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Original Article

In vitro and *in silico* Investigations on the Anti-cancer Efficacy of Eupatorin, a Polymethoxy Flavone against Ovarian Cancer PA-1 Cell Line

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ABSTRACT

Background: Ovarian cancer is a prominent contributor to cancer-related death among women residing in developed countries. The most of instances are discovered when the cancer has already developed, which results in dismal consequences. Surgery, chemotherapy, and radiation were the top priorities for first-line treatment in clinics. Traditional cancer treatments have a substantial risk of toxicity and cancer recurrence in females. Plant extracts can be utilized as an alternative to traditional chemotherapeutic drugs to solve these issues. According to recent research, plant extracts show anti-tumor, anti-cancer, and anti-proliferative effects on human tumor cell lines that have been cultivated, as well as an antiangiogenic impact. **Aim:** Eupatorin is a class of flavonoids isolated from various medicinal plants with various potent biological properties including anti-cancer, anti-inflammatory, and vasorelaxant actions. **Materials and Methods:** In the current investigation, the potential of Eupatorin as an anti-cancer agent for ovarian cancer was evaluated. In this study was determination of cytotoxicity using WST-1 assay, LDH release assay, and apoptotic cell death was detected through AO/EB dual staining, estimation of pro-apoptotic markers using ELISA method and *in silico* analysis. **Results:** The viability of PA-1 cells was reduced with an increased dosage of Eupatorin. The LDH level was increased with increased concentration of Eupatorin. The apoptosis markers levels were also increased when PA-1 cells were exposed to Eupatorin indicating apoptosis of cancer cells. The results showed that apoptosis was induced by Eupatorin in PA-1 cells by triggering the caspase pathway. In addition, the *in silico* experiment was done to examine the binding efficacy of Eupatorin with VEGF-A/VEGFR and found that Eupatorin can bind more persistently to VEGF-A/VEGFR than apratoxin thereby preventing angiogenesis. **Conclusion:** All these results suggest that Eupatorin can be used as a potent anti-cancer drug for ovarian cancer.

Keywords: Eupatorin, Ovarian cancer, Apoptosis, PA-1 cells.

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INTRODUCTION

Globally, cancer ranks as the second-fatal illness in the world with a hike in morbidities and deaths. The seventh most important cause of death and morbidities worldwide is Ovarian Cancer (OC), which has been regarded as one of the worst gynaecologic cancers. Due to OC's asymptomatic nature and the fact that 2/3 of patients are first diagnosed until the disease is in the third or fourth stage of development, it is incredibly terrifying.[1] Following breast cancer, ovarian cancer is the second most prevalent form of malignancy among women aged 40 and above, particularly in developed countries. Epithelial (most prevalent), germ cell, and sex-cord-stromal are the three primary kinds of ovarian cancer; the latter two account for only around 5% of all ovarian malignancies.[2] Ovarian cancer is associated with a number of risk factors. The prevalence, progression, and reported survival rate of the disease exhibit an increase as women get older, primarily affecting postmenopausal women. The individual's personal history of breast cancer is associated with an increased susceptibility to ovarian cancer. However, it is important to note that a family history of breast or ovarian cancer represents the most significant risk factor in this regard. It has been proven by evidence that smoking is associated with an elevated risk, specifically in relation to mucinous epithelial cancers.[3] The growth of tumors depends on cancer cell proliferation and metastasis, which can be fatal for cancer patients. Inhibiting the growth of cancer is among the most effective strategies for terminating it. A cell cycle halt or the beginning of apoptosis in malignant cells can stop the growth.[4]

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Debulking surgery, radiation therapy, hormone therapy, immunotherapy, and chemotherapy medication are some of the current methods used to treat ovarian cancer. Chemotherapeutic agents are typically administered to treat ovarian cancer. Platinum-based (carboplatin and cisplatin) and taxane-based (docetaxel and paclitaxel) medications are most frequently used.[5] The unpalatable fact is that female patients are more likely to experience recurrence because of the spread of tumor cells and medication resistance. In these situations, combining novel therapeutic techniques with established ones will help to raise the level of care provided. Botanical chemicals stand out among other therapeutic sources because of the potential for several target functions, extensive tradition of use, and global distribution. Previous research has shown that bioactive plant elements assist with treating ovarian cancer. These organic components function as a component of the initial therapy or an adjunctive alternative for maintenance therapy, therefore lessening the load of the tumor and metastatic disease.[6]

Due to their anticancer properties, flavonoids, which are polyphenolic chemicals with a wide range of pharmacological activities, are of significant interest at the moment. Some of them cause cell-cycle arrest and apoptosis, which are essential components of chemotherapeutic medicines' actions. Eupatorin (3′,5-dihydroxy-4′,6,7-trimethoxyflavone) is a flavone that has been identified earlier from several medicinal plants and has been found to stop the growth of human and murine cancer cell lines. Previous studies show that Eupatorin inhibits cell growth in breast cancer.[7] Eupatorin has an anti-proliferative effect on a variety of cancer cells via activating caspase.[8] Eupatorin has a numerous potent biological properties, including anti-inflammatory, anti-cancer, and vasorelaxant action.[9] The current study objective was to investigate the effect of Eupatorin on cell viability, LDH activity, apoptosis, and the number of apoptosis markers in the PA-1 cells. In addition to this *in silico* study was performed to predict the binding of Eupatorin with VEGF-A/VEGFR.

MATERIALS AND METHODS

Materials

Eupatorin, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), PA-1 cell line, chemicals, and other materials were procured from standard vendors Sigma-Aldrich from Missouri, United States.

Cell culture

Dulbecco's Modified Eagle Medium (DMEM) was used for maintaining the human ovarian cancer (PA-1) cell line. The medium was incubated at room temperature in an environment of humidified air while being enriched with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% antibiotics (10 µg/mL streptomycin and 100 IU/mL penicillin).

Cell viability by WST-1 Assay

For the cell viability experiment, all the cells were cultivated in 96-well plates at a final population of $1*10^6$ cells/well and they were then incubated for 24 hr. The cells were all then cultured at 37°C for a further 24 hr before being treated with Eupatorin at various concentrations (2, 4, 16, 32, 64, and 128 µg/mL). After the treatment period of 24 hr, fresh medium was utilized and 10 µL of WST-1® solution was employed in each well. A further 3 hr of incubation at 37°C followed this. The percentages of inhibition were estimated after scanning the absorbance using an ELISA microplate reader at 460 nm to evaluate the viability of the cells.[10]

LDH activity assay

By examining the amount of that leaked into the growth medium, Eupatorin's cytotoxicity was determined.[11] The media was taken and centrifuged at 3000 rpm for 5 min to obtain a cell-free supernatant treatment. A readily accessible kit from the market was used to measure the LDH activity. The test depends on the concurrent reduction of NAD and the enzymatic conversion of lactate to pyruvate assisted by LDH. The mechanism described above results in the production of NADH, which subsequently causes a change in absorbance at a wavelength of 340 nm. A 96-well plate with aliquots of medium and heated reagent was used to measure absorbance using microplate spectrophotometer equipment. The software was used to analyze the data, and the results are shown as a percentage of the control values.

%Cytotoxicity=100×(corrected reading from test well corrected reading from untreated well)/ (corrected maximum LDH release control-corrected reading from untreated well).

Examination of apoptotic cells by AO/EtBr staining approach

Apoptotic cells were identified using the AO/EtBr labelling method. PA-1 cells were loaded in wells at a population of $1*10^6$ cells and given 24 hr to proliferate. PA-1 cells were treated with Eupatorin for 24 hr at IC_{50} concentration and Doxorubicin (DOX) (10 µM) as positive control. The cells were first incubated for 24 hr, followed by a PBS wash and a 20 min fixation in a 3:1 mixture of glacial acetic acid and methanol at 4°C. Before staining with a 1:1 AO/EtBr dye for 30 min at 37°C, the cells were cleaned with PBS. Stained cells were rinsed in PBS before being seen under a fluorescence microscope.[12]

Analysis of Caspase-3, -8, and -9

To further corroborate the Eupatorin compound potential to cause apoptotic cell death, many apoptosis effectors were looked at. Using commercial ELISA test kits, we determined the protein levels of caspase -3, -8, and -9 in control cells, PA-1 cells treated with Eupatorin at the IC_{50} concentration, and Doxorubicin (positive control).[13]

In silico analysis

To understand how Eupatorin binds to VEGF-A, a docking study of Eupatorin was conducted. The Optimized Liquid Potential for Simulations (OPLS_2005) forcefield was used to minimize the three-dimensional coordinates, which were taken from the PubChem database (PubChem ID: 97214). The protein coordinates were retrieved from Protein Data Bank (PDB id: 6ZCD), which is a complex of VEGF-A/VEGFR coupled with a 15-residue peptide inhibitor. Installed software: Schrodinger Suite for Windows 11 with Intel (R) Core (TM) i3-1115G4 running at 3.00GHz and Maestro13.5.[14] By adding hydrogens, removing waters and crystallographic solvents, constructing the missing residues, and using OPLS_2005 forcefield to minimize without any constraints, the protein was created using Protein Preparation in Maestro. To compare the binding of Eupatorin, a reference molecule called apratoxin S10,[15] a VEGF-A inhibitor with experimental proof, was used. Similar to Eupatorin, Apratoxin S10 structural reduction was carried out. The receptor grid is produced at the active site i.e., the core of the bound peptide. The ligands and the binding site residues were treated as flexible in the enhanced precision docking investigations.[16] The MM-PBSA energy was calculated using the docked postures to precisely determine the ligands' binding potential to the receptor. Maestro was able to acquire the diagram of receptor-ligand associations.

Statistical analysis

The average and standard deviation of three distinct samples were used to represent the results. The statistical analysis was conducted using a one-way analysis of variance. Duncan's Multiple Range Test was used as the measurement to evaluate the variation between the variable groups. $p<0.05$ was considered to be noteworthy.

RESULTS

Cytotoxic effect of Eupatorin in PA-1 cell lines

For the WST-1 assay, cells were exposed to several dosages of Eupatorin (2, 4, 16, 32, 64, and 128 µg/mL) for a period of 24 hr. The outcomes showed that Eupatorin strongly suppresses cell growth in a concentration-dependent manner. The inhibitory concentration (IC_{50}) of Eupatorin for PA-1 cells was identified 17.19 µg/mL can be observed from the percentage of cell viability in Figure 1(a) and Doxorubicin (DOX) (10 µM) as positive control.

Effect of Eupatorin in PA-1 cell line by LDH assay

For the LDH activity, cells were exposed to different concentrations of Eupatorin (2, 4, 16, 32, 64, and 128 µg/mL) for a period of 24 hr. The outcomes showed that Eupatorin strongly increases the LDH activity in a concentration-dependent manner as depicted in Figure 1(b). The LDH activity increases concerning the cell damage and Doxorubicin (DOX) (10 µM) as positive control. This implies that Eupatorin has an impact on cell damage and causes cell death.

Apoptotic morphological alteration predicted in Eupatorin-treated PA-1 cells

Variations in cell morphology are a defining feature of cell apoptosis. Here, AO/EtBr labelling was utilized to evaluate morphological modifications produced by apoptosis (Figure 2). The ruptured nuclei of dead cells may be precisely penetrated by a red fluorescent dye known as EtBr. The green fluorescent dye of AO could only penetrate normal, non-apoptotic cells. Our results demonstrated that normal cells were selected as alive cells due to their bright green nucleus fluorescence (a). On the other hand, PA-1 cells treated for 24 hr with Eupatorin at (IC_{50} 17.19 µg/mL) revealed apoptotic cells labeled in orange and red (b). Especially, orange staining denotes early apoptosis whereas red labelling of

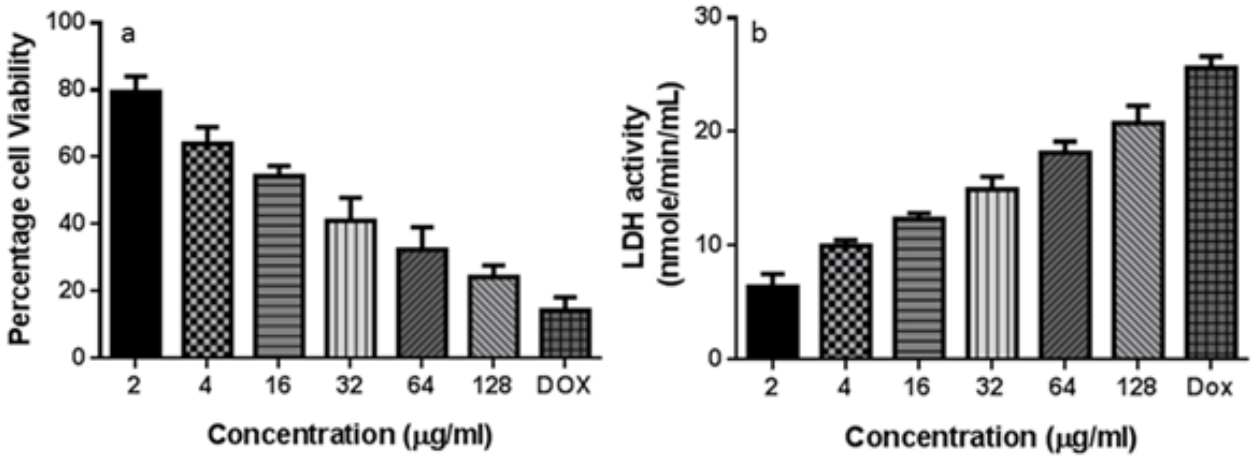

Figure 1: (a) Shows a cell cytotoxicity assay using WST-1 (b) cytotoxicity assay using LDH enzyme release assay. Doxorubicin (DOX) (10 µM) as positive control. Experiments were performed in triplicate to determine the IC_{50} value and representative data are shown here for the dose resulting in 50% inhibition of growth.

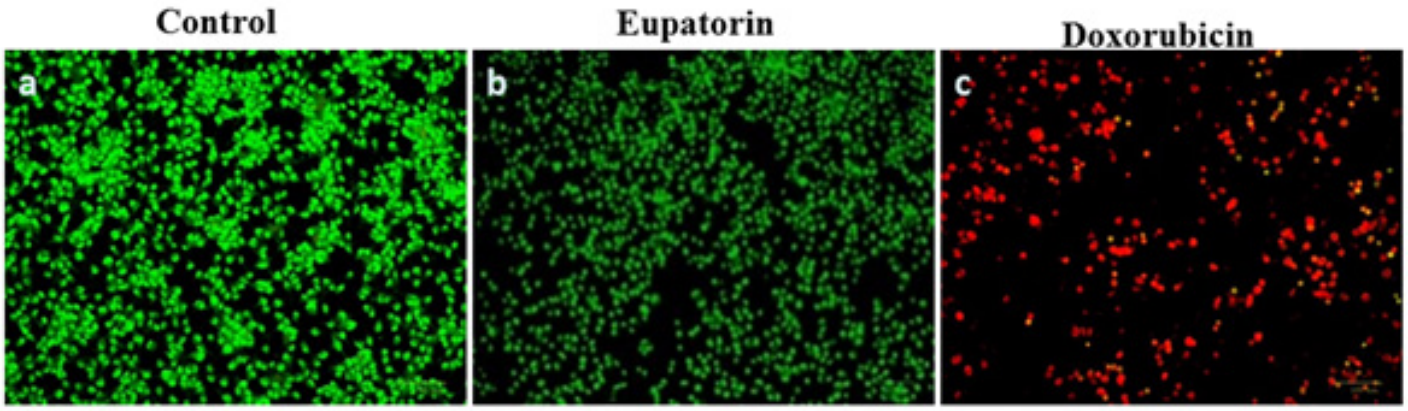

Figure 2: Effect of Eupatorin induces apoptotic induction in PA-1 cells. The cells were treated with Eupatorin (IC_{50} 17.19 µg/mL) and Doxorubicin (10 µM) as a positive control and untreated cells as a control for 24 hr, stained with dual dye AO/EB, and then analyzed by fluorescence microscopy. Control cells (a), PA-1 cells treated for 24 hr with Eupatorin (b), positive control (c).

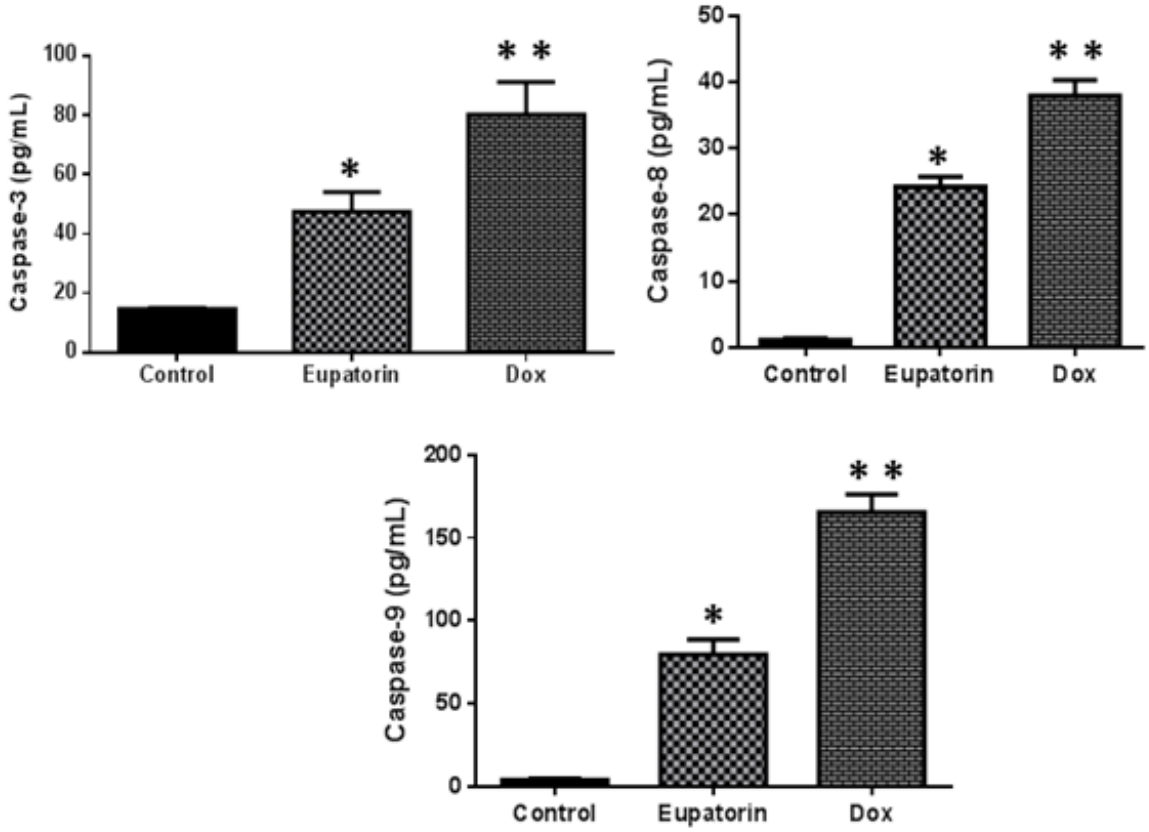

Figure 3: Eupatorin inhibits Human cancer PA-1 cell proliferation and promotes apoptosis. Levels of caspase-3, 8, and 9 in PA-1 cells were measured via ELISA. Three independent repeated tests were conducted. The results were presented as mean±standard deviation. One-way ANOVA was employed to analyze the data. Data are the mean±SD from six replicate measurements. Eupatorin treated groups vs. control group, *p<0.05 and Doxorubicin treated groups vs. control group, **p<0.001.

broken nuclei indicates late apoptosis. Doxorubicin (10 µM) as positive control (c).

Effect of Eupatorin on apoptotic markers

To ascertain if the Eupatorin causes apoptosis in PA-1 cells including the activation/repression of apoptotic markers, the parameters were assessed using the ELISA technique depicted in Figure 3. The analysis of how the Eupatorin treatment altered the levels of caspase -3, -8, and -9 is shown in Figure 3. The caspase -3, -8, and -9 levels were considerably raised in the PA-1 cells following administration of Eupatorin at (IC_{50} 17.19 µg/mL) doses. Doxorubicin (10 µM) a positive control.

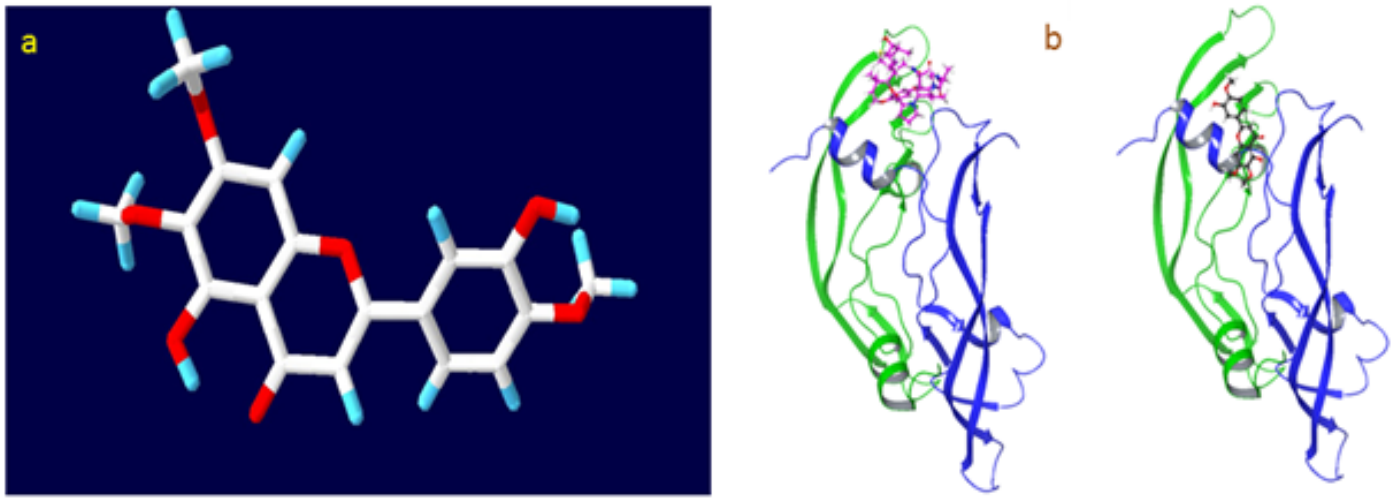

Figure 4: (a) 3D structure of Eupatorin (PubChem ID: 97214). (b) A view of the best pose of the docked conformation of Apratoxin S10 and Eupatorin. The green color ribbon depicts the 3D structure of VEGF-A and VEGFR in blue.

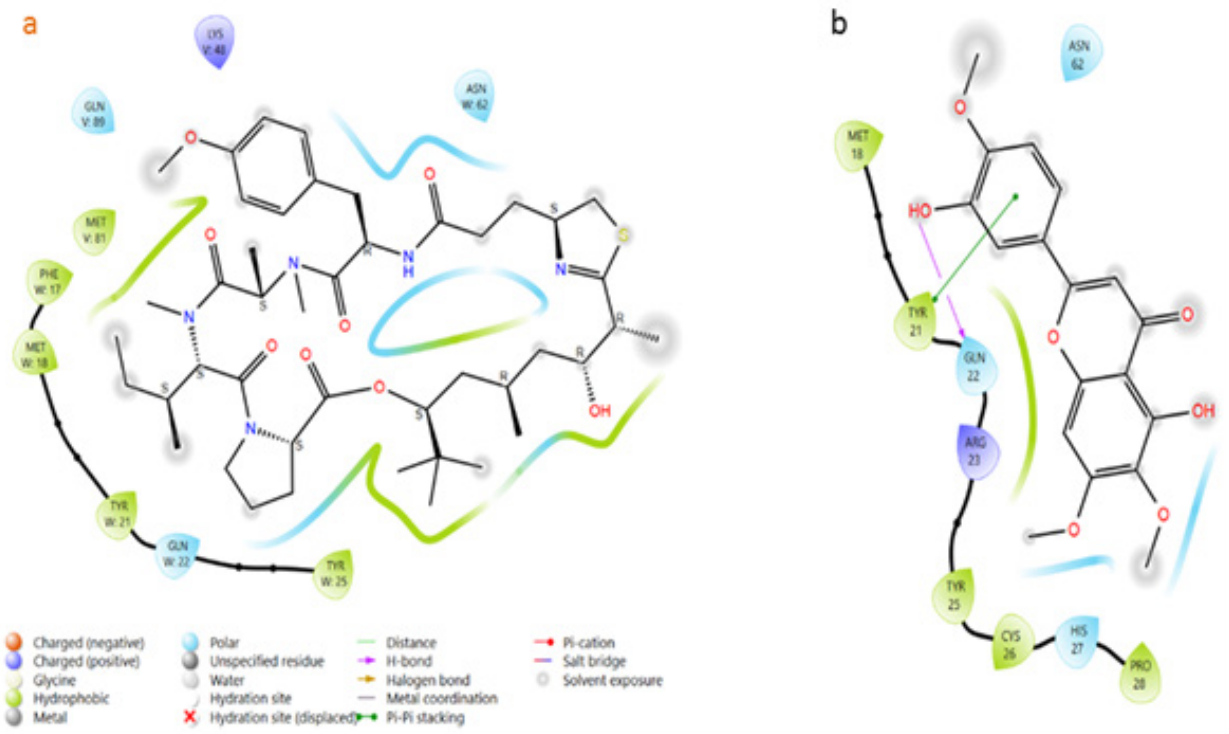

Figure 5: (a) The ligand's interaction with the VEGF-A/VEGFR complex stabilizing the bound conformation. (b) Different poses of interaction between ligands and VEGF-A/VEGFR complexes stabilize bound conformations.

In silico

The free energy of binding was calculated using Molecular Mechanics-Poisson Boltzmann Surface Area (MM-PBSA) calculations using the top-ranked postures (Figure 4(a)) as input. The docking findings demonstrate that Eupatorin's ΔG free (-37.21) was lowest when compared to Apratoxin S10 (-33.64 kcal/mol), an inhibitor with experimental support. This demonstrated that Eupatorin was a more efficient inhibitor of the VEGF-A/VEGFR complex than Apratoxin S10. The docking contacts between the Thr21 and Gln22 residues and Eupatorin are seen in Figure 4(b), where they are involved in a pi-pi stacking and a hydrogen bond, respectively. Figure 5(a) and 5(b) represents the interactions between the ligands and the VEGF-A/VEGFR complex.

DISCUSSION

When considering tumors affecting the female reproductive system, ovarian cancer is regarded as the most fatal. The majority of ovarian cancer cases are discovered at a mature and remote stage since there aren't any screening methods that are precise enough to identify it early.[17] While the majority of patients respond favourably to the first effects of current ovarian cancer therapies, these drugs frequently fail to stop tumor development and relapse and have serious adverse effects. Phytochemicals and other natural products have been suggested as a complement to conventional chemotherapy in recent years to treat ovarian cancer. Numerous research studies have demonstrated the anti-inflammatory, autophagy inducing, tumor cell cycle inhibiting, and apoptotic machinery activating properties of phytochemicals.[18]

WST-1(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono-sodium salt) assay is a common colorimetric test for determining the viability of the cells. The basic mechanism of the reaction is that WST-1 combines with mitochondrial succinate tetrazolium reductase to produce a water-soluble formazan dye.[19] In our research, treatment with Eupatorin substantially impeded PA-1 cell proliferation. The experiment revealed the Eupatorin 's IC_{50} concentration. Secondary metabolites known as flavonoids are found abundantly all over the plant world and have several biological effects including anti-inflammatory, anti-tumor, and antioxidant properties. These substances are prospective anticancer medicines due to their capacity to inhibit angiogenesis, cause apoptosis, disrupt the mitotic spindle, and halt the cell cycle.[20] Eupatorin is a flavonoid and its capacity to inhibit the proliferation and angiogenesis of a variety of tumor cells, including HeLa cervical adenocarcinoma and breast cancer cells was highlighted.[21]

Leakage of Lactate Dehydrogenase (LDH) is a sign of cell damage. The stable LDH in the cytosol is quantitatively measured by this test. Most eukaryotic cells contain the cytosolic enzyme LDH. LDH is released out of the cell when the plasma membrane integrity becomes damaged during the cell death process.[22] In our current work, it was noted that the level of LDH release in PA-1 was increased with increased concentration of Eupatorin. The effect of Eupatorin was comparable with the positive control Dox. The LDH leak into the medium serves as a signal for cell death, and the increased LDH activity is caused by the enzyme's leakage through damaged cell membranes.[23] This indicates that Eupatorin has potent inhibitory action against PA-1 cells.

A kind of genetically controlled programmed cell death called apoptosis is crucial for maintaining the growth and homeostasis of multicellular organisms by inhibiting unhealthy and excessive cells. One of the characteristics of tumorigenicity is insufficient apoptosis. A significant target for cancer treatment is the stimulation of apoptosis.[24] Acridine Orange (AO) is used to stain all of the cells, giving the nucleus a green appearance. Cells can only absorb Ethidium Bromide (EB), which gives the nucleus its red color when the cytoplasmic membrane's permeability has been compromised. Additionally, EB performs better than AO. As a result, the nucleus of viable cells is usually green, while the nuclei of early apoptotic cells are bright green with condensed or fragmented chromatin, the nuclei of late apoptotic cells are orange with condensed and fragmented chromatin, and the nuclei of cells that have died through direct necrosis are structurally normal orange.[25] In our work, it was noticed that the PA-1 cells exposed to IC_{50} of Eupatorin exhibited orange fluorescence. This indicates that Eupatorin promotes apoptosis in PA-1 cells due to its anti-oxidant activity.

Aspartate-specific cysteine proteases known as caspases play crucial roles in immunological response and apoptosis. The process of apoptosis involves caspases 3, 8, and 9. After an external stimulus activates the death receptors, Caspase-8 is a key player in the extrinsic apoptotic pathway, which causes fast cell death. Ovarian cancer appears to be very aggressive with persistent inflammation, immunoediting, and immunological resistance, which are all associated with caspase-8 downregulation.[26] The "executor" protease caspase-3, which is essential for cellular death and operates at the very end of apoptosis.[27] Caspase-3 activity is well known as a marker of apoptosis and as a powerful indicator of the effectiveness of cancer therapy. The cysteine-aspartic protease known as caspase-9 controls both healthy and abnormal tissue degeneration by acting as an activator of intrinsic apoptosis.[28] The parameters were measured using the ELISA method to determine if the apoptosis triggered by the Eupatorin on PA-1 cells involves the activation/repression of apoptotic markers. Following administration of Eupatorin at IC_{50} values, the caspase-3, -8, and -9 levels were dramatically increased in the PA-1 cells, indicating its anti-carcinogenic potential.

Recent work has demonstrated Eupatorin's anti-angiogenesis and apoptosis-inducing properties.[21,29] Vascular Endothelial Growth Factor (VEGF) and its receptor are interesting targets for the development of anticancer drugs because they are crucial in both pathologic and physiological angiogenesis.[30] Therefore, the VEGF-A/VEGFR complex was chosen as the target to study Eupatorin's binding. To ascertain the precise posture and position of Eupatorin binding as well as the interactions relevant to the binding occurrence, docking experiments were conducted by considering the ligand and the binding site residues as flexible. Modern modeling software Schrodinger is used to rank the poses of various Eupatorin conformations and the reference ligand Aparatoxin S10 in docking simulations. The free energy of binding was calculated using Molecular Mechanics-Poisson Boltzmann Surface Area (MM-PBSA) calculations using the top-ranked postures (Figure 4). The docking findings demonstrate that Eupatorin's Gfree (-37.21) was lowest when compared to Apratoxin S10, an inhibitor with experimental support (-33.64

kcal/mol). This demonstrated that Eupatorin was a more efficient inhibitor of the VEGF-A/VEGFR complex than Apratoxin S10. The docking contacts between the Thr21 and Gln22 residues and Eupatorin are seen in Figure 5, where they are involved in a pi-pi stacking and a hydrogen bond, respectively. It should be emphasized that only modest van der Waals forces were able to stabilize the Apratoxin S10-VEGF-A/VEGFR complexation. Therefore, the docking investigation has demonstrated that Eupatorin can bind to VEGF-A/VEGFR more persistently than Apratoxin S10 and may be effective in suppressing VEGF to prevent angiogenesis.

CONCLUSION

According to our research, the antioxidant and anti-cancer characteristics of Eupatorin caused apoptosis in PA-1 cells. Cell viability was reduced with an increased concentration of Eupatorin. With increased concentration of Eupatorin the level of LDH, apoptosis, and apoptosis markers were increased. From in silico, we found that Eupatorin can bind more persistently to VEGF-A/VEGFR than Apratoxin thereby preventing angiogenesis. We may thus conclude that Eupatorin can induce apoptosis in PA-1 cells and may be proved to be a potent anticancer drug. Overall, our findings support Eupatorin anticancer properties, and more research into this framework might contribute to the progress of a powerful anticancer medicine to effectively manage PA-1 cells cancer.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

pH: Potential of hydrogen; **M:** Molar; **mg:** Milligram; **µg:** Microgram; **IC_{50}:** Concentration of a compound with half-maximal cell viability; **CO_2:** Carbon dioxide; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **PI:** Propidium Iodide; **PBS:** Phosphate buffer saline; **OC:** Ovarian cancer; **PA-1:** Human ovarian cancer cell line; **AO/EtBr:** Acridine orange/Ethidium bromide; **LDH:** Lactate dehydrogenase.

SUMMARY

The seventh most important cause of death and morbidities worldwide is Ovarian Cancer (OC), which has been regarded as one of the worst gynecologic cancers. Debulking surgery, radiation therapy, hormone therapy, immunotherapy, and chemotherapy medication are some of the current methods used to treat ovarian cancer. Due to their anti-cancer properties, flavonoids, which are polyphenolic chemicals with a wide range of pharmacological activities, are of significant interest at the moment. Eupatorin is a flavone that has been identified earlier from several medicinal plants and has been found to stop the growth of human and murine cancer cell lines. In the current investigation, the potential of Eupatorin as an anti-cancer agent for ovarian cancer was evaluated. In this study was determination of cytotoxicity assay, LDH release assay, and apoptotic cell death was detected through AO/EB dual staining, estimation of pro-apoptotic markers and *in silico* analysis. The viability of PA-1 cells was reduced with an increased dosage of Eupatorin. The LDH level was increased with increased concentration of Eupatorin. The apoptosis markers levels were also increased when PA-1 cells were exposed to Eupatorin indicating apoptosis of cancer cells. The results showed that apoptosis was induced by Eupatorin in PA-1 cells by triggering the caspase pathway. In addition, the *in silico* experiment was done to examine the binding efficacy of Eupatorin with VEGF-A/VEGFR and found that Eupatorin can bind more persistently to VEGF-A/VEGFR than apratoxin thereby preventing angiogenesis. All these results suggest that Eupatorin can be used as a potent anti-cancer drug for ovarian cancer.

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