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A Scientific approach for Anti-Cancer activity of Hygrophila auriculata (L.),

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ABSTRACT: Novel Computer — helped dnig plan (CADD) methods are usually applied withinside the drug commercial enterprise to force the certain definition. The rate and benefit of making use of CADD, is ethnic and give best for pharmaceutical industry for targeted diseases. Based on the concept, Hygrophila auriculata (L) comes under the family Acanthaceae has been advocated for the treatment of variety of diseases, Molecular interpretation docking was carried by the tool Auto Dock 4.2. Alt serves to understand the restricting homes in a regular kingdom climate. ln this research, we worried SF I An as protein. The targeted protein with the ligand lupeol showing different pose energies is observed, lupeol shows good binding affinity towards 5F1A protein and this bioactive lupeol can be used as the potent inhibitor and effective for cancer disease.

INTRODUCTION

The theoretical foundation of CADD includes quantum mechanics and molecular modelling studies like shape, targeted molecules and impact on the drug design; ligand-based drug layout. Database gives significant information on looking the binding site, binding affinity which is based on targeted molecule. Drug discovery is a growing and emerging medicinal care which is a long, complicated, luxurious and particularly unstable way. To overcome this nil effect on discovery of medicine, computer-aided drug design (CADD) techniques are being widely used with inside the pharmaceutical enterprise to boost up the manner.[1] Computational systems offer a massive cost advantage when utilized during the lead optimization phase of drug development. Knowing the 3D structure of a target enables researchers to use shape-based analyses and molecular docking to accurately predict how a ligand binds within the receptor's active site. Docking programs need to find the drug mechanism; it must be look at protein-ligand complexes, i.e., approximate power of the interplay. A ligand docking test can also generate hundreds of target-ligand complicated conformations and a green scoring function is needed to rank the complexes and differentiate valid and invalid binding mode predictions.[2]

Hygrophila auriculata (L.), a widely scattered and major source with greater diversity herb belongs to Acanthaceae family, used for variety of diseases such as communicable disease and diabetes.[3] Based on literature review, *Hygrophila auriculata* traditionally active part such as roots, stem, fruits, seeds, and flowering tops and aerial parts of the selected *Hygrophila auriculata* has been widely used in the treatment of jaundice, hepatic obstruction, rheumatism, inflammation, UTI, gout and

malaria.[4] The plant has the active chemical constitutents such as flavonoids, alkaloids, aliphatic esters, minerals, sterols, triterpenes (lupeol, hentricotane, betulin, luteolin, luteolin 7-O-rutinosides).[5]

Lupeol has pentacyclic triterpene ring [also known as Fagarsterol] found in various active medicinal plants has prospective therapeutic and preventive activity for various disorders. Lupeol possesses the molecular formula $C_{30}H_{50}O$ and a calculated molecular weight of 426.72 g/mol. Characterization of its physical properties reveals a sharp melting point range spanning 215–216 °C**.** The IR spectral analysis reported the triterpene lupeol shows the hydroxyl function with the olefinic moiety at the spectrum ranges from 3235 to 1640 cm-1.[6] The molecular formula depicts methyl singlets occumulation with olefinic function, which support the therapeutic activity of *Hygrophila auriculata.* Pentacylic triterpenoidal ring in Lupeol is confirmed in 1H-NMR spectrum.[7,8] HPLC and UV detection of lupeol confirmed a characteristic pseudomolecular or fragment ion peak at m/z 409 ($M+H-18^+$), corresponding to water loss from the protonated molecule. Functionally, this pentacyclic triterpenoid exhibits a broad spectrum of pharmacological properties, demonstrating significant therapeutic efficacy across various in vitro and in vivo models. These active constituents acts against inflammation, cancer, arthritis, diabetes, heart diseases, renal toxicity and hepatic toxicity.[9]

As fever-reducing agents, antipyretics work by targeting the hypothalamus, effectively neutralizing the increase in core body temperature triggered by prostaglandins. The elevated body temperature then works to lower it, which results in a reduction in fever.[10]

This research article is about the antipyretic activity in hygrophila articulate with contains Lupeol as chemical constituents. It is found with docking method using auto dock software.[11,12]

METHODS

Auto Dock 4.2

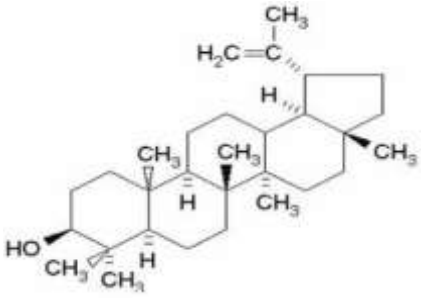
Using the Auto Dock 4.2 Tools, Molecular docking was studied. Auto docking majorly determine the interaction and binding properties between protein and ligand. This present research work we used 5F1A as protein as molecule.

The interpretation of Auto Dock was done in several steps:

Docking analysis with lupeol was prepared against antipyretic which kept in standard formats. Followed by, Auto Grid with calculated atomic affinities required for the interaction. Auto Docking was completed with the ligands. Results are analysed and interpreted with Auto Dock and Discovery Studio Visualizer are used to visualize the interactions.[13,14]

Preparation of ligands for docking

Lupeol ligands were drawn by using chemsketch. Studio visualizer 4.1 is used to discover the 2D dimensional and 3D dimensional structure and results were saved in pdb format.



LUPEOL

Preparation of protein (5F1A)

From RCSB-Protein Data Bank Crystallographic structures of selected protein was recovered with high resolution. Protein molecule binding selectivity was based on the enzymatic activity and chief prototype of a drug molecule. Docking study and interpretation correlate the biological efficacy to the selected binding domain of protein. In this experiment Auto Dock 4.2

MGL tools version 1.5.6 software packages were used for the molecular docking and to visualize the results, Discovery Studio Visualizer were used.

The water molecules deletion with ionic charges was done to avoid error. To find out the binding site Lamarckian genetic algorithm was applied. Molecular docking analysis was done by using the output file which was generated after the study.

The binding affinity was evaluated based on binding energy, inhibition constants (K_i), and the total number of hydrogen bonds. Lupeol which is one of the chemical constituent present in *Hygrophila auriculata* plant showed potent binding interactions with the protein Crystal Structure of Salicylate Bound to Human Cyclooxygenase-2 (5F1A).

Anti inflammatory Studies:

12.1 Measurement of TNFα:[123]

Background

RAW 264.7 mouse macrophages are cultured as an adherent monolayer in petri dishes, a format that allows the cells to be harvested without the use of dissociating enzymes or a cell scraper, both of which can compromise cell integrity. The harvested macrophages are subsequently resuspended, stimulated with lipopolysaccharide (LPS), and plated into 12-well culture plates already containing the test reagents. Following a 16–18 h incubation period, the conditioned medium is collected, and the cytokine profile is quantified using an enzyme-linked immunosorbent assay (ELISA).

Materials and Reagents[124]

The following materials and reagents were used in this study: RAW 264.7 mouse macrophage cell lines; a 0.1 mg/ml lipopolysaccharide (LPS) solution prepared in phosphate-buffered saline (PBS); and a Mouse TNF-alpha Quantikine ELISA kit for cytokine quantification. Cell culture was supported using high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing Glutamax, supplemented with fetal bovine serum (FBS) and penicillin-streptomycin to maintain sterility and promote cell viability. Additionally, a dedicated macrophage medium was employed for the culture and maintenance of the macrophage cell population throughout the experimental procedures.

Equipment:[125]

The experimental setup required the following equipment: 150 × 15 mm petri dishes and 12-well tissue culture-treated plates for cell culture; 10 ml capacity serological pipettes and 1.5 ml microcentrifuge tubes for liquid handling; and 50 ml sterile conical tubes for sample collection and processing. A water bath maintained at 37 °C and a pipette-aid were used to support routine cell culture procedures, while a centrifuge equipped with a swinging-bucket rotor and adaptors compatible with 50 ml conical tubes was employed for cell pelleting. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2, and cellular morphology was monitored regularly using an upright microscope equipped with a 10× objective. Following treatment, optical density was quantified using a microplate reader at a primary wavelength of 450 nm, utilizing a background correction set at either 540 nm or 570 nm.

12.2 Procedure

A. Preparation of macrophage cultures

1. Expand the RAW 264.7 mouse macrophages in macrophage medium on a petri dish. Every 2-3 days macrophage medium was changed.
2. Aspirate the culture medium from the dish upon reaching 70-80% confluence stage and add 10 ml of fresh macrophage medium.
3. Developed cells were harvested by washing the petri dish with macrophage medium multiple times to dislodge the cells using a pipette, which produces macrophage suspension.
4. The macrophage suspension were transfer into a 50 ml conical tube. Then to this additional 10 ml macrophage medium to the plate and repeat wash step.
5. Centrifuge the suspension in conical tube at 200 – 205 x G for 5 – 7 min, it produce cell pellets.

6. Resuspend the cell pellet in a known volume of macrophage medium, then count the cells.

7. Dilute the cell suspension with fresh medium to prepare a final macrophage suspension with a density of 200,000 cells/ml.

B. Preparation of assay plates

1. Determine the number of 12-well cell culture plates necessary to assay samples of interest in triplicate.

2. Transfer the test compound(s) of interest in triplicate to the appropriate wells of a 12-well plate.

3. Add macrophage medium to all wells containing a test reagent to a final volume of 500 µl. Add 500 µl macrophage medium to 6 additional wells not containing a test reagent.

4. Transfer 500 µl of the macrophage cell suspension (100,000 cells) to each of 3 wells containing 500 µl of macrophage medium only (without a test reagent). These wells will serve as unstimulated macrophage controls (Mac controls).

Macrophage stimulation with LPS:[126]

1. Add a 1:500 dilution of the 0.1 mg/ml LPS solution to the remaining macrophage suspension, then immediately pipette up and down 10–15 times to ensure even distribution and incubate the cells for 5 min at room temperature.

2. After thoroughly mixing the cell suspension, transfer 500 µL (100,000 cells) into the appropriate wells containing the test samples. Next, add 500 µL of the same suspension to three remaining wells containing 500 µL of macrophage medium only to serve as LPS-stimulated macrophage controls.

3. Gently rock the plate 3 times to ensure an even distribution of macrophages across the wells, then incubate at 37 °C for up to 24 hours. The final composition of each assay well is 100,000 macrophages in 1.0 ml of macrophage medium with 100 ng/ml LPS**,** excluding the three unstimulated control wells.

B. Collection of conditioned medium for assays of cytokine production:[127]

- Verify Activation: Inspect the cultures under a microscope to confirm proper macrophage activation before harvesting.
- Harvest Medium: Collect the macrophage-conditioned medium and transfer it into 1.5 mL microcentrifuge tubes.
- Centrifuge: Spin the collected samples for 5 minutes at 500 × g at room temperature.
- Isolate Supernatant: Carefully transfer the resulting supernatant into fresh 1.5 mL tubes.
- Use the samples immediately for the ELISA, or aliquot and store them at -80 °C**.**
- Prior to the TNF-alpha ELISA assay, the medium samples were vortexed. The ELISA was performed following the manufacturer's protocol, and absorbance was measured using a microplate reader at 450 nm with background correction set at 540 nm or 570 nm.
- Macrophage growth medium (500 ml total volume) was prepared by combining 445 mL of high-glucose DMEM containing GlutaMAX with 50 ml of fetal bovine serum (FBS) and 5 ml of penicillin-streptomycin. The complete formulation was filter-sterilised using a 0.22 µm membrane filter and stored at 4 °C for up to one month. Immediately prior to cell culture applications, aliquots of the medium were pre-warmed to 37 °C in a temperature-controlled water bath.

12.3 Measurement of Cell Proliferation with TNFα Stimulation in MTT Assay :[128]

Two non-small cell lung cancer (NSCLC) lines, HEC-1A (adenocarcinoma) and ECC (squamous cell carcinoma), were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 5% fetal bovine serum (FBS), under standard culture conditions of 37 °C and a humidified 5% CO_2 atmosphere. Cell viability was assessed by seeding equal cell numbers into 24-well plates and performing an MTT assay, a colorimetric technique in which mitochondrial reductases within metabolically active cells convert the membrane-permeable MTT dye, enabling viable-cell quantification by spectrophotometry. Purified recombinant human TNF-α (a gift from Steve Sprang, University of Texas-Southwestern, Dallas, TX) was prepared as a 1 mg/ml stock solution in PBS containing bovine serum albumin (BSA).

At specified intervals after TNF-α exposure, 200 µL of MTT reagent (5 mg/ml in PBS; Sigma Chemical Co.) was introduced into the culture medium, followed by a 3-hour incubation at 37 °C. Formazan crystal dissolution was achieved by adding 2 ml of a solubilizing solution consisting of 10% Triton X-100 and 0.1 N HCl in isopropanol to each well. Plates were subsequently agitated on a gyratory shaker for 5–10 minutes, and absorbance readings were taken at 595 nm on a microplate reader (Molecular Devices, Inc.). In select experimental groups, the pan-caspase inhibitor Z-VAD-FMK (Calbiochem) was added 30 minutes before sample pre-incubation. The corresponding results are illustrated in Figures 31 and 32 and summarized in Tables 17 and 18, while the comparative effect of TNF-α stimulation versus non-stimulation is depicted in Figure 33.

In-vitro* Anti -inflammatory Screening of Aqueous Extract *Hygrophila auriculata

Table No.13. Effect of Anti – inflammatory Activity of Aqueous extract of*Hygrophila auriculata* in Raw cell line

RAW cell line conc(ug/ml)	TNF-alpha (pg/ml)
25	481.89
12.5	687.9
6.25	2432.89
LPS	8712.63
unstimulated	6.45

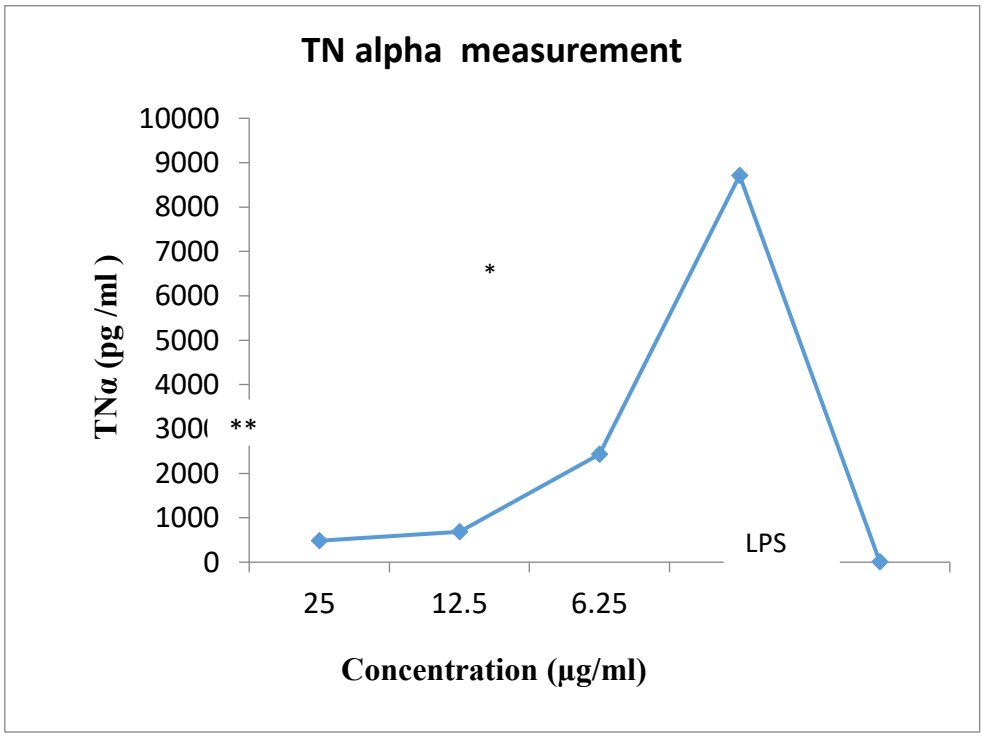


Fig.no.32 Effect of Anti inflammatory activity of Aqueous HV in TNα measurement in normal cell line, showed the most significant activity in 25µg/ml with p≤0.05 and p≤0.02 in 12.525µg/ml.

***In-vitro* Anti -inflammatory Screening With Stimulation:**

A. Measurement of Cell Proliferation with TNFα Stimulation in MTT Assay :

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Table No.17. Measurement of Cell Proliferation with TNFα Stimulation in MTT Assay(HEC 1A):

With TNF alpha (30ug/ml)		Cell line	HEC 1A				
Concentration (µg/ml)	% of cell death			Mean	SD	SEM	% Live cell
250	92.79	92.21	92.97	92.66	0.40	0.23	7.34
100	89.57	92.79	91.56	91.31	1.63	0.94	8.69
50	87.70	86.94	85.76	86.80	0.97	0.56	13.20
25	81.20	82.31	81.72	81.74	0.56	0.32	18.26
12.5	74.75	75.22	75.81	75.26	0.53	0.31	24.74
6.25	66.96	68.19	69.89	68.35	1.47	0.85	31.65
3.125	65.03	65.61	66.14	65.59	0.56	0.32	34.41
1.562	60.46	60.28	61.69	60.81	0.77	0.44	39.19
0.781	53.84	53.72	53.78	53.78	0.06	0.03	46.22

Table No.15. Measurement of Cell Proliferation with TNα Stimulation in MTT Assay(ECC -1):

With TNF alpha (30ug/ml)		Cell line	ECC - 1				
Concentration (µg/ml)	% of cell death			Mean	SD	SEM	% Live cell
250	88.96	87.73	87.98	88.22	0.65	0.37	11.78
100	83.80	82.58	83.07	83.15	0.62	0.36	16.85
50	74.23	72.88	66.13	71.08	4.34	2.50	28.92
25	64.05	64.17	63.80	64.01	0.19	0.11	35.99
12.5	63.31	61.72	62.09	62.37	0.84	0.48	37.63
6.25	46.99	49.45	46.01	47.48	1.77	1.02	52.52
3.125	31.04	33.37	34.72	33.05	1.86	1.08	66.95
1.562	15.58	16.32	15.71	15.87	0.39	0.23	84.13
0.781	12.64	6.63	3.56	7.61	4.62	2.67	92.39

Cell line	IC50(without TNF-alpha)	IC50(with TNF-alpha)

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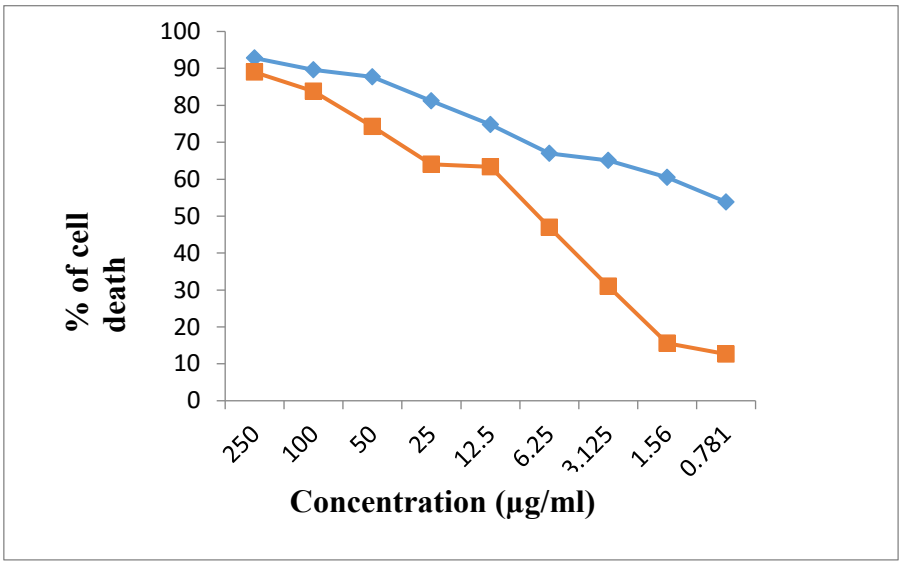
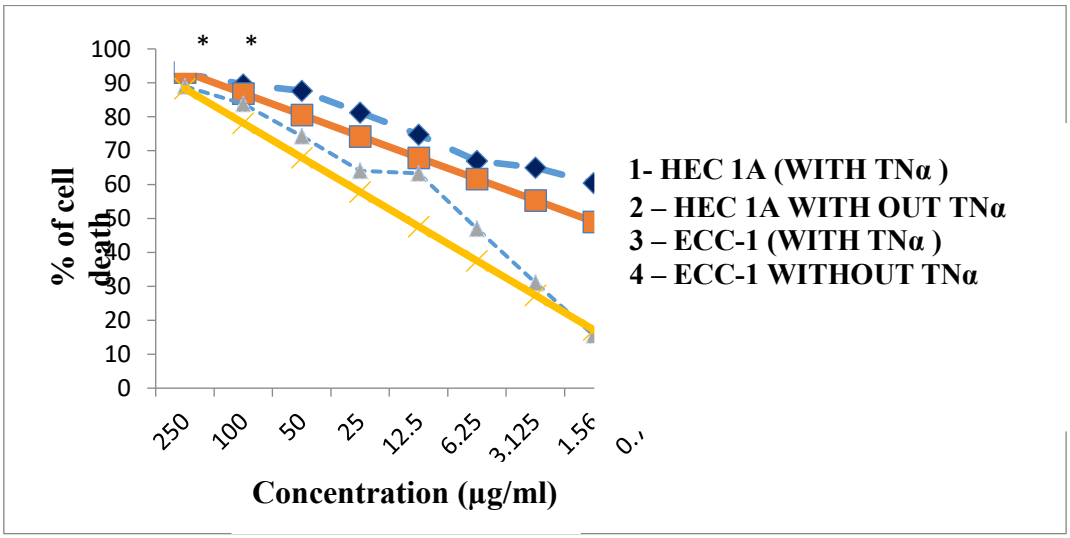
HEC 1A	12.58	9.265
ECC - 1	10.21	8.135

Fig.no33: Measurement of Cell Proliferation with TNα Stimulation in MTT Assay



Effect of cytotoxicity activity of sample with TNα Stimulation(30mg/ml) showed significant activity at 250 µg /ml.

Fig.no: 34: Comparison of Cytotoxic effect of sample with and without stimulation of TNα Stimulation:



Comparison of Cytotoxic activity of Sample with stimulation of TNFα showed most significant activity at 250 µg /ml (p≤0.05) and significant activity arised from 50 µg /ml to 100 µg /ml(p≤0.02)

Discussion: In addition to the cytotoxicity studies, the anti inflammatory studies with and without stimulation of TNFα in HEC 1A and ECC 1. Anti inflammatory studies in raw cell line has IC 50 value at 12.58 µg /ml and 10.21 µg /ml. In addition with TNFα in HEC 1A and ECC 1 has IC50 value 9.265 µg /ml and 8.135 µg /ml. In comparison of TNFα stimulation showed significant value. Even the addition of

TNFα, IC50 value decreased, it shown the aqueous extract of ***Hygrophila auriculata*** has good activity even in the presence of TNFα also. The reduction of TNFα was majorly induce the reducation of cell necrosis in the endometrial cancer.

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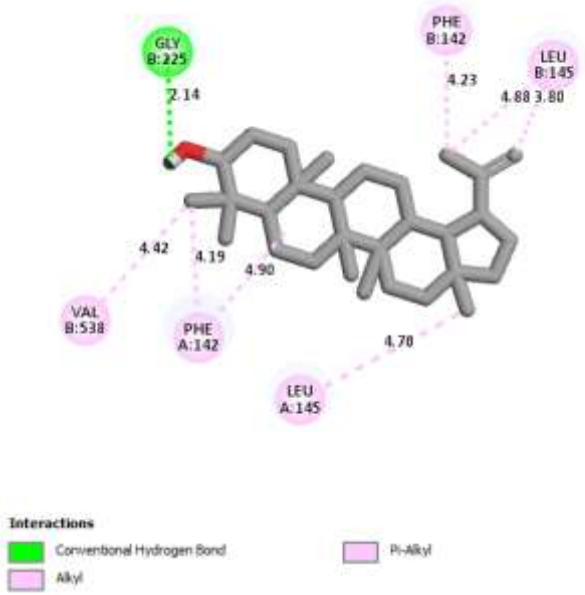
RESULT AND DISCUSSION

Binding site of 5F1A

The OH binds with GLY B:225 and he alpha methyl group with VAL B:538,PHE A:142 and the saturated aromatic carbon binds with PHE A:142,angular methyl group attached to the cyclopentane ring binds to the LUE A:145 and the alkene group attached above the cyclopentane ring binds with LUE B:145 and methyl group with PHE B:142,the bond length measurement is shown for each binding.It shows docking score of -9.1

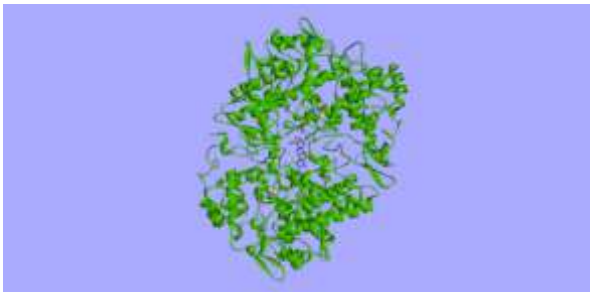
Binding lupeol with 5F1A



Protein ligand interaction using autodock 4.2

The targeted protein with the ligand lupeol showing different pose energies is observed and mentioned in the table below.The ligand showing best pose energy which depicts how strong the ligand molecule fits into the core of the receptor.

Binding affinity of Lupeol with 5F1A



Binding Measurement and groups

S.NO	BINDING RECEPTOR	MEASUREMENT	GROUP	DOCKING SCORE
1	GLY B:225	2.14	-OH	-9.1
2	VAL B:538	4.02	-CH3	

3	PHE A:142	4.90,4.91	Cyclohexane,-CH3	
4	LUE A:145	4.70	-CH3	
5	PHE B:142	4.23	-CH3	

CONCLUSION

The ligand lupeol shows good binding affinity towards 5F1A protein and this bioactive lupeol can be used as the potent inhibitor which can be further verified for the invivo study and to undergo various clinical trials for producing an effective anticancer drug.

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