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► To cite this version:

Nasser Mohamad, Hijazi Akram, Bouchra Sayed Ahmad, Jamaledine Zeinab, Ibrahim Sajida, et al.. Efficiency of combining pomegranate juice with low-doses of cisplatin and taxotere on A549 human lung adenocarcinoma cells. *Asian Pacific Journal of Tropical Biomedicine*, 2018, 8 (1), pp.19-24. 10.4103/2221-1691.221130 . hal-02618791

HAL Id: hal-02618791

<https://hal.inrae.fr/hal-02618791>

Submitted on 6 Jun 2023

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Asian Pacific Journal of Tropical Biomedicine

Journal homepage: www.apjtb.org



doi: 10.4103/2221-1691.221130

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Efficiency of combining pomegranate juice with low-doses of cisplatin and taxotere on A549 human lung adenocarcinoma cells

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ARTICLE INFO

Article history:

Received 20 September 2017

Revision 4 October 2017

Accepted 3 November 2017

Available online 1 January 2018

Keywords:

Lung cancer
Chemotherapy
Cisplatin
Taxotere
Pomegranate
A549 cells

ABSTRACT

Objective: To test the coalescence effect of two chemotherapy drugs at low effective dose (cisplatin and taxotere) combined with pomegranate juice on A549 cancer cells. **Methods:** Infrared spectroscopy method is a qualitative test that was performed to ensure the existence of the phytochemicals providing the antioxidant activity through the presence of the hydroxyl group (-OH). The viability of A549 cell line and normal MCs was tested using the neutral red uptake, Clonogenic survival, XTT and Cell migration assays. **Results:** Our results showed that this combination firstly led to a greater decrease in the viability of cells comparing to those treated with chemotherapy drugs alone, and secondly led to a significant reduction in cell migration. **Conclusions:** These data suggest a synergistic effect between the pomegranate and cisplatin which makes probably this combination a powerful option for treating lung adenocarcinoma and in parallel minimizing the systemic side effects.

1. Introduction

Lung cancer is one of the most common cancers and it is the most leading cause of preventable death. It is the leading cause of cancer related death in men and women in the United States accounting for approximately 28% of total cancer deaths in 2010 despite comprising only 15% of new cancer cases[1]. Lung cancers are aggressive tumors that are classified into two main types which are the non-small cell lung cancer (NSCLC) representing (80%-85%) and small cell lung cancer (SCLC) representing (15%-20%) based on histological, clinical and neuroendocrine characteristics[2,3].

NSCLC are subdivided into: adenocarcinomas, squamous cell, large cell carcinoma, bronchoalveolar lung cancer, and mixed histological types.

SCLC arises from neuroendocrine cells 'kulchitsky cells' of the lungs[4]. These cells make polypeptide hormones and are characterized by dense core neurosecretory granules[5], while the NSCLC arises from the alveolar cells[6,7]. Adenocarcinomas come from basal bronchial cells and type II pneumocytes that appear in the periphery of the lung, and the squamous type arises from the bronchial epithelial cells located more centrally[8]. Concerning

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How to cite this article: Mohamad N, Akram H, Bouchra SA, Zeinab JE, Sajida I, Hassan R, et al. Efficiency of combining pomegranate juice with low-doses of cisplatin and taxotere on A549 human lung adenocarcinoma cells. Asian Pac J Trop Biomed 2018; 8(1): 19-24.

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Foundation project: It was supported by Lebanese University for the financial support of this work (Project M. Nasser UL2017).

the treatment, it depends on the cancer stage, in which surgical treatment is reserved for early stages. Lebanese University for the financial support of this work (Project M. Nasser UL2017). (I and II) however such intervention seemed harmful for advanced stages (III B and IV) and treated with chemotherapeutic drugs[9]. There is no specific agent for cancer treatment which is the focal point of the cancer research. Nowadays, the traditional and complementary/alternative medicine was the insight for cancer researchers[10], and we choose the pomegranate in our study as a hope to treat the advanced stages adenocarcinoma A549 cells.

Epidemiological studies have shown that diets rich in phytochemicals and antioxidants play a major role in combating diseases and maintaining health[11]. One of the most important fruits that has been studied is the pomegranate. Besides its use as a fruit, its medicinal properties have attracted the interest of researchers of many countries. Pomegranate fruit has medicinal properties such as anti-inflammatory and antibacterial activities. The pomegranate seed oil has inhibitory effect on skin and breast cancers. The pomegranate seed oil has phytoestrogenic compounds and the fruit is rich in phenolic compounds with strong antioxidant activity. Ellagic acid is one of the main components of pomegranate with phenolic structure and antioxidant activity.

Cisplatin (CDDP) was known to be a chemotherapy drug for all types of cancer, but it was found to induce cytotoxic side effects that will harm normal cells. It is used alone or combined with other chemotherapeutic drugs, such as taxotere, which is an anti-microtubule agent. However, these conventional therapies have failed to make a major impact on the survival of NSCLC patients highlighting the need for new approaches.

In our present work, it is aimed to evaluate the effect of the combination of pomegranate juice with low dose of chemotherapeutic drugs (taxotere and cisplatin) on A549, human lung adenocarcinoma cells.

2. Materials and methods

2.1. Preparation of pomegranate

Lebanese pomegranates were peeled and the arils were mixed and then centrifuged. The supernatant was frozen at -80°C and then lyophilized and stored in a desiccator at room temperature. Lyophilized pomegranate was dissolved in complete medium prepared at different concentrations (50, 100, 150, and 200 $\mu\text{g/mL}$).

2.2. Chemical tests

2.2.1. Antioxidant test (DPPH radical scavenging method)

2,2-diphenyl-1-picrylhydrazyl (DDPH) is a dark-colored crystalline powder composed of stable free radical molecules with antioxidant activity[12]. In this study, we prepared two solutions of DPPH

(0.003 g): the first one in 50 mL of methanol and the second in 50 mL of ethanol to compare the activity of the best extract, and we prepared 0.003 g of DPPH and dissolved them in 20 mL of ethanol to monitor the change of the activity. Then we prepared our extract by measuring 0.04 g of the pomegranate powder and dissolved them in 20 mL of distilled water and we vortexed them to obtain a homogeneous solution. After that, we prepared 5 different concentrations (0.5, 0.4, 0.3, 0.2, 0.1 mg/mL) from our solution.

2.2.2. Phytochemical screening tests

Qualitative tests were performed on the juice to determine its content in compounds involved in some of the biological properties antioxidants. The presence of phenolic acid, terpenoid, flavonoid, quinone, alkaloid, tannin, resin and saponin was analysed using the methods of Farhan *et al.* (2012)[13,14].

2.2.3. Infrared spectroscopy (IR)

Pomegranate powder was mixed with potassium bromide powder, a material having no IR-active vibrations in the mid-IR range. The mixture was pressed into a transparent disk and inserted in the beam path. In this case, a sum of vibrational properties were recorded, and the functional groups would be determined. This procedure was performed to evaluate the presence of hydroxyl group which is the main functional group for antioxidant activity.

2.3. Cell culture and treatment

2.3.1. A549 cells

The A549 human lung adenocarcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin in an atmosphere of 5% CO_2 at 37°C .

2.3.2. Normal cells (control)

Mouse mesangial cells (MCs) were cultured in DMEM supplemented with 0.1 mM non-essential amino acids, 10% foetal bovine serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin at 37°C in a humidified incubator containing 5% CO_2 .

2.3.3. Treatment of cells

Lyophilized pomegranate was dissolved using vortex in the culture medium specific for each cell type, as a concentrated stock solution (10 mg/mL) and then filtered by a filter diameter of 0.2 μm , and used for the preparation of different concentrations for the treatment of cells. A total of 50% to 60% confluent cells were treated with the pomegranate for 48 h in complete medium, seeded in a 24-well plate in a concentration of 10 000 cells/well.

The evaluation of the anticancer activity was performed by measuring the viability of the A549 cell line and normal MCs (control) after a treatment for 48 h with increasing concentrations of cisplatin (4, 8, 12 and 16 $\mu\text{g/mL}$) and pomegranate (50, 100, 150, 200 $\mu\text{g/mL}$). Similarly, the A549 cells were treated with the

combination of the pomegranate (200 µg/mL) and cisplatin (4 µg/mL) with or without taxotere (5 µg/mL).

2.4. Viability test

2.4.1. Neutral red uptake assay

Neutral red assay was performed to measure the cell viability. It was used to evaluate the cytotoxicity of pomegranate and cisplatin on the A549 adenocarcinoma cells.

Neutral red uptake assay was carried out following the protocol[15]. Briefly, after 48 h treatment, the medium was aspirated and cells were washed twice with PBS and incubated for 3 h in a medium supplemented with neutral red (50 µg/mL). The medium were taken out, and it was washed twice again with PBS. Cells were subjected to further incubation for 10 min at room temperature in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. Then, the medium was transferred (100 µL/well) to 96-well plates. The plates were read at 490 nm using multi-well microplate reader.

2.4.2. Clonogenic survival assay

A549 Cells were seeded (100 cells/well) into a 6-well plate and treated with pomegranate of concentrations (50, 100, 150, 200, 400 and 1 200 µg/mL) for 24 and 48 h. After that, they were kept for 10 d in culture. After 10 d of incubation, the cells were washed twice with PBS, and then the crystal violet was added to each well and incubated for half an hour at 37 °C. Thirty minutes later, they were washed by distilled water and the colony of survival cells that were colored by crystal violet was counted.

2.4.3. XTT assay

XTT colometric-assay (Sigma, St. Lois, MO) was used to evaluate the cellular viability of A549 cells through measuring the activity of enzymes that reduce the water soluble XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reagent to an orange formazan by actively respiring cells. After treatment of A549 cells with cisplatin at concentrations (4 and 8 µg/mL), pomegranate (150 µg/mL), and a combination of both cisplatin and pomegranate of concentration (4+150) µg/mL for 48 h in 96-well mixing 5 mL of XTT with 100 µL of activator. From this mixture 50 µL was added to each well plate, and then the 96-well plate was incubated for half an hour at 37 °C and 5% CO₂. After that the absorbance was measured at an optical density of 450 nm[16].

2.4.4. Cell migration

Nearly confluent monolayers of cells were maintained in complete medium, and a track was scraped across these cells by a plastic tip. The cells were allowed to migrate through the edges of the wound at 37 °C for 24 h after treatment with or without pomegranate. The cells that have migrated through the wounded edges were photographed using a computer imaging system and the distance (in cm) was subsequently measured[17]. For migration assays, a uniform

cell-free area was created by scratching confluent monolayers with a plastic micropipette tip as previously described. Briefly, the wound area was inspected at different time intervals (0, 24 hour) to determine the distance migrated by the cells. The closer the gap, the faster the cell migrated. At each time point four photographs were taken and the widths of the wound space were evaluated by multiple measurements.

2.5. Statistical analysis

All statistical analyses were carried out with PRISM (GraphPad, San Diego), and *P* values <0.05 or 0.001 were considered significant. The one way Anova was used to estimate cell viability, and differences were analyzed by the Bonferroni test.

3. Results

3.1. Antioxidant test (DPPH radical scavenging method)

It showed that the optical density was decreased as the concentration of pomegranate juice was increased. In addition, the antioxidant activity of DPPH was more efficient with methanol than with ethanol. This antioxidant activity represents 70% with methanol compared to 33% of ethanol, and this was also observed by the transformation of violet color to yellow color as the concentration of DPPH was increased from 0.1 to 0.5 mg/mL. This indicates that 0.5 mg/mL of methanolic extract has higher antioxidant activity than that of ethanol. This finding represents that methanol has the strongest antioxidant activity in pomegranate extract[18].

3.2. Phytochemical screening test

The phytochemical screening tests were performed to evaluate the presence of secondary metabolites responsible of the antioxidants property. The obtained results (Figure 1) confirmed the existence of alkaloid (orange), flavonoid (yellow), phenolic acid (blue to green), terpenoid (violet), and quinone, which was observed by the formation of orange, yellow blue to green, violet and red color respectively, in addition to resin that was observed by the presence of turbidity.

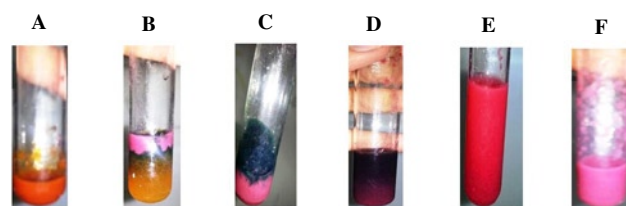


Figure 1. Phytochemical screening tests.

A: alkaloids; B: flavonoids; C: phenolic acids; D: terpenoids; E: quinones; F: resins.

3.3. Infrared spectroscopy results

Figure 2a showed the presence of broad spectrum band at 3424.96 cm^{-1} , indicating clearly the existence of hydroxyl group that played a major role in achieving the anti-oxidant activity.

3.4. Viability tests results

3.4.1. Neutral red

The optical density decreased as a function of concentration and time of treatment. Indeed, only living cells incorporated neutral red solution in their lysosomes. The decrease in optical density reflected that the number of living cells began to decrease compared to the control which was not treated.

3.4.2. Cisplatin treatment decrease cell viability in a dose dependent manner

Treatment with increased concentrations of cisplatin (4, 8, 12 and 16 $\mu\text{g/mL}$) showed a significant decrease in A549 cells viability when compared to the control cells (Figure 2b). A significant difference was seen between cells treated with (4 and 8 $\mu\text{g/mL}$) and another group cells treated. No significant difference was observed in cell viability between cisplatin 12 $\mu\text{g/mL}$ and 16 $\mu\text{g/mL}$.

3.4.3. Pomegranate treatment decrease cell viability in a dose dependent manner

Results showed a significant decrease in cell viability by comparison to the control (non-treated cells). On the other hand, no significant difference was observed between the two highest concentrations of cisplatin (300 and 400 $\mu\text{g/mL}$). Consequently, 300 $\mu\text{g/mL}$ of pomegranate was considered as a high concentration for the treatment of the A549 cell lines (Figure 2c).

3.4.4. Effect of combination of pomegranate and cisplatin in cell viability

The neutral red results showed a significant decrease in A549 cells viability with increased concentrations of either pomegranate or cisplatin, while there was a same inhibition for pomegranate (150 $\mu\text{g/mL}$) alone as that for cisplatin (4 $\mu\text{g/mL}$) alone. However, when we combined pomegranate (150 $\mu\text{g/mL}$) with cisplatin (4 $\mu\text{g/mL}$), there was a significant increase in A549 cells death. A finding that became more significant as we increased the concentration of pomegranate to 300 $\mu\text{g/mL}$. Furthermore, by increasing the cisplatin concentration to 8 $\mu\text{g/mL}$ more cell death was observed in comparison to low dose cisplatin (4 $\mu\text{g/mL}$). In order to minimize the side effects of cisplatin by decreasing its concentration without impairing its cytotoxicity

efficacy, we considered that combination of cisplatin (4 $\mu\text{g/mL}$) with pomegranate (300 $\mu\text{g/mL}$) would be the ideal concentration to face A549 cancer cells and indeed decreased cisplatin toxicity (Figures 2d and 2e).

3.4.5. Effect of the combination of pomegranate, cisplatin and taxotere on cell viability of A549

The combination of CDDP with taxotere decreases the cell viability of A549 up to 65% compared with the control cells, whereas in the presence of the pomegranate 200 $\mu\text{g/mL}$ decreased the cell viability seems to be more important compared to CDDP and taxotere alone (Figure 2f).

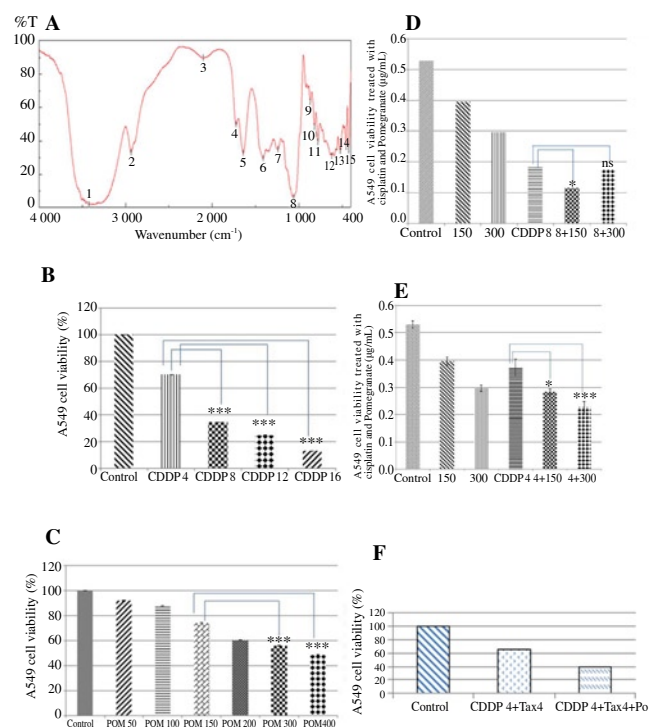


Figure 2. Infrared spectroscopy result.

(a) Infrared spectroscopy. (b) Neutral red assay (***) $P < 0.001$). (c) Cytotoxic activity of pomegranate fruit extracts. (d) Synergistic activity of pomegranate combined with cisplatin (8 $\mu\text{g/mL}$) after 48 h. (e) Synergistic activity of pomegranate with cisplatin (4 $\mu\text{g/mL}$). (f) Effect of pomegranate combined with low dose of chemotherapeutic agents on cell viability of A549 cells.

3.4.6. Effect of combination of pomegranate and cisplatin on normal cells

Results showed no significant difference for cells treated with either pomegranate, cisplatin alone or a combination of these treatment compared to those untreated control cells (Figure 3a).

3.4.7. Pomegranate reduced the migration abilities of A549 cells

Pictures taken after 24 h of treatment showed a significant decrease in migration by comparing the treated groups with the untreated

control. Indeed comparison between treatment groups showed that combination chemotherapy with pomegranate decreased the migration more than that of chemotherapy alone (Figure 3a, 3b).

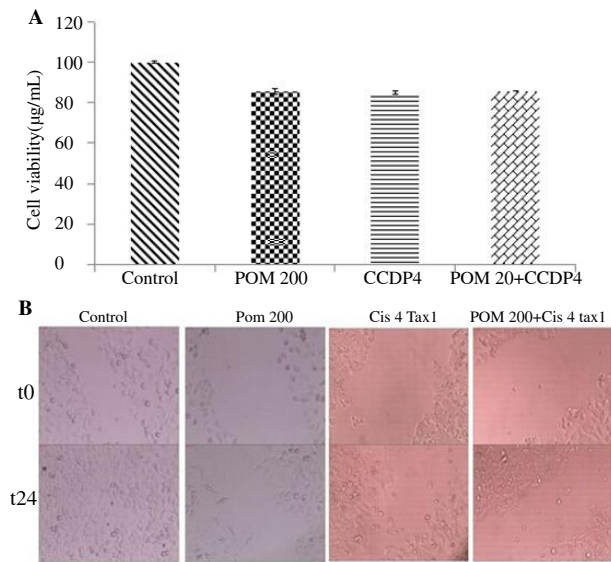


Figure 3. Effect of combination of pomegranate and cisplatin on normal cells.

(a) Effect of pomegranate (200 µg/mL) and CDDP (4 µg/mL) on cell viability of MCs. (b) Wound healing assay.

3.5. Clonogenic survival assay

The results showed a slight decrease in the number of A549 cells with increased concentration of pomegranate from 50 to 100 µg/mL at 24 and 48 h. On the other hand, increasing the concentration of pomegranate to 200, 400, and 1 200 µg/mL resulted in higher A549 cells death when compared to the control at 24 and 48 h (Figure 4).

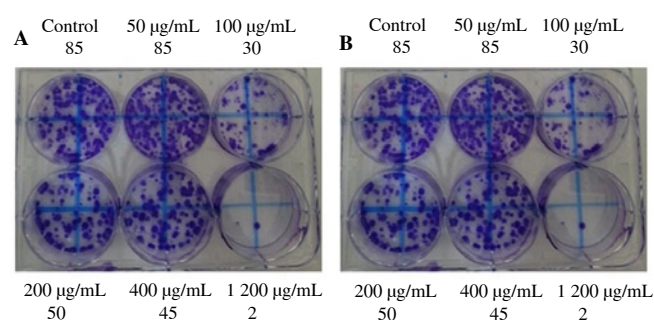


Figure 4. Clonogenic survival assay. A: Results at 24 h. B: Results at 48 h.

3.6. XTT assay

XTT assay showed a significant decrease in cell viability with increased concentration of cisplatin (Figure 5).

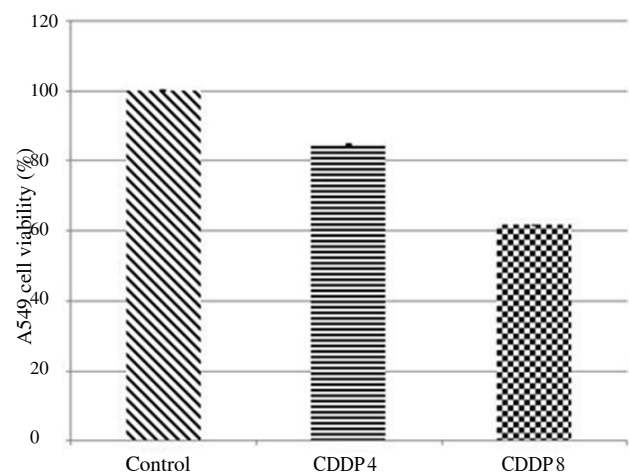


Figure 5. XTT cytotoxicity assay showing more cell death in cisplatin (8 µg/mL) than with cisplatin (4 µg/mL).

4. Discussion

Lung cancer was considered as a rare cancer in the 19th century. However in the 20th and 21st century, lung cancer became the 2nd common cancer especially in developing countries.

A dramatic change in lung cancer incidence has occurred due to several factors including smoking, pollution, radiation, ionization, and certain infections. Among these factors, tobacco smoke has been considered the main culprit of this deadly cancer. But despite the different strategies that take place to overcome this disease, lung cancer is still the most leading cause of cancer death. Multiple therapeutic modalities are now used to treat lung cancer like chemotherapy, radiotherapy, surgery, and recently genetic targeted therapy. Yet, chemotherapy, the gold standard modality for advanced stages, is associated with multiple side effects especially with platinum based chemotherapy. Combination chemotherapy regimen has been used to minimize the chemotherapy related side effects but the mortality is not decreased significantly.

In this study, we evaluated the response of human cells from non-small cell lung cancer (A549) against the pomegranate combined with a low dose of two chemotherapeutic drugs: cisplatin and taxotere. The lyophilized pomegranate has an antineoplastic effect represented by a decrease in viability of A549 cells in a dose-dependent manner.

The preliminary phytochemical screening of pomegranate showed the presence of phenolic acids, flavonoids, terpenoids, quinones, alkaloids, tannines and resins. In addition, infrared analysis confirmed the presence of compounds having the -OH group which exerts the antioxidant effect. These results suggest that these compounds are responsible for the cytotoxic effect of the pomegranate on A549 cells.

In vivo studies have confirmed this antineoplastic effect of pomegranate, patients treated with cisplatin alone or in combination with other chemotherapeutic drugs, suffer from side effects that are

not observed in the case of treatment with pomegranate. Hence, the idea to minimize the dose of chemotherapeutic drugs and compensates it by combination with the pomegranate, and then minimizing their side effects.

When combined pomegranate with a low dose of cisplatin or with low doses of cisplatin and taxotere, results showed a higher decrease in the viability of A549 cells compared to the groups treated with chemotherapy or pomegranate alone where pomegranate enhances the anticancer effect of a low dose of cisplatin[19].

It is difficult to cure lung cancer because of its ability to metastasize to distant vital organs. The ability of cancer cells to metastasize to vital organs is the most life-threatening stage of cancer, therefore inhibiting the migration abilities of cancer cells is critical for any chemotherapeutic drug. Thus, the pomegranate showed an inhibitory effect on the mobility of NSCLC A549 cells, after a 24-hour treatment and an improvement in the inhibitory effect of the chemotherapeutic combination of cisplatin and taxotere at low dose. Our results from the wound-healing assay showed an important decrease in the ability of NSCLC A549 cells to migrate after combining low dose of chemotherapeutic drugs with Lebanese pomegranate, suggesting a role in inhibiting metastasis.

In conclusion, this study has shown that the pomegranate has an inhibitory effect on the viability of A549 cells. In addition, the combination of pomegranate with a low dose of chemotherapeutic drugs exhibits a synergistic inhibitory effect against cell viability, and cell migration and then against the metastatic properties of NSCLC. Our results highlight a new therapeutic concept that offers combining pomegranate with low dose of chemotherapeutic drugs to treat lung adenocarcinoma.

Conflict of interest statement

The authors declare no conflict of statement.

Acknowledgments

The authors are thankful to Lebanese University for the financial support of this work (Project M. Nasser UL2017).

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