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Effect of total hydroalcoholic extract of *Nigella sativa* and its n-hexane and ethyl acetate fractions on ACHN and GP-293 cell linesSamira Shahraki^a, Abolfazl Khajavirad^{b,*}, Mohammad Naser Shafei^c,
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ABSTRACT

Medicinal plants are noted for their many advantages including the ability to treat diseases such as cancer. In this study, we examined the antitumor effect of the medicinal plant *Nigella sativa* on the morphology, survival, and apoptosis of ACHN (human renal adenocarcinoma) and GP-293 (normal renal epithelial) cell lines. From a hydroalcoholic extract of *N. sativa*, n-hexane and ethyl acetate fractions were extracted. Cells were treated with various concentrations of total hydroalcoholic extract and n-hexane and ethyl acetate fractions; cell viability, morphological changes, and apoptosis were then determined. Results were presented as mean \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) was applied for the statistical analysis of the data. The total extract and the fractions in a dose- and time-dependent manner reduced the cell viability in ACHN with no effect on the GP-293 cell line. In addition, the total extract resulted in more morphological changes in the ACHN cells compared to the GP-293 cells. The effect of the total extract in inducing apoptosis after 48 hours in the ACHN cell line was greater than in GP-293. In addition, the effect of the two fractions was lower than the total extract at all used concentrations. Therefore, the effect of total extract and n-hexane and ethyl acetate fractions of *N. sativa* on cell viability and apoptosis in the ACHN cell line is greater than in the GP-293 cell line. However, the effect of the total extract is higher than either of the two fractions on their own.

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1. Introduction

In recent years, medicinal plants have been paid special attention in the treatment of human diseases due to their low price, availability, and better acceptance by patients.¹ One of these plants is *Nigella sativa*, which is also known as black cumin and panacea.² *N. sativa* is a member of the Ranunculaceae family; it is approximately 20–30 cm in height and has white, yellow, pink, light blue, or red flowers.³ The plant contains various compounds including nonvolatile oils, alkaloids, saponin,⁴ oleic and linoleic acids,⁵ thymoquinone (TQ), p-cymene, t-anethole, carvacrol, 4-terpineol, and longifoline.⁶ Other components include sterols, phospholipids,

tannins, resins, hydroxyl ketones, polyphenols, tocopherols, and vitamins.⁷ TQ is an important pharmacologically active component and several effects of *N. sativa* are attributed to this ingredient.⁸ Pharmacological effects of *N. sativa* such as antioxidant, analgesic, anti-inflammatory, antimitation, anti-liver and anti-kidney toxicity, antidiabetic, antiulcer, and immunoprotective properties have also been reported in several studies.⁶

The anticancer effects of *N. sativa* have been evaluated and its strong antitumor effects against blood, lung,⁹ liver,¹⁰ breast,¹¹ and cervical¹² cancers have been shown. Ait Mbarek et al¹³ showed that *N. sativa* had anticancer effects on P815 (breast cancer), VERO (monkey renal cell carcinoma), BSR (mouse renal cell carcinoma), and sheep heart cell carcinoma (ICO1) cell lines. The effect of *N. sativa* on renal cell carcinoma (RCC) was also shown in another study.¹⁴

In a previous study, we showed that an alcoholic extract of *N. sativa* had antiproliferative and apoptotic effects on the ACHN (human renal adenocarcinoma) cell line.¹⁵ For further investigation, in the present study, we examined the effects of a hydroalcoholic

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extract of *N. sativa* and its n-hexane and ethyl acetate fractions on the morphology, survival, and apoptosis on two cell lines, ACHN and GP-293 (normal human renal epithelial cells).

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) purchased from sigma (St Louis, MO, USA). The Annexin V and propidium iodide (PI) kit was supplied by Biovision (USA). Sodium citrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and the fluorescent probe propidium iodide (PI) were purchased from Sigma.

2.2. Preparation of extract and its fractions

To obtain a hydroalcoholic extract, 100 g of dried *N. sativa* seeds were powdered and then soaked in a solution of 70% alcohol and 30% distilled water for 72 hours. In order to prepare the fractions, 10 g of the obtained extract was mixed with 100 mL of ethanol and transferred to a decanter funnel. The n-hexane solvent was added to the funnel, and the n-hexane fraction was then extracted. In the next step, the remainder of the solvent in the decanter funnel was combined with dichloromethane solvent, and the dichloromethane fraction was then extracted. At the end, the remaining solvent from the previous steps was mixed with ethyl acetate, and the ethyl acetate fraction was extracted. The total extract and n-hexane and ethyl acetate fractions were prepared after omitting the solvent.¹⁶

2.3. Cell culture

The ACHN and GP-293 cell lines were obtained from the Pasteur Institute (Tehran, Iran). The cell lines were cultured in DMEM with 4.5 mg/mL glucose, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin antibiotic in an incubator containing 5% CO₂ at 37 °C.

2.4. Cell viability

Cell viability was evaluated by using a modified MTT assay. The MTT test is a colorimetric method based on the reduction and breakdown of yellow tetrazolium crystals. It is used for calculating the percentage of live cells. After performing the trypan blue test and counting the live cells, 5000 ACHN cells and 10⁴ GP-293 cells were seeded in each well of the 96-well culture plate. For each ACHN and GP-293 cell line, three plates were tested, and for each concentration, three wells were used. After 24 hours, in order to ensure the adherence of the cells to the plate floor, the superficial medium of each well was removed. Each cell line was then incubated for 24 hours, 48 hours, and 72 hours with different concentrations (50, 100, 250, 500, 750, 1000, 1250, 1500, 1750, and 2000 µg/mL) of total extract, n-hexane fraction, and ethyl acetate fraction of *N. sativa*. After these intervals, the upper medium was removed then 200 µL of a medium consisting of 5% FBS and 0.5 mg/mL MTT solvent was added to each well. The plates were incubated for 4 hours. After incubation and emptying of the medium, 200 µL of dimethyl sulfoxide (DMSO) was added to each well and the light absorbance of each well was recorded at a wavelength of 570 nm by an Eliza reader.¹⁷

2.5. Morphological studies

After the exposure of cells to several concentrations of extract and fractions of *N. sativa* (50–2000 µg/mL) for 24 hours, 48 hours,

and 72 hours, morphological properties such as spindle shape, surface dependency, granulation, and uniform distribution were observed by using an inverted light microscope. The untreated cells served as the control group.

2.6. Assessment of apoptosis

In order to differentiate the measurement of cell apoptosis in the ACHN and GP-293 cell line, the Biovision kit was used. This kit includes Annexin V and PI. Both cell lines were treated with the extract of *N. sativa* and its n-hexane and ethyl acetate fractions 24 hours after culture for a duration of 48 hours. Then the cells were collected, 500 µL of binding buffer was added to them, and they were incubated for 5 minutes in darkness and at room temperature. In the next step, 5 µL of Annexin V and 5 µL of PI were added to the cells and incubated for 10 minutes under the same conditions. The samples were then analyzed with a flow cytometry device. Cells with primary or secondary apoptosis or necrosis were placed in separate flow cytometry plots and in this way various cell groups were separated.

2.7. Statistical analysis

One way analysis of variance (ANOVA) was used for normally distributed variables. If a significant difference was observed, the post-hoc Tukey test was applied for comparing the results between the experimental groups. The results were presented as mean ± standard error of the mean (SEM) and *p* < 0.05 was considered as statistically significant.

3. Results

3.1. Morphological study

The total extract of *N. sativa* at all concentrations had no significant effect on the GP-293 cell line at 24 hours. However, at 48 hours and 72 hours a rise in morphological changes such as number of living cells, rounding, and granulation, especially at higher concentrations, was observed. The condition of cells in a concentration of 50–1500 µg/mL of n-hexane after 24 hours was similar to controls whereas wells with 2000 µg/mL concentration had a lower number of live cells in comparison to controls. After 48 hours and 72 hours, morphological changes occurred at 1750 µg/mL and 2000 µg/mL concentrations. After 24 hours of exposure to the ethyl acetate fraction, no morphologic changes occurred in any of the studied concentrations. After 48 hours, changes took place only at the 2000 µg/mL concentration. After 72 hours, morphological changes including reduced cell number, increased intercellular distance, and nonuniform distribution of cells were evident from concentrations of 1500 µg/mL to 2000 µg/mL. Fig. 1 shows the morphological changes of the GP-293 cell line in the 2000 µg/mL concentration at 72 hours.

In the ACHN cell line, morphological study of the cells after 24 hours exposure of total extract showed that cells treated with 1250–2000 µg/mL concentration did not have a homogenous distribution and were seen in multiple colonies all around the well. Moreover, many cells had become round and granulated and had lost their spindle-like shape. After 48 hours and 72 hours, morphological changes including reduced cell number, increased intercellular distance, and nonuniform distribution of cells showed at concentrations from 1500 µg/mL to 2000 µg/mL. The ACHN cells 24 hours after exposure to the n-hexane fraction showed spindle-shaped cells, which were attached to the well bottom and had a homogenous distribution. The cells at 50–1500 µg/mL concentrations had lower cell density compared to controls and cells in 1750 µg/mL and 2000 µg/mL wells, and in addition to reduced congestion, they were round and granulated.

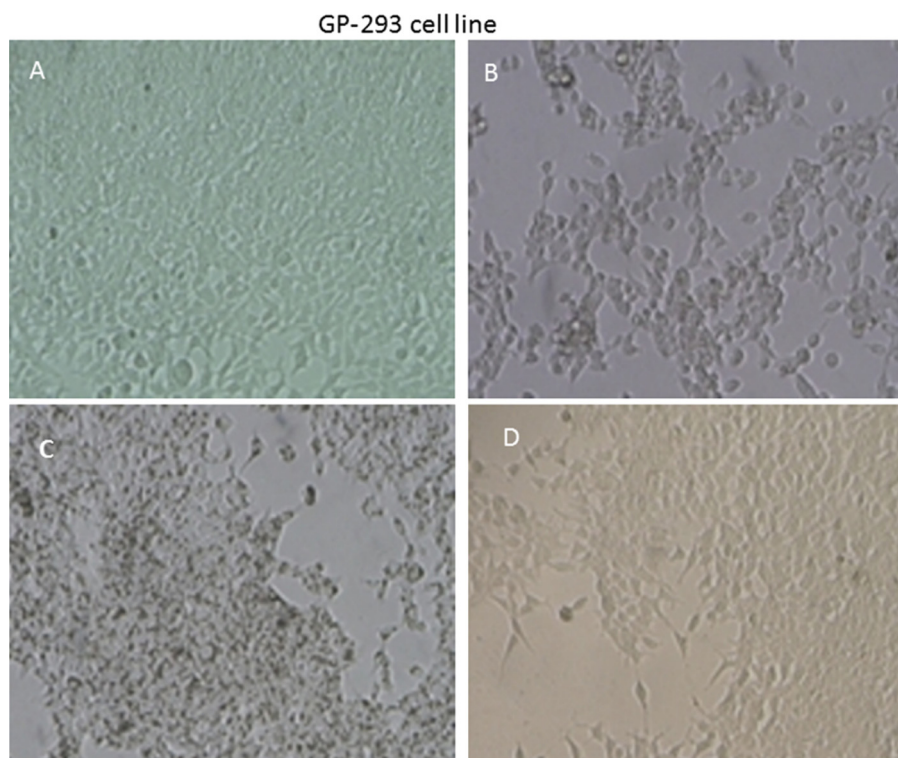


Fig. 1. Morphologic changes of the GP-293 cell line: untreated (A); treated with 2000 µg/mL concentrations of total extract of *Nigella sativa* (B), n-hexane fraction (C), and ethyl acetate fraction (D) after 72 hours.

In the ethyl acetate fraction 24 hours after treatment of the ACHN cells, morphological changes took place from the 1250 µg/mL concentration onwards mainly as round and granulated cells, whereas by increasing time and dosage, nonuniform distribution, reduced cell congestion, and cell accumulation in multiple groups were observed. The morphological changes of the ACHN cell line in the 2000 µg/mL concentration at 72 hours are shown in Fig. 2.

3.2. Effect of total extract and n-hexane and ethyl acetate fractions on cell viability

Fig. 3 shows the MTT test results for different *N. sativa* total extract concentrations on the GP-293 cell line after 24 hours, 48 hours, and 72 hours. Twenty-four hours after their exposure to total extract, a significant reduction in cell viability was seen in comparison to control wells. After 48 hours, this reduction was meaningful only in the 2000 µg/mL concentration well ($p < 0.001$). As shown, 72 hours after total extract exposure to the GP-293 cells, a significant decrease in cell viability was seen from the 1000 µg/mL concentration compared to the control group ($p < 0.001$).

Fig. 4 shows the ACHN cell viability results for different total extract concentrations after 24 hours, 48 hours, and 72 hours. At all these time points, significant decreased cell viability was recorded from the 50 µg/mL concentration compared to controls ($p < 0.001$ to $p < 0.01$).

Fig. 5 shows the GP-293 cell viability results for different n-hexane concentrations after 24 hours, 48 hours, and 72 hours. No significant decreased cell viability was recorded at any of these time points.

Fig. 6 shows the MTT test results of different concentrations of the n-hexane fraction on the ACHN cell line after 24 hours, 48 hours, and 72 hours. Twenty-four hours after exposure to n-hexane, no significant reduction in cell viability was seen in comparison to control cells. After 48 hours, this reduction was meaningful only in

the 2000 µg/mL concentration ($p < 0.001$). After 72 hours, a significant reduction in cell viability was seen from the 250 µg/mL concentration compared to the control group ($p < 0.001$ to $p < 0.05$).

Fig. 7 shows the GP-293 cell viability after exposure to different concentrations of the ethyl acetate fraction. As shown, the cell viability did not significantly change after 24 hours, 48 hours, and 72 hours.

In Fig. 8, the effect of different concentrations of the ethyl acetate fraction on the MTT test in the ACHN cell line is shown. The cell viability after 24 hours significantly decreased from the 750 µg/mL concentration in comparison to control wells ($p < 0.001$ to $p < 0.05$). After 48 hours and 72 hours, a meaningful reduction in cell viability was indicated from the 50 µg/mL concentration in comparison to controls ($p < 0.001$ to $p < 0.01$).

3.3. The effect of total extract and n-hexane and ethyl acetate fractions on cell apoptosis

The effect of total extract and n-hexane and ethyl acetate fractions of *N. sativa* on cell apoptosis percentage in the GP-293 cell line after 48 hours is shown in Fig. 9. Different concentrations of total extract and both n-hexane and ethyl acetate fractions (250–2000 µg/mL) had no significant effect on the apoptosis percentage of the GP-293 cell line in comparison to control. However, with total extract and n-hexane and ethyl acetate fractions on the ACHN cell line after 48 hours, a higher total extract concentration increased the apoptosis rate; this effect was only significant in the 2000 µg/mL concentration of total extract ($p < 0.001$; Fig. 10).

4. Discussion

The findings of this study revealed that the effect of the total extract of *N. sativa* and its two fractions on morphologic changes were dose- and time-dependent and those effects on the ACHN cell

ACHN cell line

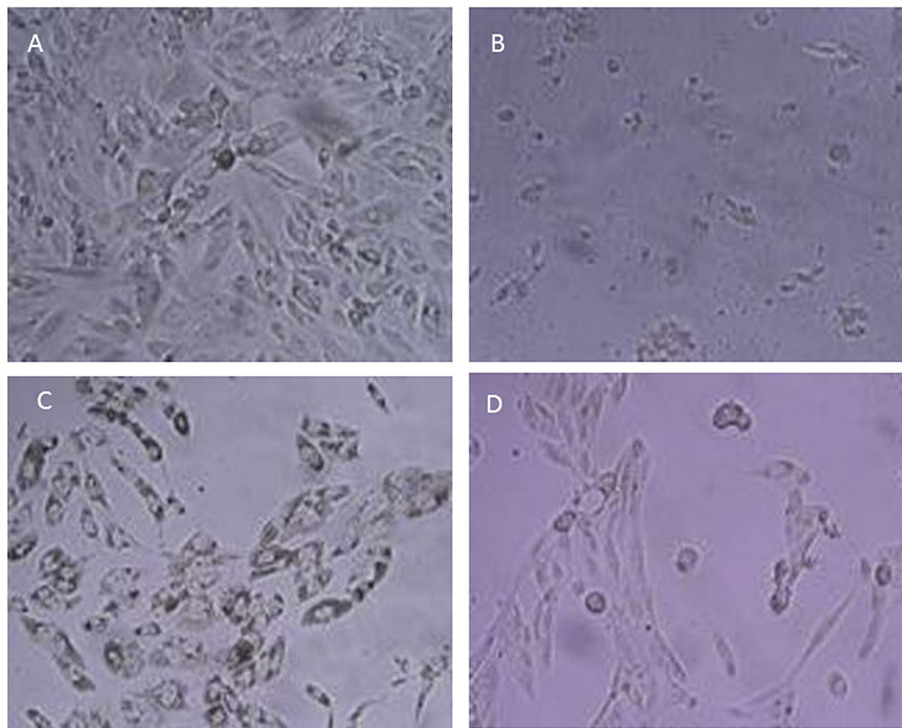


Fig. 2. Morphological changes of the ACHN cell line: untreated (A); treated with 2000 µg/mL concentrations of total extract of *Nigella sativa* (B), n-hexane fraction (C), and ethyl acetate fraction (D) after 72 hours.

line were higher than the GP-293 cell line. In addition, morphological changes induced by the total extract were stronger than with the n-hexane and ethyl acetate fractions.

Moreover, the results of this study showed that the effect of the ethyl acetate fraction, which consists of semipolar compounds,¹⁶ is higher than the n-hexane fraction, which has nonpolar compounds such as fats and lipids.¹⁸

Recently, in view of the high costs of cancer treatment and the development of new anticancer drugs that have more complications and lower efficacy in treating malignant tumors, significant progress has taken place in using herbal products for treating

various diseases.¹³ Medicinal plants are considered as valuable resources in the discovery of new medications.¹²

The application of drugs that inhibit the proliferation of cancer cells by apoptosis may be an option for treating cancer.¹³ Several studies have shown that *N. sativa* with its anticancer effects is effective in treating leukemia and renal, hepatic, prostate, breast, cervical, and skin carcinomas.^{9–11,19}

The MTT cell viability test results indicated a greater ability of the total extract in a dose- and time-dependent manner to reduce the percentage of live cells of the cancer cell line in comparison to the normal cell line. Based on the findings of the

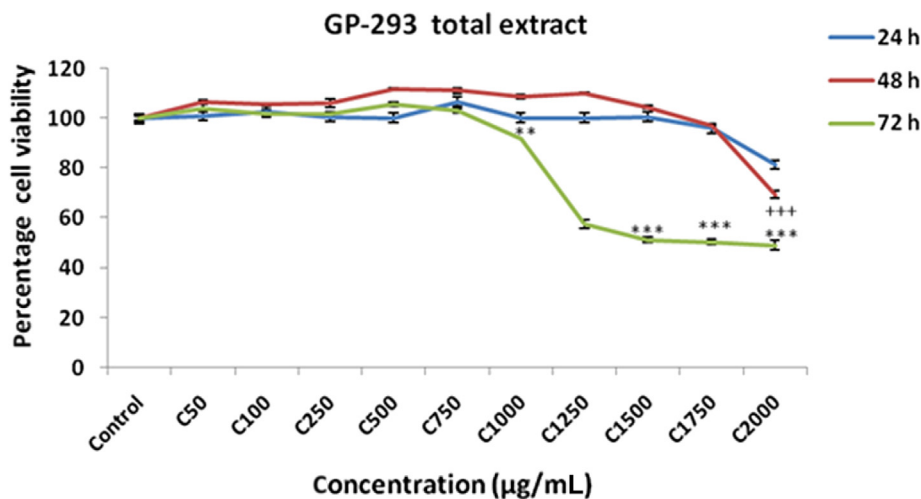


Fig. 3. The effect of the total extract of *Nigella sativa* on the cell viability of the GP-293 cell line at 24 hours, 48 hours, and 72 hours. +++ $p < 0.001$ compared to control after 48 hours. ** $p < 0.01$ and *** $p < 0.001$ compared to control after 72 hours.

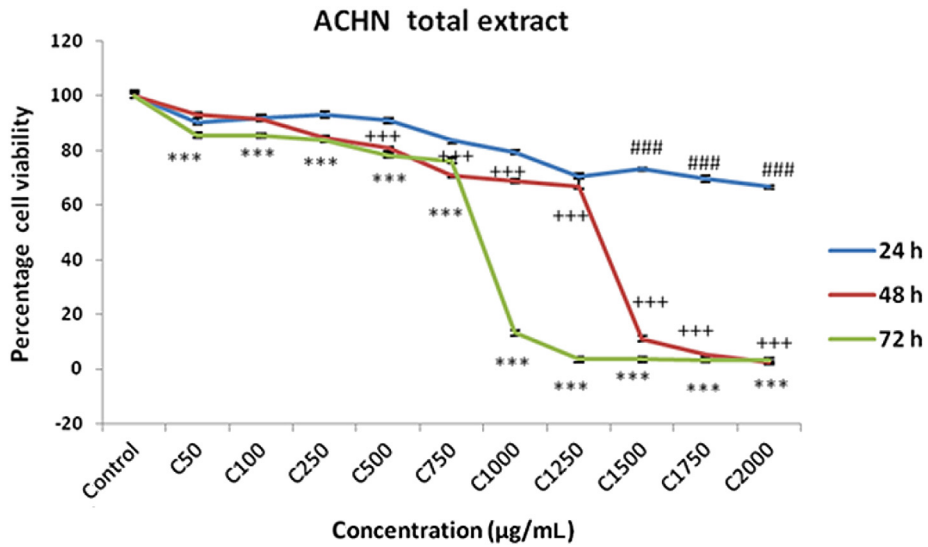


Fig. 4. The effect of the total extract of *Nigella sativa* on the cell viability of the ACHN cell line at 24 hours, 48 hours, and 72 hours. ###*p* < 0.001 compared to control after 24 hours. +++*p* < 0.001 compared to control after 48 hours. ***p* < 0.01 and ****p* < 0.001 compared to control after 72 hours.

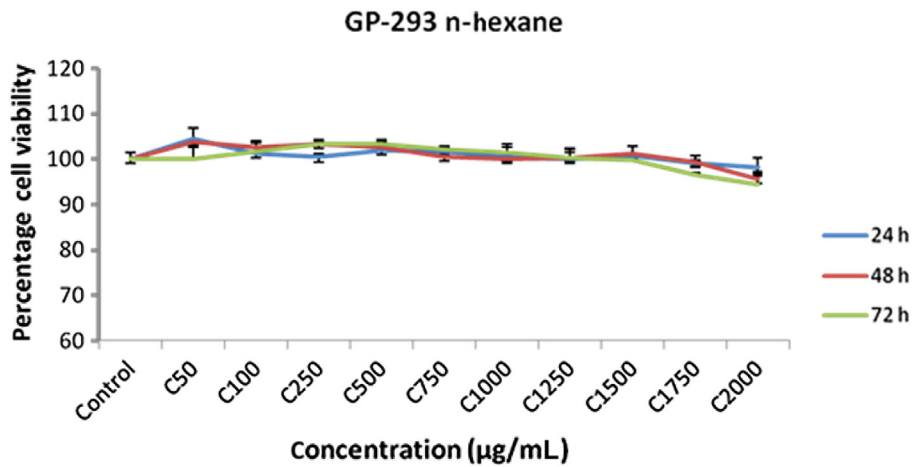


Fig. 5. The effect of the n-hexane fraction of *Nigella sativa* on the cell viability of the GP-293 cell line at 24 hours, 48 hours, and 72 hours.

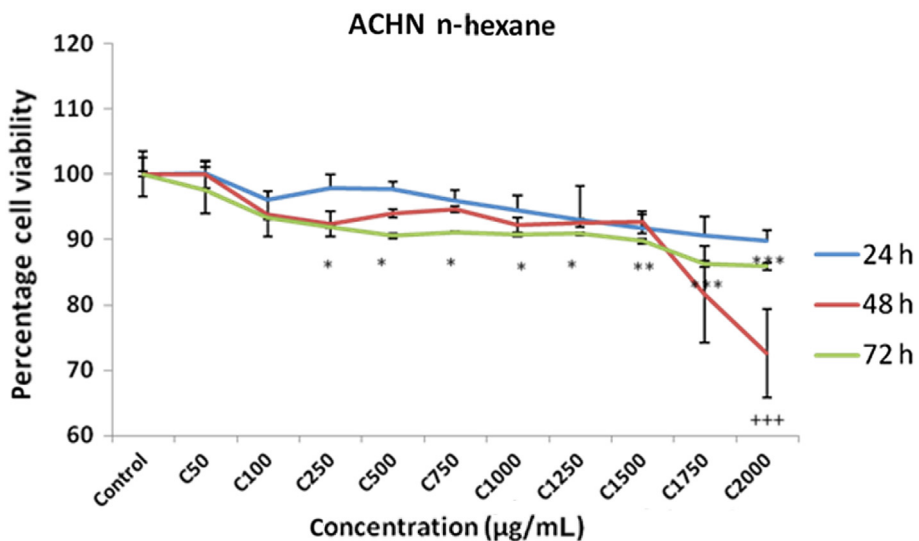


Fig. 6. The effect of the n-hexane fraction of *Nigella sativa* on the cell viability of the ACHN cell line at 24 hours, 48 hours, and 72 hours. +++*p* < 0.001 compared to control after 48 hours. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 compared to control after 72 hours.

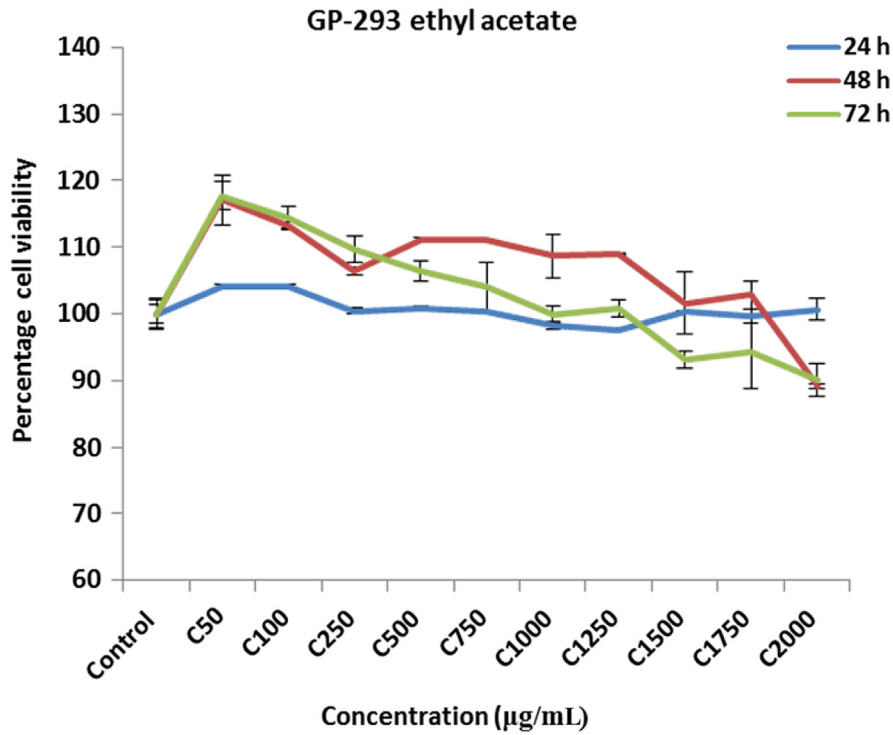


Fig. 7. The effect of the ethyl acetate fraction of *Nigella sativa* on the cell viability of the GP-293 cell line at 24 hours, 48 hours, and 72 hours.

current study, the dose- and time-dependent ethyl acetate fraction is highly toxic on the ACHN cell line in comparison to the GP-293 cell line. In a study by Swamy et al,⁵ the ethyl acetate fraction of the ethanolic extract of *N. sativa* showed a cytotoxic effect on HEPG2 (hepatic cell carcinoma), Molt4 (human lymphoblastic cell carcinoma) and LL/2 (lung cell carcinoma) cell

lines.⁵ The results of our study also indicated that the effect of the ethyl acetate fraction in growth inhibition of the ACHN cell line in a dose- and time-dependent manner was higher than the n-hexane fraction. Moreover, the effect of the total extract of *N. sativa* on the ACHN cell line was even greater than the ethyl acetate fraction.

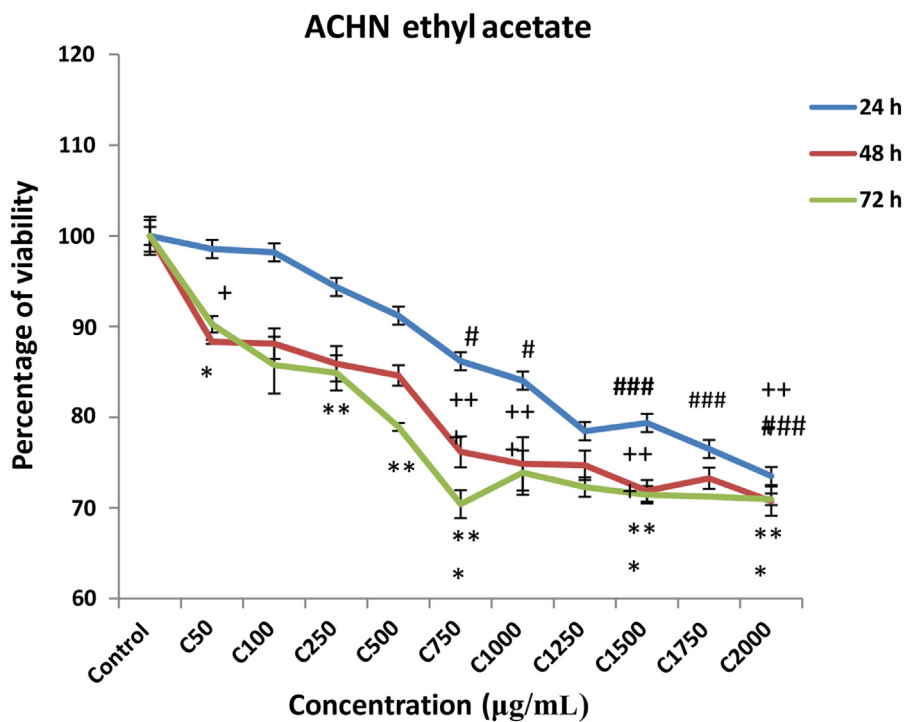


Fig. 8. The effect of the ethyl acetate fraction of *Nigella sativa* on the cell viability of the ACHN cell line at 24 hours, 48 hours, and 72 hours. #*p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001 compared to control after 24 hours. ++*p* < 0.01 and +++*p* < 0.001 compared to control after 48 hours. ***p* < 0.01 and ****p* < 0.001 compared to control after 72 hours.

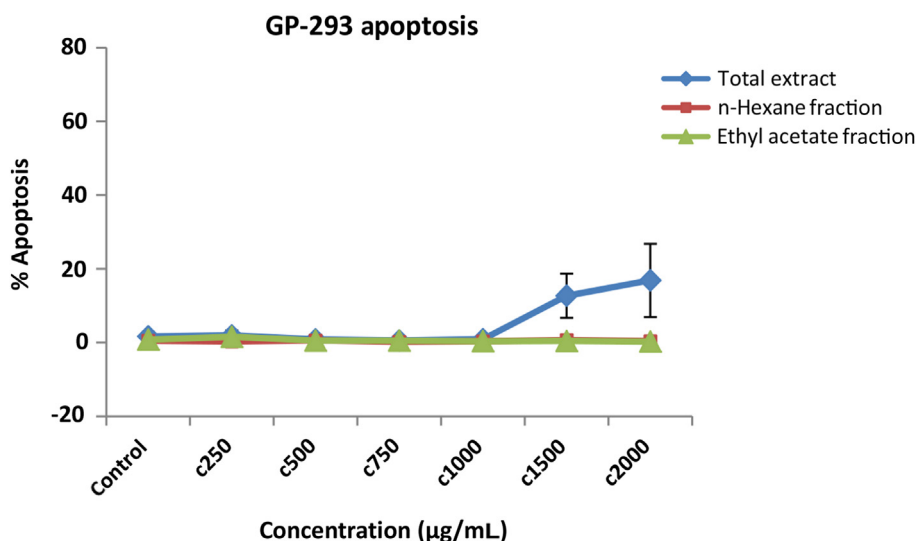


Fig. 9. The effect of the total extract of *Nigella sativa* and its n-hexane and ethyl acetate fractions on the percentage of apoptosis in the GP-293 cell line after 48 hours.

The flow cytometry results showed the mean apoptosis rate in the ACHN and GP-293 cell lines treated with total extract to be 27.5% and 5.6%, respectively. The mean apoptosis rate in the two cell lines treated with the n-hexane fraction was 9.9% and 0.41%, respectively. The values were 10.22% and 0.6%, respectively, for those treated with the ethyl acetate fraction. Moreover, it was observed that in the ACHN cell line, ethyl acetate at lower concentrations caused higher apoptosis, whereas at higher concentrations the rate of apoptosis decreased, which shows a shift in its mechanism from apoptosis to necrosis.

In the study by Shafi et al,¹² the methanol, n-hexane, and chloroform fractions of *N. sativa* were seen to induce apoptosis in the HeLa cell line (cervical cancer cells). The chloroform fraction induced greater apoptosis (42%) in comparison to the n-hexane (30%) and methanol (21%) fractions.¹²

Ait Mbarek in 2007¹⁸ reported that the *N. sativa* total extract, ethyl acetate, and butanol fractions have different cytotoxic effects on P815, Vero, BSR, and ICO1 cell lines. The cytotoxic effect of the butanol extract was less than the other fractions on the studied cell lines. The above-mentioned studies indicate that the effect of

different fractions of plant extracts can vary depending on the raw material. Moreover, this difference in fraction effects can also be due to different cell lines. The results of the current study also suggest that the ethyl acetate fraction of *N. sativa* in comparison to the n-hexane fraction has a greater ability to induce apoptosis in the ACHN cell line in comparison to the GP-293 cell line; while the ability of the *N. sativa* total extract in inducing apoptosis in the ACHN cell line is greater than each of its fractions.

Comparing the flow cytometry results with the MTT test and by considering the similar percentage of reduced cell viability and apoptosis in each concentration of the ACHN and GP-293 cell lines, apoptosis could be proposed as the prominent mechanism of cytotoxicity in both cell lines.

Nevertheless, in the current study, it could be suggested that the probable mechanism of apoptosis induction by *N. sativa* fractions is the underexpression of Bcl2, over-expression of P53, and activation of caspases 3, 8, and 9. However, determination of the exact mechanism requires further investigation.¹²

Traditional Chinese Medicine researchers believe that each herbal extract, which is a mixture of several elements in that plant,

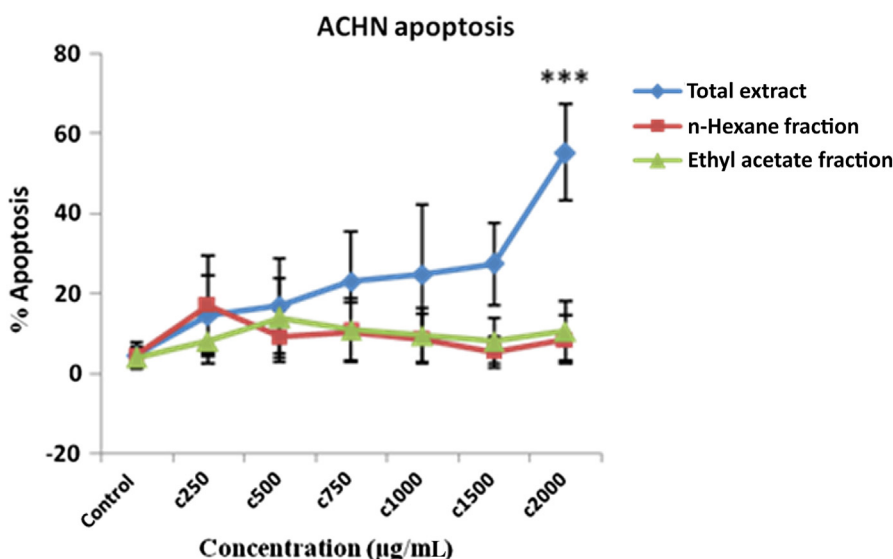


Fig. 10. The effect of the total extract of *Nigella sativa* and its n-hexane and ethyl acetate fractions on the percentage of apoptosis in the ACHN cell line after 48 hours. *** $p < 0.001$ compared to control after 48 hours.

in comparison to an extract of the pure active ingredient, has higher efficacy and lower toxicity.²⁰ In the study of Wong et al.,²⁰ the total extract of *Rabdosia* caused greater inhibition of prostate cancer cell proliferation and showed higher synergistic effects at several concentrations than pure oridin (an active ingredient of the *Rabdosia* extract). Also, in the study by Seeram et al.,²¹ the total phenolic extract of blueberry significantly inhibited the growth of two oral cancer cell lines (CAL27 and KB) and three prostate cancer cell lines (22RV1, RWPE-2, and RWPE-1), whereas the anthocyanin and anthocyanidin fractions of blueberry had less inhibitory effects on the colon and oral cancer cells.

Regarding the results of the above-mentioned studies, it could be expressed that treatment by a medicinal plant is not related to just one specific chemical agent, but it is the combination of its materialistic existence and structural integrity that gives such favorable outcomes. In the whole structure of a plant, there are certain elements with higher efficacy and positive synergistic effects and other components that neutralize side effects. Therefore, separating certain parts of a plant as a medicinal material ruins its integrity and results in reduced therapeutic effects and increased related complications.²² The finding of the current study confirms such results—the higher efficacy of the total extract of *N. sativa* and its ethyl acetate fraction suggest that the anticancer agents are distributed in different fractions of the plant. This further justifies the use of the total extract form in the treatment of common cancers.

Conclusion

In conclusion, our findings show that the total extract and n-hexane and ethyl acetate fractions of *N. sativa* have cytotoxic and apoptotic effects on ACHN and GP-293 cell lines but the effects on ACHN are stronger. In addition, the effects of the total extract on both cell lines are greater than either of its two fractions alone.

Conflicts of interest

The authors do not have any direct financial relationship with the commercial identities mentioned in this article.

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