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Characterisation and Cytotoxic Evaluation of the Compound Obtained from Petroleum Ether Fraction of the Tuber *Amorphophallus paeoniifolius*

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ABSTRACT

The usual chemotherapy approach, which employs cytotoxic drugs for cancer treatment, comes with severe adverse effects, plant-based natural products have been discovered to possess similar capabilities as synthetic anticancer agents in fighting and preventing certain cancer cell types. These natural products have shown a preference for targeting cancer cells, sparing healthy cells [25]. The present research, which centers on evaluating the cytotoxic activity of the compound derived from the petroleum ether extract of the tuber Amorphophallus paeoniifolius (Elephant foot yam), known for its potential anticancer effects on HeLa cells (cervical cancer cells) [14] holds great significance. The compound extracted from the petroleum ether column fraction was characterized using the High-Performance Liquid Chromatography (HPLC) method. The cytotoxic activity of the isolated compound was studied by in vitro experiments on HeLa cells and normal cells (L929 cells). The cell viability was determined by MTT and apoptosis assays and by means of direct observation with an Inverted Phase Contrast Microscope. The compound showed a half-maximal inhibitory concentration, IC_{50} 27.61 µg/mL for HeLa cells in the MTT assay, indicating the cytotoxic effect on cancer cells. Furthermore, the compound demonstrated a non-toxic effect on normal cells, as indicated by the observation that the viability percentage of normal cells exposed to the compound was 94.76% at the maximum tested concentration of 100 µg/mL. These findings were further supported by microscopic examination and morphological apoptosis assay. The morphological apoptosis assay, which utilized a double staining acridine orange/ ethidium bromide (AO/EB) method, demonstrated that the compound plays a crucial role in programmed cell death, as evidenced by changes in fluorescence observed in HeLa cells treated with varying concentrations of the compound. The comparative apoptosis study with normal cells also supports the selective toxicity of the compound toward cancer cells. The role of the compound in apoptosis was further established by its interaction with the apoptosis-inhibiting B-Cell Lymphoma-2 protein (PDB ID: 2W3L), which was screened using the docking procedure with AutoDock Vina.

Keywords: *Amorphophallus paeoniifolius, Ellagic acid, Apoptosis, Cytotoxic, B-Cell Lymphoma-2 protein, Docking*

1. Introduction

Cancer is a severe disease that has a significant impact on a global scale. It does not differentiate based on age or gender, although certain types of cancer are more common in one gender than the other. Roughly 95 percent of cancer cases are the result of genetic mutations driven by environmental and lifestyle factors, while the remaining 5 percent stem from hereditary genetic factors [1]. The mutated oncogene possesses the ability to evade programmed cell death, rely on self-promoting signals, resist inhibitory signals, and promote tissue invasion and angiogenesis [2]. These characteristics highlight the complexities of this deadly disease.

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There is currently a wide range of treatment options available for cancer. Chemotherapy is a successful method for treating cancer, as it helps to eliminate any remaining tumor cells or pieces left after other treatments such as surgery or radiation therapy [3-4]. Chemotherapy involves using cytotoxic drugs to slow down cell division, thus inhibiting their ability to multiply. The fast and uncontrolled growth of cancer cells makes them highly vulnerable to the harmful effects of these drugs. Nevertheless, chemotherapy drugs also affect certain parts of the body with a high turnover of new cells, leading to side effects in areas not affected by cancer.

Plant-derived natural products (NPs) are distinguished by their remarkable diversity in scaffolds and intricate structural features [7]. They generally possess a greater molecular weight, and increased quantity of sp^3 carbon and oxygen atoms [5] while exhibiting a reduced presence of nitrogen and halogen atoms. Additionally, NPs have a higher count of hydrogen bond acceptors and donors, and lower calculated octanol-water partition coefficients, which suggest enhanced hydrophilicity, and increased molecular rigidity in comparison to synthetic compound libraries [6-7]. These characteristics can be beneficial; for instance, the enhanced rigidity of NPs may play a crucial role in drug discovery, particularly in addressing protein-protein interactions [8]. NPs have been evolutionarily 'optimized' in their structure to fulfill specific biological roles, such as the regulation of innate defense systems and the interaction with other organisms [9]. This adaptation shows their significant importance in the context of infectious diseases and cancer.

These products have shown comparable efficacy to synthetic anticancer medications in combating specific types of cancer cells [10]. These natural compounds exhibit selectivity by targeting tumor cells without harming healthy cells. Moreover, most plant-based drugs are affordable and easily obtainable.

A. paeoniifolius, a significant tuber plant belonging to the Aroid family, is commonly known as the "king of tuber crops" [11]. This plant is known for its wide range of therapeutic activities, which can be attributed to its various phytoconstituents including flavonoids, triterpenoids, alkaloids, tannins, fatty acids, steroids, proteins, and carbohydrates [12]. From ancient days, people have used the tuber of this plant to treat abdominal disorders, abnormal tissue growths, inflammations, hemorrhoids, hemorrhages, flatulence, constipation, weakness, fatigue, and anemia [12]. Additionally, research has shown cytotoxic and apoptotic effects of a sub-fraction of the methanolic extract of the tuber on a liver cancer cell line, PLC/PRF/5 [13]. *In vitro*, cytotoxic investigation on HeLa cells, utilizing five distinct tuber extracts, revealed that the petroleum ether extract exhibited superior anticancer properties on HeLa cells [14]. The traditional use of the tuber against tumors, enlarged spleen, and related works suggests that the tuberous roots of the plant contain cytotoxic compounds.

2. Materials and Methods

2.1 Preparation of Plant Extract

Tubers of *Amorphophallus paeonifoliius* were harvested from the Pathanamthitta district in Kerala, India. Once the peel was removed, the tuber was cut into smaller fragments and left to dry in a shaded area. The dried tuber was then finely powdered and subjected to extraction using Petroleum Ether through the Soxhlet method. Using a rotary evaporator under reduced pressure, the extract obtained was concentrated.

2.2 Isolation of Compound using Column Chromatography

The tuber sample, which had been extracted using petroleum ether solvent, was dried under a vacuum. The finely powdered mass was then placed on the pre-packed silica column and subsequently n-hexane, chloroform, and methanol of increasing polarity, were allowed to flow at a steady rate, utilizing gravity to facilitate the fractionation of the sample extract. The resulting compounds were collected as separate fractions, each numbered consecutively in test tubes for further analysis using thin-layer chromatography. A total of 40 fractions were collected and subjected to TLC. The fractions obtained from the column, eluted with a mixture of 80% chloroform in n-hexane to 100% chloroform, were characterized using the LC-MS/MS method.

2.3 HPLC Study

The LC-MS/MS characterized fraction was re-columned and then analyzed using TLC [15] compound exhibiting fluorescence at Rf 0.80 on the TLC plate under UV light was scraped off, and the silica gel was dissolved in petroleum ether and centrifuged. The resulting centrifugate was evaporated to obtain the pure compound. The qualitative and quantitative analysis of the isolated phytocompound was carried out using the Shimadzu Corporation's prominent UFLC system in Kyoto, Japan. The stock solution was diluted with ethanol to prepare the working standards. The mobile phase system utilized a gradient of 2% glacial acetic acid in water as one solvent and 2% glacial acetic acid in methanol as another. The components were eluted using a linear gradient method at an oven temperature of 33°C, and detection was performed at a wavelength of 280 nm.

2.4 Cytotoxic Studies

The cytotoxic properties of the isolated compound were investigated using *in vitro* studies involving cervical cancer cells (HeLa cell line) and normal cells (L929 cell line). These cell lines were obtained from the Rajiv Gandhi Centre for Biotechnology (RGCB) in Trivandrum, India, and the National Centre for Cell Sciences (NCCS) in Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplied by Sigma Aldrich, USA.

2.4.1 Culturing of Cell line

The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM-Himedia), enriched with 10% heat-inactivated Foetal Bovine Serum (FBS) and a 1% antibiotic mixture comprising Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). They were cultivated in 25 cm^2 TC flasks and incubated in a Galaxy® 170 cell culture incubator (Eppendorf, Germany) at a temperature of 37°C, under a 5% CO2 atmosphere with controlled humidity.

2.4.2 Preparation of Test Sample

The test samples were initially prepared in DMEM medium at a concentration of 10 mg/mL and then sterilized by filtration with a 0.2 µm Millipore syringe filter. Following this process, the samples were diluted in DMEM medium and added to the wells containing cultured cells at final concentrations of 10, 25, 50, 75, and 100 µg/mL, respectively.

2.4.3 Direct Microscopic Observation Study

The treated and control wells were continuously monitored for a duration of 24 hours using an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICAPSTM HD camera) at a 10x magnification. The findings were recorded by photography. Any noticeable alterations in cell morphology, including cell rounding, shrinkage, granulation, and vacuolization within the cytoplasm, were interpreted as indicators of cytotoxicity.

2.4.4 MTT Assay

The assay was performed by seeding 2500 cells in each well of a 96-well plate and allowing them to adjust to the conditions of culture medium in an incubator set at 37°C with a 5% carbon dioxide environment for 24 hours. Subsequently, test samples with varying concentrations were added to the wells containing cultured cells. The plates were subsequently incubated for an extra 24 hours. After this incubation, the media in the wells were carefully aspirated and discarded. Subsequently, a 100 µL volume of a 0.5 mg/mL MTT solution in PBS was introduced into each well. The plates were incubated for an additional 2 hours to allow for the formation of formazan crystals. Upon completion of this incubation, the supernatant was removed, and 100 µL of MTT solubilization solvent (DMSO) was added to each well. The contents of the wells were gently mixed by pipetting to ensure the dissolution of the formazan crystals [16]. Absorbance values were then measured using a microplate reader at a wavelength of 570 nm. Three wells per plate, which did not contain cells, served as blanks. All experiments were conducted in thrice, and the average value were calculated. The IC_{50} value is then estimated by plotting percentage viability along the Y axis and concentration of the sample (µg/mL) along the X axis and fit the data with a straight line (linear regression). Then using the equation of the fitted straight line having slope 'm' and intercept 'c', IC_{50} is calculated as, x= (0.5-c)/m.

2.4.5 Apoptosis Assay

The identification of apoptosis through morphological changes is accomplished by employing the double staining technique, which utilizes acridine orange (AO) and ethidium bromide (EB) [17]. Different concentrations (10 µg, 25 µg, 50 µg, 75 µg, 100 µg in 100 µl of 5% DMEM) of freshly prepared samples were added to the wells containing cultured cells. The control wells were not subjected to any treatment. Following a 24-hour exposure to the samples, the cells were rinsed with cold PBS and subsequently stained at room temperature for 10 minutes using a combination of AO (100 µg/mL) and EB (100 µg/mL). The stained cells were then washed twice with 1X PBS and examined under a fluorescence microscope fitted with a blue filter (LABOMED, TCM 400, USA).

2.5 In silico Study

The apoptotic action of the phytochemical was evaluated by docking it with antiapoptotic target BCL-2 (PDB IB: 2W3L) using Auto Dock Vina software [18]. 3D crystal structure of protein in 'pdb' format was acquired from the Protein Data Bank (PDB). PyMOL was utilized for protein preparation. The ligands' 2D structures in 'sdf' format were downloaded from ZINC ligand database. Open Babel was used to convert the ligand from 'sdf' to 'pdb' format. AutoDock Vina was employed to convert the 'pdb' ligands and the prepared protein into 'pdbqt' files. Then the DOCKING INPUT file: conf.txt was prepared after generating the grid box with centre x= 40.818; y= 35.111; z= -0.898 and size x=25; size y=25; size z=25 [19]. The docking score was obtained by running the INPUT file in the Command prompt(cmd) and the docking interactions were visualized using the Discovery Studio visualizer.

3. Results and Discussion

3.1 HPLC Study

The sample exhibited retention peaks similar to those of standard ellagic acid (EA) and cinnamic acid. The calibration curve details for both the standard and sample are shown in Table 3.1 and Table 3.2. Figures 3.1, 3.2, and 3.3 display the standard peaks of EA, cinnamic acid, and sample peaks, respectively. The concentration of phytocompounds in the sample, determined by comparing with the retention peak of the standards, is presented in Table 3.3.

Table 3.1 Calibration equations and details of standards

Compounds	Retention time, Rt (min)	Calibration equation	r^2
Ellagic acid	28.35	y= 9185.5x - 8829.1	0.9979
Cinnamic acid	30.39	y= 40666x - 28679	0.991

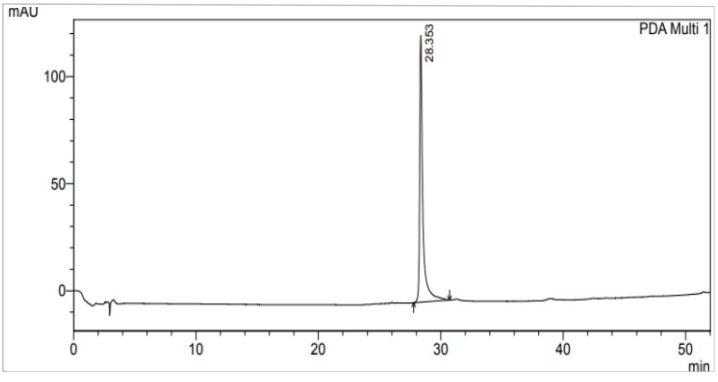

Fig.3.1 Chromatogram of standard EA

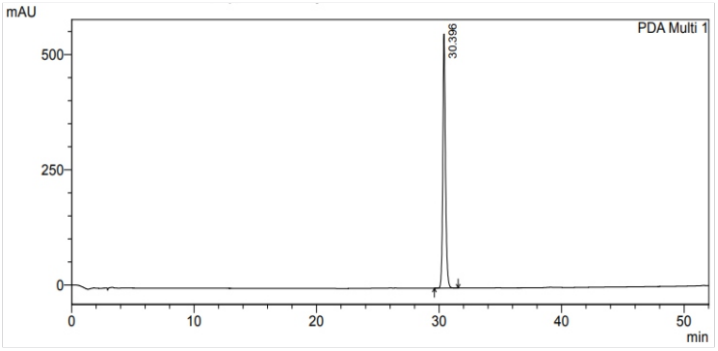

Fig.3.2 Chromatogram of standard cinnamic acid

Table 3.2 Features of the retention peak of the sample

Peaks	Rt (min)	Peak Area	Height	Area %	Height %
1	28.413	40943	2193	11.728	29.605
2	30.163	308149	5215	88.272	70.395
Total		349092	7408	100.00	100.00

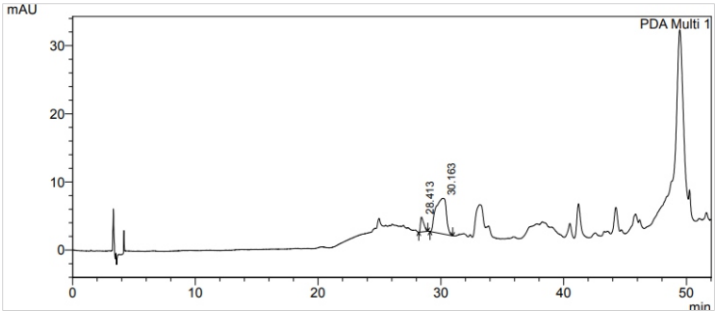

Fig.3.3 Chromatogram of sample

Table 3.3 Concentration of ellagic acid and cinnamic acid in the sample

Sample Name	The concentration of polyphenol (µg /g)	
	Ellagic acid	Cinnamic acid
AMPA1	5.418	8.282

The ellagic acid concentration measured was 5.41 µg/g, while the cinnamic acid concentration was 8.282 µg/g in the sample. Ellagic acid, a polyphenolic compound commonly found in fruits such as pomegranates, raspberries, strawberries, peaches, and plums, as well as in nuts like walnuts and almonds, and in vegetables, [20] is known to be associated with glycoside moiety or as part of polymeric ellagitannins, and has been shown to exhibit fluorescence characteristics [21]. Additionally, EA has been reported to possess diverse therapeutic properties. Hence the focus of the subsequent investigation was to evaluate the cytotoxic activity of the natural fluorophore on both cancer and normal cells.

3.1.1 Statistical Analysis

The statistical significance of the data collected in the characterization study was assessed through a regression model using the coefficient of determination (r^2). The obtained r^2 value is close to 1, indicating a strong correlation between peak area and concentration in the standard curve.

3.2 MTT Assay on HeLa and L929 Cells

The result of MTT assay of EA on HeLa cells and on normal cells are shown in Fig.3.4. and in Fig.3. 5.

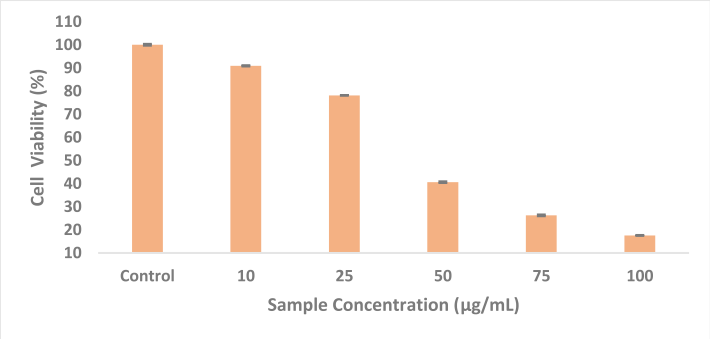

Fig.3.4 Percentage viability of HeLa cells on varying concentration of EA

The viability of HeLa cell diminished in a dose-dependent manner when exposed to different concentrations of EA. The most notable cytotoxic effect occurred at 100µg/mL, resulting in a viability of 17.58%. The IC50 value, representing the concentration at which 50% of the cells were impacted, was determined to be 27.61 µg/mL. In this study, the co-efficient of determination (r^2) is employed to evaluate the statistical significance of measured values in MTT assay. The resulting r^2 value is almost 1, providing statistical evidence for the dose-dependent reduction in the viability of HeLa cells.

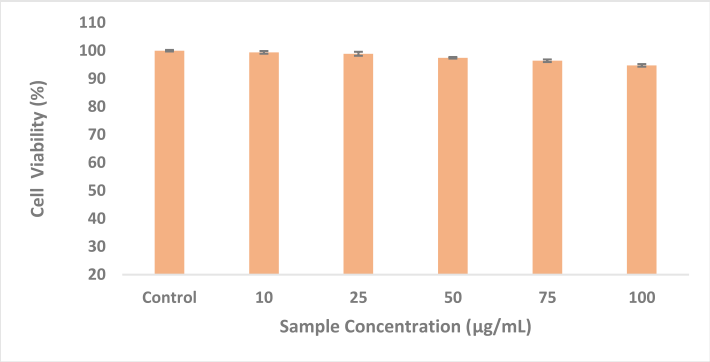

Fig.3.5 Percentage viability of L929 cells on varying concentration of EA

The MTT analysis carried out on L929 cells demonstrated that the viability of cells treated with EA did not display a notable decrease. Furthermore, it was noted that at the maximum concentration examined, 100µg/mL, EA did not lead to any substantial decrease in cell viability. These findings strongly suggest that the compound EA is non-toxic to normal cells.

3.3 Result of Direct Microscopic Observation

Images were captured to document the microscopic examination of both the control (HeLa cells) and cells exposed to varying concentrations of EA. The images obtained when HeLa cells and L929 cells are used is shown below.

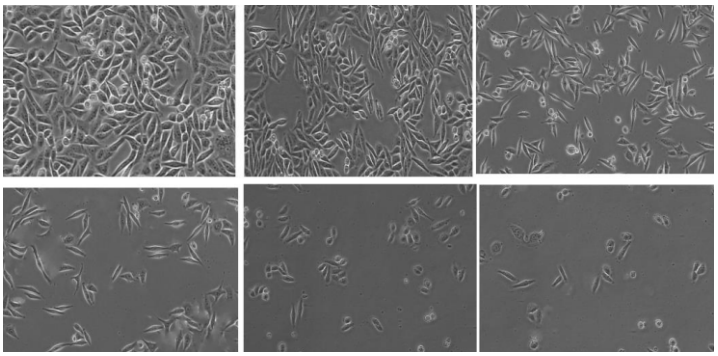

Fig.3.6 Morphology of (a) Control (HeLa Cervical Cancer cells) (b) 10 µg/mL of EA on HeLa cells (c) 25 µg/mL of EA on HeLa cells (d) 50 µg/mL of EA on HeLa cells (e) 75 µg/mL of EA on HeLa cells (f) 100 µg/mL of EA on HeLa cells

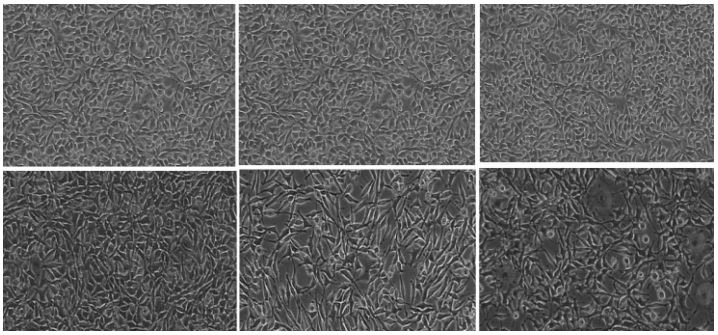

Fig.3.7 Morphology of (a) Control (L929 Murine fibroblast cells) (b) 10 µg/mL of EA on L929 cells (c) 25 µg/mL of EA on L929 cells (d) 50 µg/mL of EA on L929 cells (e) 75 µg/mL of EA on L929 cells (f) 100 µg/mL of EA on L929 cells

EA induced remarkable morphological changes in cancer cells. With increasing concentrations of EA, distinct alterations in the morphology of HeLa cells, such as cell shrinkage and rounding, were evident, cell death was observed at high concentrations, providing strong evidence of the compound's anticancer effects. In contrast, minimal changes were noted in the morphology of treated L929 normal cells, confirming the compound's non-toxic nature to normal cells.

3.4 Apoptosis Assay by Double Staining Method

The results obtained in the present morphological apoptosis study by employing Ethidium bromide (EB) and Acridine orange (AO) and using HeLa cells and normal cells treated with varying concentration of EA are shown below in Table 3.4 and Table 3.5.

Table 3.4 Apoptosis assay on HeLa cell line

Cell Line	Concentration of the sample (µg/mL)	Observation (after 24 hours)	Inference
HeLa	0 (CONTROL)	Normal Bright Green Nucleus	Normal living cells
	10	Normal Bright Green Nucleus. Green, orange, and red-stained nuclei showing condensation of chromatin	Normal living cells, early and late apoptotic cells
	25	Normal Bright Green Nucleus. Green, orange, and red-stained nuclei showing condensation of chromatin	Normal living cells (very less). Early and late apoptotic cells
	50	Green, orange, and red-stained nuclei showing condensation of chromatin	Early and late apoptotic cells
	75	Green, orange, and red-stained nuclei showing condensation of chromatin	Early and late apoptotic cells
	100	Orange and red-stained nuclei showing condensation of chromatin	Late apoptotic cells

Table 3.5 Apoptosis assay on L929 cell line

Cell Line	Concentration of the sample (µg/mL)	Observation (after 24hours)	Inference
L929	0 (CONTROL)	Normal bright green nucleus	Normal living cells
	10		Normal living cells
	25		Normal living cells
	50		Normal living cell
	75		Normal living cells
	100	Normal Bright Green Nucleus. Green and orange nuclei with chromatin condensation	Normal living cells, early and late apoptotic cells

The findings from the AO/EB staining revealed a greater number of apoptotic cells in HeLa cells treated with EA than in normal L929 cells. Additionally, it was noticed that the apoptosis rate increased with the higher EA concentration on HeLa cells. Moreover, even at high doses like 75 µg/mL, no signs of apoptosis were visible in the treated normal cells, as indicated by the presence of only normal bright green nuclei. The cellular changes induced by ellagic acid are depicted in Fig.3.8 and Fig.3.9.

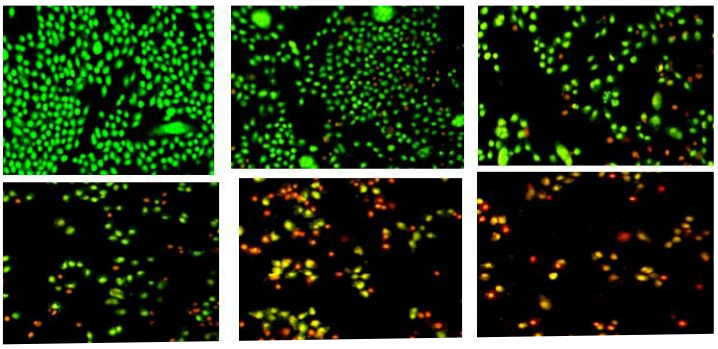

Fig.3.8 The fluorescence image of HeLa cells treated with AO/EB a) Untreated cell and cells treated with b) 10 µg/mL c) 25 µg/mL d) 50 µg/mL e) 75 µg/mL f) 100 µg/mL of EA respectively

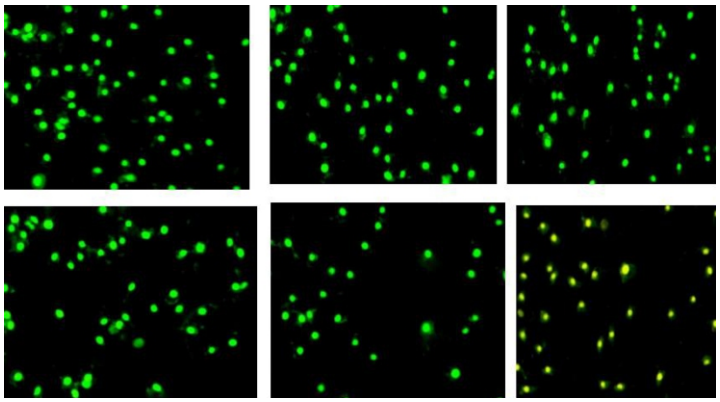

Fig.3.9 The fluorescence image of L929 cell line treated with AO/EB a) Untreated cells and cells treated with b) 10 µg/mL c) 25 µg/mL d) 50 µg/mL e) 75 µg/mL f) 100 µg/mL of EA respectively

Following AO/EB staining, HeLa cells exposed to EA exhibited noticeable nuclear fragmentation and chromatin condensation, both of which are distinct morphological signs of apoptosis. Conversely, there were no significant alterations observed in normal cells. These findings provide evidence that EA has the ability to target and eliminate cancer cells through apoptosis, without harming normal cells.

3.5 Docking Study

Scientific investigations have shown that dietary compounds exert their chemopreventive effects by inducing apoptosis [21]. Since EA can specifically activate apoptosis in cancer cells, it may serve as a valuable target for the innovation of novel anticancer therapies. Western blotting and immunofluorescence microscopy experiments [22] demonstrated the cytoplasmic expression of BCL-2 in four out of five cervical carcinoma cell lines (HeLa, CaSki, C-33A, and HT-3, excluding SiHa). The apoptosis-triggering action of EA was further evaluated by examining its binding interaction with the antiapoptotic BCL-2 protein (PDB ID: 2W3L). This involved a docking process utilizing AutoDock Vina. EA demonstrated a significant docking score of -9.1 kcal/mol, showcasing a higher quantity of favorable interactions such as hydrogen bonding, pi-cation, pi-alkyl, and amide-pi stacked interactions within the 2D configuration of the ligand-receptor complex. The docking interaction of the EA with the target protein 2W3L with their 3D and 2D structures are shown below in Fig. 3.10 and Fig. 3.11 respectively.

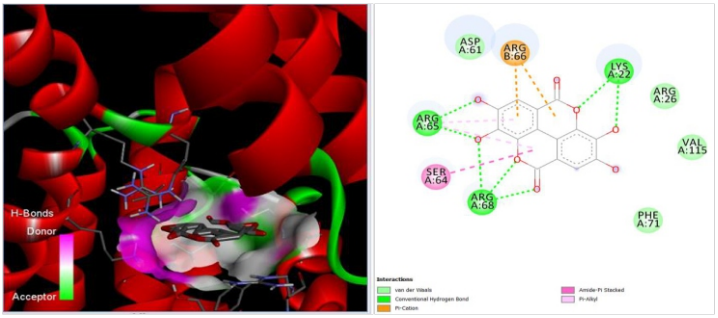

Fig.3.10 EA with target protein 2W3L
Fig.3.11 2D diagram of receptor - EA interaction

3.5 Mechanism of Antiapoptotic Action of Ellagic Acid Based on Docking Study

In the intrinsic apoptotic pathway, Bcl-2 functions as an anti-apoptotic protein, playing a vital role in inhibiting the release of cytochrome c and preserving mitochondrial integrity. Conversely, BOK, BAK, BAD, and BAX function as pro-apoptotic proteins that aid in the translocation of cytochrome c from the intermembrane space of mitochondria into the cytosol. This release triggers apoptosis initiation, potentially aiding in cancer therapeutics [23]. Both NMR and X-ray crystal structures have shown that the binding groove in Bcl-2 is receptive to small-molecule intervention. Subsequent research has proven that these molecules can sensitize tumor cells to apoptosis [24;28].

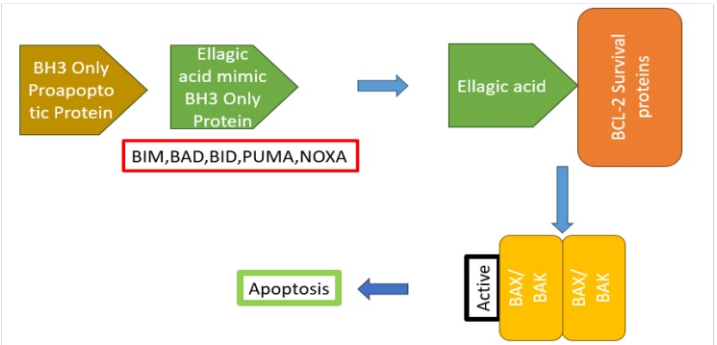

Fig.3.11 Mechanism of BCL 2 protein - EA interaction

Perhaps ellagic acid, acting as a small molecule mimic, is a better fit for the BH3 domain groove of the Bcl-2 protein, as evidenced by the favorable docking score and the numerous hydrogen bonding interactions in the 2D structure of the ligand-receptor complex. This inactivation of Bcl-2 by ellagic acid may lead to the accumulation of monomeric BAX proapoptotic protein in the cytosol, followed by the oligomerization of BAX [25-27] resulting in the liberation of cytochrome c and apoptosis-inducing factors from the intermembrane space of the mitochondria into the cytosol, which ultimately results in cell death.

4. Conclusion

The compound EA, derived from the tuber elephant foot yam, exhibited anticancer activity as revealed by direct microscopic observation, MTT and apoptosis assays. The *in silico* docking study and morphological apoptosis assay indicated that ellagic acid's anticancer activity is primarily due to its ability to induce apoptosis. Research has shown that apoptosis is the primary mechanism through which dietary compounds demonstrate their chemopreventive potential. Therefore, this study provides scientific evidence supporting the use of the elephant foot yam tuber for cancer treatment. As EA is non-toxic to normal cells, it can serve as a safer alternative to synthetic cytotoxic drugs lacking selectivity. Moreover, the presence of Bcl-2 proteins in cancer cells has been clinically recognized as a contributing factor to the resistance exhibited by pro-apoptotic drugs. Since EA demonstrates an inhibitory effect on the anti-apoptotic Bcl-2 protein, it can be strategically used in combination with conventional cytotoxic agents to address drug resistance and enhance their therapeutic outcomes. Further preclinical and clinical studies are required to gain a comprehensive understanding of the implications of using EA as a chemotherapeutic drug.

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