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Evaluation The Effect Cytotoxicity and Program Cell Death of *Bauhinia variegata* Volatile Oil

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ABSTRACT

Lung cancer is the most prevalent type of cancer found in the world, and it remains a significant global health concern, despite advancements in diagnostic and therapeutic techniques. The exploration of new anti cancer agents derived from plants has emerged as a promising trend in scientific research. Phytochemicals are considered selective in their effects and are generally less harmful to normal cells compared to conventional cancer treatments. In the present study, flowers of Bauhinia variegata were gathered from Baghdad in the spring of 2024, and volatile oil was extracted by using Clavenger. Cytotoxic effects of volatile oil were assessed through MTT assay against lung cancer cell lines A549 and REF normal cell line at 24,48and 72 h, and using four different concentrations. The result showed that the highest cytotoxicity of volatile oil at 100 µg/ml was 86.1% at 72h. While the lowest inhibition rate was 11.25% in the 25 µg/ml after 24 h of treatment. In contrast, it could hardly inhibit the proliferation of normal cells although volatility oil had slight inhibition on them as its max inhibition rate (11.7%) at 72 h was only obtained when attend with volatile oil of 100 µg/ml and min inhibition rat was also low (2.266%) at 24 h when attend with volatile oil of 25 µg/ml. A validated of reducing cell viability was down by using AO/EtBr, which means apoptosis was induced in A549cell treated with volatile oil but there is no react in REF cell. At the gene expression level, the flow cytometery technique was used to determine the increase in gene expression of P53 and so it coincided with apoptosis occurring in cancer cells exposed to the volatile oil. The volatile oil didn't cause the P53 gene to express in REF cells. activity of mitochondria was examined using Rh 123 dye during flow cytometry testing with volatile oil in the treatment of cancer cells. mitochondrial membrane potential was reduced efficacy, leading to cell death, as evidenced by the dimmer fluorescence caused by cancer cell the programmed cell dead process. Conversely, in REF cells, the mitochondria remain vibrant and active regardless of volatile oil treatment.

Keywords: *Apoptosis, Bauhinia variegata, cytotoxicity, P53, Volatile oil*

1. INTRODUCTION

In the 21st century, cancer is the leading cause of illness and death and a factor in shortening life expectancy worldwide. Cancer is caused by the uncontrolled growth of dysfunctional cells that form tumors [1-3]. According to the World Health Organization (WHO), 8.8 million people will die from cancer worldwide by 2030. In 2015, there were more than 17.5 million newly diagnosed cases [4]. The WHO estimates that in 2019, poor countries accounted for 7.0% of all cancer cases worldwide. Older adults are more likely to develop cancer than any other age group, and this number is expected to triple by 2050, from 143 million to 436 million [5]. The uncontrolled growth of abnormal cells in lung tissue is the name for lung cancer, a type of cancer that begins in the lungs. This type of cancer is both common and deadly. Lung cancer can have a variety of causes, including but not limited to smoking, environmental pollutants, occupational exposure, genetics, and family history of the disease. Most cases of lung cancer can be divided into two types: non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). Most lung cancer cases (about 80-85%) are attributed to NSCLC, while only about 10-15% are attributed to SCLC. These subtypes differ significantly in their microscopic features, growth patterns, and treatments. Symptoms of lung cancer may include chronic cough, difficulty breathing, chest discomfort, hoarseness, decreased appetite, severe fatigue, and recurrent respiratory infections. The prognosis for lung cancer is bleak because it is often not detected until the late stages [6-8]. Phytochemical components play a crucial role in assessing the functions of plants. Their existence in herbal plants imparts

their healing properties. These phyto-components exhibit pharmacological effects such as antimicrobial, antioxidant, anti-cancer, and anti-inflammatory [9,10]. The current data shows that medicinal plants as traditional therapies has become popular worldwide, are often employed to manage many disease like cancer [11]. These plants have different active constituents such as alkaloids, terpenoids tannis, flavonoids [12] Volatile oil is one of the active compounds in medical plants and it is complex of chemical components like monoterpenes, sesquiterpenes, it have many widely used as an antitumor component and many activities, and many novel multifunctional LS, such as PH-sensitive LS, and NIR thermo sensitive [13,14]. *Bauhinia variegate* which is belongs to the Fabaceae family, planted in garden, park and many regions, commonly named as orchid tree, kanchar [15]. It was growth in Asia in tropical climate. All parts (seed, flowers, stems, root) of *Bauhinia v*. contain chemical constituents', which play a vital role in promoting human health; it can be used in traditional medical. It was traditionally used in the treatment of cancer, bronchitis, tonic, and astringent. It can be us as anticancer anti-inflammatory antioxidant [16]. Apoptosis is a one of controlled genetically programmed cell death that regulates multicellular organisms and tissue development by deleting physiologically redundant, physical damaged, and abnormal cells [17]. Acridine orange/Ethidium bromide (AO/EtBr) dual fluorescent staining under fluorescent microscope is pretty helpful in differentiating between viable and nonviable cells in terms of integrity of cell membranes getting disrupted in case of apoptosis [18]. Human cells can activate p53 to induce apoptosis. for response to excessive DNA damage. Cells lacking p53 can still undergo apoptosis upon DNA damage [19].

2. MATERIALS AND METHODS

Maintenance of cell cultures

All human melanoma cells and the A549 cell line were cultured in RPMI-1640 (Thermo Fisher, Waltham, MA) supplemented with 10% Fetal bovine serum ((FBS; (Biological Industries, Beit HaEmek, ISRAEL)) and 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passages using trypsin- EDTA, reseeded at 80% confluence 2 times per week, and incubated at 37°C [20,21].

Cytotoxicity Assays

To assess the cytotoxic effect of Bauhinia variegata volatile oil, the MTT assay was conducted utilizing 96-well plates [22,23]. Cell lines were plated at a density of 1×10^4 cells/well. Following 24 hrs. Or upon reaching a confluent monolayer, A549 cells were treated with the volatile oil obtained from Bauhinia variegata. Cell viability was assessed after 24, 48, and 72 hours of exposure while the cells were incubated at 37 °C with three replicates. The absorbance was measured using a microplate reader at 492 nm; the assay was executed in triplicate. The cell growth inhibition rate (the percentage of cytotoxicity) was calculated using the following equation [24,25]

$$Inhibition\ rate\ = A - B/A\ * 100$$

where A represents the optical density of the control, and B denotes the optical density of the samples [26].

To visualize the morphology of the cells under an inverted microscope, the cells were plated into 24-well micro-titration plates at a density of 1×10^5 cells mL^{-1} and incubated for 24 hours at 37 °C. Subsequently, cells were treated with volatile oil from Bauhinia variegata for 24 hours. After the duration of exposure, the plates were stained using crystal violet stain and incubated at 37 °C for 10^{-15} minutes [24]. The stain was gently rinsed off with tap water until the dye was entirely washed away. The cells were examined under an inverted microscope at 100× magnification, and images were captured with a digital camera connected to the microscope [27].

Acridine orange- ethidium bromide staining

Compound-induced cell death of A549 and REF cells was evaluated by AO/Et Br staining (Sigma-Aldrich, USA). After 24 hours, A549 and REF cells were seeded into 24-well plates, treated with essential oil of Cercis plant, and incubated for another 20 hours. A549 and REF cells were washed twice with phosphate-buffered saline. Two equal amounts of fluorescent dyes were added to the wall for two minutes. Finally, the cells were observed under a fluorescence microscope [28, 29].

Measurement of p53 Levels by flow cytometry assay

The activation status of P53 was determined using a fluorescent P53 staining kit (Thermo Fisher Scientific, USA). A549 cells and REF cells (4×10^4 cells/ml) were cultured in 5 ml of medium at 37 °C for 24 h. The incubated cells were then exposed to Bauhinia essential oil for 24 h. The treated cells were harvested after the incubation period and washed twice with 1x ice-cold. The pellet was then collected, and the cell density was rearranged (1×10^6 cells/ml) with growth medium. The prepared cells were then incubated with (1 ml) FITC-IETD-FMK for 60 min. FITC-anti-P53 was identified at 37 °C. The cells were washed twice with 1 ml of buffer. The stained cells were then transferred to flow cytometry tubes and evaluated using flow cytometry. The data were calculated and analyzed using BD Accuri C6 software.

Mitochondrial membrane potential assay

The effect of Bauhinia essential oil on mitochondrial function in A549 cells and REF cells can be tested using the fluorescent

dye rhodamine (Rh123). This dye is used to examine mitochondrial membrane potential before and after Bauhinia essential oil treatment. Cells were treated with Bauhinia essential oil 24 hours after seeding in 96-well plates and stained with 5M.Rh123 for 2 hours at 37 °C. Cells were then detached using 0.2 mL 5% trypsin-EDTA, centrifuged at 300 rpm for 5 minutes, and resuspended in (FACS) buffer. These cells were evaluated using flow cytometry and histograms were created.

Statistical analysis

By using an unpaired t-test with GraphPad Prism6 [30], the obtained data were analyzed. And the values were exist as the mean ± SD of triplicate measurements [31].

3. RESULTS AND DISCUSSION

Cytotoxicity effect of Bauhinia variegata

a) Volatile oil on cell line

It is also called a cell growth inhibition assay; the in vitro method was used to investigate the effect of volatile oil of Bauhinia variegata flowers on cancer cell line at different concentrations. The cytotoxic effect of Bauhinia variegata volatile oil on cell line were studied. Four different concentrations were selected as follows (25-50-75-100) µL/ml and for three time periods (24-48-72) hours. The MTT assay results showed that cell viability and growth were affected by different concentrations of volatile oil of Bauhinia variegata. Exposure to volatile oil of Bauhinia variegata led to inhibition in A549 cell. However, with increasing volatile oil of Bauhinia variegata concentration has a high ability to inhibit cancer line cells in all concentrations and it has also given clear significant differences at the three times and for all selected concentrations, but in inhibitory proportions that vary according to the concentrations and time. The results demonstrated that the volatile oil of Bauhinia variegata made clear morphological changes in A549 cells lines after treated as in Figure 1 and Table 1. The highest rate of inhibition of cancer cells was 86.1% at a concentration of 100 µl after 72 hours and was significantly greater to all four concentrations in the three time. The lowest inhibition rate was 11.25% after 24 hours of treatment of cancer cells (A549 cell) with essential oil at a concentration of 25 µL. The results shown in Table 2 also showed that when treating normal REF cells with volatile oil, the highest inhibition rate obtained after 72 hours, which significantly outperformed the treatment of normal REF cells with essential oil for 24 hours and 48 hours, as well as treatment with essential oil for 48 hours, has significantly exceeded 24 hours in all concentrations, as the results showed that the inhibition rate increases directly with increasing concentration, as well as showing Table 2 that there are significant differences in the three times between the concentrations, as the higher concentrations significantly outperformed the lower concentrations significantly, as the concentration of 100 µl / ml, which is the highest concentration, has exceeded the concentration of 25 µl / ml, which is the lowest concentration.

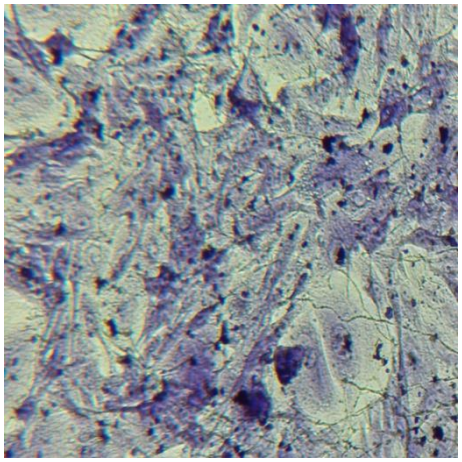
Table 1. A549cell after treatment with volatile oil of Bauhinia *variegata*

Con.	Inhibition ratio24h.	Inhibition ratio48h.	Inhibitionratio72h	LSD	P value
25	11.25	18.6	24.666	**7.452	0.0007
50	25.033	33	42.3	**8.503	0.0005
75	42.9	51.766	72.566	**8.622	0.0001
100	47.933	66.766	86.1	**10.437	0.0001
LSD	**9.346	**11.08	**9.641	-	-
P- value	0.0001	0.0001	0.0001	-	-

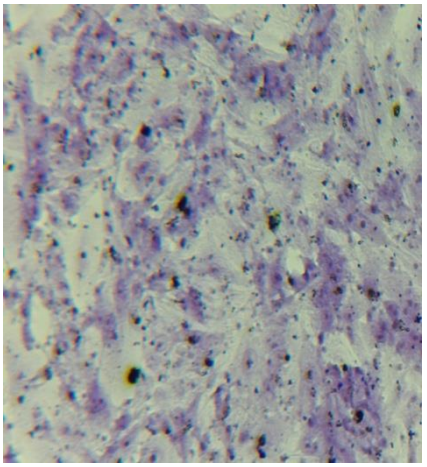
Table 2. REF cell after treatment with volatile oil of Bauhinia *variegata*

Con.	Inhibition ratio24h.	Inhibition ratio48h.	Inhibitionratio72h	LSD	P value
25	2.266	4.033	5.233	**1.706	0.0087
50	3.766	5.533	6.6	**2.154	0.0059
75	5.6	7.4	9.4	**2.605	0.0002
100	6.633	8.6	11.7	**2.457	0.0002

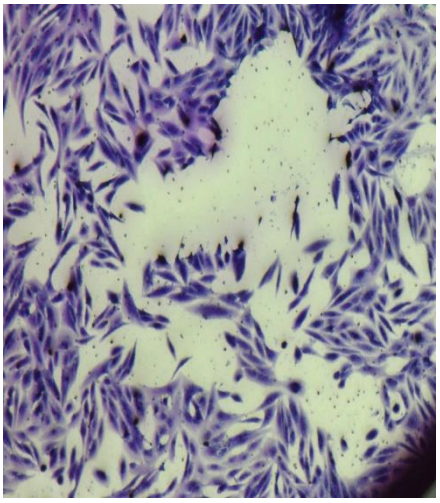
LSD	**2.074	**2.502	**3.189	-	-
P-value	0.0061	0.009	0.001	-	-



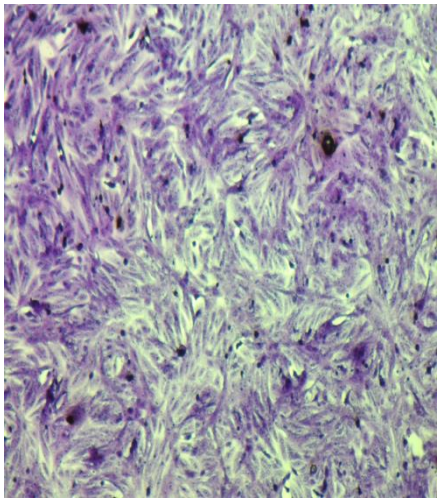
(a) REF cell treatment with volatile oil



(b) Control untreated REF cells



(c) A549cell treatment with volatile oil



(d) Control untreated A549 cells

Figure 1. Morphological changes in REF cell& A549 cells before and after treated with volatile oil of *Bauhinia variegata* of. Magnification power 10x.

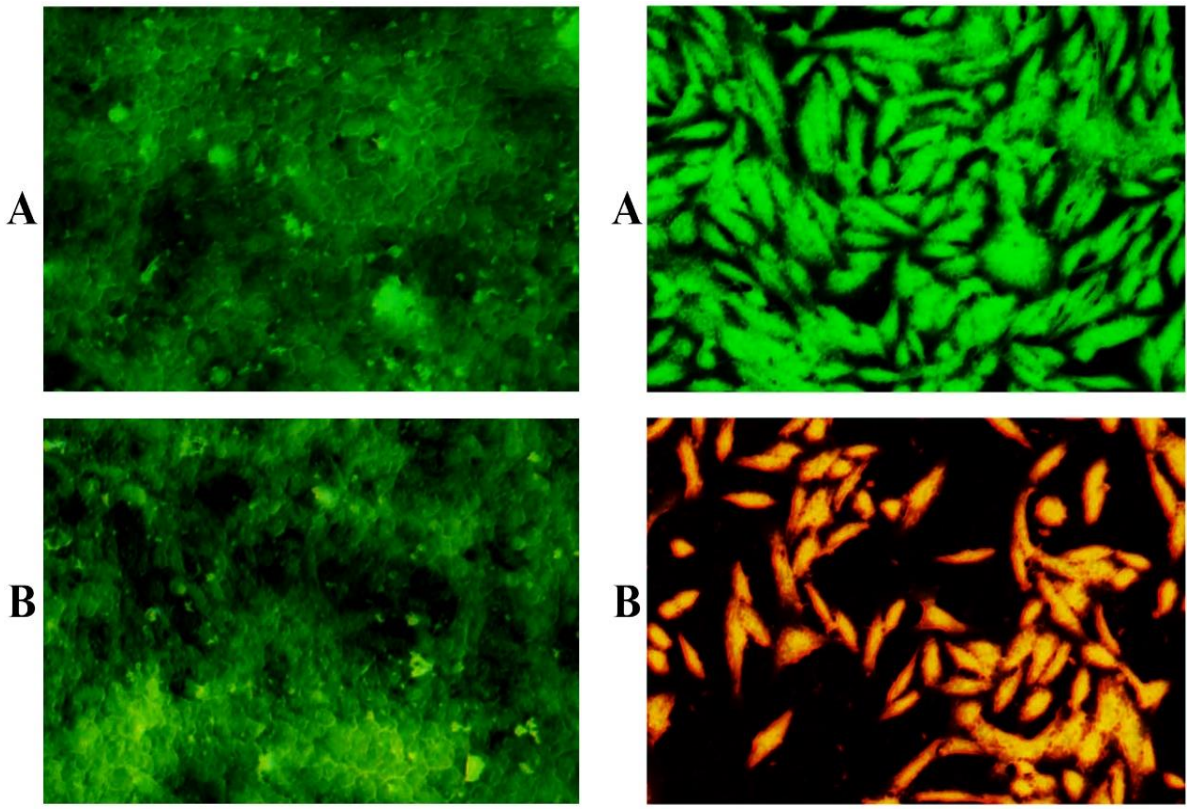
It is clear from the above that the increase in concentration and increase the time of treatment and incubation has had a significant impact on increasing the ability of the extract inhibition significantly, the greater the concentration greater the ability to inhibit at the three times. The longer the duration of the treatment, the higher the results in the inhibition ability. Bauhinia variegata extract contain bioactive compounds that gave to its effect as anticancer in dose respond manner [32]. Several biological processes and biological interactions occur between flavonoids and terpenoids and phenolic compounds can be inhibit growth of cell [33]. The high inhibition rates may be due to the presence of many active compounds in the volatile oil extracted from the of Bauhinia variegata plant grown in Iraq, as these compounds are considered safe on the line of normal cells (REF cell), but they are clearly toxic to cancer cells, as in the beta-Elemenone compound, which is a terpene compound that has an inhibitory ability for cancer cells, and this is what Alagbe [34]. Copaene compounds, which are also tricyclic sesquiterpene compounds, are volatile liquid compounds used in the manufacture of anti-inflammatory, antibacterial, anti-cancer and antioxidant drugs [35], in addition to Epoxy- linalooioxide, which is also an anti-cancer agent

[36]. Also, many studies conducted on crude alcoholic extracts are consistent with the result reached from the use of volatile oil for Bauhinia variegata itself, as the effects of cytotoxicity of the crude extract of were noted, as the study confirmed that the Bauhinia variegata has anti-cancer activity, as its use led to inhibiting the growth of cancerous tumors, including lung cancer A549 cells, as it showed that the Bauhinia variegata can be considered a safe option in the treatment of many of ontological diseases [32,37,38]. The volatile oil of Bauhinia variegata flowers has been shown to exhibit a highly significant increase in cytotoxicity against cell line, because of its strong cytotoxic action.

The effect of Bauhinia variegata

a) Volatile oil on program cell death

The results of this study showed that no obvious cell apoptosis was observed in the negative control group (REF and A549 cells), as shown in Figure 2-A. When REF cells and A549 cells (not treated with volatile oil) were stained with AO/EtBr, the double staining was analyzed under a fluorescence microscope after 24 hours, as shown in Figure 2-A. In contrast, the volatile oil of Bauhinia can induce apoptosis in lung cancer cells. The reduction in cell proliferation often leads to changes in various key signaling pathways, which is caused by the initiation of programmed cell death mechanisms that affect gene expression levels. In addition, the nuclear morphology of treated cells was evaluated using acridine orange-ethidium bromide double staining. Assessment of apoptotic cells by DNA damage. This study also examined the efficacy of redbud essential oil. AO-EB staining was used to examine the different apoptotic characteristics of nuclear changes. After AO/EtBr staining, non-apoptotic cells appear green, while apoptotic cells appear orange or red; the increase in dead cell volume results in uneven orange-red fluorescence and blurred outlines, as they appeared to be dissolving or on the verge of disintegration, as seen in Figures2-B. However, the normal cells (REF cells) treated with the volatile oil from Bauhinia variegata didn't induce apoptosis in lung cancer cells and continued to appear green in color. Double AO/EtBr fluorescent staining can identify fundamental morphological changes in apoptotic cells. Additionally, it facilitates the differentiation between normal cells, dead cells, early, and late apoptosis. Therefore, AO/EtBr staining serves as a quantitative approach to detect apoptosis [39].



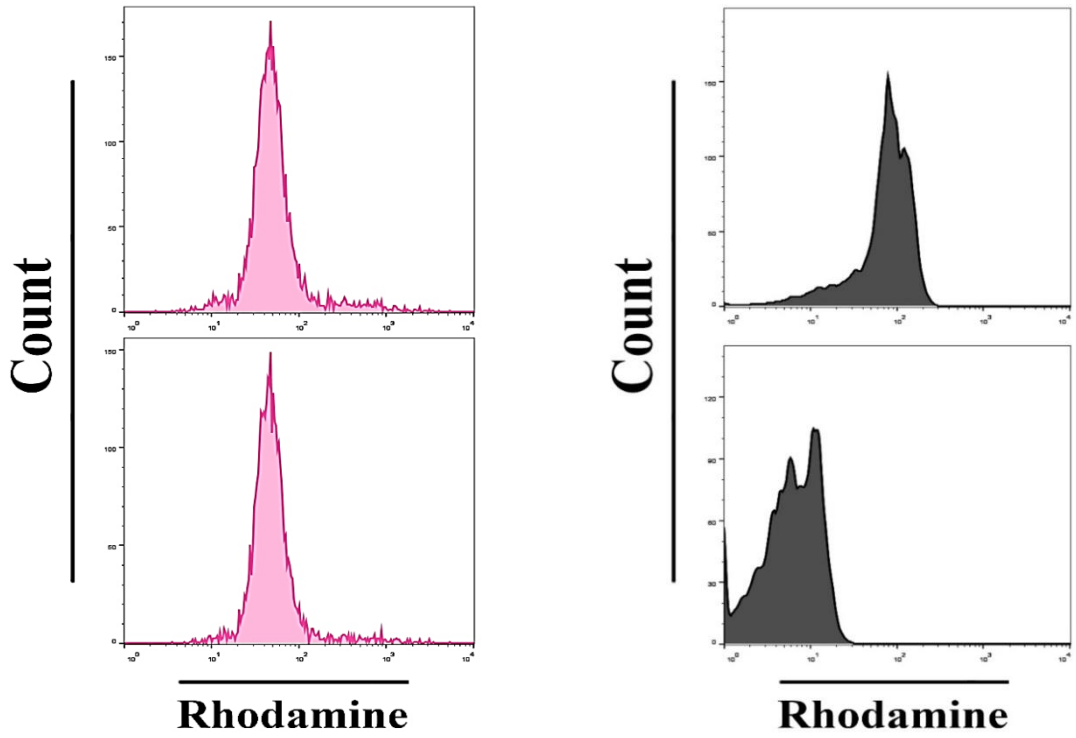
(a) REF cell **(b) A549 cell**

Figure 2. Apoptosis markers in REF & A549 cells following treatment with. volatile oil of *Bauhinia variegata* A, Control untreated REF& A549 cells. B, REF & A549 cells after been treated with volatile oil of *Bauhinia variegata*.

The effect of Bauhinia variegata

a) Volatile oil on mitochondrial cell line

Matrix metalloproteinases (MMPs) are important biomarkers that can serve as potential therapeutic targets for cancer. Mitochondrial dysfunction may be an important feature of apoptosis. Mitochondria play an important role in initiating apoptosis by various cell death stimuli. Changes in mitochondrial organelles are manifested by the loss of mitochondrial membrane potential $\Delta\psi m$ and the release of cytochrome C protein into the cytoplasm of the cell, leading to the regulation of Caspase_3 in the Caspase_9 signaling pathway. In this study, we used flow cytometry to identify MMPs. As measured by flow cytometry, A549 cells treated with essential oils for 24 hours and stained with Rh 123 (rhodamine 123) were significantly reduced, indicating that mitochondrial membrane activity was reduced, and cell apoptosis was significantly increased compared with A549 cells not treated with essential oils. A549 cancer cells after essential oil treatment are shown in Figure 3. A549 cells treated with Bauhinia essential oil for 24 hours showed a significant reduction in Rh123 staining, indicating that mitochondrial membrane potential was reduced compared with untreated control A549 cells. The normal REF cells that are not treated with volatile oil (control) have deviated to the right in the x-axis away from the y-axis, which means that the mitochondrial organelle is still active and able to carry out its vital functions, and the normal cells have shown fluoresce brilliance when treated with the volatile oil of the flowers of Bauhinia variegata plant as a result of the accumulation of rhodamine pigment, which indicates that the mitochondria is not affected by volatile oil and that it is still able to carry out its functions. As for the cancer cells A549, they were deflected to the left of the x-axis as shown in the diagram, away from the y-axis, which indicates the effectiveness and vitality of the mitochondria, but after treatment with volatile oil, their activity decreased significantly, indicating their decomposition and death, as they can be observed at the zero point and not moving towards the x-axis .The results of this study are consistent with the results of the study conducted in 2020, as its results showed the ability of the alcoholic extract of the Bauhinia variegata root to stimulate apoptosis and inhibit the activity of mitochondria, which leads to reducing cancer cells and their activity inside the body of the organism in-vivo, and the results also showed that Bauhinia variegata extract has antioxidant effectiveness because it has reducing power and the ability to remove free radicals [40] is consistent with the results obtained in this study.



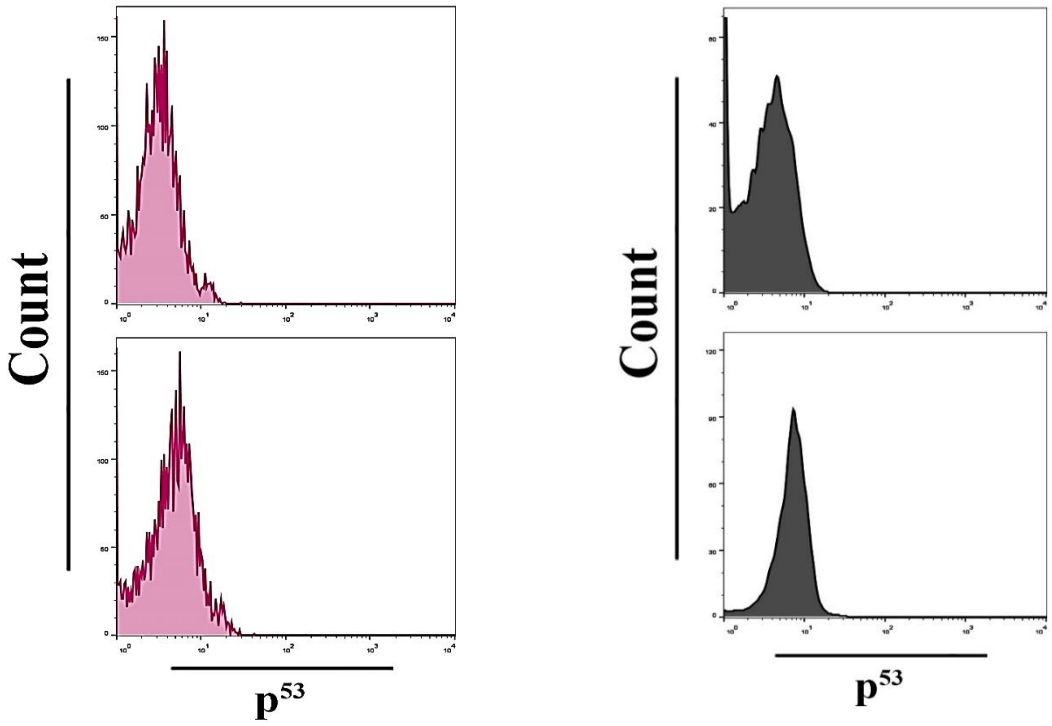
(a) REF cell **(b) A549 cell**

Figure 3. Volatile oil of *Bauhinia variegata* induces mitochondrial dysfunction A549 cells &REF. Upper panel, Control untreated A549 cells &REF. Lower panel, A549 cells & REF after been treated with Volatile oil of *Bauhinia variegata*

The effect of Bauhinia variegata

a) On p53 expression

A test was performed to measure P53 levels in the A549 cell and the REF normal cell line using FITC-anti-p53 and fluorescence P53 staining to give a diagnostic characteristic of P53 in FC. The results of the treatment of A549 cells with volatile oil of Bauhinia variegata showed that the P53 curve has deviated significantly from angle 90 towards the right away from the y-axis towards the x-axis, which leads to the formation of Apoptosome, a type of protein compound that is formed as a result of the release of cytochrome C from mitochondria to stimulate the process of apoptosis, which indicates a significant increase in the level of P53 in A549 cells with a very small percentage of cells settled in the angle at zero point. As shown in Figure 4. In this study, we measured the p53 expression level. The results showed that treatment of A549 cells with Bauhinia essential oil led to a significant increase in p53 compared to the control group. The reason for the increase in P53 in A549 cancer cells treated with essential oils is that the cells have entered the gene expression stage and programmed cell death stage, while the untreated A549 cancer cells have not moved fundamentally, that is, the cells have entered the gene expression stage and programmed cell death stage. the cancer cells were not induced to enter apoptosis and their inability to gene expression. As for the normal REF cells, when treated with essential oil, it was noted that there is almost no difference between the normal REF cells that are not treated (Control) and the treated normal cells, the normal cells do not induce P53 and therefore no gene expression occurred and therefore the normal cells treated with volatile oil didn't enter the stage of programmed cellular death, which caused the curve level to not move almost from the zero point. volatile oils, which are one of the secondary metabolic compounds, are characterized by their effective ability as antibacterial, antifungal and antioxidant, and have also proven effective in inhibiting the growth of cancer cells and regulating the process of programmed death because of their distinctive medicinal and aromatic properties [41,42].



(a) REF cell **(b) A549 cell**

Figure 4. Volatile oil of *Bauhinia variegata* induces p53 expression in REF cell & A549 cells. Upper panel, Control untreated cells. Lower panel, REF cell & A549 cells after been treated with Volatile oil of *Bauhinia variegata*

4. CONCLUSION

Volatile oil extracted from Bauhinia variegata flowers have many active compounds that have been shown to be effective in inhibiting lungs cancer cells and showed potential role in the area of cancer treatment and induce gene expression in apoptosis. For this reason forward working using volatile oil of *Bauhinia variegata* to inhibition other cell line and induce apoptosis will continue.

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CONFLICTS OF INTEREST

The authors did not disclose any conflicts of interest.

FUNDING

There was no external funding for this study.

ETHICS STATEMENTS

This research was conducted in accordance with the institutional and national ethical standards for the care and use of plants in scientific research. All procedures related to plant experiments were conducted in accordance with relevant institutional and national guidelines. Approval was obtained from the Institutional Ethics Committee at [University of Baghdad], with approval number [22-7744 in 12-12-2023]. No experiments were conducted that required special approval or were exempt from approval for justified reasons.

AUTHOR CONTRIBUTION

Study conception and design A. T. Awaed, H. M. Habeeb; data collection: A. T. Awaed; analysis and interpretation of results A. T. Awaed, H. M. Habeeb; draft manuscript preparation: A. T. Awaed, H. M. Habeeb. All authors reviewed the results and approved the final version of the manuscript.

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