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Research Article

**INSILICO AND INVITRO ANTI CANCER EVALUATION OF
CINCHONINE ALKALOID: AS APOPTOSIS INDUCING
AGENTS IN HUMAN HT- 29 CELL LINE INTRODUCTION**

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Article Received: September 2020 **Accepted:** October 2020 **Published:** November 2020**Abstract:**

To evaluate the In silico and In vitro of anti-cancer potential of Cinchonine alkaloid: as apoptosis inducing agents on HT-29 Cell line. Selection of alkaloid by molecular docking. MTT Assay on Cinchonine on HT-29 cellline. Apoptosis study by flowcytometry. In silico drug likeness and toxicity prediction of Cinchonine reveals that they are safe (no Mutagenicity, Tumorigenicity, Irritating effect, Reproductive effect). Cinchonine on in vitro method demonstrate anticancer activity on HT-29 cell line and induce the apoptosis. Further studies of Cinchonine were required to explore the mechanism of apoptosis in HT-29 cell line. cinchonine may be a promising lead molecule for the anticancer drug development on HT-29 cell line.

Keywords: In silico and In vitro of anti-cancer, Cinchonine alkaloid, molecular docking, HT-29 Cell line.

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INTRODUCTION:

Cancer is a group of diseases characterized by autonomous, uncontrolled cell proliferation, evasion of cell death, self-construction of oxygen and nutrient supply and spreading of cancerous cells through metastasis (Little, 2010). The cancer cell genotype is manifested with six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (as shown in figure 1, Hanahan and Weinberg, 2000) [1].

The cell cycle:

Actively dividing eukaryote cells pass through a series of stages known collectively as the cell cycle, two gap phases (G1 and G2); an S (for synthesis) phase, in which the genetic material is duplicated; and an M phase, in which mitosis partitions the genetic material and the cell divides [2,3].

MATERIALS AND METHODS:**In silico studies****Molecular docking study using Molegro Virtual Docker (MVD)****(a) Preparation of ligand**

The 3D structures of the phytoconstituents are retrieved from PubChem chemical databases and drawn using Chem Draw Ultra 8.0 software (Cambridge Soft Corporation, Cambridge.) and saved in mol format after minimisation of energy. The ligands are imported to the workspace and prepared them [4].

(b) Preparation of target proteins

Docking analysis is done by initially selecting the target for the disease and followed by obtaining the 3D structure of 3RCD, 5EVZ from protein data bank in pdb format. The target for docking study is selected as A-Chain of 3RCD, 5EVZ. It is well known that PDB files often have poor or missing assignments of explicit hydrogens, and the PDB file format cannot accommodate bond order information. Therefore, proper bonds, bond orders, hybridization and charges were assigned using the MVD. The potential binding sites of both the targets were calculated using the built-in cavity detection algorithm implemented MVD[5].

(c) Molegro Virtual Docker's docking search**algorithms and scoring functions**

Ligand docking studies were performed by Molegro Virtual Docker (MolegroApS, Aarhus C, Denmark). MVD is a fast and flexible docking program that gives the most likely conformation of ligand binding to a macromolecule. MolDock is a molecular docking algorithm, based on a new heuristic search algorithm that combines differential evolution with a cavity prediction algorithm. The docking scoring function of MolDock is an extension of the piecewise linear potential (PLP) including new hydrogen bonding and electrostatic terms. To further improve docking accuracy, a re-ranking scoring function is introduced, which identifies the most promising docking solution from the solutions obtained by the docking algorithm.

(d) Parameters for scoring functions (Mol Dock score)

Ignore-distant-atoms option was used to ignore atoms far away from the binding site. Additionally, hydrogen bond directionality was said to check whether hydrogen bonding between potential donors and acceptors can occur. The binding site on the protein was defined as extending in X, Y & Z directions around the selected cavity with a radius of 25 Angstroms. The docking scores of the phyto constituents are compared against the standard drug [6].

In silico assessment of drug likeness of Morin hydrate

Drug likeness of Morin hydrate was predicted using Data warrior, version 4.2.2 (Actelion Pharmaceuticals Ltd, Allschwil, Switzerland). It is a tool developed to analyze the drug likeness of substances. Structure of Morin hydrate was drawn using Chem Draw Ultra 12.0, then saved as MDL SDfiles (*.sdf) and directly uploaded into the Datawarrior prediction program to predict the drug likeness of the phyto constituents [7].

In silico acute rat toxicity of Morinhydrate

LD₅₀ values for rats with different routes of administration (oral, intravenous, intraperitoneal and subcutaneous) were predicted by GUSAR software (Department for Bioinformatics, Institute of Biomedical Chemistry of the Russian Academy of Medical Sciences, Moscow, Russia). It was developed to create QSAR models on the basis of the appropriate training sets represented as MDL SDfile contained data about chemical structures and endpoint in quantitative terms [8].

2.1. INVITRO ANTI CANCER EVALUATION OF MORIN HYDRATE BY MTT ASSAY:

Principle of assay:

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. DMSO, Isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells [9].

2.2. INVITRO EVALUATION OF MOLECULAR MECHANISM OF MORIN HYDRATE: Apoptosis by Flow cytometer:

1. The cells were seeded in a 24-well flat bottom micro plate containing cover slips and maintained at 37°C in CO₂ incubator for overnight.
2. Treat IC₅₀ value of each compound was treated at 12 hrs. After

the incubation, cells were

3. washed with PBS.
4. Centrifuge for 5 minutes at 500 x g at 4°C. Discard supernatant and resuspend the cell pellets in ice-cold 1X Binding Buffer to 1 x 10⁵ per mL.
5. Keep tubes on ice. Then add 1 µL of annexin V-FITC solution and 5 µL PI and Mix gently.
6. Keep tubes on ice and incubate for 15 minutes in the dark.
7. Add 400 µL of ice-cold 1X binding buffer and mix gently. Analyze cell preparations within 30 minutes by flow cytometry.

RESULTS & DISCUSSION:

Molecular docking study using 3RCD:

Molecular docking study was carried out to identify phyto constituents responsible for anti-cancer activity by MVD. 4BU3 is selected as target protein and shown in figure (A). Grid generated around the binding pocket of crystalized ligand, shown in figure (B). Protein with surface, shown in figure (1). Cinchonine and chlorogenic acid gained good mol dock scores - 140.279 and -156.336 respectively, shown in table 1.

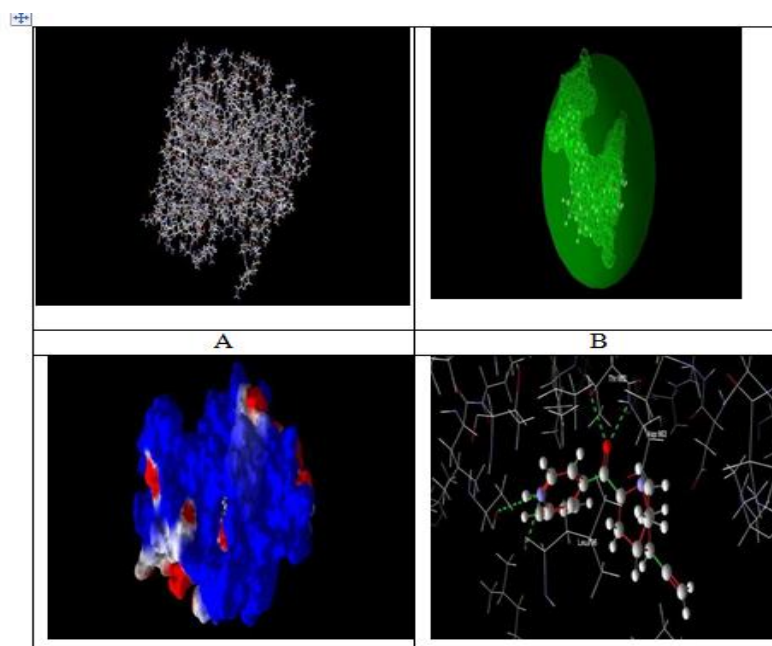


Figure 1: A. Structure of 3RCD enzyme B. Grid generated around the binding pocket C. protein surface D. 2D plot of ligand protein interaction profile by MVD. Visualization of cinchonine-3RCD enzyme, Hydrogen bond interaction (cinchonine 4- Asp 863 Thr 862 Leu 796 Met 833)

Table-1-Molecular docking study using 3RCD

Ligand	Target Protein 3RCD		
	MolDock Score	Rerank Score	H-Bond
5. Chlorogenic acid	-154.064	-109.09	-3.66332
10. Veratrine	-122.195	-29.1136	-12.2706
9. Cinchonine	-121.003	-63.1818	-7.29576
2.Morin	-107.114	-84.3467	-5.77359
1. catechin	-105.507	-88.0376	-7.73061
3. Hordenine	-98.4901	-83.9677	-3.07235
4. Isovanillic acid	-72.7352	-57.8175	0.35352
8. Cystamine	-69.4735	-57.9611	-7.24598
6. Glutarimide	-68.6678	-58.8374	-7.87074
7.Beta-pinene	-50.6347	-45.6483	-4.8931

Molecular docking study using 5EVZ

Molecular docking study was carried out to identify phytoconstituents responsible for anti- cancer activity by MVD. 5EVZ is selected as target protein and shown in figure (A). Grid generated around the binding pocket of crystalized ligand, shown in figure (B). Protein with surface, shown in figure(2). Morin hydrate and chlogenic acid gained good mol dock scores -151.81 and -118.57 respectively, shown in table 2.

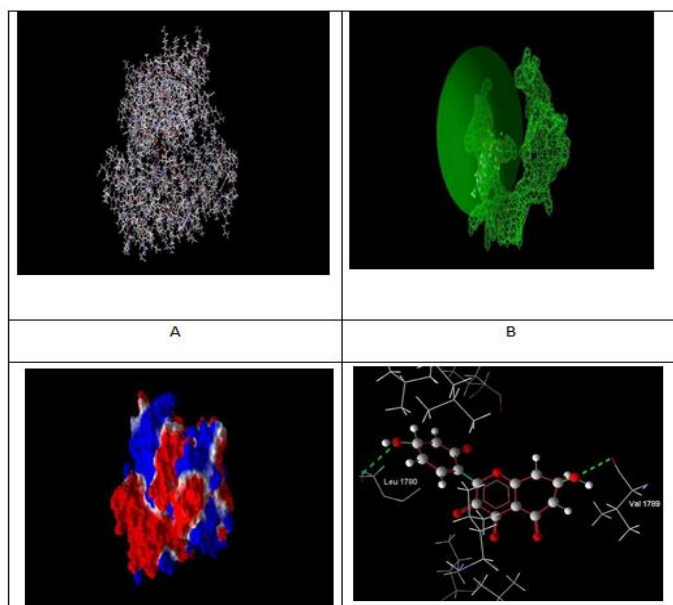


Figure 2: A. Structure of 5EVZ enzyme B. Grid generated around the binding pocket C. protein surface D. 2D plot of ligand protein interaction profile by MVD. Visualization of Cinchonine–5EVZenzyme, Hydrogen bond interaction (Cinchonin 4= Ser 300, 365- 2, Lys 296)

Table-2-Molecular docking study using 5EVZ

Ligand	Target Protein 5EVZ		
	MolDockScore	Rerank Score	H-Bond
3. Chlorogenic acid	-151.861	-130.228	-19.9587
2. Morin	-118.57	-64.7388	-17.9657
6. Cystamine	-92.6475	-72.7367	-15.6393
1. catechin	-122.706	-98.191	-11.8825
5. Glutarimide	-56.4585	-49.919	-11.0957
4. Isonanillic acid	-92.6201	-77.0395	-9.4846
1. Cinchonine	-117.412	-99.1159	-7.08643
4. Veratrine	-66.4168	299.359	-4.88472
2. Hordenine	-80.7809	-68.5607	-2.90098
7. Beta-pinene	-47.9458	-45.955	0

Molecular docking study using 5T44:

Molecular docking study was carried out to identify phytoconstituents responsible for anti-cancer activity by MVD. 5T44 is selected as target protein and shown in figure (1). Grid generated around the binding pocket of crystalized ligand, shown in figure (2). Protein with surface, shown in figure (3).

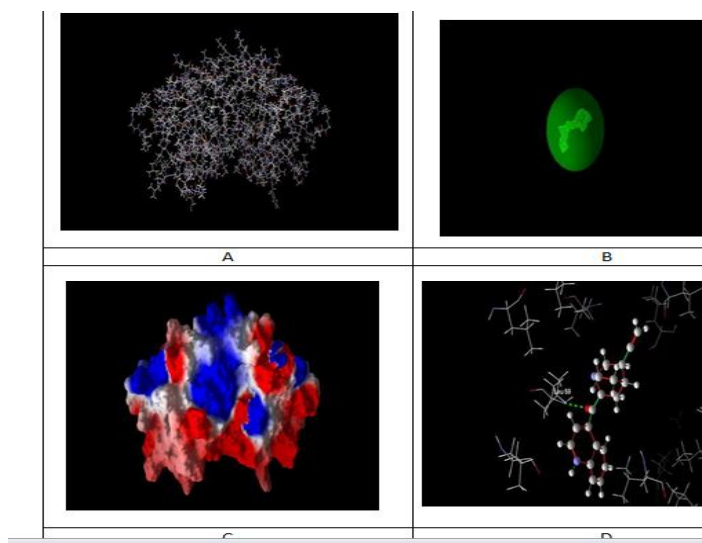


Fig-3-A. Structure of 5T44 enzyme B. Grid generated around the binding pocket C. protein surface D. 2D plot of ligand protein interaction profile by MVD.

***In silico* acute rat toxicity prediction:**

LD50 values (table 7.5.1) and Acute Rodent Toxicity Classification for rats (table 7.5.2) of phytoconstituents with different routes of administration were predicted by *in silico* approach and

it was found that their therapeutic window is wide.

Table -3-In silico prediction of LD₅₀ values with different routes of administration by GUSAR

PHYTO CONSTITUENT	Rat LD ₅₀ mg/kg			
	IP	IV	ORAL	SC
CINCHONINE	141,600	32,750	714,300	50,530

Determination of Toxic Properties:

Toxic properties also determined by employing the Data warrior. Irritating effects, reproductive effects, Mutagenicity and tumorigenicity risk of the phyto constituents was graded as high-risk, medium risk and low risk. Toxicity risk alerts are an indication that the designed/alterd structure may be harmful concerning the risk category specified. We found that they are free from major toxicity risk. Determination of acute toxicity, expressed as median lethal dose (LD₅₀), is one of the most important steps in drug discovery pipeline. Because *in vivo* assays for oral

acute toxicity in mammals are time-consuming and costly, *in silico* prediction models give preliminary idea about the safety of the drug like compounds which is economic and less time-consuming. In silico prediction of LD₅₀ values for rats with four types of administration (oral, intravenous, intraperitoneal, subcutaneous) by GUSAR software. The training sets were created on the basis of data from SYMYX MDL Toxicity Database. They include the information about ~10000 chemical structures with data on acute rat's toxicity represented on the LD₅₀ values (log10mmol/kg).

Table-4- % cell viability of different concentrations of cinchonine and control

Tested concentration(µg/ml)	% of cell viability (triplicate values)		
500	50.85	49.44	49.72
250	63.56	62.15	62.99
100	72.60	71.47	72.03
50	79.38	78.53	79.10
25	88.70	87.57	88.14
Control	100	100	100

Table-5- % cell viability of different concentrations of cinchonine and control

Tested concentration(µg/ml)	% of cell viability
500	50.00
250	62.9
100	72.03
50	79.00
25	88.13
Control	100

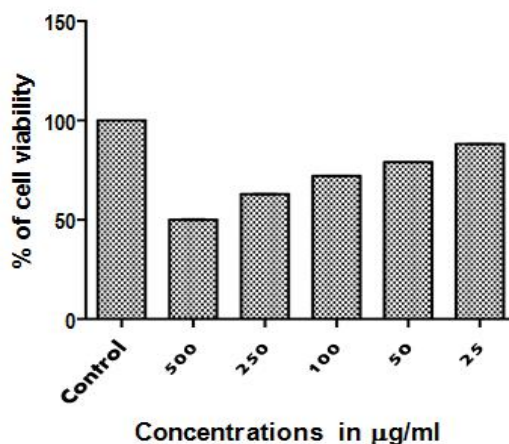


Figure-4- % cell viability of different concentrations of cinchonine with control

Determination of Cytotoxic Effect:

For determination of cytotoxic effect of morin cinchonine on HT-29 cells, a cell viability (MTT) assay was performed following 48 h of cinchonine exposure. And results were reported as relative cell viability (%). The results demonstrated that cinchonine decreased the cell viability of HT-29 cells at 250, 500 $\mu\text{g/mL}$ concentrations (62.9, 50.00%) as compared to control. The result demonstrates that cinchonine exhibits selective toxicity for breast cancer cells. In this study HT-29 cells treated with cinchonine at IC 50 concentration and stained with annexin-V-FITC/PI and determined by flow cytometry. Flow cytometric analyses reveals enhanced apoptosis of HT-29 cells treated with cinchonine in a concentration dependent manner (Figure 13), based on the formation of a significant accumulation of early apoptosis cells. In HT-29 cells, at IC 50 concentration cinchonine treatment the percentages of apoptotic cells were early apoptosis (16.92%) & amp; late apoptosis (45.30%) . This results revealed enhanced apoptosis of HT-29 cells treated with cinchonine in a time dependent Manner.

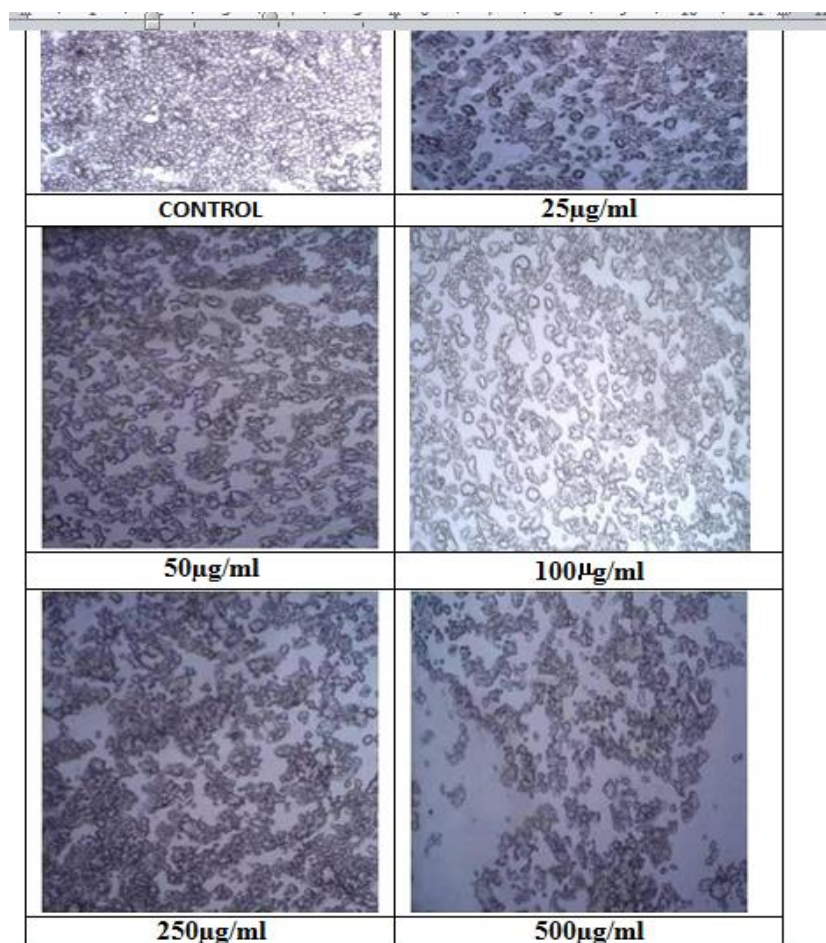


Figure-5- Effect of different concentrations of cinchonine on HT- 29

Table-6- Apoptosis by Flow cytometer

	Early Apoptosis	Late Apoptosis	Necrosis	Live
Sample	16.92%	45.30%	16.92%	36.10%
Control	0.17%	1.64%	0.28%	97.91%

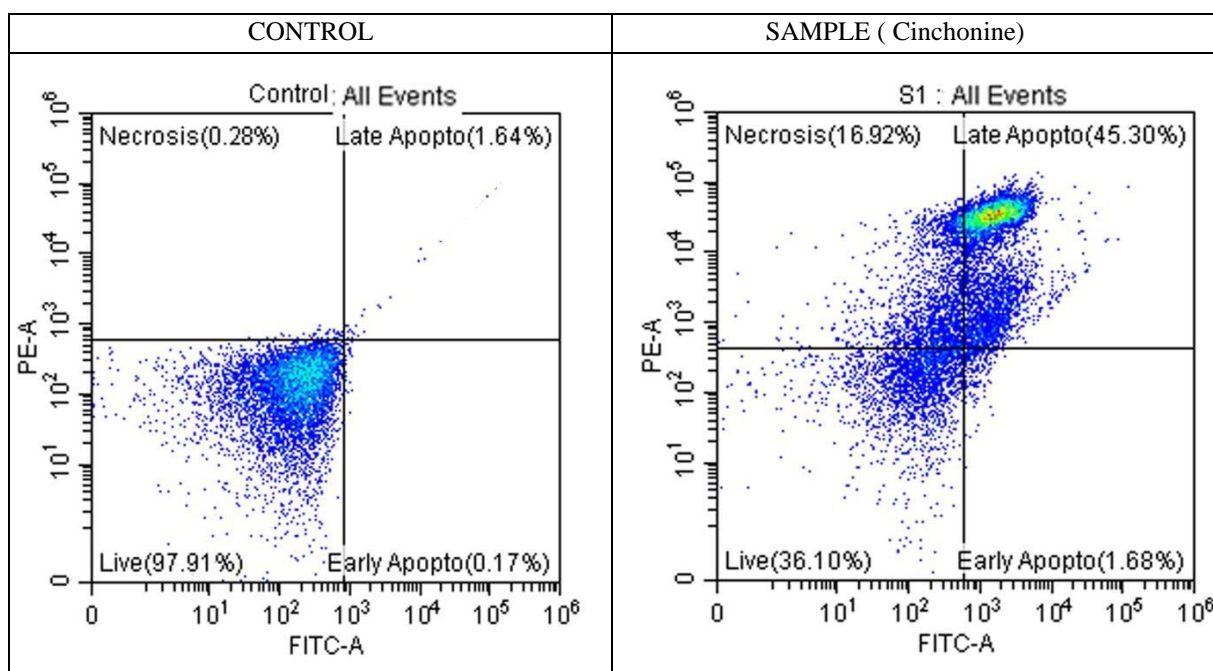


Figure -6-% Apoptosis of cinchonine and control

CONCLUSION:

In silico drug likeness and toxicity prediction of Cinchonine reveals that they are safe (no Mutagenicity, Tumorigenicity, Irritating effect, Reproductive effect). Cinchonine on in vitro method demonstrate Anticancer activity on HT-29 cell line and induce the apoptosis. Further studies of Cinchonine were required to explore the mechanism of apoptosis in HT-29 cell line. cinchonine may be a promising lead molecule for the anticancer drug development on HT-29 cellline.

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