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Evaluation of Anticancer Potential and Molecular Docking Analysis of *Crotalaria pallida* Crude Extract

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Abstract: Medicinal plants have long served as vital components of traditional healthcare systems due to their accessibility, affordability, and therapeutic efficacy. Among these, *Crotalaria pallida*, a member of the Fabaceae family, holds ethnobotanical significance and is traditionally used for various ailments. This study explores the cytotoxic and antimicrobial potential of *C. pallida* seed extracts using an integrated approach encompassing *in vitro*, *in vivo*, and *in silico* methodologies. The ethanolic extract was subjected to MTT assay against A549 human lung carcinoma cell lines, revealing a notable dose-dependent cytotoxic effect, with 58.18% cell viability at 140 µM and an IC_{50} value of 7.11 µg/ml. Molecular docking studies demonstrated strong interactions between these phytocompounds and virulence proteins from *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and A549 cell lines, with binding affinities ranging from -3.4 to -6.2 kcal/mol. Ligplot+ analysis further validated their potential as inhibitors by highlighting consistent hydrogen bonding and hydrophobic interactions. The molecular dynamics simulations affirmed structural stability in protein-ligand complexes, indicating potential therapeutic relevance. These findings not only confirm the cytotoxic efficacy of *C. pallida* against lung cancer cells but also support its antibacterial potential, laying a scientific foundation for its traditional use. The study emphasizes the value of integrating ethnobotanical knowledge with modern molecular tools to identify and validate novel plant-based drug candidates. Future investigations are recommended to further elucidate the pharmacodynamics and clinical relevance of the bioactive constituents.

Keywords: *Crotalaria pallida*, Medicinal plants, Cytotoxicity, A549 lung cancer cells, MTT assay, Molecular docking

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Introduction

Medicinal plants, also known as herbal medicines, have been integral to traditional healthcare systems across various civilizations since antiquity. These botanicals are renowned for their

therapeutic properties and have been employed to prevent and treat a wide array of ailments, support healing processes, and enhance overall well-being. Their widespread use is largely attributed to the presence of bioactive compounds such as alkaloids, flavonoids, terpenoids, and phenolic constituents, which contribute to their pharmacological efficacy. Herbal medicines are generally regarded as safe, cost-effective, and readily accessible. They are often prepared in the form of teas, oils, tinctures, or extracts, and continue to be a vital part of healthcare in many rural and underdeveloped regions where access to modern medicine is limited (Saet *et al*., 2007). India, recognized globally for its rich biodiversity, is home to approximately 45,000 plant species and 81,000 animal species, with 5,150 plant and 1,837 animal species considered endemic (India State of Forest Report, 2009). About 41% of the country's geographical area is covered by forests, which serve as reservoirs of medicinal flora. In many developing countries, traditional healers and plant-based remedies remain the primary source of healthcare for a large portion of the population. Beyond accessibility, herbal medicines are often favored due to cultural preferences, holistic healing approaches, and a perception of being safer and less invasive than synthetic pharmaceuticals. *Crotalaria*, a genus within the Fabaceae (legume) family, encompasses several hundred species distributed across tropical and subtropical regions. These plants, typically herbs, shrubs, or small trees, are characterized by their vibrant yellow flowers and nitrogen-fixing capabilities, which enhance soil fertility. Some species serve agricultural roles as fodder crops or green manure. Moreover, various *Crotalaria* species are traditionally used in folk medicine across different cultures for treating numerous health disorders. Cancer remains a leading cause of mortality worldwide, prompting the continuous search for effective treatment strategies. Conventional therapies such as chemotherapy, radiation, immunotherapy, and targeted drug treatments are widely used, yet they are often associated with severe side effects and high costs. In contrast, natural compounds from plants, fungi, and marine organisms have shown promising anticancer potential. These compounds can act through multiple mechanisms, including the induction of apoptosis, inhibition of angiogenesis, and suppression of tumor metastasis (Kamatou *et al*.,2008). As a result, there is a growing interest in identifying plant-derived agents with anticancer properties, particularly from medicinal plants traditionally used in under-resourced regions. In addition to *in vitro* and *in vivo* assessments, molecular docking has emerged as a powerful *in silico* technique in drug discovery and computational biology. It enables the prediction of the preferred orientation of small molecules (ligands) when bound to biological macro-molecules such as proteins, thereby facilitating the identification of potential drug candidates (Paul *et al*., 2018). Molecular docking provides insights into binding interactions and estimates the binding affinity, playing a critical role in understanding drug-receptor interactions and optimizing therapeutic compounds with improved efficacy and reduced toxicity. This study investigates the phytochemical and anticancer properties of *Crotalaria pallida*, with a specific focus on its cytotoxic effects and the interaction of its bioactive compounds through molecular docking techniques. The research aims to bridge traditional knowledge with modern scientific methods, offering promising perspectives for novel anticancer agent development.

Materials and Methods

Collection of Plant Sample

Frequent field surveys were carried out between May 2019 and August 2021. The excursion was taken regularly, ranging from once a month to thrice a month. The plant samples were gathered in the village of Bathalapalli in the Koundinya Forest, Tamil Nadu, India. As part of the investigation, plant samples representing various types were collected from the study region. Following the collection of plant samples from the study area, identification was carried out.

Authentication of Plant Sample

The plant materials were confirmed by two taxonomists-- (i) Dr. S. Soosairaj, Department of Botany, St. Joseph's College, Tiruchirapalli, Tamil Nadu, India,the sample was identified and authenticated as *Crotalaria pallida* Aiton (Fabaceae) and given the (Accession number 3053,dated 08.02.2022); and (ii) Dr. G. Gnanasekaran, Department of Botany, Madras Christian College (Autonomous), Chennai, Tamil Nadu, India who alsoidentified and authenticated the specimen as *Crotalaria pallida*Aiton (Fabaceae). The plant samples wereallowed to dry in the shade, powderedand then extracted for further analysis.

Anticancer Activity

Cell Culture Maintenance

The A549 cell line derived from human lung cancer was obtained from the National Centre for Cell Sciences (NCCS) in Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% (v/v) heat-inactivated fetal bovine serum (FBS), as well as 100 U/ml penicillin and 100 µg/ml streptomycin.These cells were cultured at 37°C in a 5% CO_2 atmosphere within a humidified incubator, maintaining a 95% air composition.

Cytotoxicity

Cytotoxicity denotes the possession of certain chemical substances that can induce cell death. When applied to either cancerous or normal cells, cytotoxic agents may trigger cell necrosis. Assessing cytotoxicity is a critical process in determining the potential toxicity of a substance. This evaluation helps us understand the *C. pallida*extract efficacy in inhibiting cancerous cell growth, offering valuable insights for cancer treatment. A substance that is verified to be capable of killing together normal and cancerous cells is labeled as a cytotoxic agent. Conversely, if a substance can selectively kill only cancerous cells without harming normal cells, it is considered an anticancer agent. Such agents undergo further clinical trials and tests before being used as potential treatments for cancer. Hence, identifying cell viability through cytotoxic assays is crucial to determine a substance's ability to harm normal cells, cancerous cells, or both.

The cytotoxicity of the sample was assessed with A549 cell lines using the MTT (3-(4,5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide) assay as described by Mossmann (1983). A total of 1 x 10^6 cells were seeded in each well of 96-well microplates and allowed to grow to 70-80% confluence during a 48h cultivation period at 37°C in a 5% CO_2 incubator. Following this, the cell culture medium was replaced, and the cells were exposed to different concentrations of the sample, followed by an additional 24h incubation period. After incubation, both the untreated (control) cells and the treated cells were examined using an Inverted Phase-Contrast microscope at 20X magnification, and photographs were captured to observe any morphological alterations. Subsequently, the cells were rinsed with phosphate-buffered saline (PBS) at pH 7.4, and 20 µl of MTT solution (5 mg/ml in PBS) was added to each well. The microplates were then placed in the dark at 37°C for 2 h to allow formazan crystal formation. Afterward, the formazan crystals were liquefied in 100 µl of dimethyl sulfoxide (DMSO), and the absorbance was measured spectro-photometrically at 570 nm. To determine the percentage of cell viability, the following formula was used:

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100

The graph was generated with cell viability (%) on the Y-axis and the concentration of the sample on the X-axis.

Molecular Docking

Molecular docking investigations were conducted using AutoDock software to analyze the interactions of bioactive compounds with the target protein. AutoDock is powerful molecular modeling simulation software, particularly well-suited for protein-ligand docking studies.The binding affinities of phytochemical mixtures were investigated through docking interactions using

the AutoDock software. The mixtures were derived from the ethanol extracts of *C. pallida* seeds and their interactions with the modeled proteins from *S. aureus*, *E. coli*, *P. aeruginosa*, and *B. subtilis* were analyzed. In the present study, we conducted docking simulations of certain ligands with the protein to gain insights into their binding interactions. Blind docking was conducted using both AutoDock 4.2 and AutoDock Vina software. Subsequently, the binding affinities of each ligand-protein complex were calculated and compared (Morris *et al*., 2009). *Bacillus subtilis* receptor protein with the selected 3-ethyl-1H-pyrrole and 1,9-cyclohexadecadiene ligands showed no hydrogen bond formation and yielded negative interaction results. To facilitate the docking process, grid maps were generated using Autogrid. The grid box encompassed the entire protein, with dimensions set at 56 × 48 × 52 Å and a grid-point spacing of 1 Å. The center of the grid was positioned at X = 28.098, Y = 34.923, Z = 26.723. To ensure robust results, 50 Genetic Algorithm (GA) runs were performed with a grouping size of 300. The remaining GA parameters were maintained at their default settings.

Selection and preparation of ligands

The chosen ligands were obtained from the SDF format (.sdf) from the PubChem database and it was changed to PDB format (.pdb) using the OpenBabel GUI software (version 2.3.1) (Kim, 2021). These selected phytocompounds were ensured to be Lipinski-compliant. The ligands' topological surface area (TPSA), the count of rotatable bonds and the utilization of PubChem IDs were noted. To prepare the ligands for docking, AutoDock Tools (ADT) 1.5.7 was utilized. Several steps were performed, including assigning hydrogen atoms, defining roots, identifying aromatic carbons, determining the count of torsions, and adding Gasteiger control to the ligands. Finally, the ligands were hoarded in pdbqt format, ready for docking simulations.

Preparation of protein

The crystal structures of *Escherichia coli* virulence protein (PDB ID: 2Y2T), *Pseudomonas aeruginosa*virulence protein (PDB ID: 6B8A), *Staphylococcus aureus*virulence protein (PDB ID: 2G9H) and A549 lung cancer cell virulence protein (PDB ID: 2XYG) were acquired from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (https://www.rcsb.org/) in PDB format (Berman *et al*., 2000). To prepare the proteins for docking, the downloaded .pdb files were opened using ADT 1.5.7. In the preparation process, aqueous molecules and heteroatoms were detached from the target proteins, and polar hydrogens were added. Then, Kollman charges were given to the organized proteins, and the final protein structures were saved in pdbqt format for docking simulations.

Visualization

The examination of protein-ligand complexes and the identification of the amino acid residues engaged in the binding interactions was conducted using Ligplot+ software (version 2.2.5) (Laskowski *et al*., 2002). Ligplot+ is a Java-based graphical user interface that generates two-dimensional diagrams depicting ligand-protein interactions. The focus of the analysis was on two primary types of intermolecular interactions: hydrogen bonding and hydrophobic interactions. These interactions were studied among the target protein's amino acid residues and the efficient groups of the ligands. Moreover, for three-dimensional visualization of the protein-ligand complexes, Pymol software was utilized. Pymol allowed for a comprehensive and detailed visualization of the complex interactions in a three-dimensional representation (Yuan *et al*., 2017).

Results and Discussion

Anticancer Activity

Crotalaria pallida was also subjected to MTT assay against A549 human lung cell lines as shown in (Fig. 1) which showed cell viability of 58.18% (Table 1). 24 h post-treatment morphological alterations in untreated (control) and treated

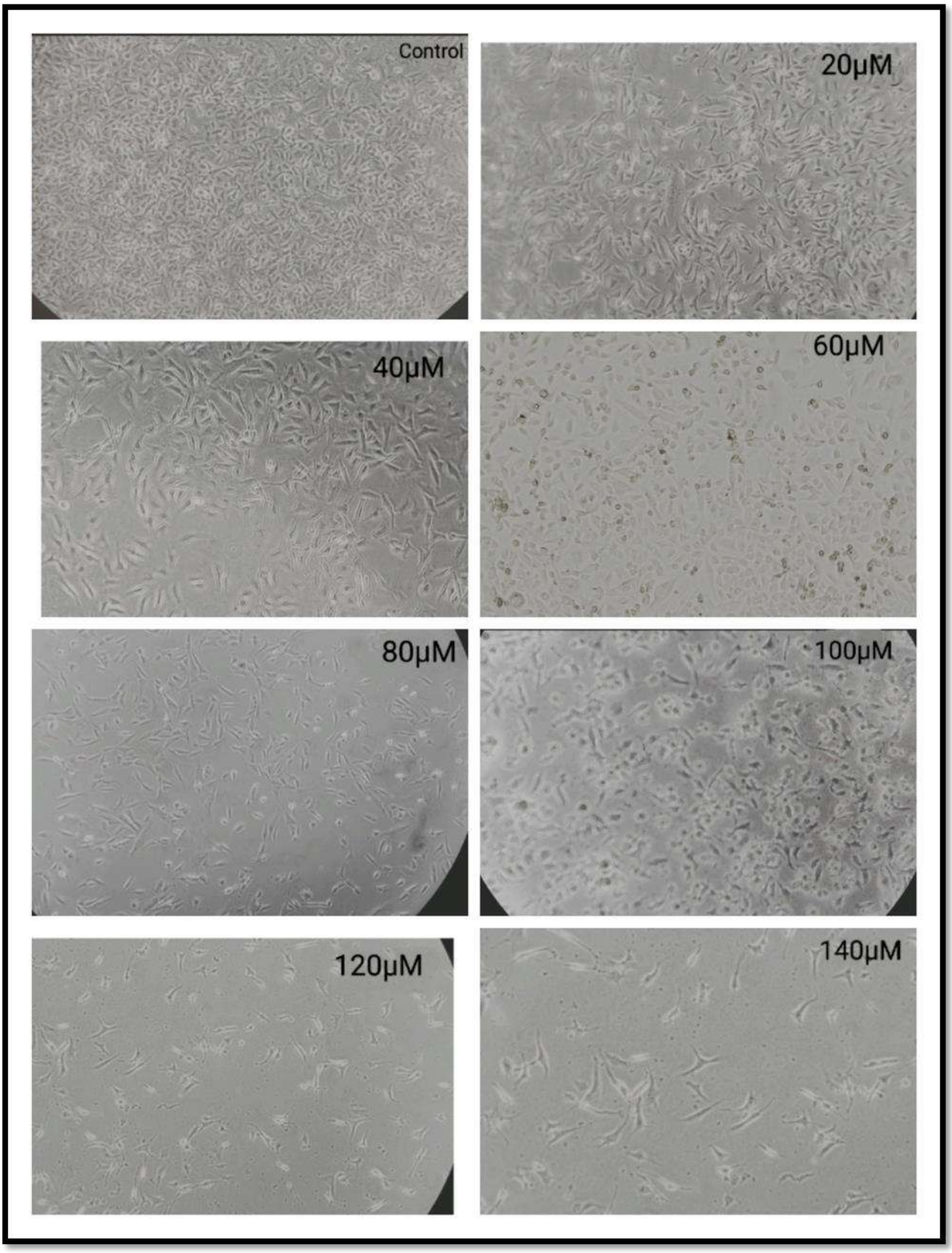

Fig. 1: *In vitro* cytotoxicity of *C. pallida* seed extract-A549 cell line.

Table 1: *In vitro* cytotoxicity of *C. pallida* seed extract - A549 cell viability

S. No.	Sample conc. (µg/ml)	A549 cell viability (%)
1	Control	100
2	20 µM	91.6
3	40 µM	87.2
4	60 µM	85.68
5	80 µM	70.98
6	100 µM	65.95
7	120 µM	61.30
8	140 µM	58.18

cellular populations were meticulously examined with an Inverted Phase Contrast microscope at 20X magnification. Photographic documentation was performed to capture visual evidence of these changes. Following that, the cells were thoroughly washed with phosphate-buffered saline (PBS, pH 7.4). Subsequently, 20 µl of a 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) in PBS was added toeach well. The microplates housing the samples were then incubated at 37ºC in a light-protected environment for duration of 2 h.

The ensuing formazan crystals were solubilized employing 100 µl of dimethyl sulfoxide (DMSO), subsequently facilitating spectrophoto-metric absorbance measurement at 570 nm. To visually represent the correlation between cell viability (%) and the varying concentrations of the test sample, a graphical depiction was constructed. On the Y-axis, cell viability (%) was indicated, and on the X-axis, the concentration of the sample was represented. Furthermore, our investigation extended to discern whether any diminution in MTT reduction could be attributed to the release of intracellular components following cellular lysis or secretion.

In light of the percentage survival analysis conducted on the A549 cell line, as observed under the phase contrast microscope, conspicuous indications of robust cell cytotoxicity were discerned at a concentration of 140 µM within the seed extract. Additionally, a state of moderate cytotoxicity became apparent at a concentration of 20 µM. Specifically, the viability of cells remained at a mere 58.18% within the 140 µM concentration, underscoring the well-defined manifestation of cytotoxic effects. The results present a dose-dependent inhibition curve that intersects the 50% threshold, yielding an inferred IC_{50} value of 7.11 µg/ml within A549 human lung cell lines.

Cancer is a significant global cause of mortality, with lung cancer particularly assuming prominence as a foremost contributor to cancer-related fatalities. The heightened incidence, accelerated advancement, and unfavorable prognosis associated with lung cancer (Assaf *et al*., 2013) underscore its gravity. Traditional therapeutic modalities, encompassing chemo-therapy and radiotherapy, have been extensively employed in lung cancer treatment. Regrettably, the application of anti-tumor agents within these regimens yields notable adversities (Force *et al*., 2008). This predicament has kindled substantial interest in the exploration and formulation of novel medicinal agents derived from botanical sources, with the express aim of addressing the exigencies posed by lung cancer treatment (Wei *et*

al., 2013).

To comprehend the underlying molecular mechanism triggering apoptosis induced by GA, an examination was conducted on various aspects within A549 cells. These assessments encompassed the quantification of reactive oxygen species (ROS) and changes in mitochondrial membrane potential (MMP), and the activation of caspase-3. It is noteworthy that GA is documented to possess a dual nature, encompassing both pro-oxidant and antioxidant attributes (Strlic*et al*., 2002). The buildup of ROS alongside heightened oxidative pressure has been linked to the etiology of numerous conditions, including various forms of cancer. A growing body indicates that the induction of apoptosis by GA is closely intertwined with oxidative stress stemming from ROS and perturbations in mitochondrial function (Inoue *et al*., 2000).

Cancer, delineated by seven fundamental hallmarks encompassing unrestrained growth, anomalous cell behavior, autonomous responsive-ness to growth stimulants, evasion of growth-restricting signals, resistance to programmed cell death, angiogenesis, an inflammatory micro-environment, and the proclivity for tissue invasion and metastasis, has been comprehensively outlined (Russo *et al*., 2012).

While cytotoxic investigations have been conducted on several species, such as *Crotalaria retusa* (showing antiproliferative properties against Jurkat cell lines), *Crotalaria verrucosa* (effective against human cervical cancer cell lines), and *Crotalaria juncea* (also targeting human cervical cancer cell lines), a dearth of cytotoxic evaluation about*C. pallida* remains conspicuous. Diverse concentrations of leaf extracts were applied to ascertain cell viability. The negative control featured 2% DMSO, against which viable cell proportions were determined. The progression of cell mortality was conspicuously linked to the escalating concentrations of plant extract. Comparative analysis involved capturing images devoid of solvents or extracts, revealing a discernible abundance of live cells. Conversely, as the culture was enriched with increasing concentrations of the plant extract, the cellular population exhibited a decremental trend. The most pronounced cell growth inhibition, exceeding 90%, was noted at a 2.0 mg/ml concentration of the leaf extract, consequently establishing an IC_{50} value of 1.25 mg/ml. The methanol extract of *C. pallida* leaf not only shows robust cytotoxic effects but also exhibits antioxidative properties. Consequently, it holds the potential to serve as an efficacious anticancer agent for addressing cervical cancer and to address conditions characterized by oxidative degeneration, such asParkinson's and Alzheimer's diseases (Nafisa, 2013).

The antimitotic potential was assessed employing *Allium cepa* root meristematic cells, widely adopted for screening antimitotic agents. The meristematic zone, situated distal to the root cap, features heightened cell division rates, akin to the cancerous division in human cells. Consequently, this region serves as an apt preliminary screening platform for compounds exhibiting potential anticancer effects. The observed effects on plant chromosomes could also extend to animal systems (Rani *et al*., 2010). The treated endophytic p-coumaric acid and coumarin cells exhibited mitotic indices of 13.6 and 19.05, respectively, while their standard counterparts displayed indices of 13.2 and 12.14, respectively. These findings are in alignment with prior studies involving plant extracts from *Ocimum gratissimum* and *Morinda lucida* (Bernice *et al*., 2009).

Cancer cells are recognized for their robust proliferation, reminiscent of the proliferation observed in germinating seed meristematic cells under favorable conditions. Control experiments evidenced the unimpeded development of sprouts and radicles. In contrast, varying concentrations of p-coumaric acid and coumarin markedly curtailed radicle lengths in all treated seeds relative to the control. This outcome echoes earlier findings that synthetic anticancer drugs entirely suppressed seed germination (Nagata, 2000; Satyanarayana *et al*., 2011) and aligns with observations by

Sadananda *et al*. (2013), where endophytic fungal lectins inhibited green gram germination. This concurrence suggests that endophytic fungi-derived p-coumaric acid and coumarin, specifically from Alternaria species-1, exert analogous effects when juxtaposed with standard p-coumaric acid and coumarin.

Molecular Docking

The drug-likeness of a ligand assumes paramount significance within the realm of drug development, warranting meticulous assessment in the ligand selection phase. In this context, the molecular weights of all ligands were investigated, revealing a collective range spanning 95–220 g/mol. The lowest molecular weight observed, amounting to 95 g/mol, was attributed to two ligands, 3-ethyl-1H-pyrrole (ligand 1) and 1,9-Cyclohexadecadiene (ligand 2). Furthermore, all ligands exhibited favorable physicochemical properties including Total Polar Surface Area (TPSA) values (<140 Å2), hydrogen bond acceptors (≤10), hydrogen bond donors (≤5), and calculated logarithmic partition coefficient (log P) values (≤5).

For the determination of binding affinities between the target protein and ligands, docking studies were steered. The initial phase employed AutoDock 4.2 to validate binding efficacies. The ligand molecules underwent docking within a grid box encompassing the entire protein. Subsequently, AutoDock Vina, employing identical grid dimensions, was executed (Dar *et al*., al., 2017). Docking scores obtained from both software platforms were compared, demonstrating that AutoDock Vina consistently provided superior and more accurate binding affinity results when contrasted with AutoDock 4.2. As a result, the docking scores derived from AutoDock Vina were selected for further investigations.

To unravel the exchanges and active site residues implicated in each ligand-protein complex, Ligplot+ analysis was undertaken. A comprehensive insights into the docking scores attained from both AutoDock Vina and AutoDock 4.2 are presented, complemented byan explanation of the relations and active site residues instrumental in the binding interactions between each ligand and the protein. The assessment of binding affinities encompassed a spectrum spanning from -3.4 to -6.2 kcal/mol. Discriminative selection of optimal compounds elevated negative binding affinity values, signifying their enhanced potential to impede the activity of the virulence protein (Torres *et al*., 2019).

Molecular Docking of E. coli virulence protein-2Y2T with selected ligands

The first phytocompound 3-ethyl- 1H-pyrrole interacted with *E. coli* virulence protein PDB ID:2Y2Tis shown in Figure 2. binding energy of -3.5 kcal/mol making 1 hydrogen bond with amino acid residues ASP75 and hydrophobic residues TYR17, ILE71, VAL77, TRP95 and PRO96 (Figs. 3, 4). The second compound 1,9-Cyclohexadecadiene interacted with the same virulence protein and binding energy -5.8 making with amino acid residues PRO96 (Figs. 5, 6).

Hydrogen bonding serves a pivotal function in mediating protein-ligand interactions, exerting notable impacts on factors such as drug binding affinity, drug selectivity, adsorption patterns, and metabolic processes (Mohan *et al*., 2020).

Molecular Docking of Pseudomonas aeruginosa virulence protein-6B8A protein with selected ligands

The initial phytocompound, 3-ethyl-1H-pyrrole, engaged with the *Pseudomonas aeruginosa v*irulence protein PDB ID is 6B8A as depicted in Figure 7. The binding energy recorded was -3.1 kcal/mol, and a single hydrogen bond was established with the amino acid residue GLY122. Additionally, interactions with hydrophobic residues including MET61, HIS121 and PHE128 were observed (Figs. 8, 9). The subsequent compound, 1,9-Cyclohexadecadiene, interacted with the *P. aeruginosa* virulence protein and binding energy -5.4 engaging specifically with the amino acid residue PHE158 (Figs. 10, 11).

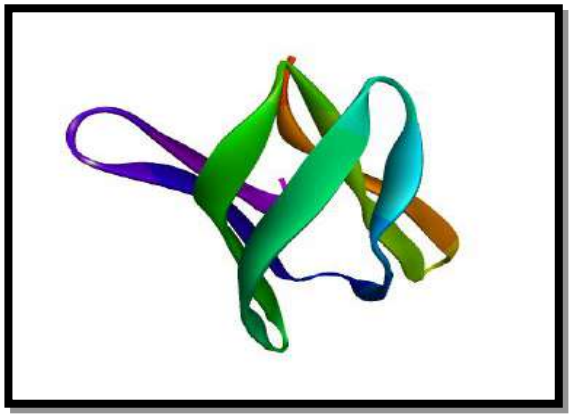

Fig. 2: PDB ID: 2Y2T *E.coli* protein receptor.

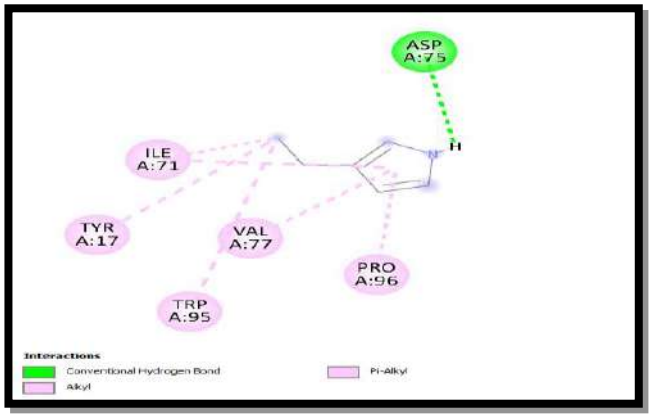

Fig. 3: 2D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with *E. coli* virulence protein receptor.

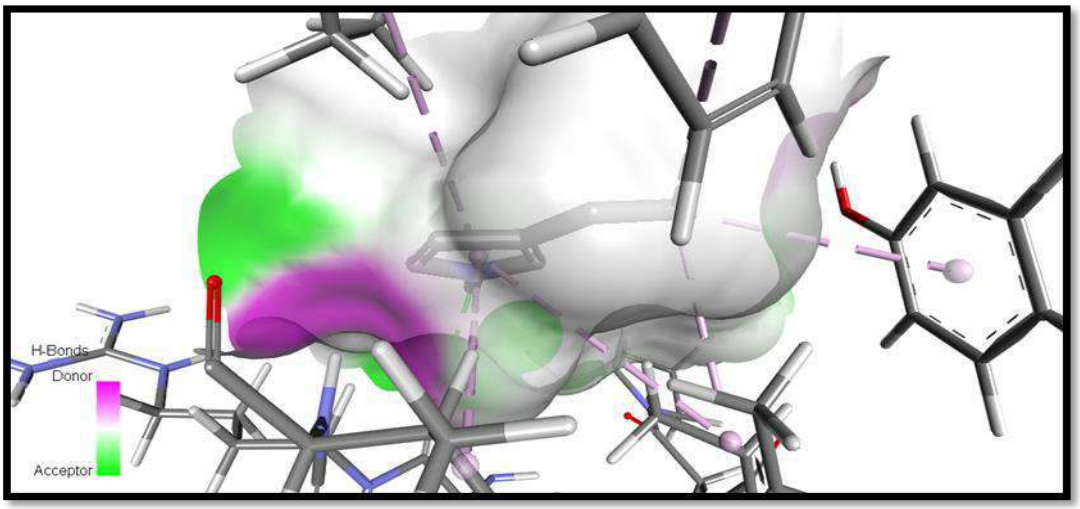

Fig. 4: 3D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with *E. coli* virulence protein receptor.

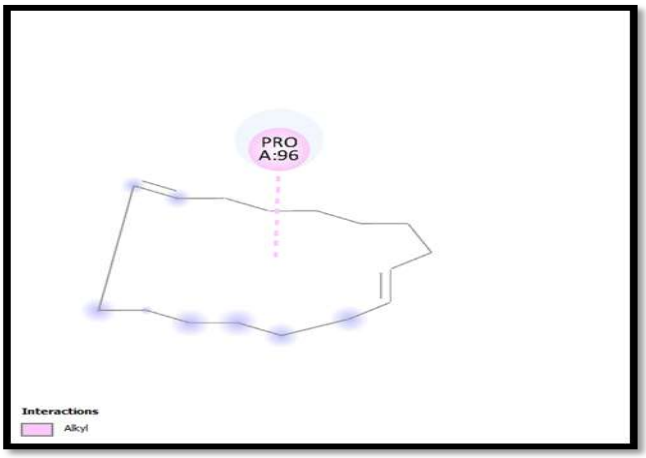

Fig. 5: 2D Binding interactions of 1,9-CyclohexadecadieneLigand compound with *E. coli* virulence protein receptor.

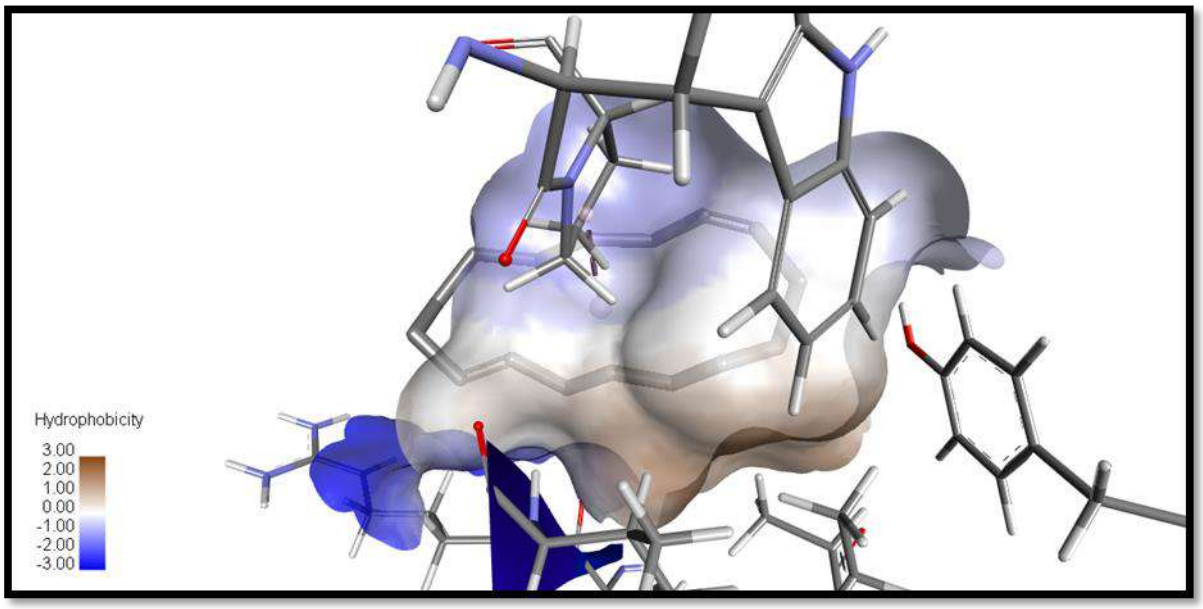

Fig. 6: 3D Binding interactions of 1,9-CyclohexadecadieneLigand compound with *E. coli* virulence protein receptor.

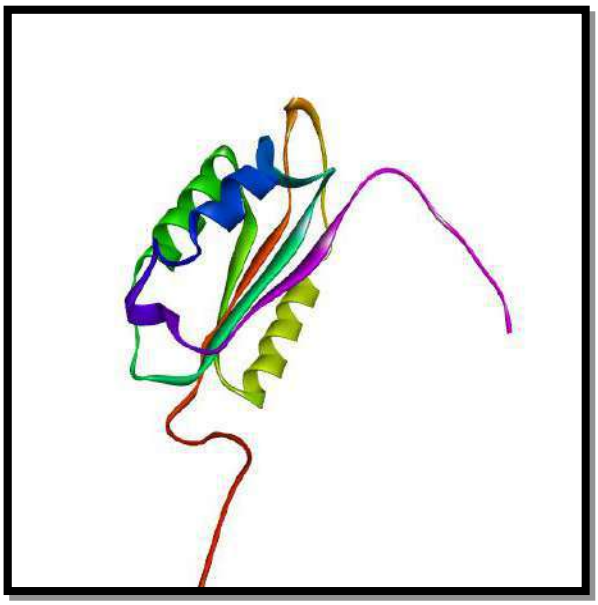

Fig. 7: PDB ID: 6B8A *P. aeruginosa* protein receptor.

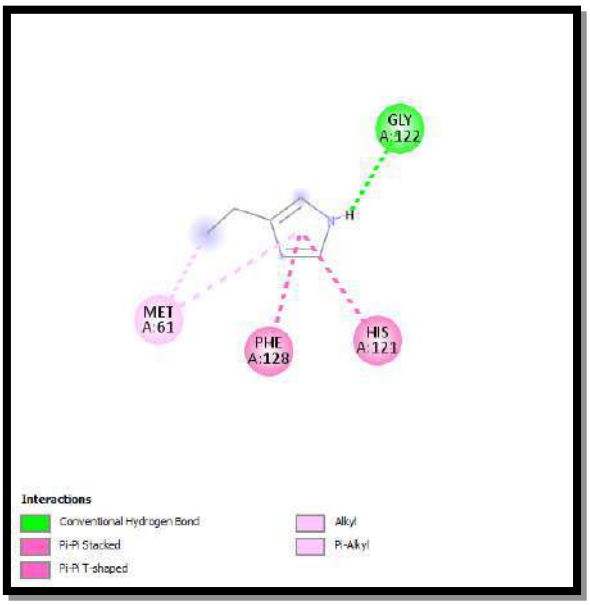

Fig. 8: 2D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with *P. aeruginosa* virulence protein receptor.

In light of this, the number of hydrogen bonds present within the protein-ligand composite was scrutinized across a 100-nanosecond trajectory (Patil *et al.*, 2010). Notably, a consistent occurrence of 1–2 hydrogen bonds was discerned within the complex over the entire duration, underscoring the robustness of the interaction at this temporal scale.

Molecular Docking of Staphylococcus aureus virulence protein-2G9H protein with selected ligands

As shown in Figure 12, the first phytocompound to interact with the *Staphylococcus aureus* virulence protein PDB ID is2G9H with 3-ethyl-1H-pyrrole. Two hydrogen bonds were formed by the amino acid residues GLY146 and ASN138 at a required energy of -3.4 kcal/mol. As shown in Figures 13 and 14, interactions with hydrophobic residues

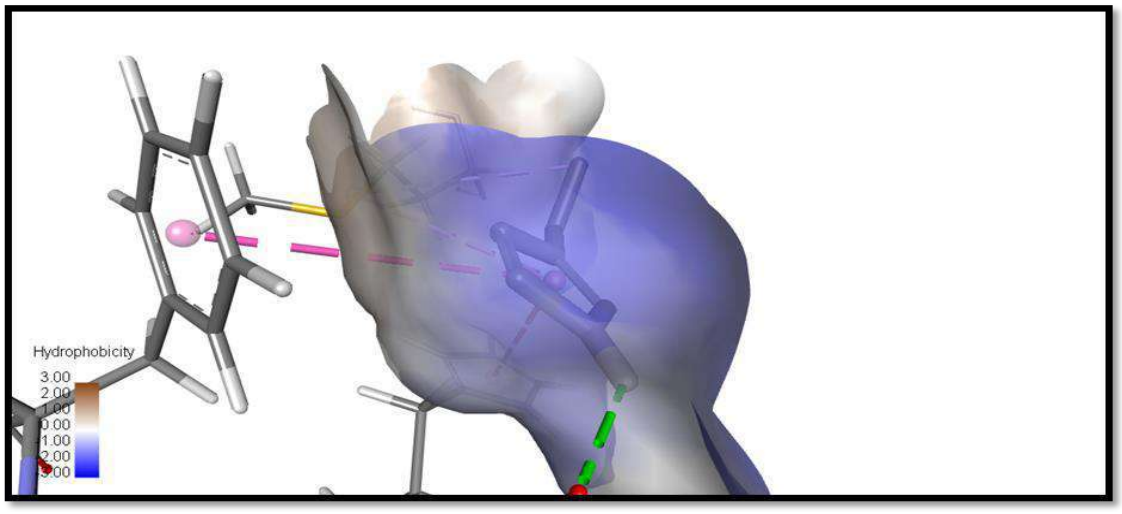

Fig. 9: 3D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with *P. aeruginosa* virulence protein receptor.

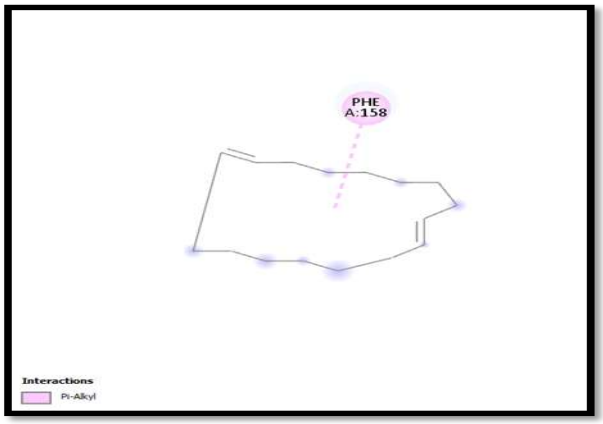

Fig. 10: 2D Binding interactions of 1,9-Cyclohexadecadiene Ligand compound with *P. aeruginosa* virulence protein receptor.

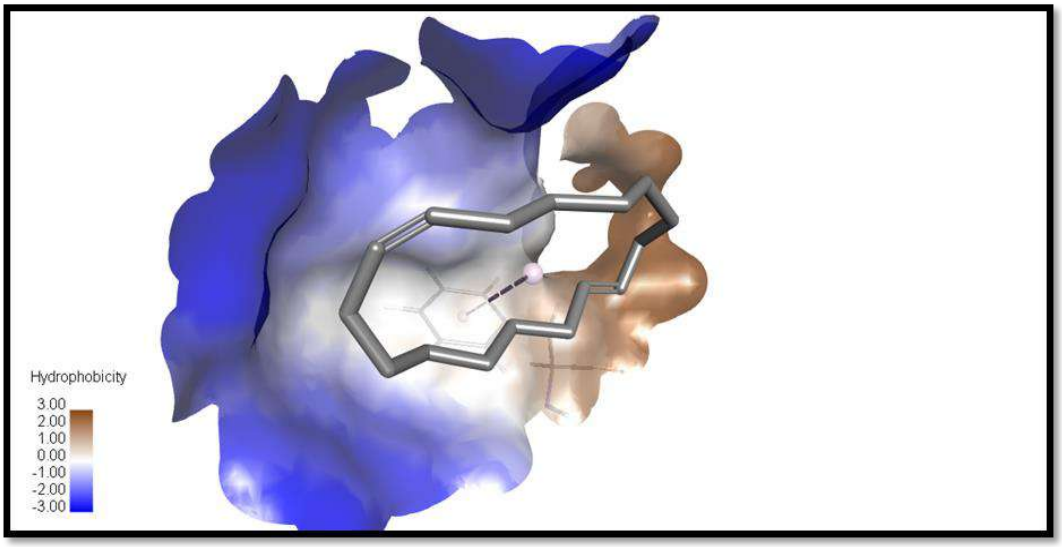

Fig. 11: 3D Binding interactions of 1,9-Cyclohexadecadiene Ligand compound with *P. aeruginosa* virulence protein receptor.

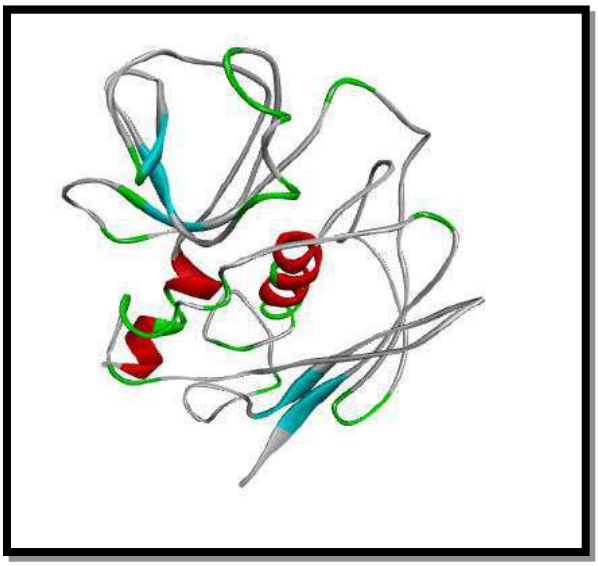

Fig. 12: PDB ID: 2G9H *S. aureus* protein receptor.

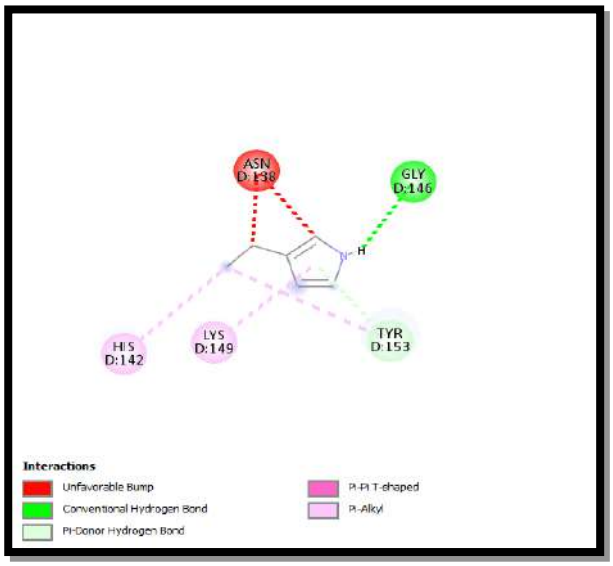

Fig. 13: 2D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with *S. aureus* virulence protein receptor.

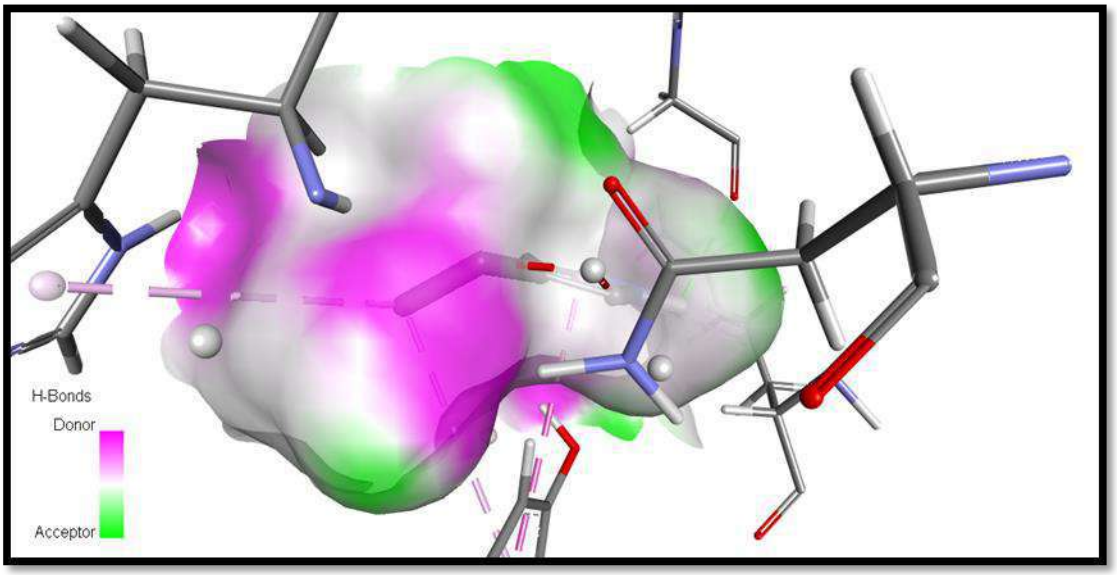

Fig. 14: 3D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with *S. aureus* virulence protein receptor.

including HIS142, LYS149 and TYR153 were also noted. The next substance, 1,9-Cyclohexa-decadiene, engaged particularly with the amino acid residue LYS76 of the *S. aureus* virulence protein and interacted with it and hydrophobic residues including ILE19 (Figs. 15, 16).

The mean radius of gyration (Rg) values computed for the unbound apo configuration and the compound formed by the protein-ligand interaction equated to 1.77 nm. A persistent and well-defined compact conformation was sustained over the designated temporal span, as evidenced by the absence of substantial fluctuations in the Rg values across the entirety of the 100-nanosecond trajectory. Notably, comparative analysis revealed that the structural compactness of the complex exceeded that of the unbound apo state (Basting *et al*., 2021).

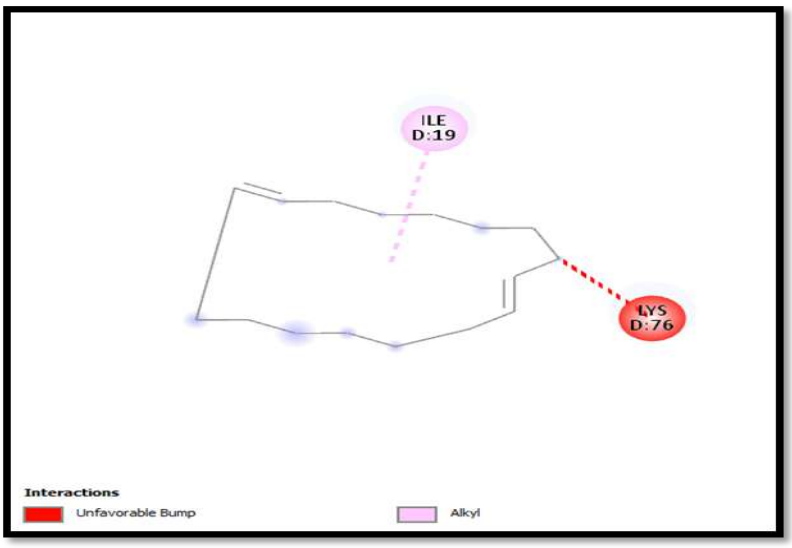

Fig. 15: 2D Binding interactions of 1,9-Cyclohexadecadiene Ligand compound with *S. aureus* virulence protein receptor.

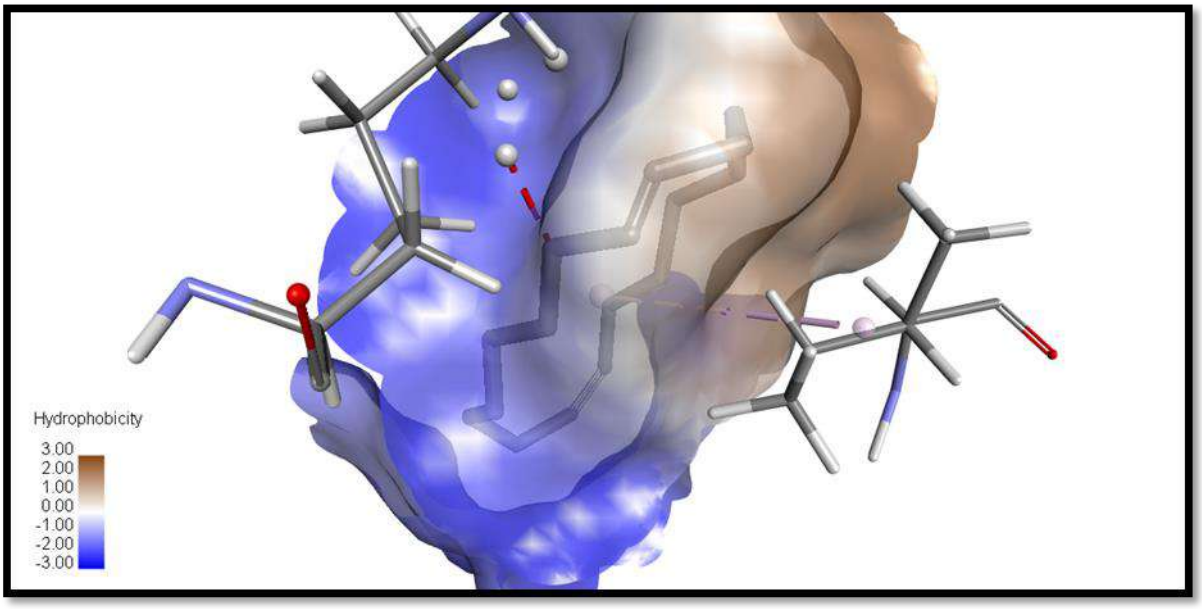

Fig. 16: 3D Binding interactions of 1,9-Cyclohexadecadiene Ligand compound with *S. aureus* virulence protein receptor.

Molecular Docking of A549 human lung cancer cell lines virulence protein-2XYG protein with selected ligands

The initial phytocompound, 3-ethyl-1H-pyrrole, engaged with the A549 human lung cancer cell lines virulence protein is identified by the PDB ID 2XYG (Fig. 17). In this interaction, no hydrogen bonds were formed exhibiting a binding energy of -3 kcal/mol (Figs. 18, 19).The interaction further encompassed hydrophobic associations with LEU136, PHE158 and ILE160. Subsequently, the succeeding compound, 1,9-Cyclohexadecadiene, displayed selective interaction and was supplemented by engagements with hydrophobic residues, including ILE 160, as illustrated in Figures 20 and 21.

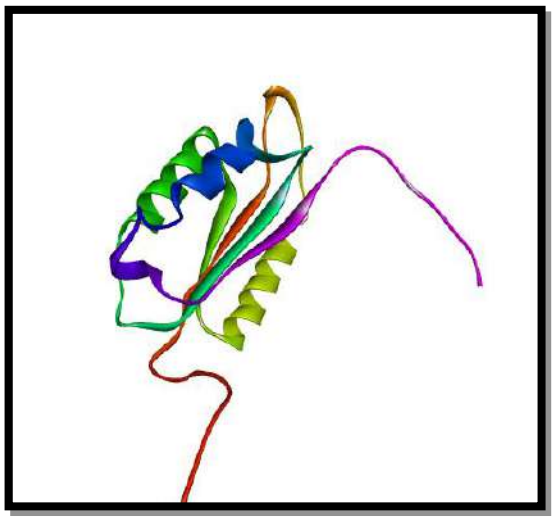

Fig. 17: PDB ID: 2XYGA549 Human lung cancer cell protein receptor.

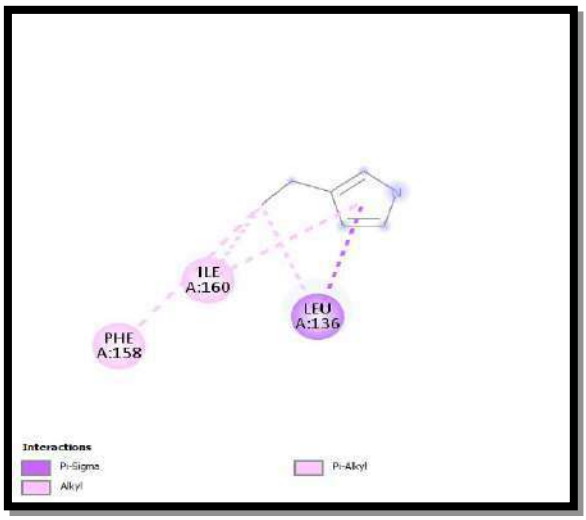

Fig. 18: 2D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with A549 Human lung cancer cell protein receptor.

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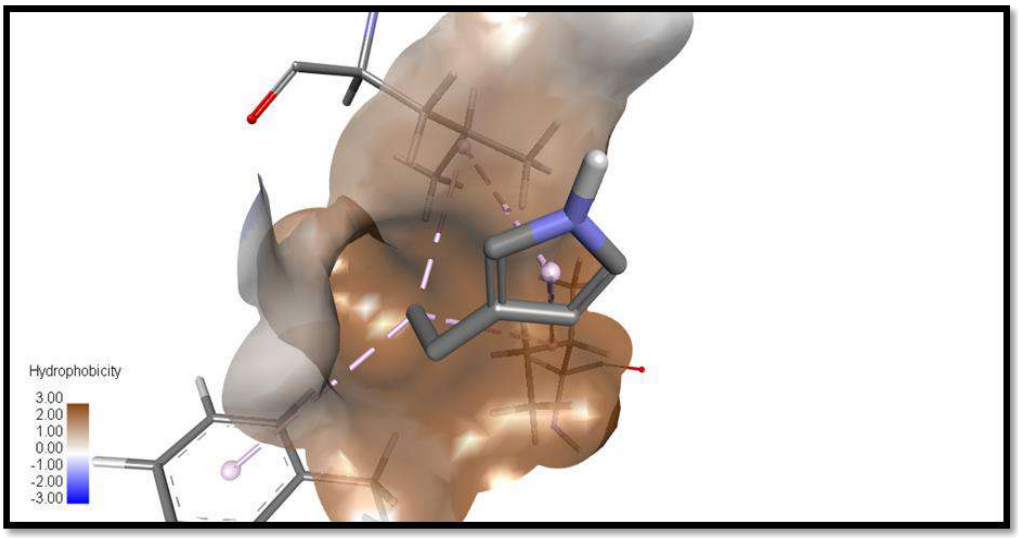

Fig. 19: 3D Binding interactions of 3-ethyl- 1H-pyrrole Ligand compound with A549 Human lung cancer cell protein receptor.

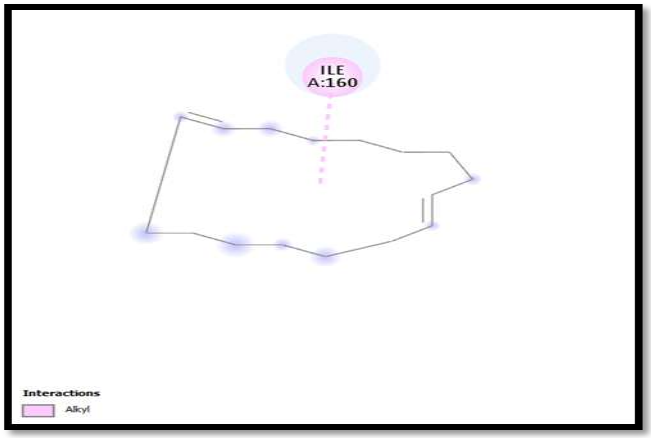

Fig. 20: 2D Binding interactions of 1, 9-Cyclohexadecadiene Ligand compound with A549 Human lung cancer cell protein receptor.

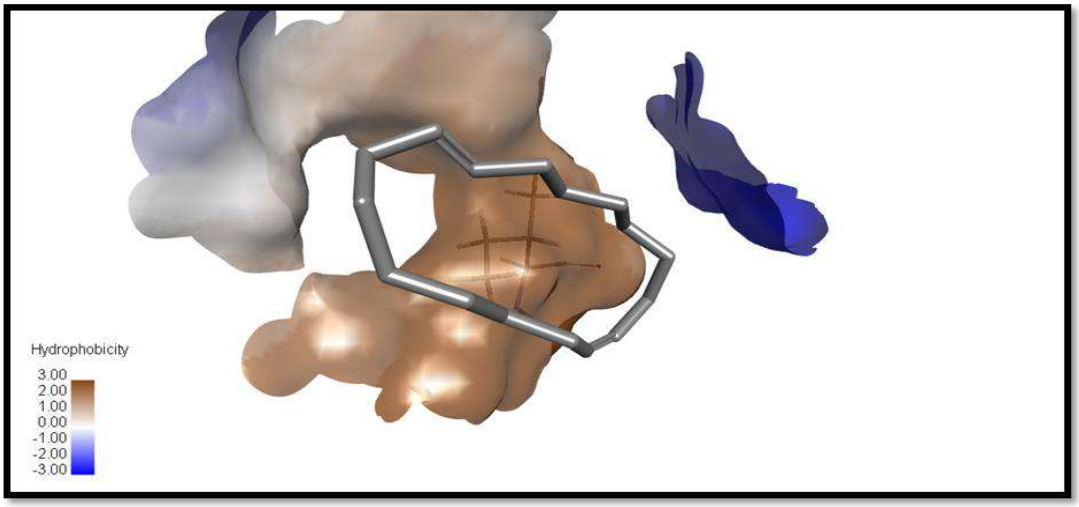

Fig. 21: 3D Binding interactions of 1, 9-Cyclohexadecadiene Ligand compound with A549 Human lung cancer cell protein receptor.

This discernible discrepancy implies that the binding event between the protein and the ligand engendered a heightened level of compactness within the protein's structure, consequently contributing to augmented overall stability of the system (Patil *et al*., 2010).

The primary goal of this study was to assess the prevailing antimicrobial efficacy exhibited by the virulence proteins of *E. coli*, *S. aureus*, *P. aeruginosa* and A549 cell lines against specific target ligands. In response to this challenge, the employment of computational methodologies has emerged as a feasible approach in the pursuit of pioneering pharmaceutical agents. The integration of computer-based techniques in the domain of drug discovery has substantially mitigated temporal constraints and alleviated the burdensome nature of repetitive tasks. The assessment of the binding free energy pertinent to the simulated protein-ligand complex was conducted over the final 20 nanoseconds of the trajectory. An increased negative value for the total binding free energy, measuring at -3.50 kcal/mol, was determined for the complex. This value signifies an elevated binding affinity between the protein and the ligand. This intensified binding affinity is attributed to the cumulative energy contributions arising from diverse interactions, which synergistically stabilized the protein complex. As a result, these findings emphasize the promising interaction potential of the virulence protein. This calls for further exploration of the inhibitory capabilities of this unique compound through comprehensive *in vitro* validation methods. Additionally, insights derived from the analysis of free energy contributions on a per-residue basis reveal that among the individual amino acid residues,Met39 and Tyr41 exhibit the most substantial energy contribution values at -2.51 kcal/mol, closely trailed by Ser104 at -2.8 kcal/mol. Notably, recent investigations have also delved into the insecticidal attributes of this compound. The present study substantiates the *in silico* antibacterial potency of the protein, thereby presenting a platform for contemplating the potential of the drug's *in vivo* or *in vitro* effectiveness against the virulence proteins of *E. coli*, *S. aureus,P. aeruginosa* and A549 cell lines.

Conclusion

The present study offers compelling evidence supporting the anticancer potential of *Crotalaria pallida* seed extracts, particularly against A549 human lung cancer cell lines. Through the MTT assay, the ethanolic extract demonstrated a

significant cytotoxic effect, with an IC_{50} value of 7.11 µg/ml, indicating its effectiveness at relatively low concentrations. Morphological changes and a clear dose-dependent decrease in cell viability reinforce its antiproliferative activity. Additionally, molecular docking studies revealed strong interactions between key phyto-compounds—3-ethyl-1H-pyrrole and 1,9-Cyclohexadecadiene—and various bacterial and cancer-related virulence proteins. These ligands exhibited favorable binding affinities, suggesting their potential as therapeutic candidates. The integration of *in vitro* cytotoxicity screening with *in silico* molecular docking offers a holistic perspective on the therapeutic relevance of *C. pallida*, paving the way for future exploration into its bioactive components. Collectively, these findings not only validate the traditional use of *C. pallida* in herbal medicine but also underscore its promise in developing novel anticancer and antimicrobial agents. Further pharmacological and mechanistic studies are recommended to elucidate its full therapeutic potential.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Department of Biotechnology, Islamiah College, Vaniyambadi, Tamil Nadu, India.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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