Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **43** (special issue, Part 3): 1079-86 (April 2024)

Article presented at the Fifth
International Scientific Conference
of Alkafeel University (ISCKU 2024)

Evaluation of Laurus nobilis, (Bay oil) anti-tumor potential on Human brain cancer AMGM-5 and Normal human HBL-100 cell lines

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***SUMMARY*. Based on MTT technology ,The cytotoxicity of laurel oil has been evaluated on normal and cancerous lines , The represented HBL-100 and AMGM-5 By exposing the cells to a series of oil concentrations(0.5, 1, 5, 10, 15, 20, 40, 80 µg /ml) for a period of 72 hours. The rate of inhibition increased with increasing concentration. The highest rates of inhibition were recorded in cell cultures treated with concentrations of 10, 15, 20, 40, and 80. It reached more than 60%, while the lowest rates of inhibition were at concentrations of 0.5, 1, and 5 micrograms/ml, as it did not exceed 20%. The half-cell inhibitory concentration (IC50) of laurel oil was obtained, as its value reached 5.493 µg/ml in the cancer cell line AMGM-5, while its value reached 4.514 µg/ml in the normal cell line HBL100. Apoptosis was detected with Ethidume bromide - acriden orange dye and An immunohistochemistry study of the programmed cell death proteins caspase 3 in cell line AMGM-5, as well as an estimation of the levels of protein expression of proteins associated with programmed cell death and quantification of gene expression of these genes will reveal the various apoptotic pathways stimulated by the action of the oil.**

RESUMEN. Basado en la tecnología MTT, la citotoxicidad del aceite de laurel se ha evaluado en líneas normales y cancerosas, representadas por HBL-100 y AMGM-5 mediante la exposición de las células a una serie de concentraciones de aceite (0,5, 1, 5, 10, 15). , 20, 40, 80 µg/ml) durante un periodo de 72 horas. La tasa de inhibición aumentó al aumentar la concentración. Las tasas de inhibición más altas se registraron en cultivos celulares tratados con concentraciones de 10, 15, 20, 40 y 80. Alcanzó más del 60%, mientras que las tasas de inhibición más bajas se produjeron en concentraciones de 0,5, 1 y 5 microgramos/ ml, ya que no superó el 20%. Se obtuvo la concentración inhibidora semicelular (CI50) del aceite de laurel, cuyo valor alcanzó 5,493 µg/ml en la línea celular cancerosa AMGM-5, mientras que su valor alcanzó 4,514 µg/ml en la línea celular normal HBL100. Se detectó apoptosis con bromuro de Etidume - colorante naranja acriden y se realizó un estudio inmunohistoquímico de las proteínas de muerte celular programada caspasa 3 en la línea celular AMGM-5, así como una estimación de los niveles de expresión proteica de proteínas asociadas a la muerte celular programada y cuantificación de La expresión genética de estos genes revelará las diversas vías apoptóticas estimuladas por la acción del aceite.

INTRODUCTION

One of the most dangerous and bizarre diseases is cancer, unchecked and invasive cell proliferation is a hallmark of cancer. The propensity of cancer cells to spread to other particular organs is one of their most unusual characteristics. As an illustration, lung cancer cells spread to the adrenal glands, liver, brain, and bones while breast cancer cells metastasis to the lungs and bones in both prostate and colon cancer The spread of tumor cells from their original sites to nearby structures and distant locations is referred to as metastasis. [1][2][3] . The challenge for researchers is also to develop safe therapeutic approaches that can distinguish between cancerous and healthy cells. The pharmaceutical industry has contributed to saving humanity from the scourge of diseases, especially the intractable ones, but it has become an obsession that haunts humanity, due to the disadvantages of these treatments, side effects and complications due to the cumulative action, in addition to the high cost of treatment. In this context, the goal of scientific experiments at the present time is to find alternative compounds to treat such diseases [4] Essential oils are defined as complex volatile secondary metabolites that are isolated from any part of plants .The French chemist Dumas (1833) performed the first systematic

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ISSN 0326 2383 (printed ed.)
ISSN 2362-3853 (on line ed.)

description of the constituents of essential oils and explained that the main characteristics of essential oils are volatile, aromatic, fluid, lipophilic, colored or colorless, generally of a density less than water, in addition to being rich in life-active components. They can be easily separated from other components of plants. Essential oils and their constituents are now gaining global attention due to their multi-purpose functional use [5] Despite numerous plant oils have demonstrated efficacy in treating the most severe diseases, independent of the pathogens associated, many plants around the world are still being researched for their active components and potential role in disease prevention.[6] Many studies have praised Laurus nobilis, its efficiency in fighting many types of cancer and combating cancer cells and limiting their spread[7] There is proof that the oils from Laurus nobilis, , and certain other plants in the Lauraceae family are effective against cancerous tumors. [8] [9] [10]

MATERIALS AND METHODS

Maintenance of cell cultures

The IRAQ Biotech Cell Bank Unit in Basrah provided the cancer cell lines, which were then kept in RPMI-1640 supplemented with 10% fetal bovine, 100 units/mL penicillin, and 100 µg/mL streptomycin. According to [11] cells were passaged using trypsin-EDTA, reseeded at 70% confluence twice or three times per week, and incubated at 37 °C and 5% CO2.

Cytotoxicity Assays

On 96-well plates, the MTT cell viability assay was performed to ascertain the cytotoxic effect. 〖1×10〗^4cells/well was the seeding density for cell lines [12] Cells were treated with a series of oil concentrations (0.5, 1, 5, 10, 15, 20, 40, 80 µg/ml) after a 24-hour period or until a confluent monolayer was obtained. Following a 72-hour treatment period, the media was removed, 28 µL of a 2 mg/mL MTT solution was added, and the cells were incubated for 2 hours at 37 °C to determine the viability of the cells. Following the removal of the MTT solution, 100 µL of DMSO (dimethyl sulphoxide) was added to the wells to solubilize the residual crystals. This was done for 15 minutes at 37 °C while the mixture was shaken The microplate was used to measure the absorbency The assay was run in triplicate, and the absorbency was measured using a microplate reader set at the test wavelength of 620 nm.[13][14]

Vitality ratio percentage = (absorbance rate of treated cells / absorbance rate of untreated cells) x 100

And The following formula was used to determine the percentage of cytotoxicity, (or the inhibition rate of cell growth)

Inhibition percentage = 100 - vitality percentage

IR=100 - PR

the inhibition rate = (absorbance rate of untreated cells \ absorbance rate of treated cells cells) x 100

IC50 or half-cell inhibitory concentration

After repeating the treatment experiments with three to five replicates for each cell line, the inhibitory concentration (IC50) value for laurel oil was extracted. Then, using the Graph Pad Prism 7 program, the inhibition percentages for all oil concentrations were calculated [15]

Detection of Cell death by staining with Acredine Orange & Ethedium Bromide (AO&EB)

Staining with Acryline Orange and Ethenium Bromide (AO&EB) to identify cell death .The cells of the cell lines were planted on the cover of the slide in the same way as mentioned in the previous paragraphs, and then the cells were exposed to a semi-lethal concentration of oil. The dishes were incubated for 24 hours at a temperature of 37°C and a humidity of 5% CO2, then they were extracted from the incubator and dyed with Ethidume bromide - acriden orange dye. They are kept in dark conditions, examined using a fluorescence microscope, and photographed using a Canon digital camera [16]

Immunological detection of programmed cell death proteins

In order to detect the programmed cell death protein caspase3 in the brain cancer cell line AMGM-5, the Ultra-Cruz® Kit was utilized to cultivate the cells on a Charge slide. After being incubated for 24 hours at 37°C, 5% humidity, and CO2 incubator, they were treated with half the inhibitory concentration (IC50) of oil when they developed a single integrated layer of cells at a rate of 80–90%. For 48 hours, the cells were fixed with cold acetone at 4°C for half an hour, then placed in 1% H2O2 for half an hour, then the cells were washed with Phosphate Buffer Saline (PBS) twice for 5 minutes each. Following an hour in Ultra-Cruz® Blocking Reagent solution, the Blocking Reagent was pulled from the cells and the primary antibody was diluted with it to a concentration of 4 µg/ml at 4°C for 24 hours. After that, the cells were twice rinsed with PBS for five minutes. After that, each time, the secondary antibody was diluted with blocking at a ratio of 1 to 25 for an hour, and three PBS washes were performed. Then it was exposed to the brown 3,3'-Diaminobenzidine (DAB) dye for 10 minutes. It was washed with distilled water to stop the reaction. Hematoxylin stain was used for double stain-

ing. It was then washed with distilled water. Then it was passed in xylene for 5 minutes. The slide cover was placed on the glass slide by placing one drop of Canada. The cells were then examined and photographed with an optical microscope [17] Pictures were taken in non-overlapping areas of the slide for each treatment for the purpose of calculating the number of dead cells, as cells stained brown with DAB dye indicate the expression of programmed cell death proteins.

Analytical statistics

To determine the IC50 for cell lines, I run a One Way Anova test using Graph Pad Prism software.

RESULTS AND DISCUSSION

Cytotoxicity of laurel oil in cell lines

Based on the MTT technique, the current study's results demonstrated that laurel oil, when applied in a series of concentrations (0.5, 1, 5, 10, 15, 20, 40, 80 micrograms/ml) for a period of 72 hours, had an inhibitory effect on both cancerous and normal cell lines (AGM-5, HBL100), with the rate of inhibition increasing with concentration. Cell cultures treated with doses of 10, 15, 20, 40, and 80 exhibited the highest rates of inhibition, with values above 60%. If the concentration does not surpass 20%, the lowest rates of inhibition were seen at 0.5, 1, and 5 micrograms/ml, as shown in Figures (1) and (2) One way anova was used in the statistical analysis, and the findings indicated that low concentrations (0.5, 1, 5) did not appear inhibition, but there were significant differences between the high concentrations (10, 15, 20, 40, 80) and the low concentrations. High (10, 15, 20, 40, 80) demonstrated notable distinctions between them at the probability threshold P≤0.05, as illustrated in Figures (1) and(2) The half-cell inhibitory concentration (IC50) of laurel oil was obtained, as its value reached 5.493 µg/ml in the cancer cell line AMGM-5, while its value reached 4.514 µg/ml in the normal cell line HBL100.

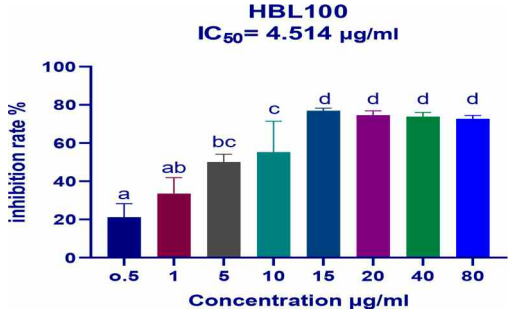


Figure (1) Average rate of inhibition for HBL100 cell lines treated with a series of concentrations of Bay oil after an exposure period of 72 hours based on the MTT assay method, (P≤0.05, N=4, SD±Mean)

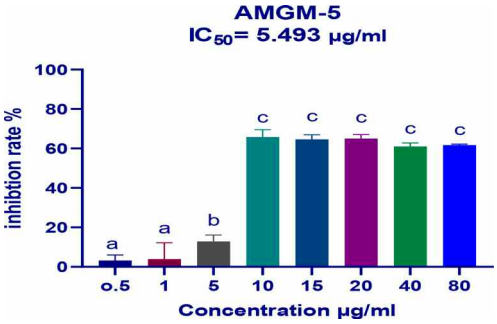


Figure (2) Average rate of inhibition for AMGM-5 cell lines treated with a series of concentrations of Bay oil after an exposure period of 72 hours based on the MTT assay method, (P≤0.05, N=4, SD±Mean).

Determining cell vitality using crystal violet dye

Test the effect of stimulating programmed cell death using laurel oil on cell proliferation and density and identify differences in cell vitality using Crystal Violet dye. The treated cells separate On the surface of the tissue culture dishes as a result of their death, the violet dye appears in a light color due to the lack of live pigmented cells. This was more evident in the treated cancer cells than in normal cells, compared to the color of the control group, which appeared in a dark violet color, which indicates the vitality of the cells. The effects were exacerbated in all cancerous and normal cell lines when treated with a mixture of study materials. This was evident from the color of the dye, as in the figure (3)

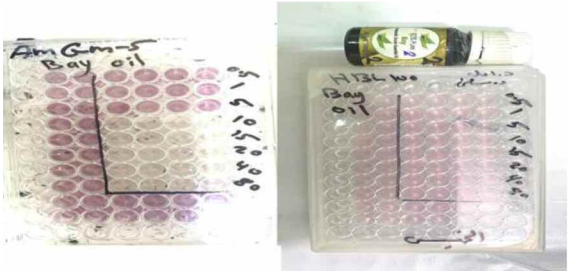


Figure (3) MTT test for cancerous and normal cells treated with a series of laurel oil concentrations, Use crystal violet dye to determine the vitality of cells after treatment for 24 hours

Apoptotic assay(AO/EB stain)

The normal and cancer cell lines HBL100and AMGM-5were stained with AO/EB for 48 hours. The findings of the fluorescence microscope examination revealed that the untreated cells were dyed green with AO dye, indicating that the cells were safe and unaffected. Conversely, some cells treated with oil at an IC50 concentration were stained yellow, signifying that they had undergone an early stage of apoptosis. A

further level of programmed cell death(Late apoptosis) has been reached by the cells, as seen by the red staining of other cells with the EB dye.

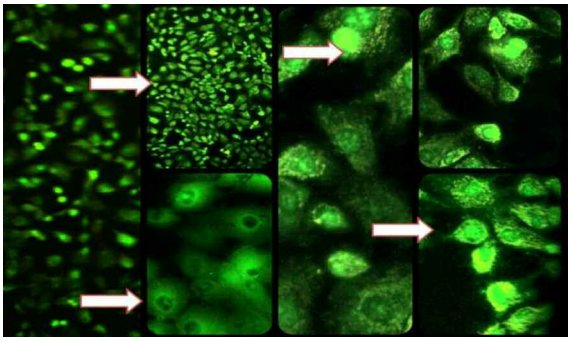


Image (3-1) HBL-100 cell line (Untreated oil-treated) after staining with AO/EB stain which appears in green, which is indicated by arrows 400X magnification

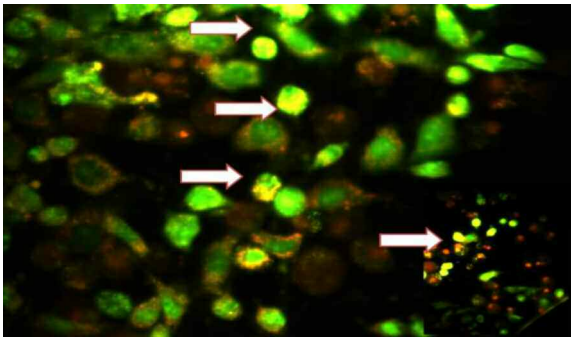
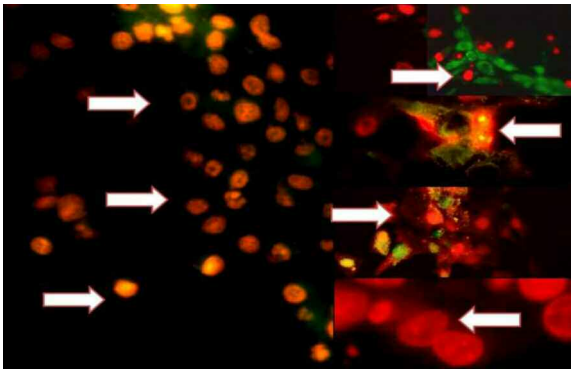


Image (3-1) Cells treated with an IC50 concentration of laurel oil were dyed yellow, indicating a state of early apoptosis that the cells had undergone. 400X magnification.



Image(3-3) Cells treated with IC50 half the lethal concentration of laurel oil reached an advanced stage of programmed cell death(Late apoptosis), so they were dyed red for the EB stain.

3-4 Immunohistochemistry

The results of microscopy showed that the caspase3 protein, a hallmark for programmed cell death, was immunodetected. In cancer cells AMGM-5 treated with half the lethal concentration IC50 of laurel oil This indicates the occurrence of protein expression of programmed cell death proteins The presence of programmed cell death proteins is indicated by the dark brown staining of some cells with DAB dye.

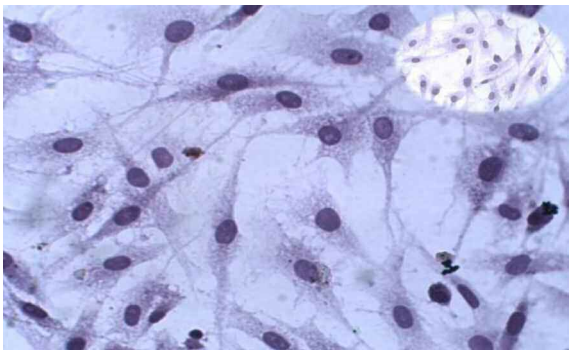


Image (3-4) AMGM-5 cell line stained with DAB and immunohistochemistry (Caspase3) control group 400X magnification

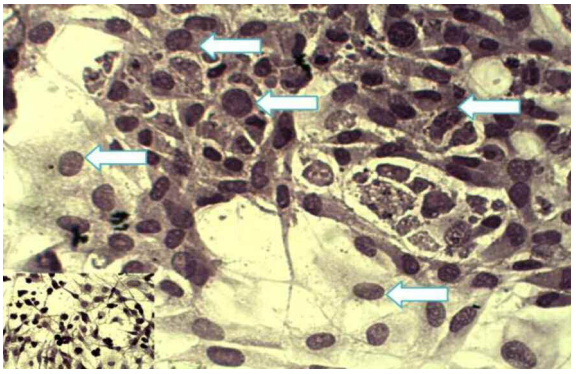


Image (3-5) AMGM-5 cell line treated with IC50 of laurel oil stained with DAB stain and by immunohistochemistry technique (Caspase3) takes on a dark brown color indicating the induction of cell death in these cells. 400X magnification

There are two mechanisms that lead to programmed cell death: the extrinsic and intrinsic pathways. Both processes utilise caspases, which are proteases specialized to cysteine, to activate several intracellular proteins and cause programmed cell death [18] Both the intrinsic and extrinsic pathways of programmed cell death are triggered by intra- or extracellular signals [19] The intrinsic pathway is known as the mitochondrial pathway, and the extrinsic pathway is known as the death receptor pathway. A wide range of factors can trigger these pathways, including DNA damage or uncontrollably multiplying cells. When the signal for programmed cell death starts, changes start to occur within the cell. Among these alterations is the activation of caspase proteins, which break apart cellular substances required for regular cellular operations, including cellular and nuclear proteins [20] The Caspases protein activation triggers the beginning of

cell death. According to [21] cells are programmable to contract, causing alterations in their plasma membrane that serve as a signal for macrophage cells to respond. According to [22] the efficiency of caspase proteinswhich can break apart hundreds of different proteins correlated with the success of programmed cell death. The caspase proteins are categorized into two groups: the four types of initiator caspases (caspase2, 8, 9, 10) and the three types of executor caspases (caspase3, 6, 7). Target proteins are broken down by executioner caspases [23] which causes the cell to undergo programmed cell death.It is crucial to understand that cancer cells can evade programmed cell death by blocking the action of caspase proteins, expressing more anti-apoptotic BCL-2 family proteins, inhibiting the proteins that trigger programmed cell death, blocking the activation of tumor-stimulating mutations in the BCL-2 family, or overexpressing BCL-2 anti-apoptotic genes, which have been found to be overexpressed in over half of all cancers, regardless of type [24] Cell death is carried out via the intrinsic programmed cell death mechanism using mitochondria and mitochondrial proteins. The BCL-2 family of proteins, some of which are present in the cytoplasm, regulates this route by controlling the permeability of the mitochondrial membrane [25] By triggering the death-activating cell activators BAX, BID, BAD, BIM, and BAK, downregulation of this group of organisms induces apoptosis [26][27]. The tumor suppressor gene p53 also activates BAX [28] When BAX and BAK are active, the mitochondrial membrane (MOMP) becomes completely permeable, which renders the cell irreversibly damaged [29] The release of mitochondrial components such cytochrome c and second mitochondria-derived active caspase (SMAC) is made possible by the complete permeability of the mitochondrial membrane. Through the interaction of mitochondrial proteins with the apoptosis activation molecules procaspase9 and APAF-1, these proteins create the apoptosome in the cytosol [32] [33] Executioner Caspase (Caspase3,6,7) is triggered when Procaspase9 in the apoptosome is activated to Caspase9 [33] Caspase, the executioner, starts to break apart intracellular proteins, which results in cell death. As for the roles that are played through Programmed cell death is induced via the extrinsic pathway death receptors belonging to the Tumor Necrosis Factor (TNF) family are bound by live signals, also known as death ligands. FAS-L and TFN-α are examples of death ligands According to the adaptor protein attaches itself to the endogenous origin death receptor [34] show [35] that there are two different types of adaptor proteins: TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADDNext, the adaptor protein death-inducing signaling complex (DISC) and procaspases-8 and 10 were discovered DISC initiates the process of fragmenting intracellular proteins and cytoskeletal proteins to finish cell death by activating procaspases8, 10 to caspase8, 10. This is followed by the activation of caspase3, 6, 7 When caspase signaling is weak, mitochondria participate in the extrinsic path [36] The BID protein is divided into tBID by caspases 8 and 10, and the latter triggers death through mitochondria, as in the intrinsic pathway, by activating the BAX and BAK proteins [37]. These results are consistent with several scientific studies that have employed laurel oil to suppress cancer cells and suggest that bay leaf may have potential as a therapeutic agent with anti-cancer capabilities for a range of cancer cells. Laurel oil has been shown to have anti-cancer action by testing on numerous cancer cell lines. This is because the monoterpene molecule in Laurel oil causes toxicity when applied to cancer cell lines. As a result, Laurel oil can be utilized as a cancer treatment [38]. Monoterpene Hydrocarbons and Oxygenated Monoterpenes According to a study that identified the active compounds and their concentrations in the oil .Many compounds were also isolated and characterized in the study-Monoterpenes may be effective anticancer treatments because they have anti-metastatic, anti-proliferative, and It is certain and proven that The quality of the oil used medicinally is determined by the ratio of a monoterpene with the required biological activity the most significant components found in laurel oil were. oxygenated sesquiterpenes, monoterpene hydrocarbons, and oxygenated monoterpenes. And 1,8-cineole was dominant. The effectiveness of the Laurus nobilis oil was attributed to1,8-Cineole [39-41]. 1,8-cineole is the main component of the volatile It is known to play an effective role in programmed cell death pathways ,For this Many studies have addressed the effective role of 1,8-cineole inhibiting types of cancerous lines [42-44]. The exact process by which 1, 8-cineole kills cells is unknown. . However, some research traces the path of apoptosis by... [45][46][47][48][49] One kind of inhibitor of apoptosis (IAP) protein that aids in the repression of apoptosis is survivin. IAPs negatively regulate apoptosis, which is how they help neoplastic cells survive. They are strongly expressed in the majority of malignancies. Numerous investigations demonstrate that poor patient survival, a greater susceptibility to chemotherapeutic drugs, a lower apoptotic score, and high survivin expression in malignancies are all related. A survivin antisense oligonucleotide specifically inhibited the production of survivin in several cancer cell lines, resulting in multinucleated cells, cell cycle arrest in the G2-M phase, and

caspase-3-dependent death When 1, 8-cineole is used to inhibit survivin expression, it causes apoptosis and growth arrest in human colorectal tumors. A member of the MAP kinase family, p38 MAP kinase is triggered by a range of cellular stressors [50-52]

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