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Article

Influence of the Extraction Solvent and of the Altitude on the Anticancer Activity of Lebanese *Eucalyptus camaldulensis* Extract Alone or in Combination with Low Dose of Cisplatin in A549 Human Lung Adenocarcinoma Cells

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Abstract: Background: Lung cancer is the second most common cancer worldwide. *Eucalyptus* plant extract has been shown to have anti-neoplastic effects. We investigated the antitumor effect of ethanolic and aqueous extracts of *Eucalyptus camaldulensis* collected at different altitudes on A549. In addition, we evaluated the additive effect of its combination with low-dose cisplatin (CDDP). Methods: Qualitative and quantitative analyses of secondary metabolites present in the plants were carried out. The antioxidant and cytotoxic activities of the different extracts on A549 were evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and neutral red assay, respectively. The cytotoxic effect of the combination of certain extract concentrations with low-dose CDDP on A549 cells was studied. Results: In the Ethanolic extract, a higher number of active substances and antioxidant activities were observed. The four *E. camaldulensis* extracts showed cytotoxic activity on A549 cells, with a higher cytotoxicity for the Ethanolic extract and the sea-level altitude species. Moreover, the dual exposure of cells to both *E. camaldulensis* extracts and a low dose of Cisplatin showed an additional cytotoxic effect on A549 cells in certain concentrations. Conclusions: This study opens novel therapeutic options in combinational therapies of *Eucalyptus camaldulensis* with low-dose CDDP for the treatment of adenocarcinoma cells of human lungs

Keywords: *Eucalyptus*; cisplatin; anti-tumor effect; A549 cells; anti-oxidant effect; cytotoxic effect



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1. Introduction

Lung cancer represents a major public health problem worldwide. It killed approximately 1,590,000 individuals in 2012 and currently is the first leading cause of cancer death in men and the second one in women [1]. Most patients with lung cancer eventually die of this disease or its treatment-related complications [2]. Since conventional therapies have failed to make a major impact on survival, alternative approaches are necessary in the battle against lung cancer [3]. Plant derivatives or extracts offer a valuable source of new medicinal agents, including anti-cancer ones. There are thousands of medicinal plants

species in the world, but only a small percentage have been tested for their anti-oxidant and anti-neoplastic activities, and only a very small percentage of these tested plants have been studied in clinical trials. Eucalyptus, a widely distributed plant over the Australian continent, is one of those medicinal plants [4]. Its medicinal properties have attracted the attention of researchers in many countries. Essential oils of the leaves of certain *Eucalyptus* species have antimicrobial, anti-inflammatory, and cytotoxic activities [5]. Eucalyptol (1,8-cineole), an essential oil isolated from *Eucalyptus* species, has been shown to have anti-inflammatory and antioxidant effects in various diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [6]. There have been numerous studies on the anti-tumor effect of *E. camaldulensis* on cancer cells [7,8]. In parallel, a previous study showed that Lebanese plants were subjected to changes in ingredients with altitude and, therefore, may lose their medical values [9]. Therefore, another study showed the influence of light quality on the antioxidant activity in callus cultures of *Rhodiola imbricate* [10]. Another study has shown that the climate (as influenced by altitude) is the main factor influencing yarrow composition and properties. *Achillea collina* leaf extracts from the higher experimental site showed a 2–4-fold increase in the chlorogenic acid level [11]. Cisplatin (CDDP) is a small platinum compound used for treatment of various human cancers including lung cancer, primarily non-small cell lung cancer (NSCLC), yet, side effects are not uncommon [12]. Therefore, the use of low-dose CDDP in combination with medicinal plants with anti-neoplastic activity may represent a potential safe alternative approach on non-cancer cells, while maintaining similar efficacy on cancer cells [13]. In this study, we investigated the effect of ethanol or water extracts obtained from two different altitudes of Lebanese *Eucalyptus* species alone or in combination with low doses of Cisplatin on adenocarcinoma cells of human lungs.

2. Results

2.1. Chemical Testing Results

2.1.1. Extraction Yields of Crude Extracts *E. camaldulensis*

Table 1 below shows the yield percentage of active substances extracted from both the aqueous and ethanoic extracts of *E. camaldulensis*. The results confirm that the active substances are more readily extracted in the ethanoic solvent than the aqueous solvent.

Table 1. Extraction yields of *E. camaldulensis* crude extract.

| Extraction Solvent | Initial Weight (g) | Final Weight (g) | Extraction Yield (%) |
|--------------------|--------------------|------------------|----------------------|
| Ethanol | 10 | 0.91 | 9.1 |
| Water | 10 | 0.69 | 6.9 |

2.1.2. Phytochemical Screening Tests

Table 2 shows the results of the phytochemical screening of the ethanoic and aqueous extracts of *E. camaldulensis* collected at two different altitudes. No qualitative difference in the chemical composition of the plants of two different altitudes was observed. On the other hand, an important difference in composition was observed between the ethanolic and aqueous extract of the same studied plant. The ethanolic and aqueous extract contains approximately the same proportion of glucides, tannins, and lignins. Alkaloids, amino acids and proteins, flavanones, anthocyanins, and fixed oils and fats are absent in both extracts. The ethanolic extract contains more resins, diterpenes, sterols, phenols, and flavonoids than the aqueous extract. Reducing sugars, terpenoids, saponins, and quinones are present only in the ethanolic extract. Glycosides, phlobatannins, and anthraquinones are present only in the aqueous extract.

Table 2. Phytochemical screening tests of the ethanolic and aqueous extracts of *E. camaldulensis*.

| Altitudes Components | Ethanol | | Water | |
|-----------------------------|---------|-----|-------|-----|
| | 345 m | 0 m | 345 m | 0 m |
| Alkaloids | – | – | – | – |
| Tannins | + | + | + | + |
| Resins | +++ | +++ | + | + |
| Saponins | + | + | – | – |
| Phenols | +++ | +++ | ++ | ++ |
| Terpenoids | + | + | – | – |
| Flavonoids | ++ | ++ | + | + |
| Glucides | + | + | + | + |
| Reducing Sugars | + | + | – | – |
| Quinones | + | + | – | – |
| Sterols | ++ | ++ | + | + |
| Glycosides | – | – | + | + |
| Diterpenes | +++ | +++ | + | + |
| Anthraquinones | – | – | + | + |
| Proteins and amino acids | – | – | – | – |
| Lignins | + | + | + | + |
| Phlabotannins | – | – | + | + |
| Anthocyanins | – | – | – | – |
| Flavanones | – | – | – | – |
| Fixed Oils and Fats | – | – | – | – |

+ = low amount, ++ = moderate amount, +++ = high amount, – = null amount.

2.1.3. Chemical Quantitative Tests

Total Phenolic, Flavonoids, and Tannins Contents

The TPC, TFC, and TTC of the two altitude-different *E. camaldulensis* species were estimated. As shown in Table 3, the sea-level (0 m) altitude plant contains slightly more TPC and TFC than that of the 345 m altitude. Meanwhile, both species contain a moderately low content of tannins. Moreover, the ethanolic extract contains a medium amount of flavonoids, which is higher than the aqueous extract. In addition, both extracts contain a high percentage of phenols, with a higher amount in the ethanolic extract. However, both extracts contain a relatively low amount of tannins. These results suggest that *E. camaldulensis* species contain high contents of phenols in their parts.

Table 3. Quantification of total phenolic (TPC), flavonoids (TFC), and tannin (TTC) contents in different extracts.

| Altitudes Total Contents (mg/g) | Ethanol | | Water | |
|------------------------------------|---------|-------|-------|------|
| | 345 m | 0 m | 345 m | 0 m |
| TPC | 100.4 | 110.8 | 74.6 | 85.4 |
| TFC | 46.7 | 54 | 32.3 | 40.2 |
| TTC | 29.3 | 30 | 31.4 | 31 |

Percentages of Alkaloids, Lipids, and Saponins

As shown in Table 4, both extracts contain a relatively low quantity of saponins with a higher amount in the sea-level altitude species. In addition, both extracts contain small percentages of lipids and alkaloids.

Table 4. Percentages of alkaloids, lipids, and saponins in *E. camaldulensis*.

| Altitudes Percentages (%) | 345 m | 0 m |
|---------------------------|-------|-----|
| % of Alkaloids | 10 | 10 |
| % of Lipids | 10 | 10 |
| % of Saponins | 20 | 30 |

Percentages of Moisture and Ash

According to Table 5, there is no remarkable difference in the percentages of moisture and ash between the two altitude-different *E. camaldulensis* species. Both contain a low percentage of moisture and a high percentage of ash.

Table 5. Percentages of moisture and ash in *E. camaldulensis*.

| Altitudes Percentages (%) | 345 m | 0 m |
|---------------------------|-------|-----|
| % of moisture | 10 | 9 |
| % of ash | 90 | 91 |

Antioxidant Activity

The antioxidant effect of the increasing concentrations of the prepared extracts was measured using DPPH reagent (Table 6 and Figures 1 and 2). As shown in Figures 1 and 2, the DPPH radical scavenging activity increases as the concentration of the extract increases. The data showed that the ethanolic and aqueous extracts of *E. camaldulensis* (0 m) have relatively higher DPPH radical scavenging activities compared to *E. camaldulensis* (345 m). Moreover, the ethanolic extract in both plants exhibits a higher scavenging activity than the aqueous extract, but the comparison was much more significant in the case of *E. camaldulensis* (345 m).

Table 6. DPPH absorbance of the different concentrations of ethanolic and aqueous extracts.

| Altitudes Concentration of the Extract (g/mL) | Ethanol | | Water | |
|---|--------------|--------------|--------------|--------------|
| | 345 m | 0 m | 345 m | 0 m |
| Control | 0.792 ± 0.09 | 0.81 ± 0.04 | 0.669 ± 0.08 | 0.64 ± 0.2 |
| 0.005 | 0.768 ± 0.1 | 0.773 ± 0.1 | 0.573 ± 0.04 | 0.552 ± 0.08 |
| 0.01 | 0.725 ± 0.07 | 0.697 ± 0.03 | 0.436 ± 0.05 | 0.388 ± 0.05 |
| 0.02 | 0.605 ± 0.2 | 0.54 ± 0.06 | 0.188 ± 0.02 | 0.153 ± 0.02 |
| 0.03 | 0.482 ± 0.06 | 0.43 ± 0.2 | 0.112 ± 0.01 | 0.118 ± 0.03 |
| 0.04 | 0.334 ± 0.02 | 0.24 ± 0.1 | 0.107 ± 0.02 | 0.109 ± 0.02 |
| 0.05 | 0.145 ± 0.05 | 0.085 ± 0.02 | 0.103 ± 0.01 | 0.103 ± 0.04 |

2.1.4. Results of Antiproliferative Assay

Exposure of A549 Cells to the Aqueous Extract

The data shown in Figures 3 and 4 demonstrate that there is a significant decline in the percentage of cell proliferation in reference to the control cells in aqueous extract (345 m) at concentrations ≥ 250 $\mu\text{g/mL}$ after 24 or 48 h of treatment. Meanwhile, this remarkable decrease is observed at concentrations ≥ 200 $\mu\text{g/mL}$ after 24 or 48 h of treatment in case of aqueous extract (0 m). The 50% inhibitory concentrations (IC_{50}) upon treatment with extract from the 345 m altitude plant are 480, 390, and 275 $\mu\text{g/mL}$ at 24, 48, and 72 h, respectively, whereas, the IC_{50} upon treatment with the 0 m plant extract are 350, 275, and 230 $\mu\text{g/mL}$ at 24, 48, and 72 h, respectively. Additionally, at concentrations ≥ 200 $\mu\text{g/mL}$ in both species, the antiproliferative effect is time-dependent between 24, 48, and 72 h. Before this concentration, a remarkable effect is observed at 72 h. Since the treatment with aqueous extracts from the two different-altitude plants at 72 h induces a significant decrease in cell

proliferation from a low concentration (25 $\mu\text{g}/\text{mL}$). On the other hand, we do not have the same effects at 24 and 48 h. Further analysis confirmed that the variation between the two altitudes is significant at certain levels (see below). Thus, exposure of A549 cells to the aqueous extract is dose- and time-dependent at certain levels. The last three concentrations with the highest effects are used for later combined treatment with CDDP.

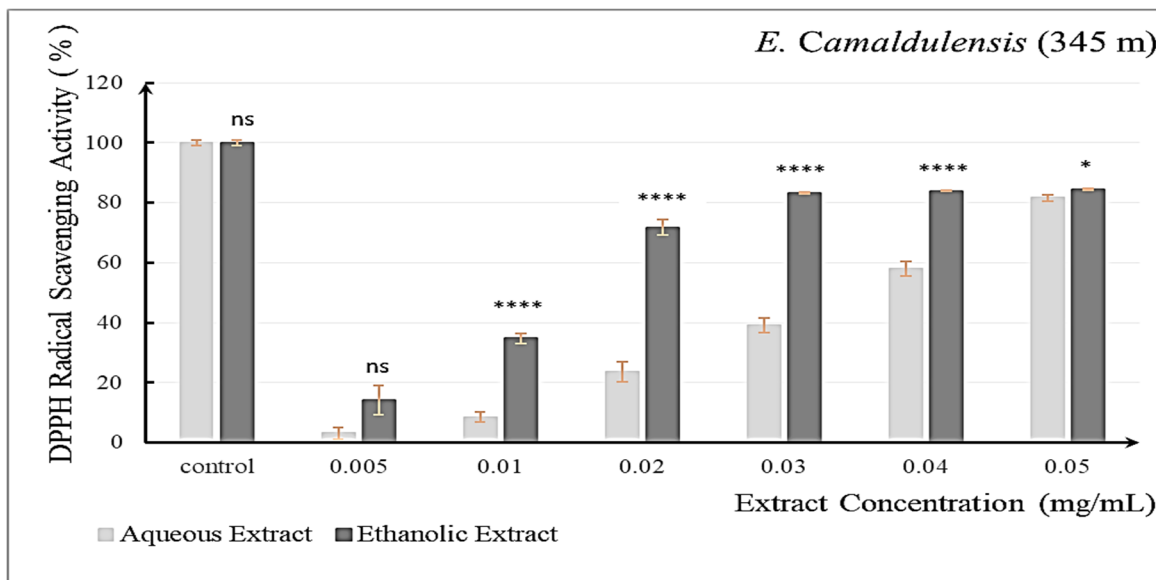


Figure 1. Radical scavenging activity of the ethanolic and aqueous extracts of *E. camaldulensis* (345 m). Peak values are represented as mean \pm SD. Statistical analysis: Unpaired *t* test or Mann–Whitney test. * $p < 0.05$; **** $p < 0.0001$. ns refers to non-significant results.

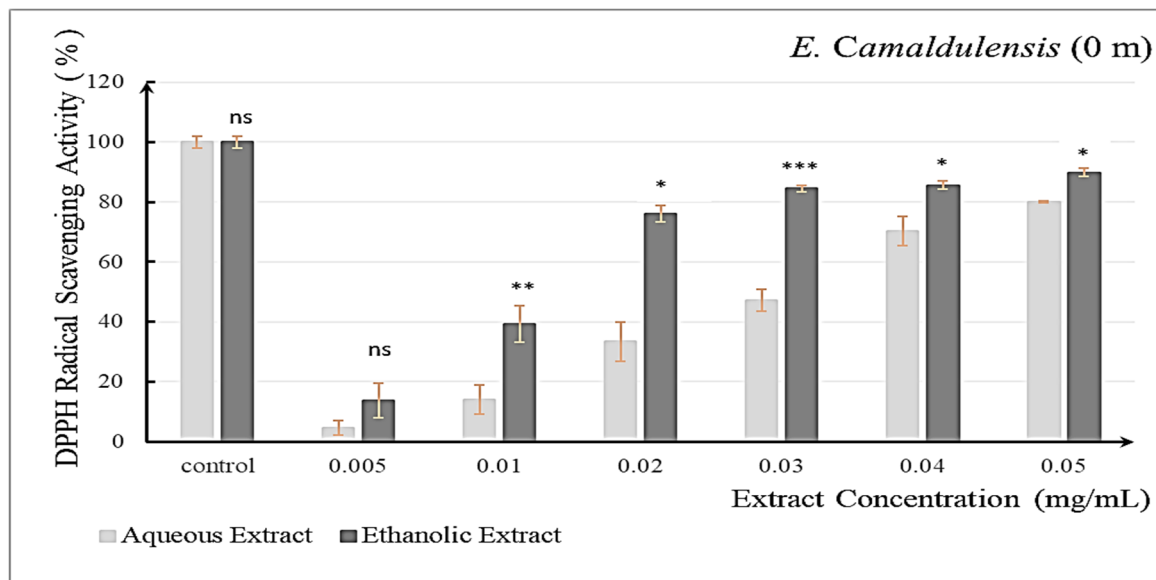


Figure 2. Radical scavenging activity of the ethanolic and aqueous extracts of *E. camaldulensis* (0 m). Peak values are represented as mean \pm SD. Statistical analysis: Unpaired *t* test or Mann–Whitney test. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. ns refers to non-significant results.

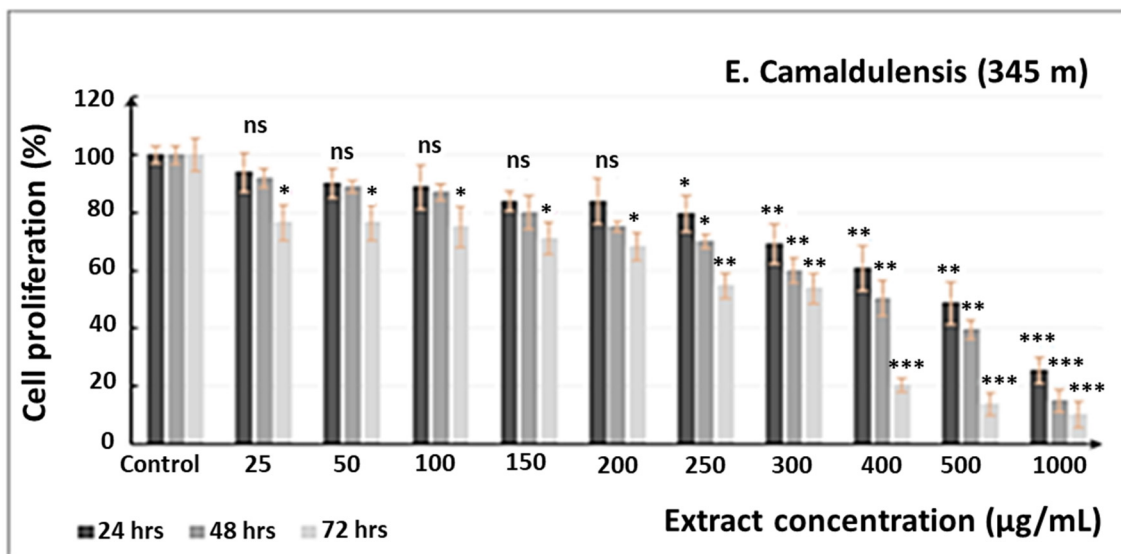


Figure 3. Cell proliferation of A549 cells after the exposure to the aqueous extract of *E. camaldulensis* (345 m) for 24, 48, and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in which percentage of proliferation of treated cells is compared with that of control at 24, 48, or 72 h. ns refers to non-significant result.

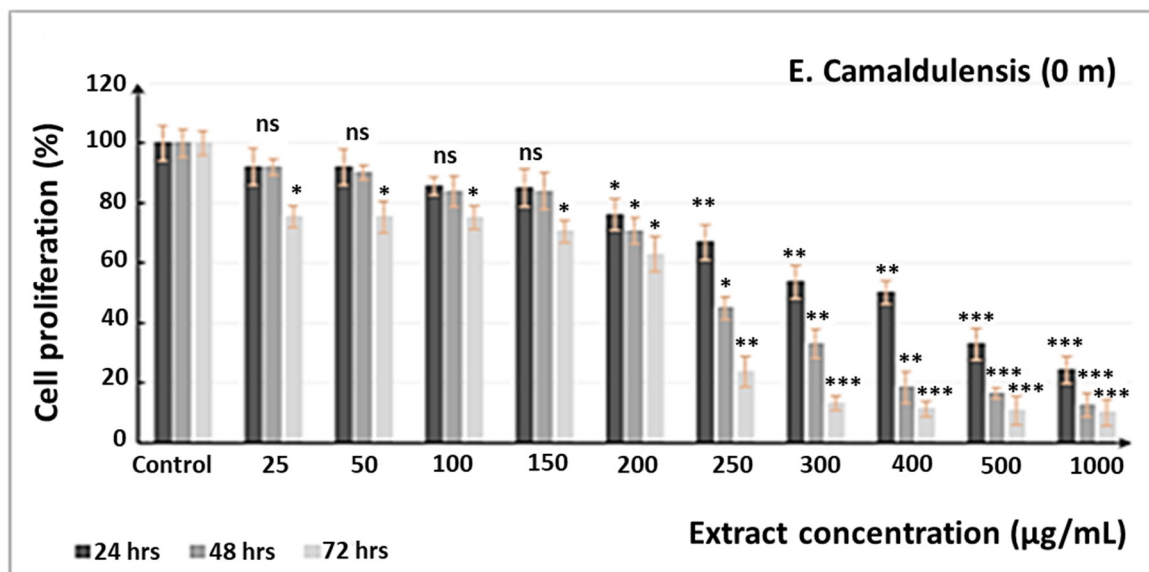


Figure 4. Cell proliferation of A549 cells after the exposure to the aqueous extract of *E. camaldulensis* (0 m) for 24, 48, and 72 h. Values are represented as mean \pm SD. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in which percentage of proliferation of treated cells is compared with that of control at 24, 48, or 72 h. ns refers to non-significant result.

Exposure of A549 to the Ethanolic Extract

The data in Figures 5 and 6 show that there is a noticeable decline in percentage of cell proliferation in reference to the control-DMSO in the ethanolic extract (345 m) at concentrations ≥ 150 $\mu\text{g/mL}$ after 24, 48, or 72 h of treatment. Meanwhile, this remarkable decrease is observed at concentrations ≥ 100 $\mu\text{g/mL}$ after 24, 48, or 72 h of treatment in the case of the ethanolic extract (0 m). The 50% inhibitory concentrations (IC_{50}) upon treatment with the 345 m plant extract are 300, 275, and 175 $\mu\text{g/mL}$ at 24, 48, and 72 h, respectively, whereas, the IC_{50} upon treatment with the 0 m plant extract are 275, 200, and 150 $\mu\text{g/mL}$ at 24, 48, and 72 h, respectively.

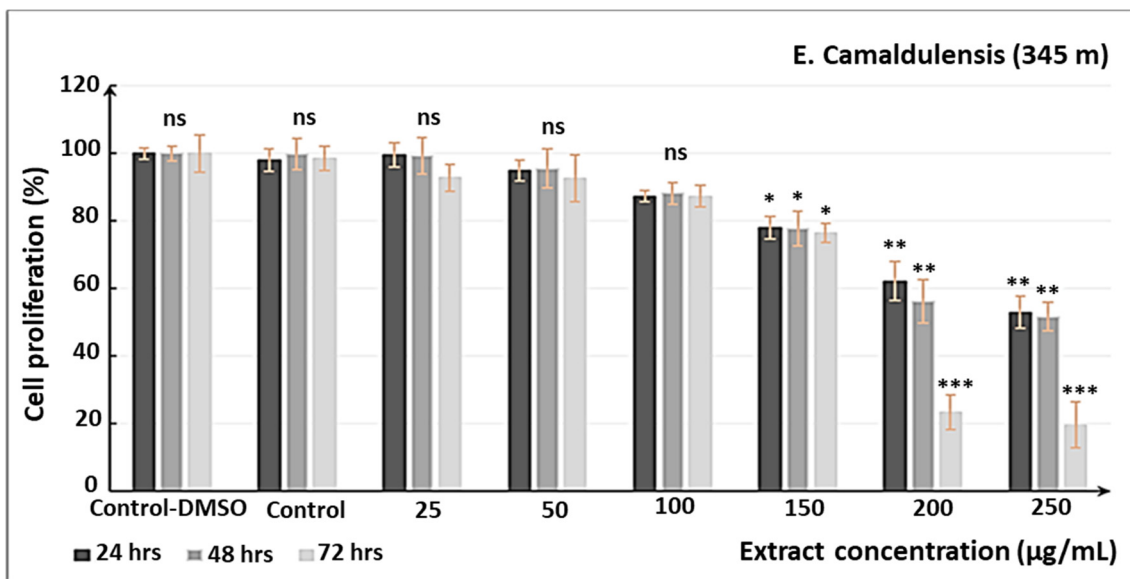


Figure 5. Cell viability of A549 cells after the exposure to ethanolic extract of *E. camaldulensis* (345 m) for 24, 48, and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in which percentage of proliferation of treated cells is compared with that of control-DMSO at 24, 48, or 72 h. ns refers to non-significant result.

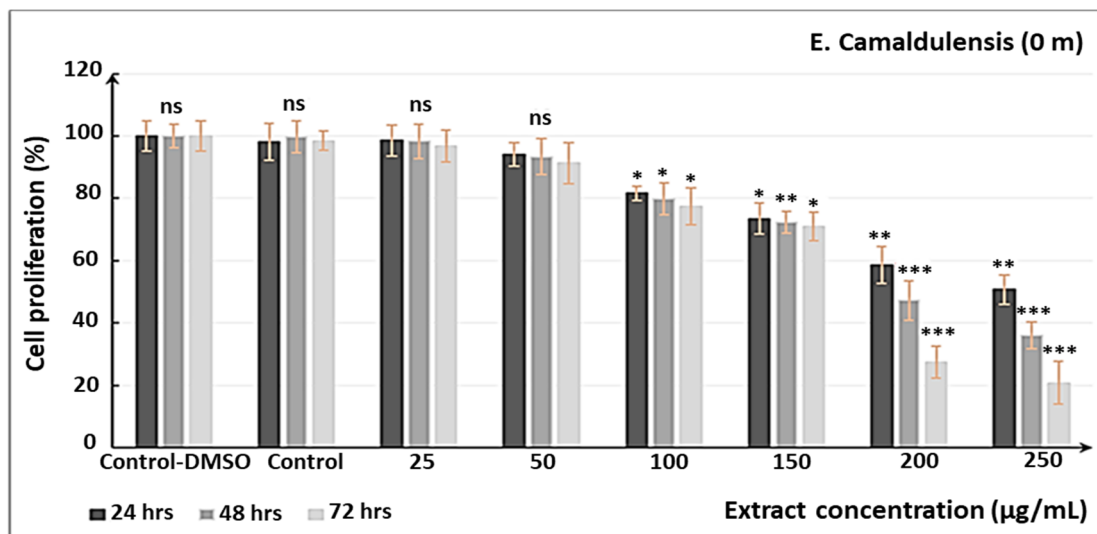


Figure 6. Cell proliferation of A549 cells after the exposure to ethanolic extract of *E. camaldulensis* (0 m) for 24, 48 and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in which percentage of proliferation of treated cells is compared with that of control-DMSO at 24, 48, or 72 h. ns refers to non-significant result.

Additionally, at concentrations ≥ 200 $\mu\text{g/mL}$ in both species the antiproliferative effect is time dependent. Further analysis confirmed that the variation between the two altitudes is significant at certain levels as shown below. Thus, treatment of A549 with the aqueous extract is dose- and time-dependent at certain levels. The last three concentrations with the highest effects are chosen for later combination with CDDP.

Treatment of A549 Cells with CDDP Alone

As can be seen in Figure 7, the antiproliferative effect caused by Cisplatin is dependent on the dose. In other words, the percentage of cell proliferation declines noticeably as the

dose of cisplatin increases in reference to the control. Moreover, are IC_{50} upon exposure to CDDP are 10, 6, and 5.5 $\mu\text{g}/\text{mL}$ at 24, 48, and 72 h, respectively. Treatment with 4 $\mu\text{g}/\text{mL}$ CDDP for 24 and 48 h caused a small decline in the percentage of cell proliferation in reference to the control. Thus, this least reactive concentration to the A549 cells is chosen for later dual treatment combined with the *E. camaldulensis* extract.

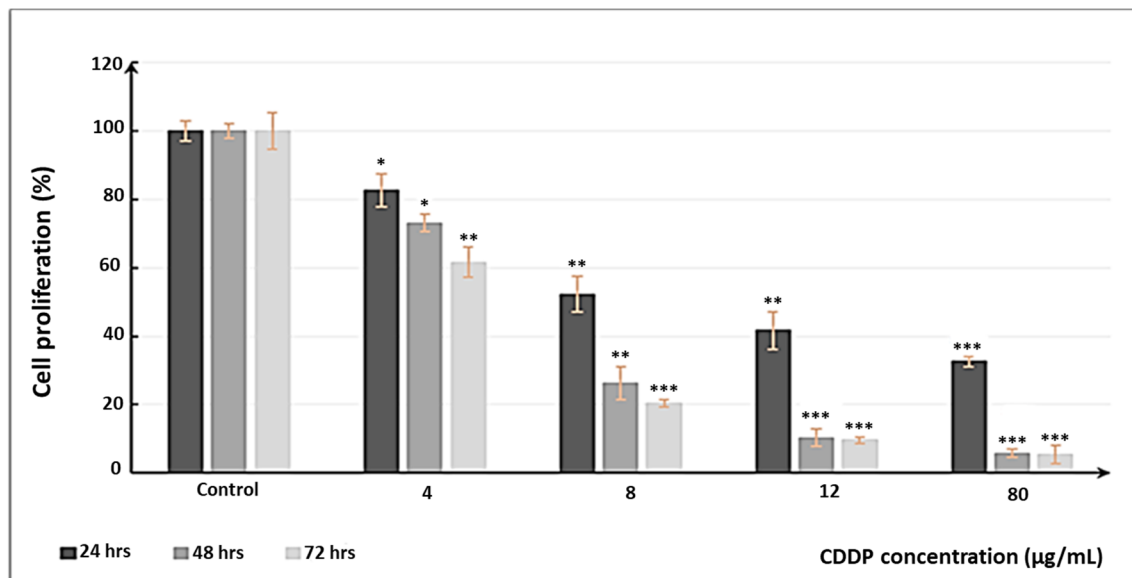


Figure 7. Cell proliferation of A549 cells after exposure to gradual increasing concentrations of CDDP alone for 24, 48, and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in which percentage of proliferation of treated cells is compared with that of control at 24, 48, or 72 h.

Exposure of A549 Cells to a Combination of Cisplatin and Aqueous Extract

As our previous study showed that Eucalyptus extract had a protective role on normal cells when treated with cisplatin, the combination treatment was not tested in this study on normal cells.

As shown in Figure 8, the percentage of cell proliferation of A549 cells after exposure to the 0 m aqueous extract in concentrations of 400 or 500 $\mu\text{g}/\text{mL}$ is significantly less than that of the 345 m aqueous extract. There is no significant difference in the percentage of cell proliferation between the two different-altitude species at concentration 1000 $\mu\text{g}/\text{mL}$. In addition, the cell proliferation of A549 cells after treatment with combination of CDDP and the 345 m aqueous extract in concentrations of 400 or 500 $\mu\text{g}/\text{mL}$ is significantly less than that of the extract alone at 24 and 48 h. Meanwhile, at 72 h, whether the combination is in the concentration of 400 or 500 $\mu\text{g}/\text{mL}$, it yields a significantly higher percentage of cell proliferation than that with extract alone, suggesting in that case an antagonism between CDDP and the extracts. Moreover, the cell proliferation of A549 cells following treatment with a combination of CDDP and the 0m aqueous extract in concentrations of 400, 500, or 1000 $\mu\text{g}/\text{mL}$ is significantly less than that of extract alone at 24 h. Meanwhile, at 48 and 72 h, similar combinations yield a significantly higher percentage of cell proliferation than that with extract alone for concentrations of 400 or 500 $\mu\text{g}/\text{mL}$ and a non-significant difference for 1000 $\mu\text{g}/\text{mL}$.

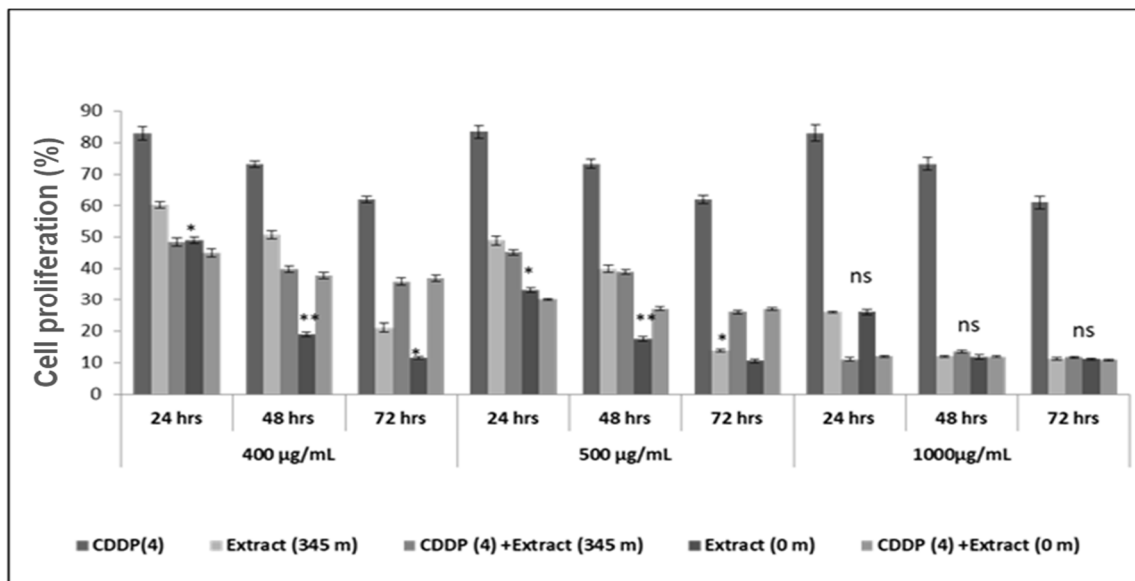


Figure 8. Comparison between the cell proliferation of A549 cells after treatment with CDDP, water extract, and a combination of both at 24, 48, and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Unpaired *t* test or Mann–Whitney test. * $p < 0.05$; ** $p < 0.05$ in which percentage of proliferation of cells treated with 0m extract is compared to that of cells treated with 345 m extract. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.05$; ** $p < 0.05$ in which percentage of cell viability of cells treated with CDDP–extract is compared to that of cells treated with extract alone. ns refers to non-significant results.

Treatment of A549 Cells with a Combination of CDDP and Ethanolic Extract

As illustrated in Figure 9, there is a noticeable decline in the percentage of cell proliferation of A549 cells after exposure to combinations of CDDP (4 μ g/mL) and different ethanolic extract concentrations (150, 200, and 250 μ g/mL) in reference to the control.

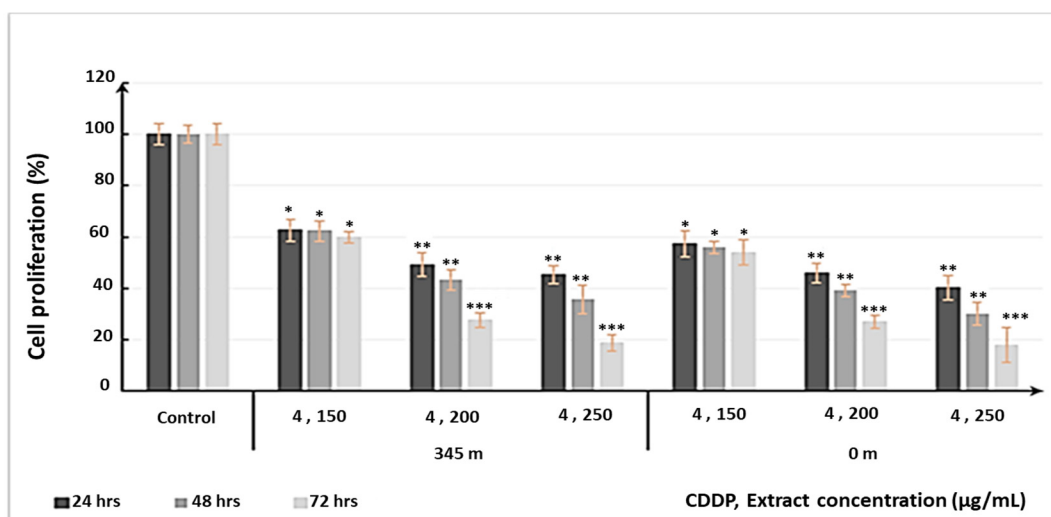


Figure 9. Cell proliferation of A549 cells after exposure to gradually increasing concentrations of Cisplatin and ethanolic extract for 24, 48, and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Paired *t* test or Wilcoxon test. * $p < 0.01$; ** $p < 0.01$; *** $p < 0.001$ in which percentage of proliferation of treated cells is compared with that of control at 24, 48, or 72 h.

Regarding the two different-altitude species, there is no remarkable difference in the percentage of cell proliferation.

As shown above in Figure 10, the percentage of cell proliferation of A549 cells after treatment with the 0m ethanolic extract in concentrations of 150, 200, or 250 µg/mL is significantly less than that of the 345 m ethanolic extract in all cases, excluding 200 and 250 µg/mL at 72 h. In addition, the percentage of cell proliferation of A549 cells after exposure to a combination of Cisplatin and the 345 m or 0m ethanolic extracts in concentrations of 150, 200, or 250 µg/mL is significantly less than that of extract alone, except at 72 h for 200 and 250 µg/mL.

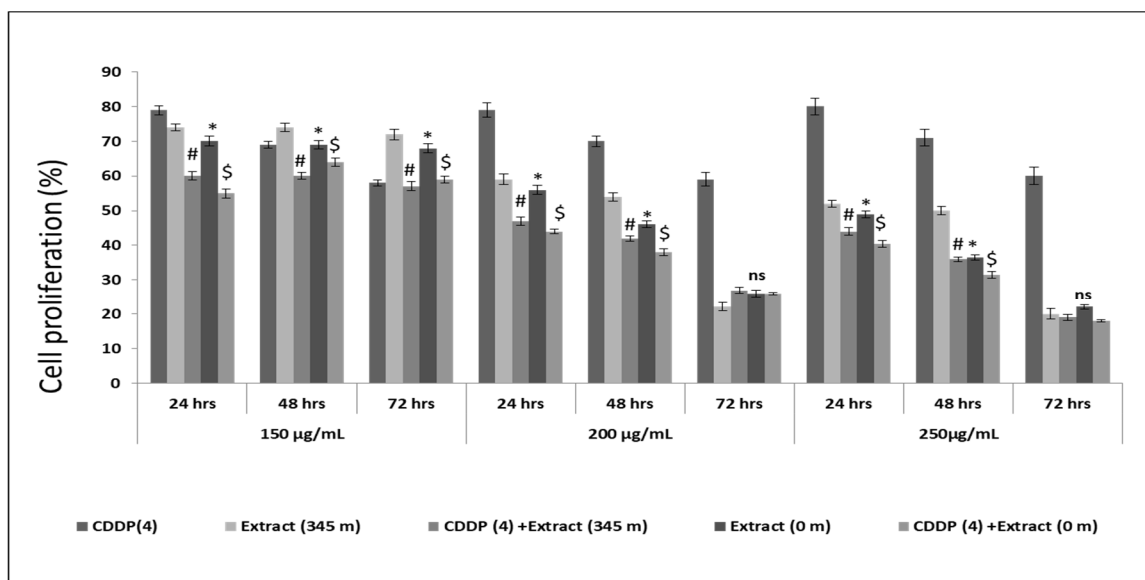


Figure 10. Comparison between the cell proliferation of A549 cells after treatment with CDDP, ethanolic extract, and a combination of both at 24, 48 and 72 h. Peak values are represented as mean \pm SD. Statistical analysis: Unpaired t test or Mann–Whitney test. * $p < 0.05$ in which percentage of proliferation of cells treated with 0 m-extract is compared to that of cells treated with 345 m-extract. Statistical analysis: Paired t test or Wilcoxon test # $p < 0.05$ in which percentage of proliferation of cells treated with a combination of CDDP–extract 345 m is compared to that of cells treated with extract 345 m alone. Paired t test or Wilcoxon test \$ $p < 0.05$ in which percentage of proliferation of cells treated with a combination of CDDP–extract 345 m is compared to that of cells treated with extract 345 m alone. ns refers to non-significant results.

3. Discussion

Many traditional medicinal plants were investigated for their anti-tumor effect in many cancers, and anti-tumor substances were found in them. As an illustration, an early study demonstrated that treatment with pomegranate fruit extract results in a decline in the viability of A549 cells but had only minor effects on normal human bronchial epithelial (NHBE) cells as assessed by the MTT(3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) and Trypan blue assays study [14]. In addition, in vitro studies on human chronic myelogenous leukemia cells (K562 cells) confirmed the cytotoxic effect of ethanolic extract of *E. camaldulensis* on the latter. [15]. Regarding the cytotoxic effect of essential oils of *Eucalyptus*, the data are remarkably restricted. A study on Jurkat (J774A.1) (immortalized T lymphocytes cell line) and HeLa cell lines (cell line of cervical tumour cells) proved the cytotoxic effect of essential oils of *E. benthamii* leaves on the latter. Moreover, *E. sideroxydon* and *E. torquata* were investigated for the cytotoxic effect of the volatile oils and extracts of their stems, leaves, and flowers against the human breast adenocarcinoma cell line (MCF7), and their effect was confirmed. [16]. In this study, the ethanolic and aqueous extracts of two different-altitude *E. camaldulensis* species were evaluated for their antioxidant effects. Then, qualitative and quantitative chemical analyses of active constituents of these four different extracts were performed. Finally, the cytotoxic effect of the four different extracts alone or combined with a low dose of chemotherapeutic drug (CDDP) on A549 cells was

studied. The chemical compositions of Eucalyptus extract for the two altitudes revealed a complex structure with different traces of monosaccharides. The different colorimetric assays confirmed that both altitudes were composed primarily of fucose. The molar ratios indicated that the monosaccharide composition was equal in both extracts, similarly to the molar ratio of fucose with respect to sulfate, which was 1:0.1 in both cases (data not shown).

The percentage yield of most plant extracts proves that the extent of the extraction capacity of ethanol was higher than other solvents. In this study, it was demonstrated that the ethanolic extract resulted in a higher yield than the aqueous one. As a confirmation, another study showed approximately the same yield percentages [17]. Phytochemical screening is an important method to investigate the involvement of a certain herbal substance in the prevention or treatment of a disease. In this study, qualitative testing confirmed that the two different-altitude plants have the same secondary metabolite profile, where it was concluded that both extracts contain glucides, tannins, lignins, resins, diterpenes, sterols, phenols, and flavonoids. As has been noted, the last five metabolites are richer in the ethanolic extract. Alkaloids, proteins and amino acids, anthocyanins, flavanones, and fixed oils and fats are absent in both extracts. The ethanolic extract contains reducing sugars, terpenoids, saponins, and quinones. Glycosides, phlobotannins, and anthraquinones are present only in the aqueous extract. In aqueous extracts, some active compounds were present in small amounts compared with the ethanolic solvents. A possible reason behind this is that these compounds (such as alkaloids and oils) are insoluble in water and thus cannot be revealed by the aqueous extraction. Phenols, terpenoids, and sterols were used as anti-oxidants, anti-cancer, anti-viral, anti-inflammatory, anti-yeast, and anti-microbial compounds. Tannins were reported to have important anti-oxidant and anti-cancer activities. Moreover, flavonoids were confirmed to have several biological effects and an apoptosis-inducing effect. Certain toxins produced from resins in the roots and rhizomes of plants are the starting material for the semi-synthesis of anti-cancer agents. Furthermore, quantitative analysis confirmed that both species contain a high amount of phenols, a medium amount of flavonoids, and a low amount of tannins. Nevertheless, it was shown that the 0 m altitude species contains a slightly higher amount of phenols and flavonoids, whereas higher contents of flavonoids and phenols were found in the ethanolic extract than the aqueous extract.

With respect to altitude and its effect on the chemical composition of a plant and the presence of secondary metabolites, a study on the Lebanese *E. creticum* grown both on 300 m and 1200 m altitudes proved that the chemical composition of the plant differed significantly depending on the altitude of growth. The change in the chemical composition included decreased amounts of secondary metabolites (especially flavonoids, phenols, and polyphenols) in plants growing in higher altitudes (1200 m). This leads to a consequent decrease in antioxidant activity, anti-inflammatory activity, and anti-proliferative activity of the plant extracts. This effect may be due to the changes in temperature, atmospheric pressure, oxygen content, and the amount of rain received by the plant at different altitudes, which may directly alter the chemical composition of the plant to accommodate for these changes [9]. Therefore, another study showed the influence of light quality on the antioxidant activity in callus cultures of *Rhodiola imbricate* [10]. Another study has shown that the climate (as influenced by altitude) is the main factor influencing yarrow composition and properties. *Achillea collina* leaf extracts from the higher experimental site showed a 2–4-fold increase in the chlorogenic acid level [11].

Furthermore, it was proven that the ethanolic extract and the 0 m altitude species have a higher DPPH radical scavenging activity than the aqueous extract and the 345 m altitude species, respectively. Concerning the Neutral Red assay, as time of treatment increases, the IC_{50} decreases, regardless the type of solvent or the altitude of the plant. For both solvents, a noticeable decline in the percentage of cell viability is observed at a lower concentration in the 0 m altitude species compared to the 345 m altitude one. For both species, the ethanolic extracts results in a noticeable decline in cell viability compared to that of the

aqueous one. For both extracts, the decline in the percentage of cell viability is dependent on time at high concentrations. Moreover, the dual treatment with the aqueous extract and Cisplatin combined decreased the percentage of cell viability less than the extract alone, but only after 24 h of treatment. This might be related to the effect of combination in the long term. In addition, the combination of ethanolic extract with CDDP brings down the percentage of cell viability to be less than the extract alone, but only after 24 h or 48 h of treatment. A higher effect is observed at some levels for both extracts after combinational treatment for 48 or 72 h. This might be related to CDDP resistance after this prolonged time. Hence, we can conclude that the ethanolic extract and the 0 m altitude species have higher cytotoxicity than the aqueous extract and 345 m altitude species, respectively. Additionally, the combination with CDDP is the best at 24 h of treatment. Furthermore, we have previously studied the effect of eucalyptus extract and cisplatin in combination on normal cells. We found that eucalyptus extract played a protective role on peripheral blood mononuclear cells (PBMC), as cell viability had increased when the former was added to cisplatin [8]. Thus, we can conclude that there exists a relationship between the anti-oxidant activity, the secondary metabolite content, and the anti-tumor effect of plant extracts. Cancer patients under treatment with Cisplatin, alone or simultaneously with any other chemotherapeutic drugs, suffer from various side effects. In this context, organometallic compounds, which are defined as metal complexes containing at least one direct, covalent metal–carbon bond, have been found to be promising anticancer drug candidates [18,19]. Hence, to minimize the side effects is the idea of treatment with cisplatin combined with the extract of *E. camaldulensis*. The results of the combinations proved that *E. camaldulensis* extracts intensify the anti-tumor effect of low-dose CDDP. This opens a new era in the manufacture of pharmaceutical drugs containing *E. camaldulensis* as an active ingredient.

4. Materials and Methods

4.1. Plant Collection and Preparation of Powder

Fresh leaves of Lebanese *E. camaldulensis* (a member of the family Myrtaceae), with a voucher specimen number 1902 [20], were collected from Beirut (at an altitude of 0 m, Coordinates: 33°49' N 35°31' E) in January 2017 and from south Lebanon, Irkay district (altitude of 345 m above sea level; Coordinates: 33.4623° N, 35.4151° E), between February and March 2017 (Figure 11). Leaves were washed carefully with distilled water to remove any impurities and then dried in the shade, at ambient temperature, and inside a zone free of moisture. Then, dried leaves were grinded manually using mortar and pestle to be transformed into powder. The powder was preserved into clean plastic containers, kept away from light, heat, and moisture until use.



Figure 11. *Eucalyptus camaldulensis* Tree.

4.2. Preparation of Crude Extracts

Extraction by ultrasound was used to prepare the crude extracts. The raw material immersed in the solvent was subjected to the action of ultrasound. It is a simple technique with a very short extraction time. Then, 10 g of powdered leaves of *E. camaldulensis* were placed in a flask containing 150 mL of selected solvent: (Ethanol or water). The flask was then placed in an ultrasound generating apparatus containing water maintained at a temperature of 60 °C for 60 min. Then, the extracts were filtered by Buchner funnel under reduced pressure to remove insoluble residues, condensed by a rotary evaporator, frozen, and finally introduced into a freeze-dryer until completely dried to powder, ready to be used in numerous tests.

The obtained residue was weighed, and the percentage yield was determined according to the following formula:

$$\% \text{ Yield} = (\text{obtained mass of sample extract} / \text{initial mass of powdered sample used}) \times 100$$

4.3. Chemical Analysis

4.3.1. The Qualitative Tests (Phytochemical Screening)

To study the chemical composition of the extracts taken from the plants, a qualitative detection of secondary metabolites was performed:

- Detection of alkaloids: The detection and determination of alkaloids was practiced by the use of the Dragendorff's reagent. Five drops of Dragendorff's reagent were then added to 1 mL of each filtered extract. The appearance of a red orange precipitate indicated the presence of alkaloids.
- Detection of tannins: 1 mL of each filtered extract was mixed with 1 mL of a 1% ferric chloride solution. The appearance of a blue color indicated the presence of tannins.
- Detection of resins: 1 mL of each extract was mixed with acetone and shaken with a little water. The presence of resins was indicated by the observation of a turbidity of the mixture.
- Detection of the saponins: 2 mL from each extract was shaken vigorously for 5 min in a vortex. The presence of saponins was indicated by the appearance of foam.
- Detection of phenols: In beakers, 5 mL of each of the filtered extract was mixed with 1 mL of FeCl₃ (1%) and 1 mL of K₃(Fe(CN)₆) (1%). The appearance of a greenish blue color indicated the presence of phenols.
- Detection of terpenoids: The Salkowski test was applied by adding 2 mL of chloroform and 3 mL of concentrated sulfuric acid to 1 mL of each of the filtered extracts. The presence of a reddish-brown color on the surface of the mixture indicated the presence of terpenoids.
- Detection of flavonoids: In test tubes containing 5 mL of each extract, 5 mL of 50% potassium hydroxide solution was added. The observation of a turbid yellow color indicated the presence of flavonoids.
- Detection of carbohydrates: α -naphthol and then concentrated sulfuric acid were added to the extract. The formation of a violet ring indicates the presence of sugars.
- Detection of reducing sugars: 0.5 mL of each extract was dissolved in 1 mL water and mixed with 5–8 drops of Fehling's (A+B) then boiled for a few minutes. The formation of a brick red precipitate indicated the presence of sugars.
- Detection of quinones: 1 mL of each extract was mixed with concentrated hydrochloric acid. The formation of a yellow precipitate indicated the presence of quinones.
- Detection of sterols and steroids: 1 mL of each extract was mixed with 2 mL of chloroform, and then 2 mL of concentrated sulfuric acid was added. The red coloration of the upper layer and a fluorescence greenish yellow in the acid layer indicated the presence of sterols and steroids.
- Detection of glycosides: 2 mL of each extract was mixed with 1 mL of glacial acetic acid in a test tube, and a drop of 5% ferric chloride solution was added to this tube.

Then, 1 mL of the concentrated sulfuric acid was carefully added. A greenish brown ring forming at the interface indicated the presence of glycosides.

- Detection of diterpenes: copper acetate test: the extracts were dissolved in the water, and then a few drops of copper acetate solution were added. The formation of a green color crystal indicated the presence of diterpenes.
- Detection of anthraquinones: 1 mL of 10% hydrochloric acid was added to 1 mL of each extract present in a tube. This tube was placed in a water bath and was boiled. Then, it was filtered and cooled. Chloroform was then added and stirred while drops of ammonium (10%) were added and boiled. The formation of pink color indicated the presence of the anthraquinones according to Siddiqui.
- Detection of proteins and amino acids: The ninhydrin test: 0.25% of the ninhydrin reagent was added to 1 mL of each extract and boiled for a few minutes. The formation of blue color indicated the presence of amino acids.
- Detection of the lignins: 2 mL of each extract was mixed with safranin. A pink color indicated the presence of the lignins.
- Detection of Phlobatannins: 1 mL of each extract was mixed with 1 mL of 1% hydrochloric acid and boiled for 5 min and then cooled. The formation of a red precipitate indicated the presence of phlobatannins.
- Detection of anthocyanins: 1 mL of each extract was mixed with 1 mL of 10% sodium hydroxide solution. Change in color to blue indicated the presence of anthocyanins.
- Detection of Flavanones: 1 mL of each extract was mixed with 1 mL concentrated sulfuric acid solution. Formation of purple red color indicated the presence of the flavanones.
- Detection of fixed oils and fats: The Spot Test was used to detect fixed oils and greases. A small amount of extract was placed between two filter papers. Oil spots produced with any extract indicated the presence of fixed oils and fats.

4.3.2. Chemical Quantifications of Secondary Products

- Total Phenolic Content (TPC):

The Folin–Ciocalteu reagent method was used to estimate TPC. First, 100 μ L of each extract (1mg/mL), 0.5 mL of Folin–Ciocalteu reagent (1/10 dilution in water), and 1.5 mL of Na_2CO_3 (2%) were mixed. The mixture was incubated in the dark at room temperature for 30 min. The absorbance of blue color solution was measured at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometer. The blank was formed by 0.5 mL of the used solvent and 1.5 mL Na_2CO_3 (2%). The results were expressed in mg of Gallic Acid Equivalent (GAE) per gram of dry weight of plant powders [20]

$$\text{TPC} = \text{GAE} \times V \times D/M$$

where GAE is the Gallic Acid Equivalence (mg/mL); V is the volume of the extract (mL), D is the dilution factor; and M is the mass (g) of the pure extract of the plant.

- Total Flavonoids Content (TFC):

The aluminum chloride method was used for the determination of the TFC. First, 1 mL of each extract (1mg/mL) was mixed with 1 mL of 2% methanolic aluminum chloride solution. After an incubation period of 15 min in the dark at room temperature, the absorbance of all samples was determined at 430 nm using Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg/g of rutin equivalent. The blank was formed of 1 mL of the used solvent and 1 mL (2%) methanolic aluminum chloride solution [20]:

$$\text{TFC} = \text{RE} \times V \times D/M$$

where RE is rutin equivalent (mg/mL), V is the total volume of sample (mL), D is the dilution factor, and M is the mass of the sample (g).

- Total Tannins Content (TTC):

The tannins were determined by the Folin–Ciocalteu method. First, 0.1 mL (10 mg/mL) of the plant extract was added to 7.5 mL of distilled water and 0.5 mL of Folin–Ciocalteu reagent. Then, 1 mL of 35% Na₂CO₃ solution was added, and the mixture was diluted to 10 mL with distilled water. The mixture was stirred well and kept at room temperature for 30 min. A series of gallic acid reference standard solutions (20, 40, 60, 80, and 100 µg/mL) was prepared in the same manner as described above. Absorbance of tested and standard solutions were measured relative to the blank at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometer. The blank was formed of 0.5 mL of the used solvent, 0.5 mL distilled water, and 1 mL 35% Na₂CO₃. The total tannins content was expressed in terms of mg of GAE/g of extract:

$$\text{TTC} = \text{GAE} \times V \times D/M$$

where GAE is the Gallic Acid Equivalence (mg/mL); V is the volume of the extract (mL), D is the dilution factor; and M is the mass (g) of the pure extract of the plant.

- Determination of the Percentage of Alkaloids:

The quantification method for alkaloids determination was used according to Harborne method with some modifications. First, 100 mL of 10% acetic acid in ethanol was added to 1 g of dry powdered plant, and then the extracts were covered and allowed to stand for 4 h. After that, the extracts were filtrated and concentrated in a water bath to 25 mL of their original volumes.

Droplets of concentrated ammonium hydroxide were added to the extract until the precipitation of the whole solution. It was allowed to settle, and then the precipitates were washed with dilute ammonium hydroxide and then filtered using Whatman filter paper of 0.45 µm pores. The residue was dried in the oven at 40 °C and weighed. The alkaloid content was determined using the following formula:

$$\% \text{ alkaloid} = [\text{final weight of the sample}/\text{initial weight of the extract}] \times 100$$

- Determination of Percentage of Lipids:

The percentage of total fat (lipids) was estimated by adding 200 mL of petroleum ether (40–60 °C) to 10 g of powders and then extracted using ultrasound apparatus containing water maintained at 60 °C. After that, the solvent was filtered using a Buchner funnel under reduced pressure and then evaporated using a rotary evaporator at 40 °C. Finally, the weight of lipids was calculated, and then the percentage of lipids was calculated according to the following formula:

$$\% \text{ of lipid} = (\text{mass of lipid}/\text{mass of powder}) \times 100$$

- Determination of the Percentage of Saponins:

First, 1 g of powdered herb was added to 100 mL of 20% ethanol solution, and then heated for 4 h at 45 °C under continuous spinning. The mixture was filtered, and the residue was then extracted with another 100 mL of 25% ethanol solution. The combined extracts were concentrated using a rotary evaporator at 40 °C to obtain approximately 40 mL. The concentrated extract was transferred to a separatory funnel and then extracted two times with 20 mL dichloromethane solution. The dichloromethane layer was thrown while the aqueous phase is maintained, then it was re-extracted with 30 mL chloroform. The chloroform extracts were washed twice with 10 mL of 5% sodium chloride solution. The remaining solution was evaporated by a rotary evaporator. After evaporation, the samples were completely dried in a water bath at 60 °C.

The percentage of saponins was calculated according to the following formula:

$$\% \text{ of saponins} = [\text{final weight of the sample}/\text{initial weight of extracts}] \times 100$$

- Estimation of Ash Content:

First, 2 g of powder were placed in a crucible silica and then burned in a muffle furnace for 5 h at 550 °C until the appearance of the gray color. The resulting ash was cooled and then weighed. The percentage of ash was estimated in relation to the initial mass of the plant powder according to the following formula:

$$\% \text{ Ash} = (\text{final mass}/\text{initial mass}) \times 100$$

where:

- Initial mass = mass of powder + mass of the crucible silica (before drying);
- Final mass = mass of powder + mass of the crucible silica (after drying).
- Determination of moisture content:

The moisture content was determined using the drying method. First, 1 g of precisely weighed powder was transferred to a previously weighed crucible silica that was dried in an oven at 100 °C for 1 h, then cooled in a desiccator for 30 min and reweighed. Before and after drying, the difference in weight was calculated and expressed as a percentage of the moisture content.

The percentage of humidity was calculated according to the following formula:

$$\% \text{ Humidity} = \{(\text{initial mass} - \text{final mass})/\text{mass of powder}\} \times 100$$

where:

- Initial mass = mass of powder + mass of the silica crucible (before drying);
- Final mass = mass of powder + mass of the silica crucible (after drying).
- Evaluation of Antioxidant Activity:
- Operating mode:

For each of the prepared extracts, the antioxidant activity was evaluated according to the method of Rammal et al., with slight modifications using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Increasing concentrations of each extract (0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 mg/mL) were prepared using serial dilution. Next, 2.62 mg of DPPH reagent was weighed and dissolved in 50 mL methanol. Then, 1 mL of each prepared concentration was added to 1 mL of the prepared DPPH solution. After agitation, the solutions were incubated for 30 min in the dark at room temperature, and then the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer [21].

The antioxidant activity was calculated according to the following equation:

$$\% \text{ Antioxidant activity} = [(\text{ABS control} - \text{ABS sample})/\text{ABS control}] \times 100$$

The ABS control is the absorbance of DPPH + solvent; ABS sample is the absorbance of the DPPH + sample.

The control was prepared by mixing 1 mL of DPPH with 1 mL of the solvent (water/ethanol), and the blank was composed of 1 mL of solvent and 1 mL of methanol. The ascorbic acid was used as a positive control.

4.4. Culture and Treatment of Cancer Cell Line

4.4.1. A549 Cell Line

The A549 human lung adenocarcinoma purchased from American Type Culture Collection (ATCC) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS), and incubated in an incubator under a controlled atmosphere of 5% CO₂ at a temperature of 37 °C.

4.4.2. Preparation of the Tested Concentrations

For the ethanolic extract, the powder was dissolved with the aid of vortex in the DMSO solvent as a concentrated stock solution (60 mg/mL) and then filtered by a filter

of diameter 0.2 μm . Proceeding that, the other 6 concentrations were prepared by serial dilution. Then, each concentration was diluted 200 times such that the DMSO concentration did not exceed 0.5% to avoid cell toxicity. The obtained treatment test concentrations for the ethanolic extract were: 25; 50; 100; 150; 200; and 250 $\mu\text{g}/\text{mL}$.

For the aqueous extract, the powder was dissolved with the aid of vortex in DMEM as a stock solution (5 mg/mL), and then filtered by a filter of diameter 0.2 μm . Proceeding that, the other 10 concentrations were prepared by serial dilution. The obtained treatment test concentrations for the aqueous extract were: 25, 50, 100, 150, 200, 250, 300, 400, 500, and 1000 $\mu\text{g}/\text{mL}$.

By serial dilution, increasing concentrations of CDDP (4, 8, 12, and 80 $\mu\text{g}/\text{mL}$) were prepared.

Combinations of *E. camaldulensis* extracts and CDDP were also prepared for each extract.

4.4.3. Treatment of the Cells

A total of 70 to 80% confluent cells were seeded in a 96-well tissue culture microplate at the density of 104 cells/well and then treated with the prepared for 24, 48, and 72 h. Evaluation of anticancer activity was performed by measuring cell proliferation of the A549 cell line, after treatment with increasing concentrations of the prepared *E. camaldulensis* extracts and low-dose CDDP (4, 8, 12, and 80 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 h. The combination ratios of Eucalyptus extracts and Cisplatin were chosen based on a previous study [8].

Similarly, the A549 cells were treated with combinations of the *E. camaldulensis* extracts and CDDP. Of note, untreated cells were used as positive control in all the performed experiments.

4.4.4. Neutral Red Uptake Assay

Neutral red (NR) stock solution was prepared by measuring 40 mg of NR powder and dissolving in 10 mL PBS such that the concentration was 4 mg/mL . NR medium used for staining the cells was prepared from the stock solution using DMEM in a way that its final concentration was 40 $\mu\text{g}/\text{mL}$. Neutral red medium was filtered to reduce NR crystals.

On the other hand, the NR desorb solution used for cell lysis was prepared to obtain the following: 1% Glacial acetic acid solution, 50% Ethanol, and 49% water.

When the processing time of the seeded cells was completed, the culture medium was aspirated, and wells were rinsed with pre-warmed PBS (1 \times) using 200 μL for each well. After removing the PBS, 200 μL of the prepared neutral red medium were added to each well and cells were incubated for 3 h, during which the lysosomes of living cells were colored. Once the incubation period had passed, neutral red was removed by washing with PBS (200 $\mu\text{L}/\text{well}$). Next, NR desorb solution (100 $\mu\text{L}/\text{well}$) was added to achieve cells' lysis. Shake micro-titer plate was shaken rapidly on a micro-titer plate shaker for 20–45 min to extract NR from the cells and form a homogeneous solution. Plates were protected from light by using a cover during shaking.

Finally, the optical density (OD) of NR extract was measured at 490 nm in a microplate reader spectrophotometer, using blanks containing no cells as a reference [14].

4.4.5. Statistical Analysis

All experiments described above were performed in triplicate. The mean and standard error of mean (SEM) were calculated for each experimental group. Data are presented as means \pm SEM. Normality test for our data was performed using the Kolmogorov–Smirnov (KS) test. Changes in the percentage of DPPH radical scavenging activity between treated cells with ethanolic and aqueous extracts or changes in the percentage of cell viability between treated cells with extract (345 m) and extract (0 m) were studied using Unpaired t-test or Mann–Whitney test.

Changes in the percentage of cell viability between treated and control cells were determined using Paired t-test or Wilcoxon signed rank test.

Changes in the percentage of cell viability between treated cells with combination of CDDP–extract and treated cells with the extract alone were determined using Paired *t*-test or Wilcoxon signed rank test. *p* value < 0.05 was considered significant for all test. Graph Pad prism was used to carry statistical analysis.

5. Conclusions

Medicinal plants have a variety of natural compounds such as flavonoids and phenolic acids which are of a great important for their anti-oxidative properties. Anti-oxidants from natural origin have the ability to scavenge free radicals and are not carcinogenic, opposite to synthetic anti-oxidants, which have restricted uses due to this.

The obtained results showed that the extracts from the plants from both altitudes, and in both aqueous and ethanoic solvents have an anti-tumor effect on A549 cells, with a greater effect for the ethanoic extract and the 0 m altitude species. In addition, the combination of the *E. camaldulensis* with low-dose CDDP exhibits an additive cytotoxic effect at certain levels against cell proliferation of A549 cells.

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