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Anti-cancer activity of lavender oil and Newcastle disease virus on human glioblastoma: an in vitro study

Zainab K. Shaheen*, Ali A. Al-Ali, Sarmad A. Al-Asadi

Department of Biology, College of Education for Pure Sciences, University of Basrah, Basrah, Iraq.

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A B S T R A C T

Glioblastoma is one of the most prevalent brain tumors and was the leading cause of cancer death. Since this cancer is an aggressive, incredibly invasive, and neurologically debilitating tumor that does not respond well to chemotherapy, a group of researchers looked at alternative, more efficient therapies. The current study focused on using biological agents and their products (Lavender essential oil and Newcastle disease virus(NDV)) to test their toxicity against the human cerebral glioblastoma (AMGM-5) as a cancer cell model by 3-(4, 5- dimethylthiazol-2-yl-2-) 2.5-diphenyltetrazolium bromide (MTT) viability assay. In brief, the cells were exposed to several concentrations of lavender oil and serial multiplicity of infection (MOI) of NDV for 72 hours. The result shows significantly decreased cell viability treated with lavender oil and NDV in a concentration-dependent manner. The inhibitory concentration that kills 50% of the cells (IC_{50}) of lavender oil was 4.471µg/ml, and the IC50 value of NDV was 0.00473MOI. Also, the morphological study was conducted by the traditional method by using hematoxylin & eosin (H&E) and apoptotic assay by using Acridine orange/Ethidium bromide (AO/EB), the changes are apoptosis and degeneration, as well as cell the emergence of necrotic cells. The compounds of lavender oil were probably responsible for the cytotoxicity of lavender oil. Our results revealed the antagonistic effect of combined essential oil and NDV. Essential oil and NDV have the same toxicity potential for glioblastoma.

1. Introduction

Cancer is one of the most common causes of death in the world, which is why researchers pay great attention to it [1]. The most prevalent brain tumor and one of the deadliest of all human cancers knowing glioblastoma multiforme, which it is an aggressive, incredibly invasive, and neurologically debilitating tumor that does not respond well to chemotherapy [2]. Many different methods of cancer treatment are used today, such as surgery, radiotherapy, and chemotherapy,

*Corresponding author email : zshaheenjisab@gmail.com

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however, because of the non-selectivity of drugs used in this procedure, a huge proportion of healthy cells are also eliminated along with cancerous cells [3]. The most difficult challenge in cancer treatment is eliminating tumor cells in the presence of healthy cells at the same time avoiding harming healthy cells [4]. So, the search for alternative drugs that have activity against a variety of cancers has become one of the most fascinating topics in cancer research [5] According to research, natural plants are one of the finest solutions for treating or preventing cancer, mainly due to the classes of active substances possessed by plants that work against many types of cancer [6]. These compounds can be extracted and used alone or in combination with other anti-cancer therapies [7]. Lavandula is a plant that produces essential oils, these oils are secondary metabolites, and have multiple functions in plants. Lavender has been used before in the field of herbal medicine, traditional medicine, and alternative medicine. There are a variety of biological activities associated with it, among them its anti-cancer properties [8, 9]. Several viruses have been discovered that can kill cancer cells while avoiding normal cells [10]. These viruses are referred to as oncolytic or tumor-killing viruses (OVs) [11]. One of these viruses is the Newcastle disease virus (NDV). A wide range of birds is infected with this virus, which belongs to the Avian Paramyxviridae family [12].The current study intends to assess the role of lavender oil and the NDV LaSota strain in suppressing cancer cell proliferation by measuring the cytotoxicity of studied materials on cell lines and determining the inhibitory concentrations that kill 50% of the cells (IC_{50}). Additionally, the determination of the combined effect of lavender oil and NDV was one objective of the current study.

2. Materials and methods:

2.1 Cell line culture:

The human cerebral glioblastoma (AMGM-5) and human breast cell line (HBL100) were cultured at 37°C in a humidified incubator supplied with 5% CO_2. These cell lines were obtained from the Cell Culture laboratory at the Department of Biology, College of Education for pure sciences, University of Basrah. RPMI-1640 (US biological, USA) containing 10% fetal bovine serum (Biowest, USA) and 2.5 µg/ml gentamycin were used to grow and maintain the cell lines. The cells were passaged using trypsin-EDTA (US biological, USA) [12]

2.2 Cytotoxicity assay

The cytotoxicity of lavender essential oil (Purelyblack, Australia) and NDV LaSota strain (Volvoc®, Germany) on the AMGM-5 cancer cell line and HBL100 normal cell line were investigated based on the inhibition rate of cell growth. In brief, 1×10^4 cells/well were seeded into 96-well plates, then treated with various doses of lavender oil alone (1, 5, 7, 10 µg/ml) and the NDV alone (0.001, 0.0015, 0.01, 0.03 MOI) or a combination of both NDV and lavender oil (1µg/ml+0.001MOI, 3µg/ml+ 0.003MOI, 5µg/ml+ 0.005MOI, 7µg/ml+0.007MOI, 9µg/ml+ 0.009MOI, 11µg/ml+0.01MOI and 13 µg/ml+ 0.025MOI) in 100µl of serum free media and incubated at 37°C. The virus was allowed to remain with the cells for 2 hours at room temperature to ensure virus penetration, this was followed by washing the cells with phosphate-buffered saline and then adding oil concentrations. 10µl of "3-(4, 5- dimethylthiazol-2-yl-2-) 2.5-diphenyltetrazolium bromide(MTT)" was added after 72 hours of exposure and incubated until 2 hours at 37ºC. It was followed by removal of the MTT solution. 100 µl of dimethylsulfoxide (DMSO) was added to the wells, then incubated in darkness until 20 minutes at 37°C. Control group represented by untreated cells. At 492 nm (test wavelength), the absorption of both untreated (control) and treated cells was measured. Utilizing the formula $\frac{t-co}{co} * 100$ the rate of inhibition for cells was calculated, where co stands for the absorbance of cells that haven't been treated, whereas t stands for the absorbance of cells that have been treated. From the plotted curve, the IC_{50} of lavender oil and NDV can be measured [12].

2.3 Determination of cell viability using crystal violet dye

The cells seeded in a 24-well plate at a density of 2×10^4 were exposed to the IC_{50} concentration of the study materials and incubated at 37ºC and 5% CO_2 humidity for 48 hours, then stained with crystal violet for 20 minutes at room temperature and then washed with water.

2.4 Morphological changes

To observe cell morphology under a microscope, the cells were seeded at a density of 5×105 cells/well on cover slides in 6-well plates. The cells were exposed to lavender oil concentrations, and NDV titers and compared with the untreated cells. After that, the plate was sealed with adhesive paper, and it was kept warm and humid with 5% CO_2 at 37ºC. The cells were stained with H&E dye and viewed under a light microscope (Lieca) 48 hours later.

2.5 Apoptosis assay '' Acridine Orange/ Ethidium Bromide, AO/EB''

Previous steps for cell seeded were repeated. The cells was stained with AO (5mg/ml)/ EB (3mg/ml) and then examined with a fluorescence microscope (Lieca) 48 hours later. The percentage of dead and living cells depending on the color of cells under the fluorescence microscope was analyzed. In brief, three non-overlapping images were selected from each cover slide, and dead and living cells were counted. The percentage of dead and living cells is calculated depending on the formula $\frac{mean\ of\ dead\ or\ living\ cells}{mean\ of\ total\ cells} \times 100\%$

3. Statistical analysis

Cytotoxicity data were analyzed using Graph Pad prism software, and variance was analyzed using the one-way ANOVA tests.

4. Results

4.1. Cytotoxicity assay

The MTT assay showed that lavender oil and NDV inhibited AMGM-5 and HBL100 cell growth after 72 hours. There was a dose-dependent response to lavender oil and NDV, so the effect was more effective at higher concentrations (Fig.1, 2). The value of inhibition for a high concentration of lavender oil (10µg/ml) on the AMGM-5 cell line was 65.23% and 39.3% on the HBL100 cell line, whereas the value of inhibition for a high dose of NDV (10MOI) on the AMGM-5 was 67% and 37.5% on the HBL100. In the current study, lavender oil has an IC_{50} value of 4.471µg/ml on AMGM-5, whereas NDV has an IC_{50} value of 0.00473 MOI on AMGM-5. In the normal HBL100 cell line, lavender oil and NDV were not able to reach 50% inhibition rates, so IC_{50} was not recorded.

4.2. The combined effect of lavender oil and NDV

Combination index (CI) data for 7 combined concentrations of both lavender oil and NDV were obtained after 72 hours of exposure using the compusyn Isobologram software version 7 (where CI<1 indicates a synergistic effect, whereas CI>1 indicates an antagonistic effect). The results showed there is a synergistic effect between lavender oil and NDV on the AMGM-5 cell line and the HBL100 cell line at one point (1). As for the rest of the points, they showed an antagonistic effect (Table 1 and 2).

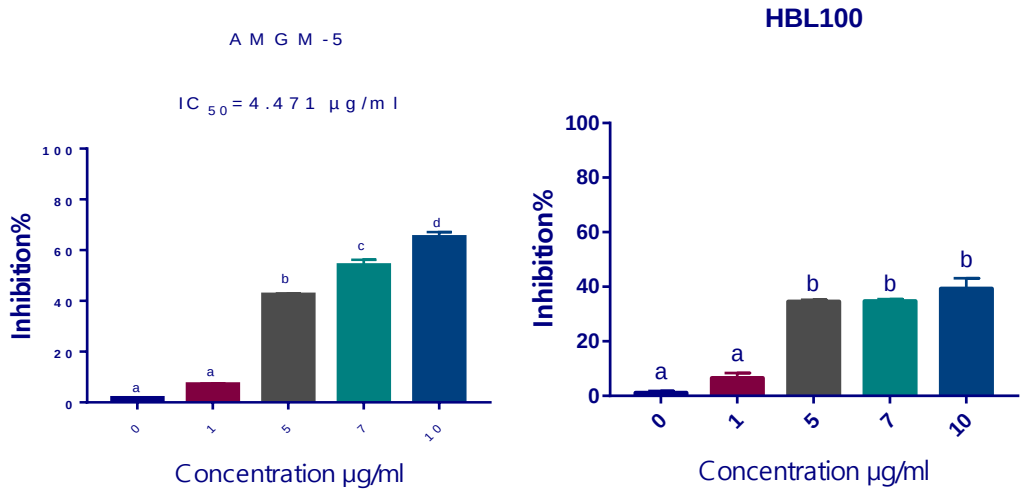


Fig. 1. The significant effect of concentrations of lavender oil on the inhibition rate of cell lines after 72 hours. P≤0.05, (n=3), SD ± Mean. The letters (a, b, c, and d) referred to the significant effect.

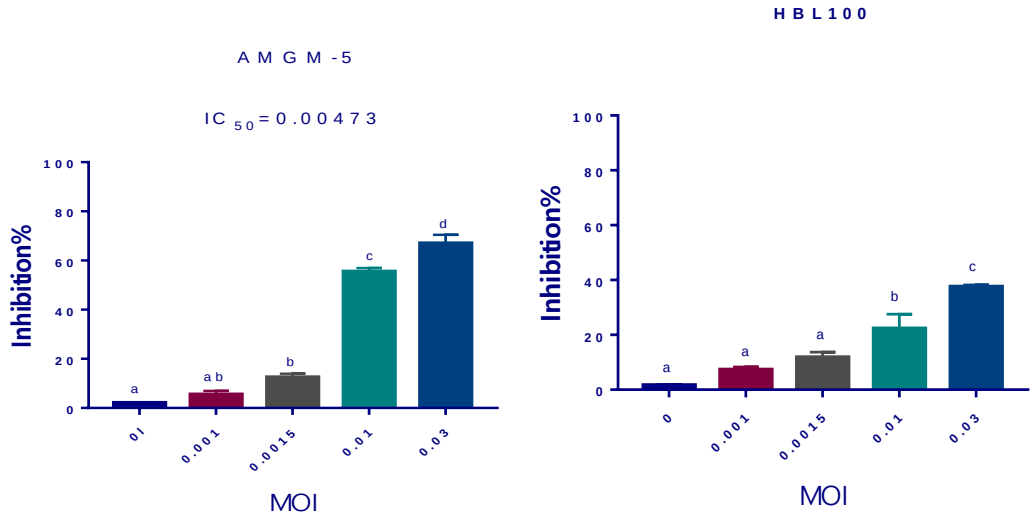


Fig. 2. The significant effect of MOI of NDV on the inhibition rate of cell lines after 72 hour. P≤0.05, (n=3), SD ± Mean. The letters (a, b, c, and d) referred to the significant effect.

Table 1. Combination effect of lavender oil and NDV on AMGM-5 cell line**.**

Dose oil	Dose NDV	Effect%	Combination index
1µg/ml	0.001moi	19	0.06405
3µg/ml	0.003moi	29	1.22389
5µg/ml	0.005moi	33	1.75207
7µg/ml	0.007moi	43	1.73549
9µg/ml	0.009moi	60	1.27868
11µg/ml	0.015moi	67	1.39726
13µg/ml	0.025moi	68	1.89474

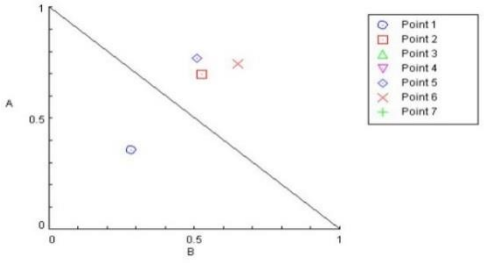
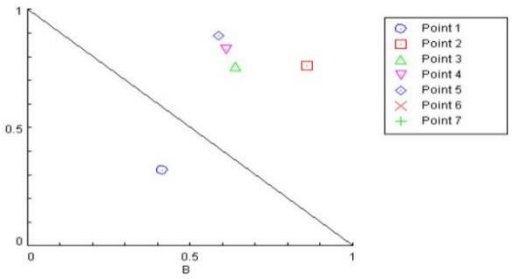


Table 2. Combination effect of lavender oil and NDV on HBL100 cell line**.**

Dose oil	Dose NDV	Effect%	CI
1µg/ml	0.001moi	12.6	0.73520
3µg/ml	0.003moi	16	1.62416
5µg/ml	0.005moi	26	1.40260
7µg/ml	0.007moi	32	1.44530
9µg/ml	0.009moi	37	1.47747
11µg/ml	0.015moi	39	1.88936
13µg/ml	0.025moi	43	2.21791



4.3. Determination of cell viability using crystal violet dye

To explore the influence of lavender oil and NDV on cell proliferation and density, as well as changes in cell viability when exposed to death-inducing study materials, crystal violet dye was used. The cells exhibit less density of crystal violet dye than the control group due to the cells' death (Fig. 3).

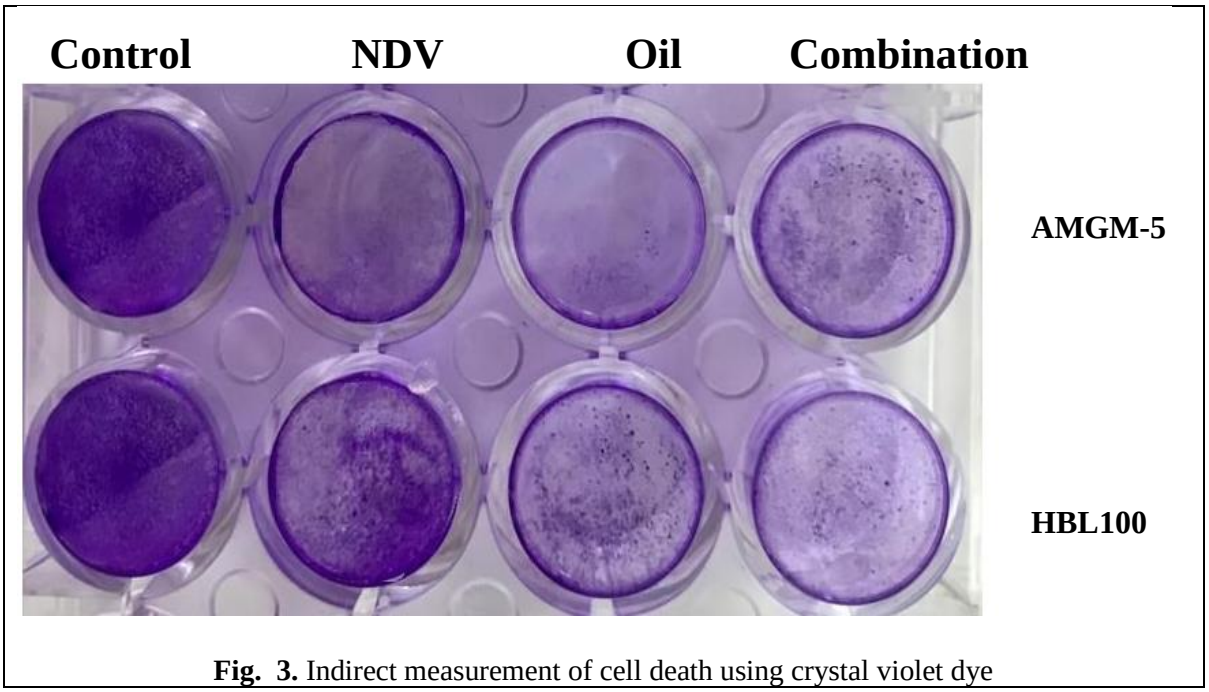


Fig. 3. Indirect measurement of cell death using crystal violet dye

4.4. Morphological changes

The cytopathological changes were studied in the AMGM-5 cancer cell line and HBL100 normal cell line treated with NDV and lavender oil alone or in combination. The pathological changes included cell shrinkage, degeneration, necrosis, and aggregation of cells that lost contact with neighboring cells (Fig. 4, 5).

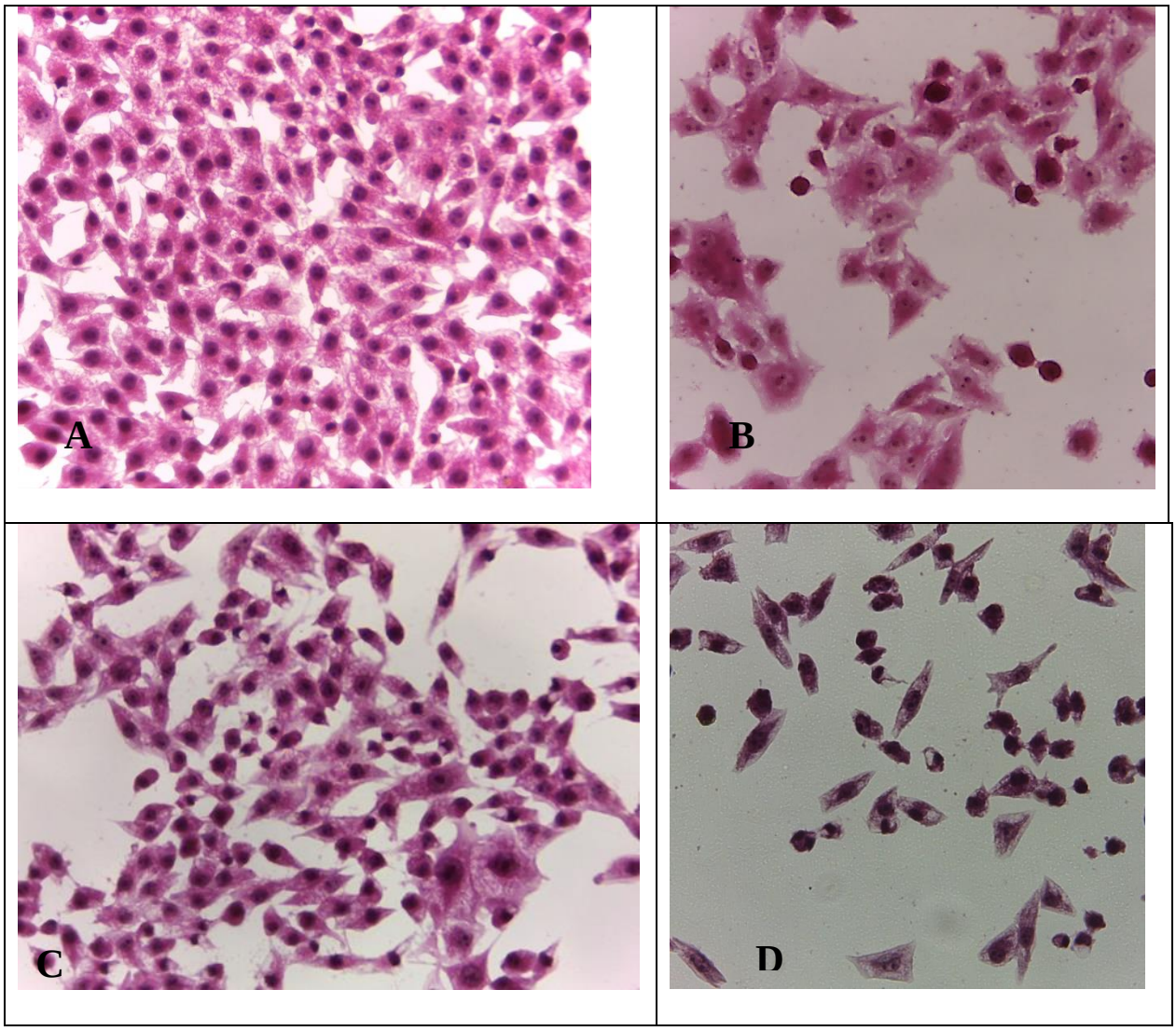


Fig. 4. HBL100 cell line morphological analysis after staining with hematoxylin and eosin after 48 hours. **(A)** Untreated confluent monolayer cells,40x. (**B**) Treated cells with IC_{50} of lavender oil, 40x. **(C)** Infected cells with IC_{50} of NDV, 40x. (**D)** Treated cells with combination of NDV+ lavender oil, 40x.

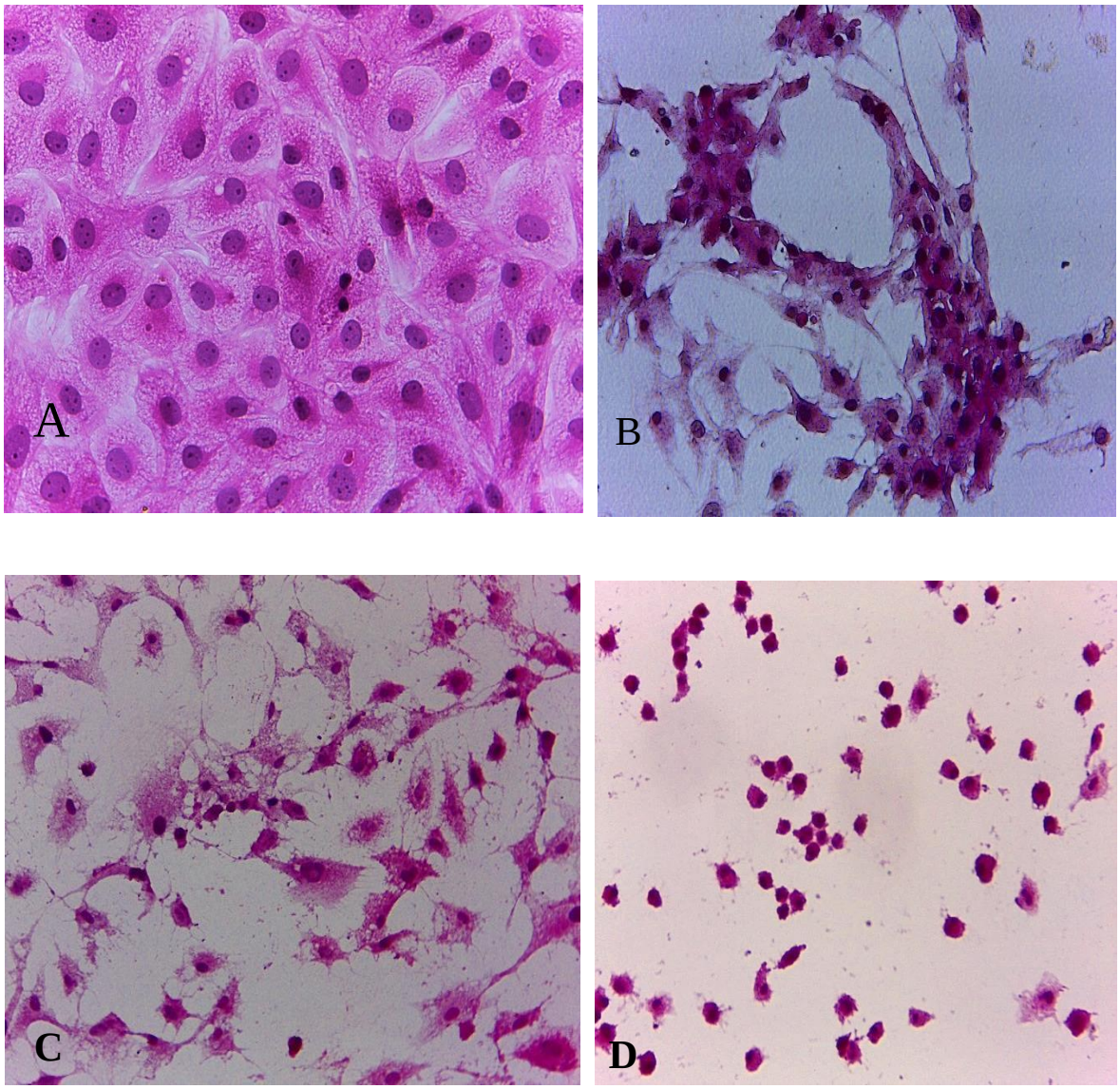


Fig. 5. AMGM-5 cell line morphological analysis after staining with hematoxylin and eosin after 48 hours. **(A)** Untreated confluent monolayer cells,40x. **(B)** Treated cells with IC_{50} of lavender oil, 40x. **(C)** Infected cells with IC_{50} of NDV, 40x. **(D)** Treated cells with combination of NDV+ lavender oil, 40x

4.5. Apoptosis assay

To distinguish between live cells, and the early or late phases of apoptosis after 48 hours of exposing the cell line to the lC_{50} doses of lavender oil and NDV, AO/EB dye was used and depending on the difference in dye permeability. Viable cells allow the AO dye to pass through their selective membranes, so the cells appear green, whereas, EB dye only passes through the damaged membranes of dead cells and thus appears in red (Fig. 6, 7).

Table 3. The percentage of apoptotic cells in comparison to control cells.

Cell lines	Type of treated	Dead cells%	Viable cells%
	Control	0	100
HBL100	Oil	35.5	64.5
	NDV	33.3	66.7
	Combination	40.8	59.2
	Control	3.1	96.9
AMGM-5	Oil	59.8	40.2
	NDV	52.3	47.7
	Combination	60	40

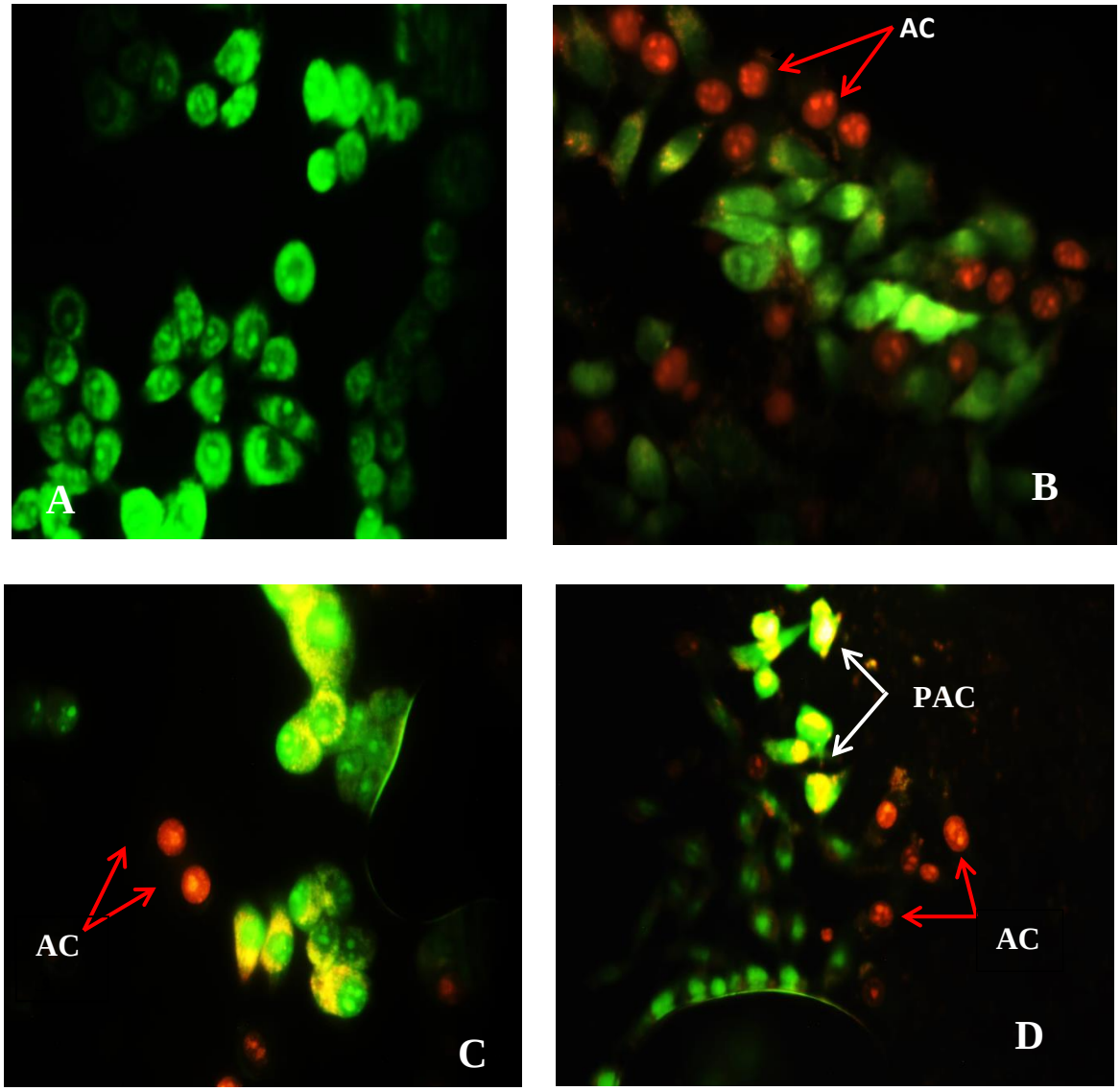


Fig. 6. Morphological characteristics of the HBL100 cell line following AO/EB staining after 48 hours. **(A)** Untreated cells, 40X. **(B)** Oil-treated cells 40X. **(C)** NDV- treated cells, 40X. **(D)** Cells were treated with a combination of NDV and oil, 40X. SN (AC=Apoptotic cells, PAC= Pre-apoptotic).

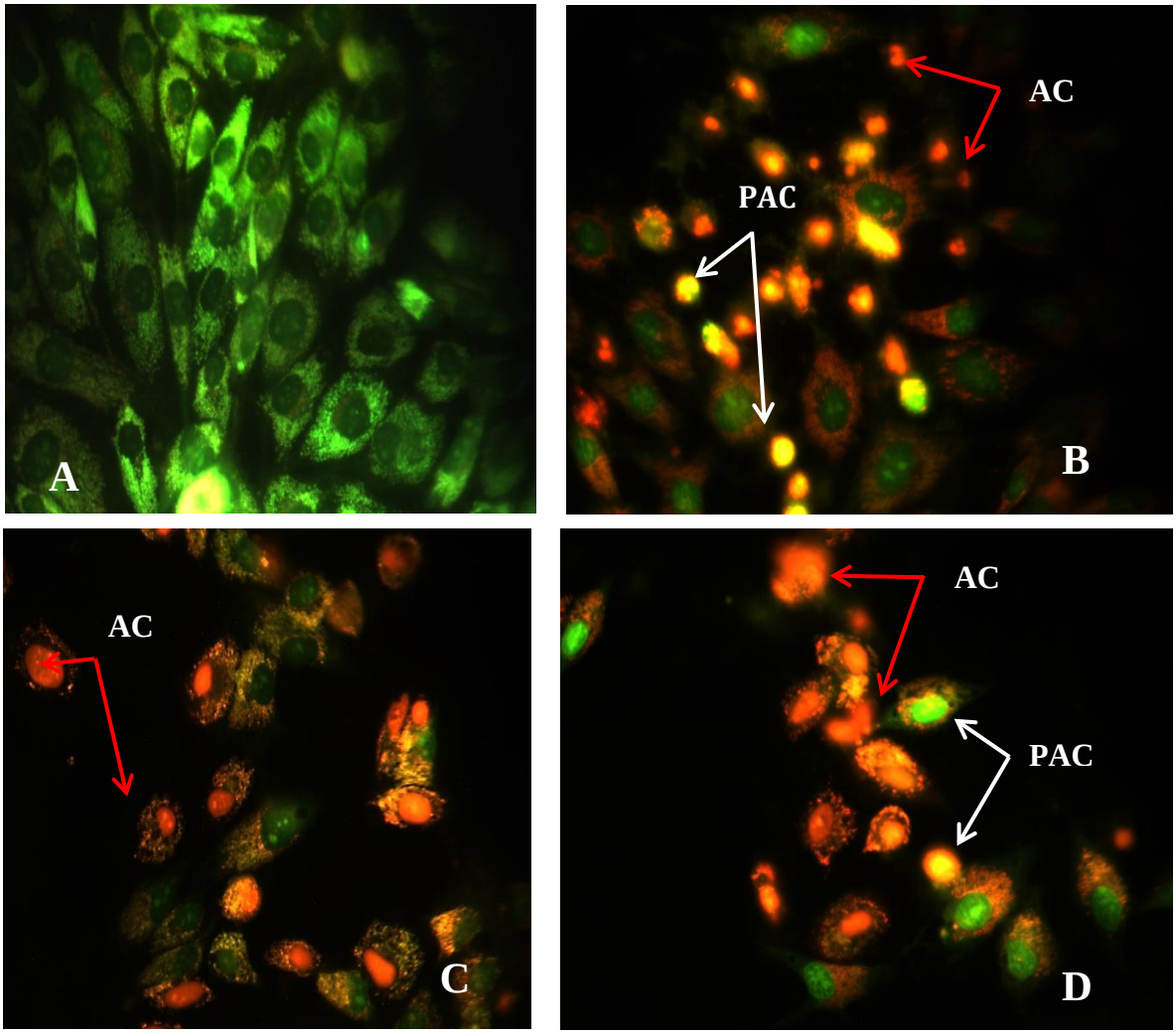


Fig. 7. Morphological characteristics of the AMGM-5 cell line following AO/EB staining after 48 hours. **(A)** Untreated cells, 40X. **(B)** Oil-treated cells 40X. **(C)** NDV- treated cells, 40X. **(D)** Cells were treated with a combination of NDV and oil, 40X. SN (AC=Apoptotic cells, PAC= Pre-apoptotic).

5. Discussion

Due to the negative effects of medications used to treat cancer, as well as the rise in cases of resistance to these therapies, researchers are working to improve the chances of survival for cancer patients by prioritizing the search for alternatives therapies derived from natural sources [8]. Recently, there has been a notable increase in the use of natural resources such as lavender oil and NDV in many areas of life. The applications of such natural resources have sparked interest to study both their beneficial and detrimental effects [13]. Numerous natural plant products have positive effects on human health and, in some situations, can cause apoptosis in various cancer cell types [14, 15]. Lavender oil is considered a safe oil in many research on the effectiveness of cytotoxicity [16], [17], [18]. In the current study, lavender oil was employed without separating its active components, and the results showed that lavender oil has an inhibitory activity on cell viability. Therefore, the anti-cancer activity is due to all its chemical components [19]. Several research has investigated that chemical analysis of components of lavender oil and were successful in classifying them [20, 21]. The toxicity of lavender oil varies between the normal cell line

HBL100 and the cancer cell line AMGM-5, with the normal line being less harmed. In contrast to the previous study's findings, the cytotoxicity of lavender oil did not differ between the normal and cancer cell lines [21]. Oncolytic viruses are a promising therapeutic technique for cancer treatment [12], [22]. The findings of this investigation agreed with prior findings that the NDV LaSota strain has anticancer activity on cancer cells [23-25]. NDV was found to be less cytotoxic to a normal HBL100 cell line than in cancer cells in the present study. This finding was consistent with prior findings revealing the NDV's preference for cancer cells [25-27]. The NDV's selectiveness against tumor cells is a result of its lack of interferon, which allows it to replicate efficiently in tumor cells [28]. Chemotherapy and essential oils have been studied synergistically and antagonistically in recent studies [11]. In contrast, NDV is employed in combination therapy with other medications or materials [12], [29]. As part of this study, lavender oil and NDV were combined to assess their potential as a combination therapy. In the current investigation discovered a synergistic impact of lavender oil and NDV on the cell lines at one point, however, it was ineffectual. While the remaining points demonstrated antagonistic behavior. The antagonistic effect may be due to the fact that lavender oil has an inhibitory effect on the NDV. Previous research has demonstrated the efficacy of essential oils to prevent viral infections[30, 31]. The morphology of AMGM-5 cells is affected by exposure to study materials since the loss of their regular shape with shrank edges, loosen adhesions, more dead floating cells, and decreased cellular density which suggests the antineoplastic action of lavender oil and NDV. HBL100 cells underwent similar morphological changes, though in a less severe manner since the materials were less toxic on the normal line. Treating AMGM-5 and HBL100 cells with NDV and lavender oil and analyzing the results with acridine orange staining shows the presence of necrotic cells, pre-apoptotic cells, and apoptotic cells. The studied materials cause pathological effects on cells by stimulating apoptosis [8], [12].

6. Conclusion and Recommendation

The use of natural resources plays an effective role in limiting the spread of tumors through their toxic properties can in inducing programmed cell death, and therefore they can be used as anti-cancers or as models to produce high-efficiency anti-cancer drugs.

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نشاط زيت اللافندر وفيروس مرض نيوكاسل المضاد للسرطان ضد ورم الأرومة الدبقية البشري: دراسة خارج جسم الكائن الحي

زينب كاظم شاهين*، علي عبد اللطيف عبد الحسن العلي، سرمد عواد موزان الاسدي

قسم علوم الحياة، كلية التربية للعلوم الصرفة، جامعة البصرة، البصرة، العراق.

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الملخص

يعد ورم الأرومة الدبقية أحد أكثر أورام الدماغ انتشارًا و السبب الرئيسي للوفيات الناتجة من السرطان. ونظرًا لأن هذا النوع من الاورام عدواني وغاز بشكل لا يصدق وموهن عصبيًا ولا يستجيب جيدًا للعلاج الكيميائي ، فقد اتجهت انظار الباحثين نحو ايجاد علاجات بديلة أكثر كفاءة. ركزت الدراسة الحالية على استخدام العوامل البيولوجية ومنتجاتها (زيت اللافندر وفيروس مرض نيوكاسل) لاختبار سميتها ضد ورم الأرومة الدبقية البشري (AMGM-5) كنموذج للخلايا السرطانية باستعمال اختبار (3-(4, 5- dimethylthiazol-2-yl-2-) 2.5-diphenyltetrazolium bromide (MTT)). باختصار ، تم تعريض الخلايا لتراكيز متعددة من زيت اللافندر والتعدد المتسلسل للعدوى (MOI) من فيروس مرض نيوكاسل لمدة 72 ساعة. تظهر النتيجة انخفاضًا كبيرًا في حيوية الخلايا المعالجة بزيت اللافندر و NDV بطريقة تعتمد على التركيز. كان التركيز المثبط الذي يقتل 50٪ من الخلايا (IC_{50}) من زيت اللافندر 4.471 ميكروغرام / مل ، بينما كانت قيمة IC_{50} لفيروس مرض نيوكاسل 0.00473 MOI. أجريت الدراسة المورفولوجية بالطريقة التقليدية باستخدام (hematoxylin & eosin, H&E) ودراسة موت الخلايا المبرمج باستخدام (acridin orang/ ethidium bromide, AO / EB) ، تراوحت التغيرات ما بين موت الخلايا المبرمج والتنكس ، وكذلك ظهور الخلايا الميتة. تعود السمية الخلوية لزيت اللافندر للمركبات الكيميائية التي يحتويها. كشفت نتائجنا عن التأثير المضاد لزيت اللافندر و NDV. أيضا كشفت الدراسة ان زيت اللافندر و فيروس مرض نيوكاسل لهما نفس احتمالية السمية للورم الأرومي الدبقي.

***Corresponding author email :** zshaheenjisab@gmail.com

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