MTT (1 mg/5 mL) was added to all wells, and the plate was incubated at 37°C for 3 hours. After that, 180 μL of the upper culture medium was carefully removed so that foramazone crystals would not peel off the bottom of the wells. Then, 150 μL of DMSO was added to each cell and gently pipetted to dissolve foramazone crystals. Care was taken not to produce bubbles during the pipetting. Immediate light absorption of the solutions was read by an ELISA reader device (STATFAX-2100, USA) at the wavelength of 540 nm (22).

Plotting the growth curve of cell lines
First, a suspension of HDF cells with the concentration of 50×10^4 cells per mL was prepared in DMEM, and then 180 mL of the suspension was poured into each row of 8 wells of 5 separated 96 well-plates to determine the growth rate on the days 0, 1, 2, 3, and 4. At the end of each day, 20 μL MTT was immediately added to the relevant plate, and after 3 hours of incubation, the light absorption by the cell suspensions was read at 540 nm wavelength. Subsequently, the growth curve of the cell line was plotted based on the data obtained in different incubation times (22).

Cytotoxicity assay
For this purpose, 180 μL of culture medium was poured into the first row with 8 wells (A1-H1), and 180 μL of the cells suspended in H_2O_2 was poured into each remaining well. After 24 hours of incubation, 20 μL of different concentrations of treating compounds were added to the fourth to twelfth rows, and 20 μL of culture medium was added to the first and second rows. The first row and the second row were considered as blank and negative control, respectively. Next, 20 μL of doxorubicin with a 200 μg/mL concentration was added to the third row, which was considered positive control. The plate was incubated for 72 hours at 37°C in the presence of 5% CO_2. Then, it was removed from the incubator, and 20 μL MTT was added to the wells and again incubated for 3 hours at 37°C. Then, the upper medium was gently removed, and DMSO was add to the wells to dissolve the foramazone crystals. Finally, the light absorption of the solutions was read by the ELISA reader device at the wavelength of 540 nm (22).

Statistical analysis
All experiments were performed in 4 replicates, and collected data were analyzed by SPSS software version 25. Descriptive statistics were presented in graphs and tables with reports of minimum, maximum, and average values ± standard deviation. Each experimental group contained 50×10^4 HDF cells per mL (22). The mean HDF cell survival was compared with the control group using Dunnett test in the inferential section. In addition, observations in two independent groups were compared using the Mann-Whitney U test, and changes in observations in several independent and dependent groups were examined by the Kruskal-Wallis H test (Mann-Whitney post hoc test) and Friedman test (by Wilcoxon post hoc test), respectively. The significance level was considered as 5% in all tests.

Results
The growth curve of the HDF cell line
Figure 1 illustrates the growth curve plotted based on the cell counts in DMEM supplemented with streptomycin-penicillin and FBS. As observed, the exponential growth phase occurred in 24–72 hours after inoculation of the cells into the culture medium; therefore, this time point was selected for recording the results in the next steps of the study (Figure 1).

A delitescence was seen at the 0–24 hours, and the exponential growth occurred during 24–72 hours. Moreover, the cells entered to stationary phase 72 hours after the inoculation.

Changes in HDF cell line survival percentage at different times by different experimental groups
The results are presented in Table 1. The comparison between different groups are as follows.

Comparison between control and H2O2-received groups
Figure 2 presents changes in the survival percentage of HDF cells treated with different concentrations of hydroalcoholic plant extracts (*N. sativa*, *C. carvi*, and *T. officinale*), royal jelly, and metformin after 24, 48, and 72 hours based on MTT test. According to the presented results, time changes had statistically significant effects on the bioavailability changes of all experimental groups, but the mean bioavailability of HDF cells did not change significantly in the control and H_2O_2-received groups during 72 hours. The highest percentage of cell survival (99.13 ± 3.22%) was observed in the treated group with a dose of 25 mg/mL royal jelly during 72 hours of exposure.

Comparison between the groups receiving plant extracts and royal jelly and the group receiving metformin
In the treated group with doses of 15 mg/mL hydroalcoholic extract of *N. sativa*, the percentage of viability (96.22 ± 5.47%) within 72 hours was significantly higher than those within 48 hours (86.53 ± 6.16%) and 24 hours (73.42 ± 4.31%) (*P* = 0.013). This difference was not significant for the treatment with 10 mg/mL hydroalcoholic extract of *N. sativa* (80.72 ± 4.23% for 72 hours, 80.28 ± 15.82% for 48 hours, and 79.22 ± 9.95% for 24 hours; *P* = 0.234). Mean cell survival in the group receiving 15 mg/mL *C. carvi* hydroalcoholic extract in 72 hours (97.87 ± 3.25%) was statistically higher than that in 48 hours (85.45 ± 10.36%) and 24 hours (76.33 ± 11.23%)
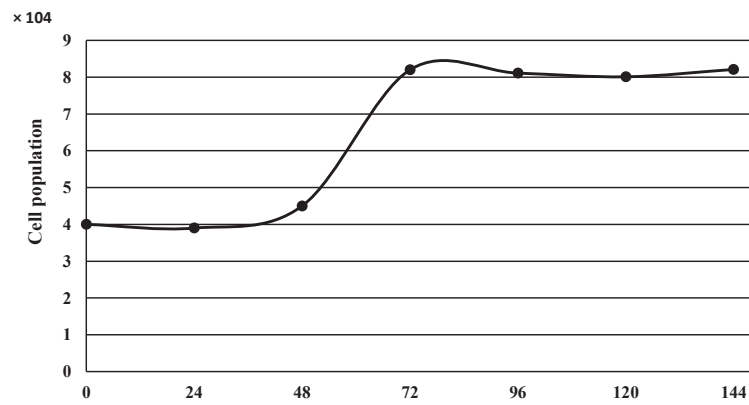
Figure 1. The growth curve of the HDF cell line. *Note.* HDF: Human dermal fibroblasts.
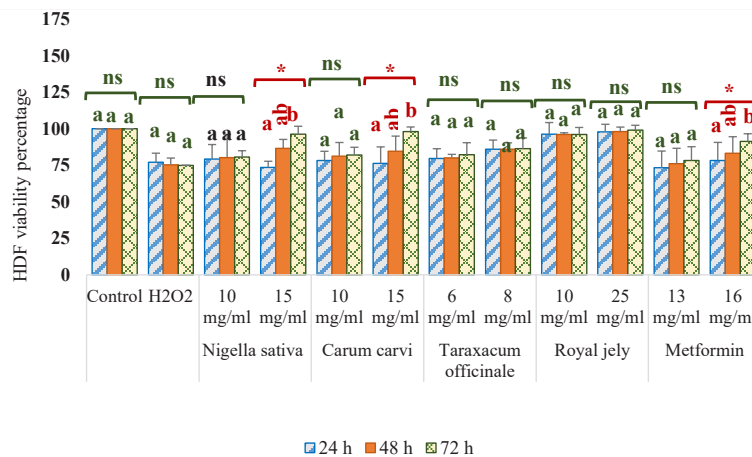

☒24 h ■48 h ☒72 h

Figure 2. Comparison of mean HDF cell survival percentage between different experimental groups after 24, 48, and 72 hours of treatment. *Note.* HDF: Human dermal fibroblasts; ns: Non-significant; * $P<0.05$ and ** $P<0.01$. In each group, the mean differences in cell viability among incubation times that have at least one similar English letter are not statistically significant.

Table 1. Comparison of the percentage of HDF cell survival within different experimental groups after 24, 48, and 72 hours of treatment

Group	Incubation Period (h)			P value
	24	48	72	
Control	100±0.00	100±0.00	100±0.00	-
H₂O₂	77.06±6.19%	75.30±4.60	74.95±0.10	0.720
N. sativa 10 mg/mL	79.22±9.95	80.28±15.82	80.72±4.23	0.234
N. sativa 15 mg/mL	73.42±4.31	86.53±6.16	96.22±5.47	0.013*
C. carvi 10 mg/mL	78.22±6.34	81.23±9.35	81.98±5.33	0.162
C. carvi 15 mg/mL	76.33±11.23	85.45±10.36	97.87±3.25	0.019*
T. officinale 6 mg/mL	79.73±6.59	80.12±2.32	82.13±8.32	0.082
T. officinale 8 mg/mL	85.92±6.31	86.02±4.31	86.31±7.44	0.723
Royal jelly 10 mg/mL	96.22±8.01	96.13±1.09	96.14±4.72	0.821
Royal jelly 25 mg/mL	97.81±5.22	98.01±3.12	99.13±3.22	0.761
Metformin 13 mg/mL	73.28±11.45	76.05±10.57	78.24±9.48	0.224
Metformin 16 mg/mL	78.33±12.31	83.22±11.31	91.23±5.34	0.027*

Note. HDF: Human dermal fibroblast; *N. sativa*: *Nigella sativa*; *C. carvi*: *Carum carvi*; *T. officinale*: *Taraxacum officinale*. The results are presented as mean value±standard deviation. * The differences in means of cell viabilities between three incubation periods are statistically significant at the level of 5%.

treatments ($P=0.019$). The cell viability in the treated group with 10 mg/mL doses of *C. carvi* hydroalcoholic extract in 72 hours ($81.98\pm5.33\%$) was not significantly different from the cell viability in 48 hours and 24 hours ($81.23\pm9.35\%$ and $78.22\pm6.34\%$, respectively; $P=0.162$).

In the groups receiving the doses of 8 mg/mL *T. officinale* hydroalcoholic extract, the percentage of HDF cell survival in 72 hours ($86.31\pm7.44\%$) was not significantly greater than those in 48 hours and 24 hours ($86.02\pm4.31\%$, and $85.92\pm6.31\%$, respectively) ($P=0.723$). This result

was also obtained in the group treated with *T. officinale* hydroalcoholic extract of 8 mg/mL concentration ($82.13 \pm 8.32\%$ for 72 hours, $86.02 \pm 4.31\%$ for 48 hours, and $79.73 \pm 6.59\%$ for 24 hours; $P = 0.082$). Compared with the three different times (i.e., 24, 48, and 72 hours) of treatment with plant extracts, the treatment duration time of 72 hours, especially in relation to the treatment with 15 mg/mL *C. carvi* and 15 mg/mL *N. sativa*, had the most effects on the cell's survival. Regarding royal jelly, the highest percentage of HDF cells survival was observed in the group receiving the doses of 25 and 10 mg/mL ($99.13 \pm 3.22\%$ and $96.14 \pm 4.72\%$, respectively) within 72 hours; however, these concentrations of royal jelly did not have significant effects on the cell survival during 48 hours ($98.01 \pm 3.12\%$ and $96.13 \pm 1.09\%$, respectively) and 24 hours ($97.81 \pm 5.22\%$ and $96.22 \pm 8.01\%$, respectively) of exposures ($P = 0.761$ for the concentration of 25 mg/mL and $P = 0.821$ for the concentration of 10 mg/mL). Metformin with the concentration of 16 mg/mL showed the most significant cell survival during 72 hours ($91.23 \pm 5.34\%$) of exposure compared to 48 hours ($83.22 \pm 11.31\%$) and 24 hours ($78.33 \pm 12.31\%$;) ($P = 0.027$). Table 2 depicts the comparison of the HDF cell survival levels between different experimental groups with the most effects after 24, 48, and 72 hours of treatment. Compared with *N. sativa* (15 mg/mL), *C. carvi* (15 mg/mL), *T. officinale* (8 mg/mL), and metformin (16 mg/mL), royal jelly (25 mg/mL) exerted the most significant effect on the cell survival after 24, 48, and 72 hours of exposure ($P = 0.016$, $P = 0.028$, and $P = 0.032$, respectively).

Discussion

The present study investigated the antioxidant effect of 3 plant extracts compared with royal jelly and the drug metformin on reducing the effect of oxidative stress induced by hydrogen peroxide in fibroblast cells. The mean survival percent of HDF cells was analyzed through the MTT test. Using 25 and 10 mg/mL royal jelly as well as 15 mg/mL *N. sativa* and *C. carvi* extracts within 72 hours of exposure revealed high levels of cell survival. The plants selected for this study were from the most potential plants which have been used for their bioactive effects due to the effective compounds found in their contents. It was found that the compounds extracted from *N. sativa* seeds such as linoleic acid, palmitic acid, and citronella have antioxidant and free radical scavenging effects (23). Monoterpenes and carvone were detected as the major bioactive compounds present in *C. carvi*,

while Sesquiterpene lactones, terpenes, phytosterol, and coumarin were found as the most frequent effective compounds in *T. officinale* (24).

Today, it is believed that oxidative stress, an imbalance between antioxidants and free radicals, is a causative agent for many diseases such as cardiovascular disease (25), cancer (26), peripheral nerve disorders, and diabetes or their complications (27). Different antioxidant therapeutic procedures have been proposed in many studies. These protocols mostly include the removal of $O2^{\bullet-}$ which inhibits its reaction with $\bullet NO$ and the consequent formation of $ONOO^-$, as well as the removal of H_2O_2 before its conversion to $\bullet OH$ or HOX (28). In this study, HDF cells were exposed to oxidative stress induced by using H_2O_2. It was found that cells undergo oxidative stress by the mechanisms performed in mitochondria which protect cellular components and themselves against oxygen free radical damages. Mitochondria perform this process by different ways mainly including potential oxygen consumption, antioxidant defense mechanisms, and using cellular bioenergetics to consume the compounds produced following degradation processes. On the other hand, exposure to external H_2O_2 with high concentrations had immediate effects on the cells including reaction with the present reactive molecules and deactivation of the existing antioxidant defenses. Degradation of H_2O_2 by catalase enzyme, greatly increased the concentration of O_2 resulting in the generation of oxygen radicals. These events cause excessive damages which exceeds cell repair mechanisms and may lead to cell death (29,30).

Bee products (e.g., honey, propolis, bee wax, pollen, and royal jelly) have been used from ancient times for their therapeutic and cosmetic properties. At present, these products are applied in apitherapy which is a branch of complementary and alternative medicine for treatment of different non-communicable and communicable diseases as well as treatment of wounds and burns (31). Among different effective compounds responsible for therapeutic effects of royal jelly, its free fatty acids have antioxidative and anti-inflammatory effects (32). Some compounds such as 10-hydroxy-trans-2-decanoic acid present in royal jelly induced the transforming growth factor production. As a result, collagen protection by fibroblast cells is stimulated, which is an important factor for skin production (33). In the present study, the exponential phase of the HDF cells' growth occurred 24–72 hours after inoculation into DMEM. Royal jelly with the concentration of 25 mg/mL showed the highest protection effect ($99.13 \pm 3.22\%$) on

Table 2. Comparison of the percentage of HDF cell survival between different experimental groups with the highest effects after 24, 48, and 72 hours treatment times

Incubation Period (h)	Control	H_2O_2	*N. sativa* 15 mg/mL	*C. carvi* 15 mg/mL	*T. officinale* 8 mg/mL	Royal jelly 25 mg/mL	Metformin 16 mg/mL	*P* value
24	100 ± 0.00	77.06 ± 6.19	73.42 ± 4.31	76.33 ± 11.23	85.92 ± 6.31	97.81 ± 5.22	78.33 ± 12.31	0.016*
48	100 ± 0.00	75.30 ± 4.60	86.53 ± 6.16	85.45 ± 10.36	86.02 ± 4.31	98.01 ± 3.12	83.22 ± 11.31	0.028*
72	100 ± 0.00	74.95 ± 0.10	96.22 ± 5.47	97.87 ± 3.25	86.31 ± 7.44	99.13 ± 3.22	91.23 ± 5.34	0.032*

Note. HDF: Human dermal fibroblast; *N. sativa*: *Nigella sativa*; *C. carvi*: *Carum carvi*; *T. officinale*: *Taraxacum officinale*. The results are presented as mean value ± standard deviation. *The differences in means of cell viabilities between three incubation periods are statistically significant at the level of 5%.

the cell survival against oxidative stress induced in the cells by H_2O_2. This effect was significantly higher than the highest concentrations of the extracts and metformin at different exposure times ($P = 0.016 - 0.032$). The results of present study indicated an increase in the cell count in the presence of plant extracts and royal jelly by time duration. This might be due to the fact that these extracts have growth stimulants that can lead to the stimulated growth of the cells in vials (34).

Conclusion

Royal jelly with the concentration of 25 mg/mL had the highest effect on the survival of HDF cells under oxidative stress. Among the used plant extracts, the most protective effect on the cells was seen in *T. officinale* hydroalcoholic extract with the concentrations of 6 and 8 mg/mL although all the three extracts were effective on the cell's survival within 72 hours of exposure. These bioactive compounds are proposed for clinical usage for skin under stressed conditions such as wounds and burns after clinical studies for probable side effects.

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