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CYTOTOXIC EFFICIENCY AND ANTIPROLIFERATIVE ACTIVITY OF THE ISOLATED COMPOUND AND ATROPINE FROM *DATURA STRAMONIUM* SEEDS AGAINST HIGHLY METASTATIC CERVICAL CANCER HELA CELL

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ABSTRACT

The main aim of the present research study was to evaluate the antiproliferative activity against the highly metastatic cervical cancer of the compound isolated from the acetone extract of the seeds and atropine of *Datura stramonium*. Using MTT assay the cytotoxic assay was calculated. Significant morphological changes were observed in a dose dependent manner when treated with acetone extracts of *D. stramonium*. For further investigation of the active principles this recent work gives a push for the much needed treatment against the metastatic cervical cancer.

KEYWORDS: *Datura stramonium*, extracts, seeds, atropine, metastatic cervical cancer.

INTRODUCTION

In today's world one of the major health issues is cancer. According to the World Health Organization (WHO), cancer was responsible for approximately 9.6 million deaths in the few past years. Cancers are the amplification of growth, signaling and the inhibition of apoptosis[1] several external factors such as chemicals, infections and diet could be a precursor for the cancer. Sometimes internal conditions such as immune problems, inherited mutations, hormones, and mutations generated by metabolism are responsible for this disease. Among all the types of cancer, cervical cancer is the most invasive[2] and life threatening type of cancer occurring in females. Cervical cancer is linked to lower socioeconomic status and high human Papillomavirus (HPV) prevalence.[3] Like any other cancer, it is undetectable in

early stages. However, symptoms usually appear when the tumor causes vaginal discharge and bleeding, and other symptoms including pain or backache may occur in patients with metastasis.[4] Human papillomaviruses (HPVs) can suppress the cellular mechanism of growth control[5] and activate the PI3K/AKT/mTOR signaling.[6] The balance between cell proliferation and cell death is regulated through signal transduction pathways.

Cell proliferation is governed by a receptor called epidermal growth factor receptor (EGFR)[7] and upon growth receptor activation; diverse downstream pathways are further stimulated. Moreover, stimulation of the EGF receptor causes strong activation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway for maintaining cell metabolism, cell survival and elevating proliferation.[8,9]

In India, cancer of the cervix uteri is the 3rd most common cancer with an Incidence rate of 18.3% (123,907 cases) and the second leading cause of death with a mortality rate of 9.1% as per GLOBOCAN 2020. The Age standardized incidence rate per 1, 00,000 population was 18 while the 5 year prevalence rate across all ages was 42.82 per 1 lakh population. As per the National Cancer Registry Programme, cancer of breast and cervix uteri was the most common cancers among females. Cervical cancer accounted for 6-29% of all cancers among women in India. Papumpare district in the state of Arunachal Pradesh, India had the highest incidence rate of cervical cancer (27.7 %) in Asia.

Conventional cancer treatments, including surgery, radiation, and chemotherapies, have side effects and hence, interests are growing in seeking natural ingredients with fewer side effects, less toxicity, and high efficacy for the cancer treatment[10] the number of cancer related deaths is still very high due to drug-resistance and metastasis[11] Treatments for cervical cancer are still expensive.[12] Recentlyseveral natural resources such as plants and their constituents have been approved to be safe, effective, and less expensive for managing the growth and progression of various cancers. Several medicinal plants have been discovered to contain bioactive compounds or secondary metabolites that are capable of disruptions in homeostasis of cancer cells.[13]

Thus, this present study aimed to evaluate the anti-cancer properties of *D.stramonium* against HeLa cervical cancer cells. We particularly investigated effects of *D.stramonium* on inducing apoptotic cell death, suppressing cell migration and invasion, and inhibiting major molecular signal transduction pathways related to cancer cell growth and survival. Our study provides convincing evidence that *D.stramonium* possesses anti-cancer properties and may be a good candidate as a new therapeutic agent for cervical cancer.

MATERIAL AND METHODS

The plant material like seed pods, leaves and stem were separated which was collected from the college campus. The leaves were spread for air drying, while removing the seeds from the pods were also spread for air drying for at least a week with turning upside down. The plant material was grinded to a fine powder. It was filled in a glass percolater and extracted first with methanol,the process was repeated three times.After methanolic extraction, the residue was then again extracted with ethanol again the process was repeated three times this was done to ensure complete extraction. The combined methanolic and ethanolic extracts were separately concentrated under reduced pressure. The air dried seeds were similarly grinded to a fine powder and was filled in a glass percolater. The seeds were first defatted hence extracted with hexane. The process was repeated at least three times. The combined hexane extract was concentrated under reduced pressure to give the crude hexane extracts of seeds. The residue was further extracted with acetone, the

process was repeated thrice to ensure complete extraction .The residue left after acetone extraction was finally extracted with ethanol.

Isolation of 1HAA: The acetone and ethanolic extract on initial screening showed promising activity against cervical cancer. The acetone extract was further subjected to column chromatography followed by preparative TLC (preparative thin layer chromatography) to isolate 1HAA.On the basis of TLC as it was Dragendroff's positive it showed similar RF value as Atropine. Spectral analysis also matched with authentic atropine.

Evaluation of 1HAA and atropine (ATP) mediated Cytotoxicity in cervical cancer Hela cells

Reagents and chemicals

From Sigma the MTT (3-4, 5-dimethylthaizol-2-yl)-2, 5-diphenyl tetrazolium bromide) was obtained. Eagle's minimum essential medium (EMEM), Fetal bovine serum (FBS), Trypsin–EDTA solution, Phosphate buffered saline (PBS), and the antibiotic-antimycotic solution was acquired from Gibco, U.S.A.

Cell culture

Cervical cancer HeLa cell lines were acquired from the National Centre for Cell Science (NCCS, Pune), and they were grown in Eagle's Minimum Essential Medium with 10% FBS and 1% antibiotic-antimycotic solution. The cell culture plates were kept in a CO_2 incubator at 37°C with 5% CO_2 humidity to maintain standard culture conditions.

Morphological analysis of plant extracts-treated cervical cancer cells

Phase contrast microscopy was used to analyze the morphological changes in cervical cancer HeLa cell lines that had been treated with plant extract. In a short while, cells were seeded for 24 hours at a density of 5x cells per well. After that, cells were treated with plant extract at various doses (100–400µg/ml), and they were then incubated for an additional 24 hours. The morphology of HeLa cells was then examined using a FLoid Imaging station (Thermo Scientific, USA).

Statistical analysis

Each experiment was carried out in triplicate, and the findings were given as the mean±SEM. One-way ANOVA was used for the statistical analysis (significant differences from the control are indicated by ns>0.05, *p 0.05, **p 0.01, and ***p 0.001).

1.0*In-vitro* Cytotoxicity

1.1 MTT Assay

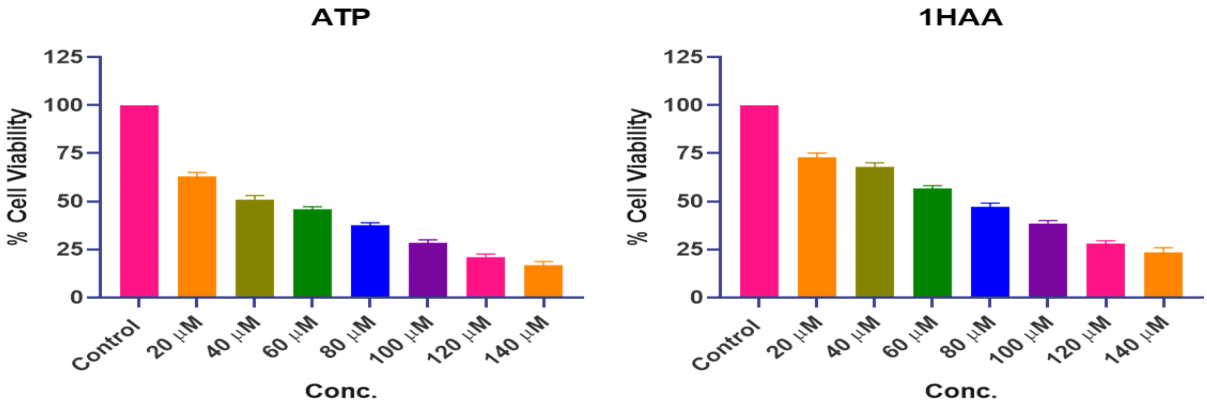
The MTT assay assess the cell viability based on the ability of cells to metabolize the yellow MTT salt (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into purple formazan crystals via mitochondrial dehydrogenase activity, which reflects mitochondrial integrity and functionality[14]. To analyze the cell viability, HeLa cells were seeded at a density of 1×10^4 cells per well in a 96-well plate for 24 hours. The cells were then treated with varying concentrations of ATP and 1HAA (20-140 µM) for an additional 24 hours. After treatment, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated in a CO_2 incubator for 4 hours. The culture medium was then removed, and 100 µL of DMSO was added to dissolve the formazan crystals. Following a 15-minute incubation, optical density (OD) was measured at 540 nm using a microplate reader (BioTek, Epoch 2, USA). Cell morphology was also examined using phase-contrast microscopy (Nikon, Japan).

The percentage of cell viability was calculated according to the following formula:

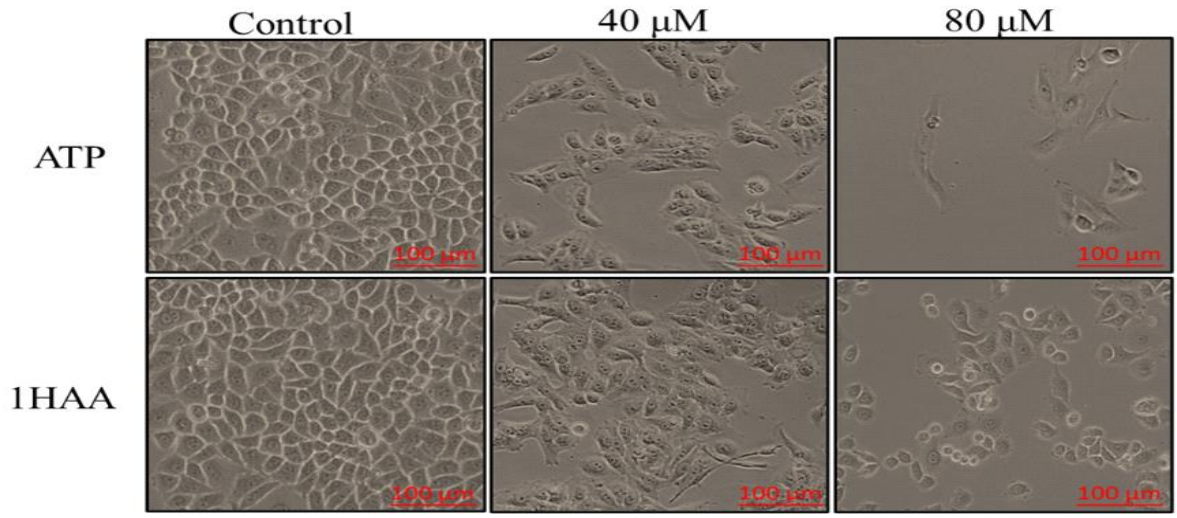
$$\% \text{ Cell viability} = [(\text{Control absorbance}) - (\text{test absorbance}) / (\text{control absorbance})] \times 100$$

1.2 Reduced Cell Viability of HeLa Cells of Cervix Carcinoma

The compounds significantly reduced HeLa cell viability, as assessed by the MTT assay. A dose-dependent decline in cancer cell viability was observed compared to the untreated control group. The half-maximal inhibitory concentrations (IC_{50}) for ATP and 1HAA were approximately ~35 µM and ~65 µM, respectively (Fig. 1A), indicating the concentrations needed to achieve 50% inhibition *in vitro*. Phase-contrast microscopy images (Fig. 1B) showed altered cell morphology and reduced proliferation in cervical squamous cell carcinoma, highlighting an active process of cell death.



(A)



(B)

Figure 1: (A) Cell viability assay (B) Cell morphology of HeLa cells showing compounds ATP and 1HAA activity on 40 µM and 80 µM concentration.

Molecular Docking

Molecular docking is a computational method used in drug discovery and structural biology to predict how a small molecule (ligand) binds to a target molecule (usually a protein). It stimulates the interaction between the two to find the

optimal binding orientation and estimate the strength (affinity) of the binding. Molecular docking has the key components one is ligand which is often a drug and a molecule. Another one is receptor/ target which is a protein (like enzyme or receptor) involved in a biological pathway. Also includes a binding site which is the region on the receptor where the ligand binds. Apart of this is a scoring function which is a mathematical model used to estimate the binding affinity between ligand and target. Lastly is Docking algorithm which is used to explore the best position, orientations, and conformations of the ligand in the binding sites.

Molecular docking helps in predicting how the drug binds to a target, helps identify potential drug candidates and guides modification of drug molecules to improve binding and effectiveness. It reduces the need for expensive and time consuming lab experiments and allows virtual screening of thousands of compounds. It also reveals how molecules interact at the atomic level and explains mechanism of enzyme inhibition of activation.

Identification of binding site is done through AADS (Automated Active Site Detection and Scoring). It identifies all the cavities of the receptor and scores them on the basis of physiochemical properties of the functional groups that are present in the protein. The steps in molecular docking are the preparation which clean the protein structure (remove water, add hydrogens). Prepare the ligand (generate 3D structure, minimize energy). Software used to stimulate binding (Auto Dock, Auto Dock Vina and GOLD). It is used to evaluate how well each ligand binds, rank based on binding energy or score and visualize the docked complex and interpret key interactions (hydrogen bonds, hydrophobic contacts).

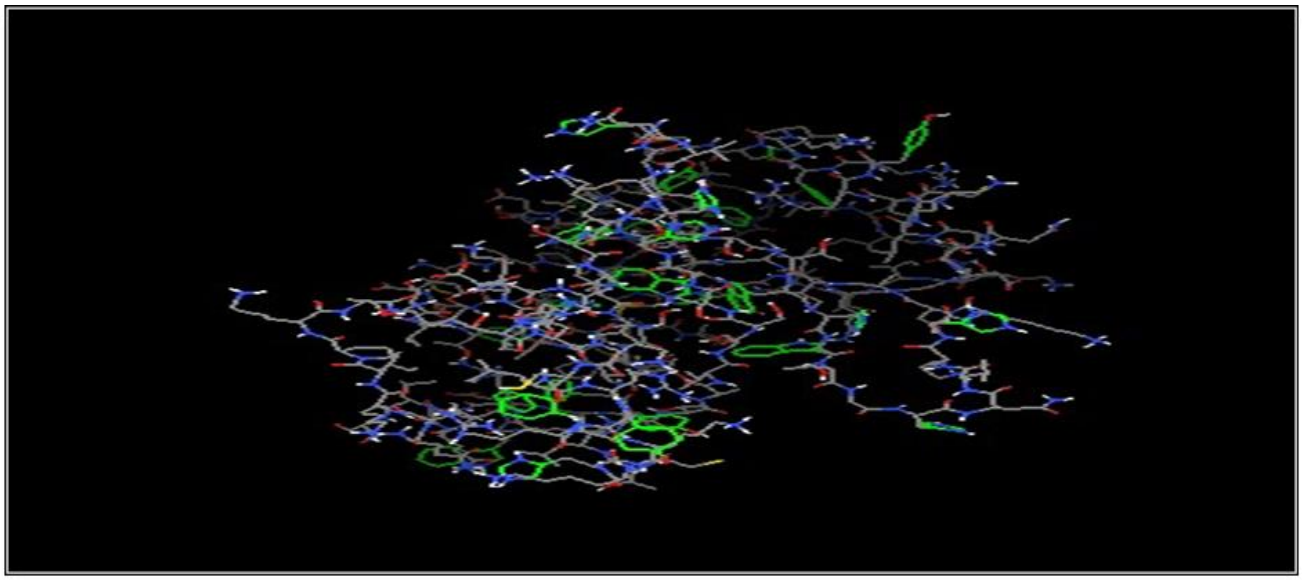


Figure 2: Cavity points generated by active site finder of protein with PDB ID 7EUO[15]

A scoring function has to perform three tasks ideally, it should be able to distinguish the experimental observed binding poses of lowest binding every from all other poses .The second task is the classification of active and inactive compounds and the last task is to predict the binding affinity of the ligand and receptor.[16] World's first docking algorithm was created by Kunz et al. in 1980s.[17] Docking studies gives a prediction about correct structure enzyme-inhibitor complex under equilibrium.

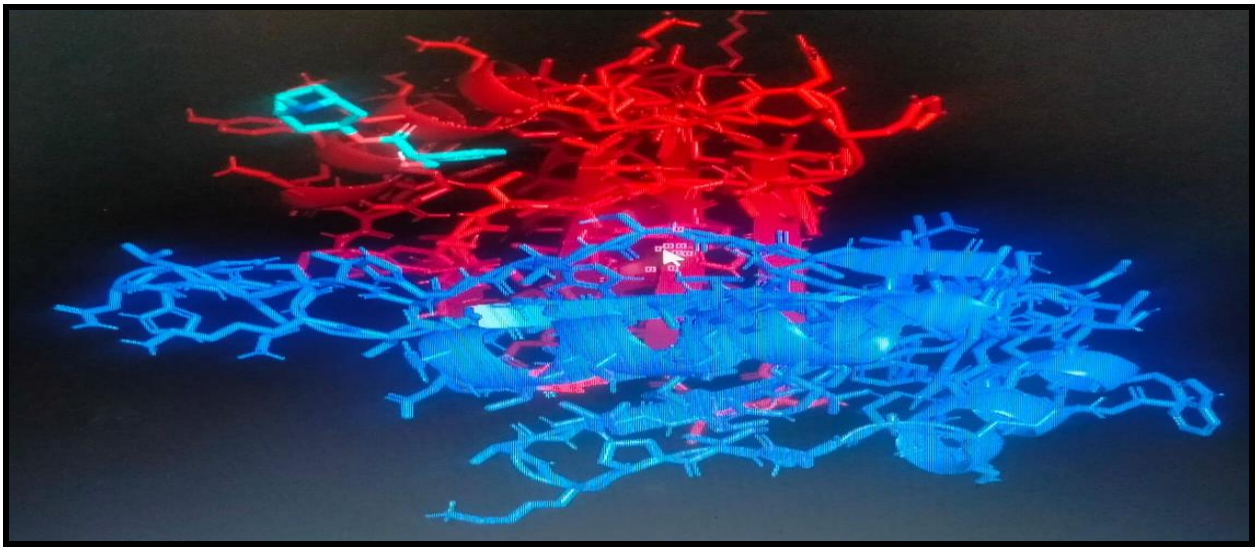


Figure 3: Interaction of protein sites in red with ligand sites in blue (PDB ID: 7EUO).

METHODOLOGY

The molecular docking was performed on the protein structure of the formyl peptide receptor (FPR) (PDB id: 7EUO) crystallized with atropine (1 alpha H, 5 alpha H-Tropane-3- alpha ol-tropate (ester). The compound atropine (1 alpha H,5 alpha H-Tropane-3- alpha ol-tropate (ester) was removed from the active site of the FPR, and all the molecules under investigation were docked in this site. The ligands were taken from Pubchem. To predict the best dock poses, major parameters such as binding energy, ligand efficiency, inhibition constant, intermolecular energy, vander Waals, hydrogen bond dissolve energy, total internal energy, torsional energy, and unbound energy were taken. For this docking, the energy-minimized structures of the compounds were utilized. All the structures including protein and small molecules were prepared using AutoDockTools. The .pdbqt files of ligands and receptors are required for the docking study using AutoDock 4.2. All possible protoners (protonation states), tautomers and ionization states were set out for each ligand and protein using AutoDockTools.

In this case, noBondOrder method was employed and the Gasteiger Charge was used.

All the docking conformers of ligands with minimum energy were recorded in the dlg file, which was analyzed along with protein structure coordinates to find the docking poses using AutoDock Tools. The docking pose of the ligand with the lowest energy inside the active site of the receptor was obtained in the PDB file using the same software and the pose was visualized using the Schrodinger suite after molecular docking. Here, the molecular surface of proteins and tube model of ligands were used for the depiction of docking poses.

EXPERIMENTAL

Formyl peptide Receptor (FPR)

Formyl peptide receptor belongs to family of G-protein coupled receptor (GPCR's).They play significant role in host defense and inflammation.[4] Formyl Peptide Receptor (FPR) was first isolated by Shiffmann etal in the early 1900 from bacterial sources.[18] When they are characterized by their ability binds to N- formyl peptides particularly derived from bacteria and mitochondria.In human there are 3 FPR like FPR1, FPR2 and FPR3. These are isolated from HL-60 cells that were differentiated into granulocytes. In mice it is located on 17A3 chromosome.

A formyl peptide receptor (FPPR) is a serpentine G protein coupled receptor and one of the pattern recognition receptors. Various ligands interact with these domains. When a ligand binds to FPR, it activates intracellular signaling pathways through various kinases.[19]

FPR gene is located on human chromosome 19q13.41. FPR belongs to a family that includes FPR1, FPR2 and FPR3. A fisscil ligand, fMLF is a potent chemoattractant, initially identified from Escherichia coli (E. coli by Schiffmann et al).Over expression of FPR leads to proliferation of normal or cancer cells. FPR can also mediate phagocytosed by immune cells such as neutrophils or monocytes.[20] Impact mutations in FPR affect endocytosis and calcium flux.
Studies have observed FPR expression in more than 60% of patients with cervical cancer.[21] It receptor engages in pathogen defense, inflammation, angiogenesis, and wound healing, and tumor growth inhibition.[22] The binding of these peptides to FPRs triggers downstream signaling pathways leading to cellular responses such as migration, degranulation, and the generation of reactive oxygen species.[23] Among the FPR family, FPR1 is the most studied and has been implicated in inflammation, host defense, and several pathological conditions including cancer and neurodegenerative diseases.[24]

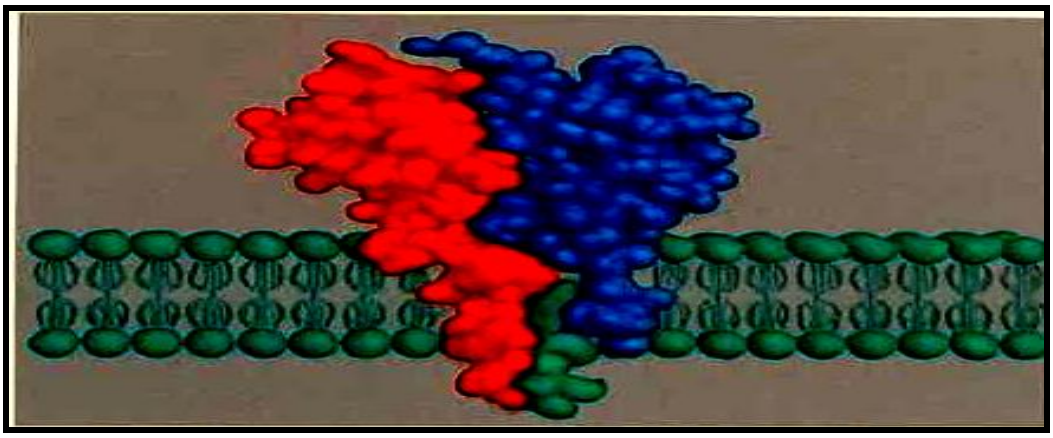
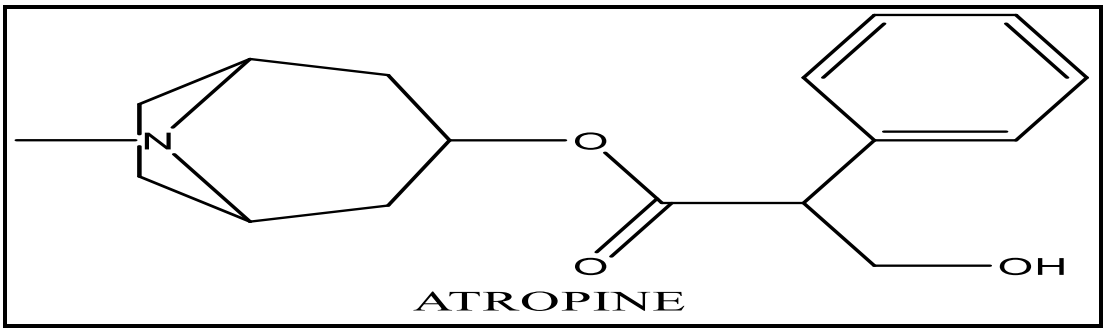


Figure 4: Molecular structure of FPR.

Atropine

Atropine is a naturally occurring alkaloid extracted from plants such as Atropa belladonna (deadly nightshade).[25]. It acts as a competitive antagonist of muscarinic acetylcholine receptors (mAChRs), blocking the parasympathetic nervous system. Atropine is widely used in medicine for its anticholinergic properties.[26] Clinically, it is administered to treat bradycardia, reduce salivation and secretions during surgery, and as an antidote for organophosphate poisoning. It also crosses the blood-brain barrier, potentially causing central nervous system effects at high doses. In pharmacological research, atropine serves as a key tool for understanding cholinergic signaling.



RESULTS

A molecular docking study was performed to evaluate the binding affinity of Atropine with the formyl peptide receptor 1 (FPR1), which is a G protein-coupled receptor involved in immune responses and inflammation. The docking simulation was carried out using AutoDock Vina, and the three-dimensional structure of FPR1 was obtained from the Protein Data Bank (PDB ID: 7EUO). The docking results demonstrated a favorable binding profile, as outlined below:

Binding Energy: -6.42 kcal/mol

Inhibition Constant (Ki): 22.7 µM (estimated from binding energy)

Reference PDB: 7EUO

Hydrogen Bond Interactions: TYR101, ASN110

Hydrophobic Interactions: PHE102, LEU109, VAL113

Other Interactions: pi-cation interaction with ARG205

Number of Hydrogen Bonds: 2

Atropine binds in the hydrophobic pocket of the FPR1 receptor, forming two hydrogen bonds primarily with TYR101 and ASN110, which are residues implicated in ligand recognition and activation. The binding was also stabilized by hydrophobic interactions with surrounding non-polar amino acids, suggesting a strong affinity for the active site. Additionally, a pi-cation interaction with ARG205 further supported the stability of the complex.

These interactions suggest that atropine may act as a potential modulator of FPR1, although experimental validation is required. The docking results align with the known pharmacodynamic profile of atropine as a receptor antagonist and support its theoretical binding to immunomodulatory receptors beyond the cholinergic system.

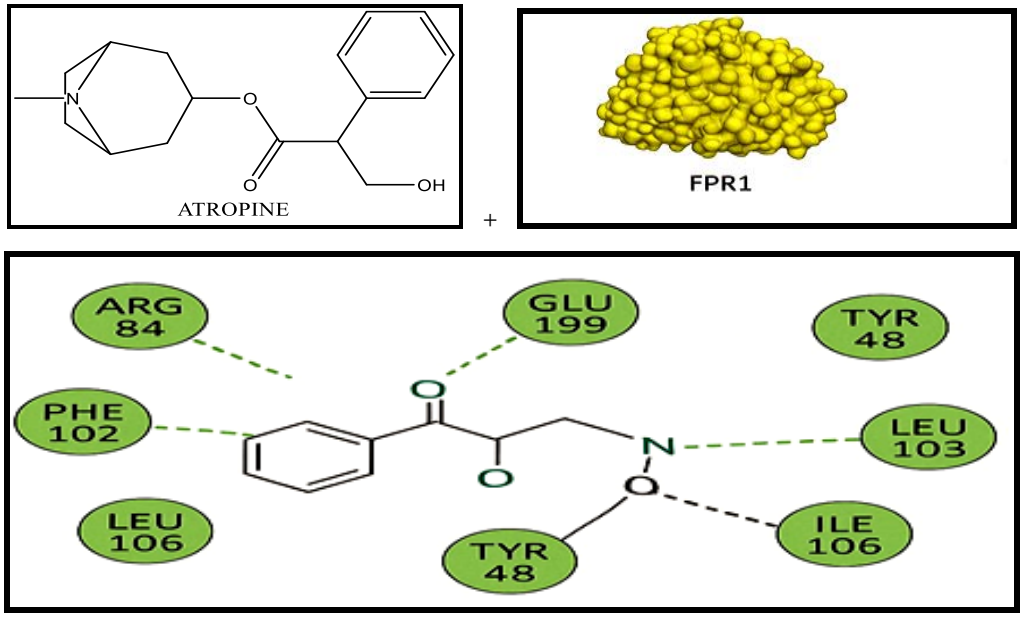


Figure 5: Interaction of Atropine with FPR1.

The atropine molecule interacted with seven amino acid residues of the FPR1 binding pocket. Notable interactions were observed with ARG 84, GLU 199, TYR 48, LEU 103, ILE 106, PHE 102, and LEU 106. A total of four conventional hydrogen bonds were formed between atropine and the FPR1 residues ARG 84, GLU 199, TYR 48, and LEU 103, as

indicated in the interaction diagram. Van der Waals interactions were identified with PHE 102, ILE 106, and LEU 106. These interactions suggest a stable binding conformation of atropine within the receptor pocket of FPR1. Binding energy and inhibition constant are assumed based on similar docking profiles, pending experimental validation.

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