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Anticancer Activity of *Peganum harmala* and *Haloxylon salicornicum* Leaf Extracts on Lung Cancer A549 and Prostate Cancer PC-3 Cell Lines

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Abstract

Cancer is a worldwide disease that ranks as the second-largest cause of death after cardiovascular disease. In 2019, the estimated number of cancer deaths was around 10 million worldwide and 600,000 in the United States. Due to the high side effects of the available treatments for cancer, such as chemotherapy and radiotherapy, a demand for alternative treatments has been a goal for scientists with less toxicity and side effects. Lung cancer is the most common type of cancer worldwide and the leading cause of death in the United States. In addition, prostate cancer is the second-leading cause of cancer death in American men after lung cancer. Natural sources discovered to have therapeutic medicinal properties against human diseases are the plants *Peganum harmala* and *Haloxylon salicornicum*. The aim of this study is to test the anticancer activity of leaf extracts of *Peganum harmala* and *Haloxylon salicornicum* on lung cancer (A549) and prostate cancer (PC3) cell lines. The anticancer activity of *P. harmala* and *H. salicornicum* was studied using the assays: crystal violet viability, lipid peroxidation, and caspase-3 techniques, which were evaluated *in vitro* on two human cancer cell lines. The results of the study showed that both plants' extracts reduced the viability of both cancer cell lines, which depended on the concentration of the extract. Also, *P. harmala* showed more potent activity against both types of cancer than *H. salicornicum*. *P. harmala* and *H. salicornicum* decreased the lipid peroxidation that induces cancer as well as increased the expression of caspase-3, which causes apoptosis and cell death. This study concluded that *P. harmala* and *H. salicornicum* leaf extracts showed significant anticancer properties, which might be due to the presence of phytochemicals, including flavonoids and phenolics.

Keywords

Peganum harmala, *Haloxylon salicornicum*, Lung Cancer, Prostate Cancer, Lipid Peroxidation, Caspase-3, Apoptosis

1. Introduction

The burden of cancer morbidity and mortality is rapidly growing worldwide. Cancer ranks as the second largest cause of death after cardiovascular disease [1] [2] [3] [4]. In 2019, it was estimated that the cases of cancer were around 19.3 million new cases with 10 million death cases worldwide and 1.7 million new cases with 600,000 death cases in the United States [3] [5]. Despite the effort to treat the cancer patients from the disease with the conventional methods, the mortality is still high due to the toxicity of the current methods such as chemotherapy and radiotherapy [6] [7] [8] [9] [10]. As a result, the use of medicinal plants is an alternative safer method that can treat cancer [11] [12] [13].

There are different types of cancers in which abnormal cells divide uncontrollably and can infiltrate and destroy normal body tissues [14]. The most common types of cancers are skin cancer, lung cancer, breast cancer, prostate cancer, pancreatic cancer, and colorectal cancer [5] [15]. The leading cause of cancer death is lung cancer in both males and females with approximately 2.2 million new cases and 1.8 million death cases in 2020 worldwide [16]. In the US, it was reported more than 200,000 new cases of lung cancer and 139,000 deaths in 2019 [5]. In addition to lung cancer which causes death to males and females, prostate cancer is the fifth leading cause of cancer death among men worldwide, with an estimated 1.4 million new cancer cases and 375,304 deaths in 2020 [17] [18]. However, prostate cancer is the second leading cause of cancer death in American men after the lung cancer. The American Cancer Society's estimates for prostate cancer in the United States for 2023 are 288,300 new cases of prostate cancer and 34,700 deaths [19].

Although the rate of cancer death has declined in the past years because of the increased awareness around prevention, screening, and early diagnosis, cancer is still the second leading cause of death globally due to the toxicity of the available treatments including chemotherapy, radiotherapy, immunotherapy, and hormone therapy [7] [20] [21]. These therapies cause different side effects such as cardiac side effects, neutropenia, infection, organ-related inflammation, and neurocognitive deficits that include impaired learning, memory, attention and speed of information processing [22] [23] [24] [25] [26]. Various reports illustrate that chemotherapy often fails in the clinic and accounts for more than 25% of mortality in cancer patients [27]. Because of the numerous side effects of cancer therapies, researchers have been looking for alternative cancer therapies such as medicinal plants with less toxicity on healthy non-cancerous cells and minor side effects on the cancer patients.

Plants have been used for medical purposes since the beginning of human history and are the basis of modern medicine. Medicinal plants and plant-derived medicine are widely used in traditional cultures all over the world and they are becoming increasingly popular in modern society as natural alternatives or supplements to synthetic chemicals. Well-known examples of plant-derived medicines include quinine, morphine, codeine, colchicine, atropine, reserpine, and digoxin. Important anticancer drugs such as paclitaxel (taxol) and vincristine have been developed from plants [28] [29]. Several parts of the plants (leaves, roots, stems, seeds, fruits, rhizomes) have been reported to have anticancer/antitumor agents including *Allium sativum* (garlic), *Alpinia galangal* (blue ginger), *Asclepia scurassavica* (Tropical milkweed), *Curcuma longa* (Turmeric), *Morus nigra* (Blackberry), *Litchi chinensis* (lychee), and so many other plants [30]-[36]. These parts of the plants possess different chemical compounds such as polyphenolic compounds including flavonoids, tannins, curcumin, resveratrol and gallacatechins and are all considered to be anticancer compounds [37].

This study focuses on the effect of two plants, *Peganum harmala* and *Haloxylon salicornicum*, which are both commonly known as having alkaloids, flavonoids and anthraquinones [38] [39] [40]. *P. harmala* and *H. salicornicum* leaf extracts were tested on lung cancer A549 cell line and prostate cancer PC-3 cell line. The overall outcomes showed that both leaf extracts of these plants significantly reduced the cell growth in both lung cancer A549 cell line and prostate cancer PC-3 cell line due to the increase of caspase-3 expression, as well as the decrease of lipid peroxidation.

2. Materials and Methods

2.1. Crude Extraction

Peganum harmala's and *Haloxylon salicornicum's* leaves were prepared and extracted independently, but the same technique was used for both plants. Firstly, the leaves were washed with water and placed in separate jars before drying for 24 hours at room temperature. Secondly, the samples were collected and transferred to a separate plastic chemical-resistant 50-millimeter centrifuge tube which was then frozen at −80˚C for three days. After freezing the leaves for 3 days, the dried/frozen samples were removed from the tubes and separately grounded using a mortar and pestle to maximize the surface area of the leaves and extraction solvents. Then, the crude extract was infused with methanol to extract the components, which were subsequently distilled in a Soxhlet for six hours. A rotary evaporator was used to evaporate the methanol. Afterward, all crude extracts were weighed and dissolved in dimethyl sulfoxide (DMSO). Finally, serial dilution was performed to make seven different concentrations of each extraction.

2.2. Serial Dilution

The process of diluting a compound in solution was done step by step. The crude

and aqueous extracts of *P. harmala* and *H. salicornicum* were separately serially diluted by 0.5 ml in DMSO (Crude) to get the final concentration. The dilutions were in the range of 1 to 1/64.

2.3. Cancer Cell Culture

To investigate cytotoxicity and antiproliferative activity, the cancer cell lines were supplied by the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin. Lung cancer cell line A549 and prostate cancer cell line PC3 were grown in Dulbecco's modified eagle's medium (DMEM), a low glucose variant (Gibco), which contained 2 mM L-glutamine, non-essential amino acids, penicillin-streptomycin, 10% fetal calf serum (Atlanta Biologicals), and supplemented with 0.01 mg/mL insulin and 1mM sodium pyruvate as well as other growth factors. In a humidified incubator with 5% CO_2 at 37˚C, cancer cells were passaged biweekly for a total of 40 passes after achieving 60% - 80% cell confluence.

2.4. Cell Proliferation Assay

Cell viability was determined using the crystal violet method. A549 and PC3 cell lines were plated on a 96-well plate. The medium containing cells was added to three wells. The cells were incubated for 18 - 24 hours at 37˚C for the cells to adhere to the wells and proliferate. After the cells had reached 60% - 80% confluence, the old medium was removed and replaced with new media containing different concentrations of the extracts. Then, cells were incubated in the optimal conditions, 18 - 24 hours at 37˚C. Following that, the culture media was discarded from the plate and the cells were rinsed with 1 × PBS and fixed with 100 µL of 100% methanol for 10 min. 50 µL of 0.5% crystal violet staining solution was added to each well and incubated for 10 - 20 minutes at room temperature. At last, the plate was washed four times with a stream of running water. The optical density was measured at 550 nm by a plate reader. DMSO was used as control in the experiment.

2.5. Lipid Peroxidation Assay

Lipid peroxidation was used to determine the function of oxidative damage in a pathophysiological process. The production of extraordinarily reactive and unstable hydroperoxides in both saturated and unsaturated lipids occurs due to lipid peroxidation. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which is a product of polyunsaturated fatty acid (PUFA) hydroperoxides, have generally been used to measure lipid peroxidation. Moreover, to quantify aldehydes, sensitive colorimetric assay was performed. Lipid hydroperoxide (LPO) assay kit (437639-1KIT Sigma) performs redox reactions with ferrous ions to assess lipid hydroperoxides directly. Hydroperoxides are very unstable, and they rapidly interact with ferrous ions to form ferric ions. The thiocyanate ion was used

as a chromogen to identify the ferric ions that arise.

2.6. Caspase-3 Assay

Caspase-3 assay kit (ab39401 Abcam) is a practical method for determining caspases' activity that recognizes the sequence DEVD in various samples. Aspects of the assay include the spectrophotometric detection of the chromophore (p-NA) and the determination of the concentration of the assay substance. When measured at 400 nm the p-NA light emission can be quantified using either a spectrophotometer or a microtiter plate reader. The absorbance of p-NA from an apoptotic sample was compared to the absorbance of p-NA from a control, allowing the estimation of the fold increase in caspase-3 activity.

In six-well plates, the A549 and PC3 cells were cultured for 4 - 5 days, or until the cells were 60% - 80% confluent, before being harvested. Discarding of media from the six-well plates was followed by adding DMSO as a control and extracts from *P. harmala* and *H. salicornicum* in separate wells of serum-free medium. The samples were incubated for 6 h and washed with 1 × PBS. Cells were then resuspended in 500 µl cell lysis buffer and incubated in ice for 10 min. They were next centrifuged at 10,000 × g for 60 sec and the supernatant that has the cytosolic extract was transferred to a new tube containing 100 µl Reaction Buffer in the ice. After thoroughly mixing, 10 µl of the 4 mM DEVD-p-NA substrate with final concentration of 200 mM was added and incubated for 60 - 120 min at 37˚C. Finally, the product was measured at OD 400 by a microplate reader.

2.7. Assay Process and Sample Preparation

The A549 and PC3 cancer cell lines were individually treated with *P. harmala* and *H. salicornicum* extracts. The cells were plated on a six-well plate for 4 - 5 days or until the plated wells were 60% - 80% confluent. The media was then discarded. DMSO was added as a control to the first two wells, and three concentrations of the extracts were applied to the remaining three wells in a serum-free medium. The medium was withdrawn after 24 h incubation and the wells were then washed with 1 ml of 1 × PBS. After washing, the plate was incubated with 600 µl trypsin for 3 min and all cells were scraped into microcentrifuge tubes with 600 µl of the medium and centrifuged at 3000 × g for 10 min. The supernatant was discarded, and the pellet was stored at −80˚C. The pellets were taken out of the freezer and placed in an ice bucket. A sonicator instrument was used to sonicate the pellets, and 110 µl of 1 × PBS was added to the pellets. The lipid hydroperoxide (LPO) assay kit was modified by adding R1 solution in a tube and was incubated for 5 min. Microplate reader measured the absorbance of the samples at 500 nm in 96-well plates. A standard curve was used to determine the lipid hydroperoxide (LPO) levels.

2.8. Statistical Analysis

StatView 5.0.1 was used to do a one-way ANOVA study to discover the statistical differences (SAS Institute Inc., Cary, NC). Statistical significance was defined

as $P < 0.05$.

3. Results

3.1. The Effects of *Peganum harmala* Leaf Extracts' Concentration on Cell Viability of PC-3 and A549 Cancer Cell Lines

The effect of various concentrations of *P. harmala* leaf extracts on the proliferation of prostate cancer cell line PC-3 and lung cancer cell line A549 was investigated. The cells were initially plated in a 96-well plate at a density of 1×10^4 cells per well and were treated with various concentrations of the extracts (0.3, 0.15, 0.0375, 0.01875, and 0.00468 μg/ml) to determine their sensitivity. The results were compared with cells treated with DMSO, which is the control in the study.

3.1.1. The Effect of *P. harmala* on the Proliferation in Prostate Cancer Cell Line PC3 and Lung Cancer Cell Line A549

The overall results showed that increasing the concentration of the leaf extracts reduced the viability of the prostate cancer cell line PC3 as well as lung cancer cell line A549. The most significant reduction in cell viability was found when the cells were treated with 0.3 μg/ml of the leaf extract. In comparison, the lowest concentration of 0.00468 μg/ml of the leaf extract still indicated a substantial reduction compared to the control (**Figure 1**). Based on statistical analysis, the ANOVA test indicated a significant association between *P. harmala's* extract and the proliferation of A549 and PC3 cell line ($P < 0.05$).

The cells were plated at 1×10^4 cells per well and subsequently treated with *P. harmala* concentrations (0.3, 0.0375, and 0.01875 μg/ml) for 24 h. **Figure 2** represents the measurement of lipid peroxidation levels in both cell lines. It was observed that *P. harmala* significantly reduced lipid peroxidation levels compared with the control (DMSO).

3.1.2. The Effect of *P. harmala* on Caspase-3 Activation in PC3 and A549

The expression of caspase-3 in A549 and PC3 cancer cell lines was determined in different concentrations of leaf extracts. The cells were initially plated in a six-well plate until the plated wells were 60% - 80% confluent. They were subsequently treated with two different concentrations of *P. harmala* (0.3 and 0.01875 μg/ml), which were compared to the control (DMSO). The expression of caspase-3 increases when the concentration of leaf extract increases. The ANOVA test found that the expression of capase-3 was significantly associated with *P. harmala's* extract ($P < 0.05$) (**Figure 3**).

3.2. The Effects of *Haloxylon salicornicum* Leaf Extracts' Concentration on Cell Viability of PC-3 and A549

The effect of *H. salicornicum* leaf extracts' concentrations was examined on the proliferation of PC-3 and A549 as well. The cells were initially plated in a 96-well plate at a density of 1×10^4 cells per well and tested on different concentrations of the extracts (48, 24, 12, 6, and 3 μg/ml). The results were compared with the results from cells treated with DMSO, experiment's control.

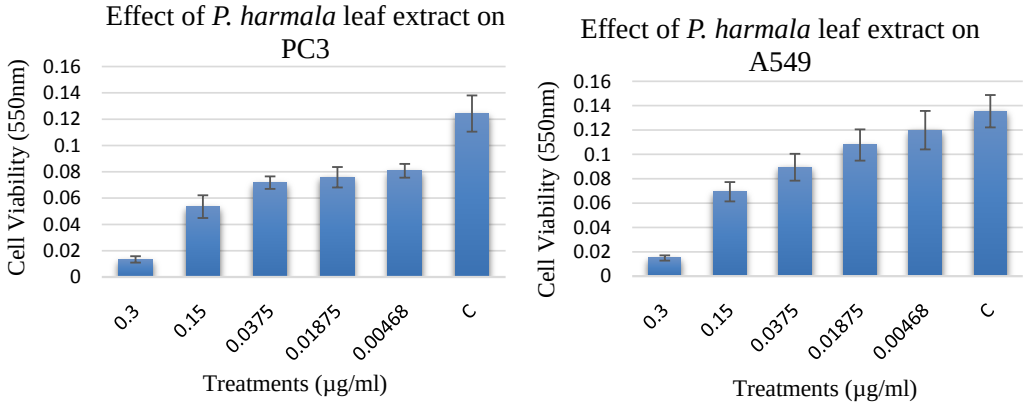

Figure 1. Viability analysis of prostate cancer cell line PC3 (on left) and lung cancer cell line (on right) after exposure to *P. harmala.* Values are presented as means (n = 3) ± SD. Statistical difference ($P < 0.05$).

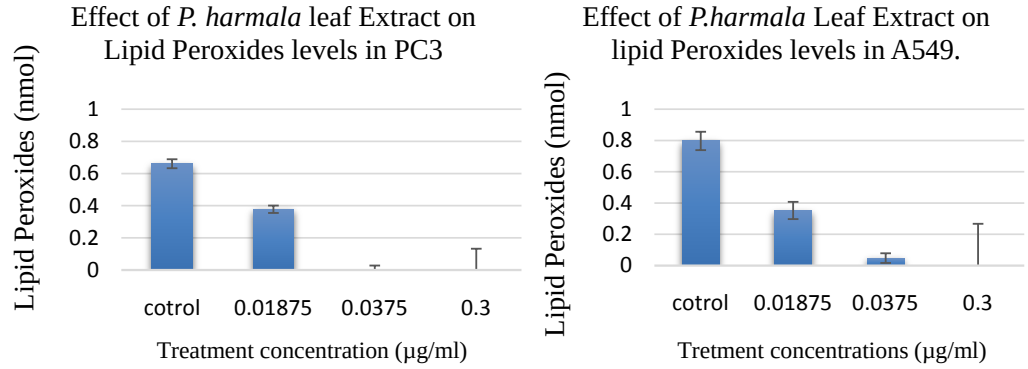

Figure 2. Effect of different concentrations of *P. harmala*'s leaf extract on lipid peroxides levels on PC3 (on left) and A549 (on right).

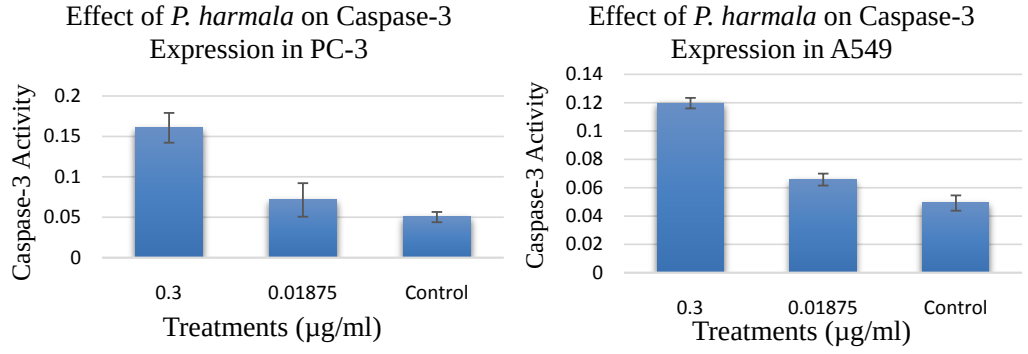

Figure 3. Effect of different concentrations of *P. harmala*'s leaf extract on the expression caspase-3 levels on PC3 (on left) and A549 (on right).

3.2.1. The Effect of *H. salicornicum* on the Proliferation in PC3 and A549

In *H. salicornicum* leaf extract, it was found that increasing the concentration reduced the viability of the PC3 as well as A549. The most significant reduction in cell viability was found when both cell lines were treated with 48 µg/ml. In comparison, the lowest concentration of 3 µg/ml of the leaf extract still indicated a substantial reduction (**Figure 4**). The ANOVA test indicated statistically significant association between *H. salicornicum* extract and the proliferation A549

and PC3 cell lines ($P < 0.05$).

3.2.2. The Effect of *H. salicornicum* on the Lipid Peroxidation Levels in PC3 and A549

The cells were plated at 1×10^4 cells per well and subsequently treated with *H. salicornicum* concentrations (10.16, 1.270, and 0.6351 µg/ml) for 24 h. **Figure 5** represents the measurement of lipid peroxidation levels in both cell lines. It was observed that *H. salicornicum* reduced lipid peroxidation levels compared with the control (DMSO).

3.2.3. The Effect of *H. salicornicum* on Caspase-3 Activation in Human Cancer Cell Lines A549 (Lung) and PC3 (Prostate)

The expression of caspase-3 in A549 and PC3 cancer cell lines was determined in different concentrations of leaf extracts. The cells were initially plated in a six-well plate until the plated wells were 60% - 80% confluent. They were subsequently treated with two different concentrations of *H. salicornicum* (48 and 6 µg/ml), which were compared to the control (DMSO). The expression of caspase-3 increases when the concentration of leaf extract increases. The ANOVA test found that there was a statistically significant difference ($P < 0.05$) (**Figure 6**).

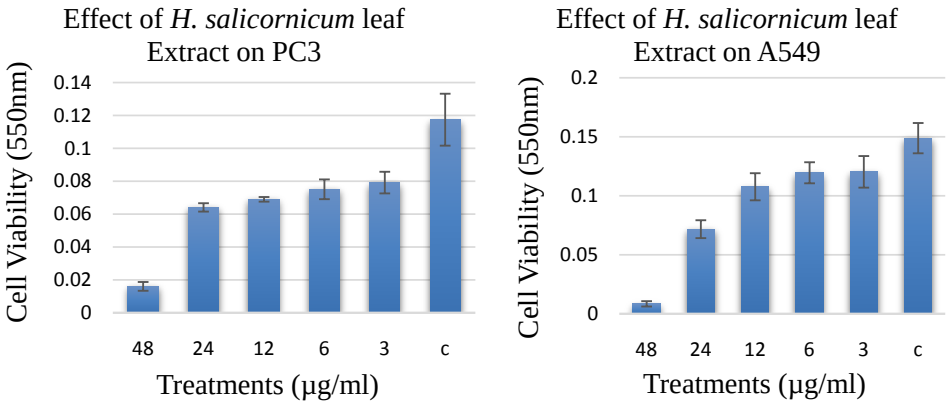

Figure 4. Viability analysis of prostate cancer cell line PC3 (on left) and lung cancer cell line (on right) after exposure to *H. salicornicum.* Values are presented as means (n = 3) ± SD. Statistical difference ($P < 0.05$).

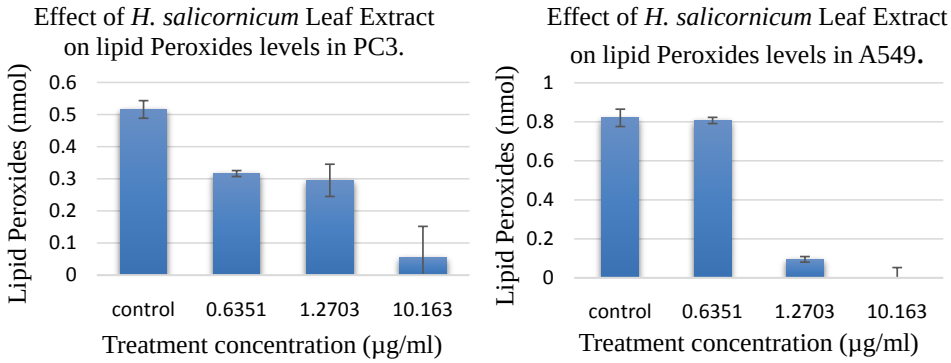

Figure 5. Effect of different concentrations of *H. salicornicum*'s leaf extract on lipid peroxides levels on PC3 (on left) and A549 (on right).

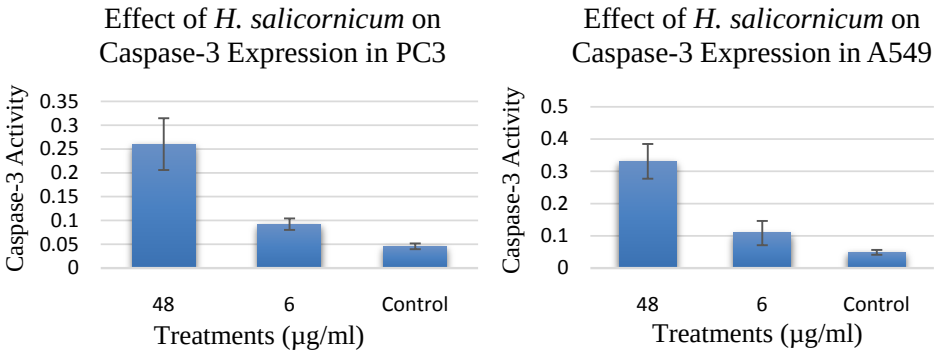

Figure 6. Effect of different concentrations of *H. salicornicum*'s leaf extract on the expression caspase-3 levels on PC3 (on left) and A549 (on right).

4. Discussion

4.1. The Effect of Different Concentrations of *P. harmala* and *H. salicornicum* Leaf Extracts on the PC-3 and A549 Cancer Cell Lines

Both plants' extraction showed an effect on both cancer cell lines' proliferation. The main factor that affects the viability of cancer cells is the concentration of the extract, that is, as the concentration increases, the proliferation of the cancer cells decreases. Also, it's been shown that *P. harmala* extract has a better reduction of A549 and PC-3 cancer cells at low concentration than *H. salicornicum*'s extract. At high concentrations, *P. harmala*'*s* extract needed 0.3 µg/ml to decrease cancer cell viability to practically zero percent in PC-3 and A549 cancer cell lines compared to 48 µg/ml of *H. salicornicum*'*s* extract needed to vanish both of cell lines.

Previous studies have focused on *P. harmala*'s seed extract viability on different cancer cell lines and positive results have been obtained. A study tested *P. harmala* seed extract on cervix cancer cell lines and showed that different doses as well as different times affect the viability of the cancer cells [41]. Another study also tested *P. harmala*'s seed as well as root extract on multiple human cancer cell lines including myeloid leukemia (HL-60) cell line, human colorectal cancer (HCT-116), prostate cancer (PC-3), lung adenocarcinoma epithelial cell line (A549), and the breast cancer cell line (MCF-7) and concluded the significant anticancer properties of *P. harmala* seed extract against multiple human cancer cell lines [42] [43]. Similarly, *Haloxylon salicornicum* extract has been tested for anticancer properties. It was found that *H. salicornicum* could minimize/prevent liver cancer and disease as well as reduce reactive oxygen species (ROS) and late apoptosis [44]. Based on previous studies, *H. salicornicum* has been focused on antioxidant, antimicrobial, and antibiofilm activities more than anticancer/antitumor activity [44] [45] [46]

4.2. The Effect of *P. harmala* and *H. salicornicum* Leaf Extracts with Varying Concentrations on PC-3 and A549 on Lipid Peroxidation Levels

Measuring oxidative stress is essential since it is usually related to certain types

of diseases such as cancer when stress levels are high [47] [48]. It is vital to reduce the levels of lipid peroxidation in cancerous cells since low levels of (MDA) and (4-HNE) suggest that the cells are not under oxidative stress. Comparing the effects of *P. harmala* and *H. salicornicum* leaf extracts on PC-3 and A549 cancer cell lines, the study found that *P. harmala* is more effective on lipid peroxidation levels than *H. salicornicum.* Both extracts lowered the levels of lipid peroxidase in all tested concentrations, however, at concentrations of 0.3 µg/ml and 0.0375 µg/ml *P. harmala*, lipid peroxidation levels were reduced to almost zero in both PC-3 and A549 cancer cell lines, while 48 µg/ml *H. salicornicum* was needed to reduce lipid peroxidase levels to practically zero only in A549 cancer cell lines.

Oxidative stress reduction of *P. harmala* has been studied on different diseases. Seed extract of *P. harmala*' was tested on rats with Parkinson's diseases [49] as well as rats with complete Freund's adjuvant (CFA) induced arthritis [50] and showed a significant reduction of oxidative stress which may possibly be due to boosting the intracellular antioxidant defense. In addition, isolations of flavonoids from *Haloxylon salicornicum* were used to treat acute kidney injury induced by Cisplatin, an effective chemotherapeutic drug used for the treatment of many types of cancers and causes serious side effects such as nephrotoxicity. The study found that flavonoids isolated from *H. salicornicum* prevented oxidative stress [51].

4.3. The Effect of *P. harmala* and *H. salicornicum* on Caspase-3 Activation A549 and PC3 Cancer Cell Lines

There are different groups of caspases in which some are involved in regulating pyroptosis, a form of programmed cell death that inherently induces an inflammation, and others are involved in regulating apoptosis, a cellular suicide. Caspase-3 is a protease that regulates apoptosis. The research finding showed that the leaf extracts of *P. harmala* and *H. salicornicum* enhance the expression of caspase-3 in PC-3 and A549 cells which significantly reduces the viability of cancer cells a study has shown an elevation of caspases 2, 6, 8, 9 which all are also associated with apoptosis on various cell lines treated with *P. harmala* leaf extract [52]. Furthermore, *P. harmala* seed extract was tested on human breast cancer and up-regulation of caspase-8 as well as other genes responsible for apoptosis such as p21 and p53 was detected as *P. harmala* decreases the growth rate of the cancer cell line through inducing apoptosis mechanism [53]. Additionally, the use of some natural compounds, such as LLE or MOG, on PC-3 cells has been observed to induce apoptosis by causing down-regulation of bcl-2 and up-regulation of Bax proteins. This mechanism leads to the initiation of significant and irreversible endoplasmic reticulum stress, followed by an end in the G1 phase of the cell cycle, ultimately resulting in apoptosis specifically in these cancerous cells [54].

5. Conclusion

In conclusion, *P. harmala* and *H. salicornicum* leaf extracts were tested and

showed significant anticancer properties that might be due to the presence of phytochemicals, including flavonoids and phenolics. The results demonstrated significant anticancer activity against lung and prostate human cancer cell lines assessed through lipid peroxidation and caspase assay. *P. harmala* was more effective against A549 and PC3 cell lines than *H. salicornicum*. In addition, the outcome showed an elevation in caspase-3 that decreases the growth rate of cancer cell lines treated with *P. harmala* or *H. salicornicum* leaf extracts. Therefore, our results could be promising to develop adjuvant phytotherapeutics for cancer treatment by using *P. harmala* and *H. salicornicum* leaf extracts.

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Data availability Statement

All data generated or analyzed during this study are included in the published article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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