Original Research Article

Assessing the Chemosensitizing effect of Neferine on Cisplatin-resistant Colorectal Cancer Cells through Molecular Docking Studies

ABSTRACT

Colorectal cancer (CRC) is the second deadliest diseases next to lung cancer. Cisplatin is the first generation platinum based alkylating agent using for treatment of advance CRC patients. But the development of cisplatin-resistance due to the continuous usage limits its therapeutic efficacy. Recent research is focused on studying the chemotherapeutic efficacy of the phytochemicals as they are less toxic compared to the conventional chemotherapeutic drugs. Neferine is a bisbenzylisoquinoline alkaloid extracted from the embryo of *Nelumbo nucifera.* The anticancer and chemosensitizing effect of neferine has been well reported in several cancer cells. However, there are no reports on the chemosensitizing effect of neferine on cisplatin-resistant colorectal cancer cells (CRCs). Hence, the present study aims at identification of target proteins responsible for cisplatin-resistance in colorectal cancer cells. The present investigation elucidates the specific interaction of neferine with various cell surface receptor proteins related to cisplatin-resistance, multi-drug resistance (MDR) proteins, signal transduction protein and transcription factors *via* molecular docking approach. The interaction between neferine and the target proteins of cisplatin-resistant colorectal cancer was analyzed through Schrodinger Maestro 11.9 module. From our docking studies we could suggest that neferine is most active for insulin-like growth factor-1 receptor (IGF1R), fibroblast growth factor receptor-2 (FGFR2), zinc finger protein SNAI1 (SNAIL1), signal transducer and activator of transcription-3 (STAT3) and transforming growth factor beta receptor-1 (TGFβR1) when sorted according to their docking score.

Keywords: Colorectal cancer; Neferine; Molecular docking; Cisplatin; IGF1R; Multi-drug resistance

1. INTRODUCTION

Globally, cancer is the leading cause of death and severely affects the quality of life. As per the estimate of World Health Organization (WHO) in the year 2025 there will be 21.9 million new cancer incidences and 11.4 million cancer deaths in the world [1]. Colorectal cancer (CRC) is the third most common and second lethal disease around the world. The current treatment for CRC mainly includes radiotherapy, surgical resection and chemotherapy [2]. Even though better treatment options

have improved overall survival rates in the early stages, 40-50% of all CRC patients have metastasis at the time of diagnosis or as a recurrent disease after receiving chemotherapy [3].

Chemotherapy is the one of the main components in the treatment of cancer. However, the curative responses of chemotherapeutic agents are limited due to drug resistance and therapeutic side effects like non-targeted organ toxicities [4,5]. Most of the cancer cells which initially respond to the traditional chemotherapeutic drugs, eventually develop resistance over a period of treatment. Cancer cells can develop resistance to the treatment through various molecular mechanisms such as- decreased drug uptake, elevated drug efflux, alteration of drug target, detoxification, increased DNA repair, apoptosis inhibition and epithelial to mesenchymal transition [6]. Cisplatin remains the most traditional chemotherapeutic drug for solid tumour treatments, including CRC [7]. It is a platinum-based drug which binds with DNA, forming a DNA-Platinum adduct, leading to inhibition of transcription and translation, inducing mitochondrial-mediated cell death [8]. Unfortunately, continuous treatment of cisplatin leads to cell resistance which is a frequent occurrence in CRC clinical chemotherapy.

Recent studies show that the combination of phytochemicals with traditional chemotherapy improves the curative response in cancer patients [9]. *Nelumbo nucifera* is commonly called as lotus used in traditional Indian and Chinese medicine to treat cardiovascular disease, neuronal disorder and insomnia [10]. Neferine, a bisbenzylisoquinoline alkaloid derivative from *Nelumbo nucifera* seed embryo (Figure - 1), exhibits various pharmacological effects including, anti-oxidant, anti-inflammatory [11], cardioprotection [12], anti-cancer [10], and chemosensitizing ability in cancer cells [11]. A previous study from our lab showed that neferine sensitizes the doxorubicin-resistant A549 lung cancer cells by increasing accumulation of inter/intracellular doxorubicin through downregulation of lung resistance protein (LRP) mediated by nuclear factor erythroid 2–related factor 2 (NRF2) inhibition [13]. Another study demonstrated that neferine downregulates the multi-drug resistance (P-glycoprotein) gene expression in the hyperthermal state that synergistically reverses the multi-drug resistance in human gastric cancer cells [14]. Combinatorial treatment of neferine with oxaliplatin has been shown to increase the chemotherapeutic sensitivity and suppress the epithelial to mesenchymal transition by inhibiting Snail protein expression in hepatocellular carcinoma cells [15]. Our preliminary study found that neferine reverses the cisplatin-resistant CRCs by inducing apoptosis, but the molecular mechanism of reversal of cisplatin-resistance is still unclear. In this present *in-silico* study, we aim to investigate the effect of neferine against cell surface receptor proteins, MDR proteins, signal transduction protein and transcription factors which are related to cisplatin-resistance *via* molecular docking approach.

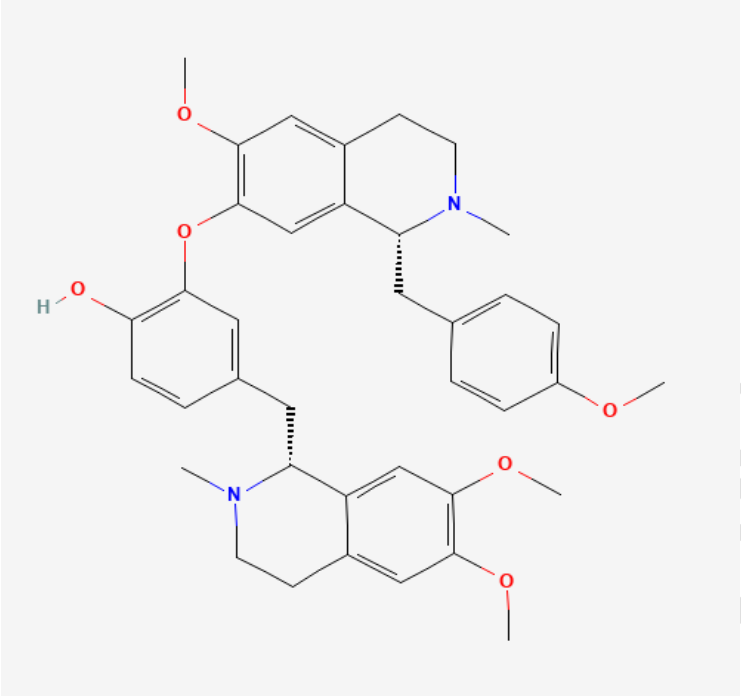

Fig.1. The structure of neferine

2. METHODOLOGY

2.1 Chemicals and Reagents

Cisplatin were purchased from Sigma Aldrich, USA. RPMI 1640 media was purchased from Gibco, USA. Fetal Bovine Serum (FBS), antibiotics and other fine chemicals were purchased from HiMedia Laboratories (Mumbai, India).

2.2 Animal cell culture maintenance

Human colorectal cancer cells (HCT-15) cell line was purchased from National Centre for Cell Science (NCCS), Pune, India. HCT-15 cells and cisplatin-resistant HCT-15 were cultured in RPMI-1640 media supplemented with 10% FBS and Penicillin (100 Units/ml), Streptomycin (30 µg/ml) and Gentamycin (20 µg/ml).

2.3 Cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed for assessing the effect of cisplatin on HCT-15 cells. The 1 x 10^5 cells/well were seeded in 96 well plates and allowed to attach by incubating overnight at 37°C in CO_2 incubator. After 24 h of treatment time, media (cisplatin) was replaced with 20 µl of (MTT 5 mg/ml) dissolved in of phosphate buffer saline (PBS) and incubated for 4 h in CO_2 incubator. Then, 200 µl of Dimethylsulfoxide (DMSO) was added to dissolve purple formazan crystals formation, and the optical density (OD) was observed at 570 nm wavelength in a microplate reader (BioTek, USA). And the results were expressed in terms of percentage viability.

2.4 Preparation of Protein and Receptor Gird

The proteins considered for the study are IGF-1R (PDB id: 2OJ9), FGFR2 (PDB id: 3B2T), SNAIL1 (PDB id: 3W5K), STAT3 (PDB id: 6QHD), TGFBR1 (PDB id: 3KCF), α5β6 (PDB id: 4UM9), TGFB1 (PDB id: 3KFD), SMAD (PDB id: 1U7F), α5β3 (PDB id: 1L5G), TGFBR2 (PDB id: 5E8V), Glycogen synthase kinase 3 beta (GSK3β; PDB id: 1I09), Forkhead Box-O3 (FOXO3; PDB id: 2K86), Gamma-Secretase (PDB id: 4R12), MDR1/ABCB1 (PDB id: 4Q9L) and ATP Binding Cassette Subfamily G Member 2 (ABCG2; PDB id: 5NJG). The protein coordinates were collected from PDB (Protein Data Bank, www. (http://www.rcsb.org/pdb/-home/home.do). Preparation of the proteins prior to docking was done using the protein preparation tab of Maestro 11.9 module of the Schrodinger suite. Corrections such as addition of hydrogen, assignment of bond orders, searching overlaps and water molecules within a range of 5 Å were deleted. Root Mean Square Deviation (RMSD) minimization up to 0.03 Å and OLPS-2005 (optimized potential for liquid simulation) was used to perform the minimization [16].

The receptor grid was prepared using the grid generation tool of Maestro 11.9 of the Schrödinger suite. The ligand is able to bind forming the achievable conformation using this receptor grid which also highlights the active site of the protein as with the co-crystallized ligand molecule. This co-crystallized ligand will then be expelled from the active site to be occupied by our ligand of interest [16]. Scaling factor of 1.0 Å and van der waals radius of 0.25 Å were used to prepare the grid while other parameters were set to default.

2.5 Ligand Preparation

The structural definition file (sdf) of Neferine was retrieved from PubChem followed by the ligand preparation by the LigPrep wizard Maestro 11.9 (Glide). Corrections such as 2D to 3D conversion, addition of hydrogen, stereochemistry, low energy state, corrections of bond lengths and bond angles, ring conformations along with minimization and optimizations were done using OPLS3 force field [17].

2.6 Molecular Docking

Maestro-GLIDE module of the Schrödinger suite was used to carry out the molecular docking using the optimized ligand, which was docked flexibly within the grid box of each protein considered. The best scoring pose of the ligand were ranked using the G-Score (Glide Score) and H-bond formation along with their corresponding binding affinities. Visualizations were done using the XP (extra precision) module of GLIDE for analyzing each protein-ligand interaction.

3. RESULTS AND DISCUSSION

3.1 Establishment of cisplatin – resistant CRCs

To establish the cisplatin-resistant CRCs, 50% cell proliferation half maximal inhibitory concentration (IC50) of cisplatin was determined by cytotoxic (MTT) assay. HCT – 15 cells were

treated with cisplatin (0 – 60 µM concentration) for 24 h and the results showed that it induces cytotoxicity in a dose-dependent manner. The IC50 value of cisplatin for HCT-15 was found to be 32 ± 3.4 µM conc. (Figure – 2A).

The HCT-15 cells were treated with IC50 dose (32 µM) of cisplatin continuously for six months to attain the cisplatin-resistant CRC cells and were termed as HCT – 15/R cells. Later, to confirm the resistance of cisplatin and resistance index, the HCT – 15/R cells were subjected to cytotoxic (MTT) assay. The HCT – 15/R cells were treated with different concentration of cisplatin from 0 – 200 µM for 24 h. The IC50 value of cisplatin-resistant HCT-15/R cells was found to be 120 ± 5.1 µM. The resistance index was 3.75 fold increase compared to cisplatin-sensitive HCT-15 cells (Figure – 2B).

Figure – 2:

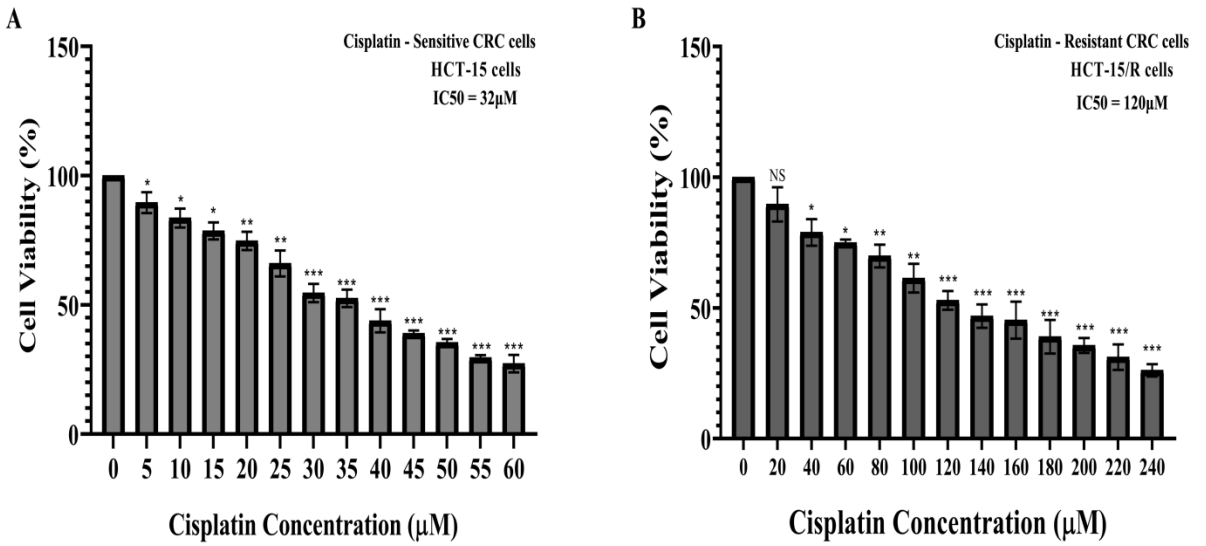

Fig.2. Establishment of cisplatin-resistant colorectal cancer cells: (A & B) the cell viability was determined by MTT assay and the results reveal the IC50 value of cisplatin-sensitive HCT-15 cells and cisplatin-resistant HCT-15/R cells to be 32 µM and 120 µM respectively. Results shown are mean ± SEM, which are three separate experiments performed in triplicate. *p<0.05, **p< 0.01 and ***p< 0.001 verse control (One-way ANOVA followed by Tukey's multiple comparison test).

Molecular docking is an in-depth investigation using bioinformatics tools and is based on the theoretical simulation approach [18]. Molecular docking studies reveal the specific interaction of molecules such as proteins with ligands and proteins with proteins, as well as the details of affinity, binding orientation, and biological activity of a drug and its target proteins [19].

Insulin-like Growth Factor type-1 Receptor (IGF1R) is a transmembrane receptor, belong to the receptor tyrosine kinase class, which is a crucial factor in the IGF signalling pathway. Overexpression of IGF1R was frequently observed in various cancer cells, including CRCs [20]. The key roles of IGF1R are cell proliferation, differentiation, survival, apoptosis, anchorage-independent

growth, angiogenesis and metastasis. The cell survival pathways like phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase (MAPK) pathways were mediated by activating IGF1R by ligands like IGF1, IGF2 and insulin [21]. A previous study reported that overexpression and nuclear translocation of IGF1R was associated with resistance to conventional chemotherapy in metastatic CRC patients [22]. Targeting IGF1R by small molecules has been considered as a novel therapeutic option to overcome chemoresistance in cancer by inhibiting tyrosine kinase activity [23].

Fibroblast Growth Factor Receptor 2, a class of tyrosine kinase receptor family (FGFR2), is one of the vital receptors in the fibroblast growth factor (FGF) signalling pathway [24]. It controls multiple physiological processes, including cell proliferation, endocrine homeostasis, cell survival, and wound healing via activation of mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK1/2), PI3K/AKT pathways, and signal transduction protein (STAT3) [25]. Mutation in FGFR2, abnormality in FGFR binding ligands, overexpression, and nuclear translocation leads to cancer progression [26]. In CRC patients, a high level of expression of FGFR2 is positively correlated with the advanced stage of cancer progression and cancer cell metastasis [27]. Recent studies reported that activation of pro-survival transcription factor (STAT3) was associated with the abnormal expression of FGFR2 and FGFR4 in 5-fluorouracil and oxaliplatin-resistant CRCs [28,26].

Transforming growth factor (TGF-β) is a multifactorial cytokine with three isoforms TGF-β1, 2 and 3 encoded by TGFB1, TGFB2 and TGFB3 genes, which play essential roles in cell proliferation, differentiation, migration, stem cell maintenance, epithelial to mesenchymal transition and apoptosis [29]. These mechanisms are the result of the sequential activation of numerous components of the TGF-β pathway, which leads to gene expression regulation [30].The TGF-β signaling is activated by TGF-β cytokines binding to type 1 and type 2 TGF-βreceptors (TGFβR1 and TGFβR2), respectively [31]. The normal growth of colonic crypt and villi was regulated by TGF-β signalling, frequent dysregulation of TGF-β leads to the loss of mothers against decapentaplegic (SMAD) proteins and TGF- β receptor-2 mediated cell cycle dysregulation in CRC [32].

The overexpression of TGF-β1, TGFβR1 and TGFβR3 induce the stem cell phenotype through the increased expression of stem cell markers such as Snai1, CD44, CD133, Sox-2, N-cadherin and Twist1 [33]. The overexpression of proteins TGFβR1 and TGFβR2 correlates with the upregulation of various stem cells markers such as Snai1, CD44, Sox-2, N-cadherin, CD133 and Twist1, causing stem cell phenotype [31].

In order to understand the possible inhibition pattern of neferine, molecular docking studies were used to predict a ligand-receptor interaction. In this present study, docking was performed for various cell surface receptor proteins and downstream proteins responsible for cancer progression, drug resistance, migration and invasion. Abnormal expression of IGF1R [34], FGFR2 [35], TGFβR1, TGFβR2, TGFβ1 [36,37], α5β6, α5β3 [38] and MDR1/ABCB1 [39] were the pivotal surface proteins found to be responsible for cisplatin-resistance in various cancers. Likewise, SNAIL1 [40], STAT3

[39], SMAD [36], GSK3β [41] and FOXO3 [42] are the crucial proteins involved in signal transductionwhich were found to be expressed abnormally in various cisplatin-resistant cancer cells.

3.2 Molecular docking results

The compound neferine was docked individually to the active site of each protein using Maestro-GLIDE module of the Schrödinger suite (Table 1). Post docking analyses were based on docking score, Glide evdw (Van Der Waals energy), Glide energy, ecoul (Coulomb energy) and the interacting residues forming hydrogen bond. The highest docking score was observed for the cell surface protein IGF1R (PDB ID = 2OJ9) showing hydrogen bonds with Leu975, Asp1056 (Figure - 3). It has been observed that overexpression of many components of the IGF family seem to be involved in tumourigenic mechanisms. IGF1R, IGF1 and IGF2 are frequently overexpressed in a large number of tumor types including CRCs [43]. IGF1R is also associated with resistance to both radiation and chemical based therapies [44]. Thus, compound neferine could be a potential candidate for targeting IGF1R in CRCs which however requires further studies.

Table 1. Molecular docking results of neferine along with the hydrogen bonding residues

Ligand	Nature of Protein	Protein name	PDB ID	Docking score	No of hydrogen bonds	Interacting residues
Neferine	Cell surface receptor	Insulin-like growth factor 1-receptor (IGF1R)	2OJ9	-7.376	2	LEU975, ASP1056
		Fibroblast growth receptor 2 (FGFR2)	3B2T	-6.813	3	LEU 481,LYS517, ASP644
	Transcription factor	Zinc finger protein (SNAIL1)	3W5K	-6.221	2	GLY623,SER621
		Signal Transducer And Activator of Transcription 3 (STAT3)	6QHD	-6.216	4	GLN247,ILE258, GLU324,ARG325
	Cell surface receptor	Transforming growth factor beta receptor 1 (TGFβR1)	3KCF	-6.068	1	PHE216
		Integrin (α5β6)	4UM9	-5.955	1	VAL 226
	Cytokine	Transforming growth factor beta 1 (TGFβ1)	3KFD	-4.581	1	MET104
	Signal transducers	SMAD Family Member (SMAD3/4)	1U7F	-4.319	2	THR 371, GLU397
	Cell surface receptor	Integrin (α5β3)	1L5G	-4.281	4	GLN 327, LYS330, ASN 332
	Signal transducers	Transforming growth factor beta receptor 2 (TGF B R2)	5E8V	-3.862	3	LYS 252, GLN334, ARG423
		Glycogen synthase kinase-3 beta (GSK3β)	1I09	-3.354	3	Asp106, TYR171
	Transcription factor	Forkhead box O-3 (FOXO3)	2K86	-2.943	1	GLU171
	Cell surface receptor	Gamma-Secretase (γ-Secretase)	4R12	-2.909	0	NIL
	Multi-drug	Multi-drug receptor or ATP Binding Cassette Subfamily B Member 1 (MDR1/ABCB1)	4Q9L	-2.857	1	ASP173

	resistance	ATP Binding Cassette Subfamily G Member 2 (ABCG2)	5NJG	-2.216	0	NIL

Figure – 3:

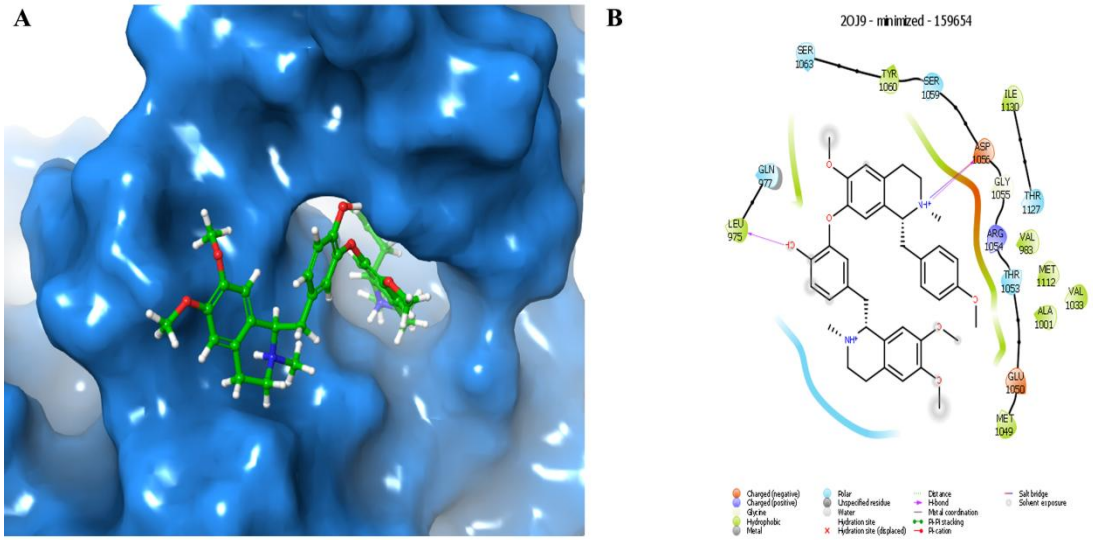

Fig.3. Docking studies of Neferine with insulin-like growth factor 1-receptor (PDB id: 2OJ9). (A) Neferine within the active site **(B)** Hydrogen bonding residues Leu975, Asp1056 with the ligand.

Then we studied the interaction of neferine with FGFR2 (PDB ID = 3B2T), a fibroblast growth factor receptor found to be deregulated in cancer. The docking score of this interaction was found to be -6.813, involves the formation of three hydrogen bonds with amino acid residues Leu481, Lys517 and Asp644 respectively within the active site (Figure - 4). Lys517 has been reported to be a highly conserved residue of the αC [45] which shows that our compound has the potential to interact specifically with the conserved area of the protein which may possibly lead to its disorientation for its inhibition. Further, protein targeted according to docking score was the zinc finger (ZF) protein SNAIL1 (PDB ID = 3W5K) with a docking score of -6.221 forming two hydrogen bonds with Gly623, Ser62. Ser621 (Figure - 5) is one of the prominent residue of the three zinc fingers namely zinc finger 2, 3 and 4 which are interwoven to form a compact ball structure with a tight and compact base for importin-β interaction [46]. Thus, our compound has been shown to interact specifically with the very core interaction site in the Snail1 ZF–importin complex.

Figure – 4:

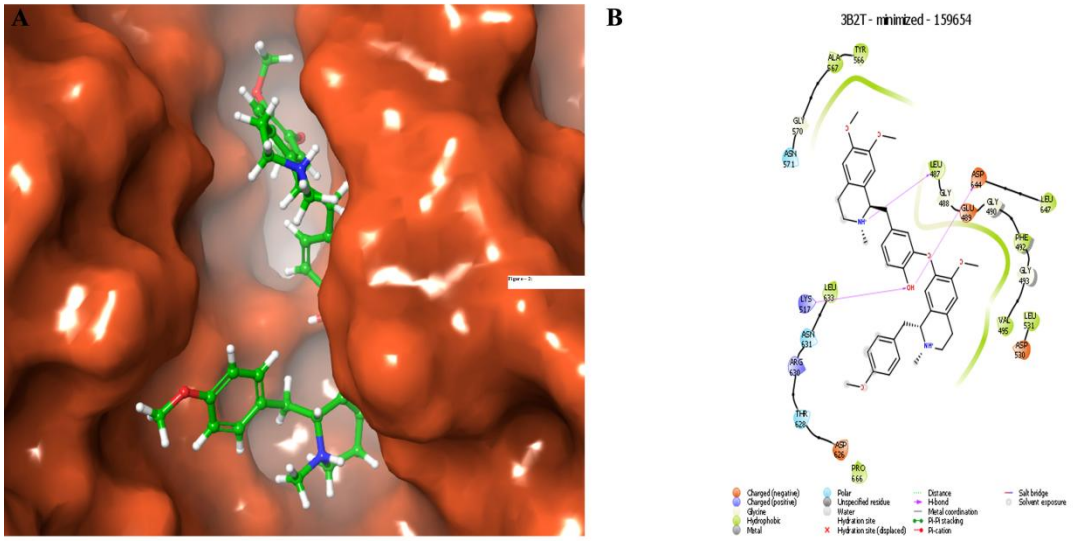

Fig.4: Docking studies of neferine with fibroblast growth factor receptor (PDB id: 3B2T). (A) Neferine within the active site **(B)** Hydrogen bonding residues Leu481, Lys517, Asp644 with the ligand.

Figure – 5:

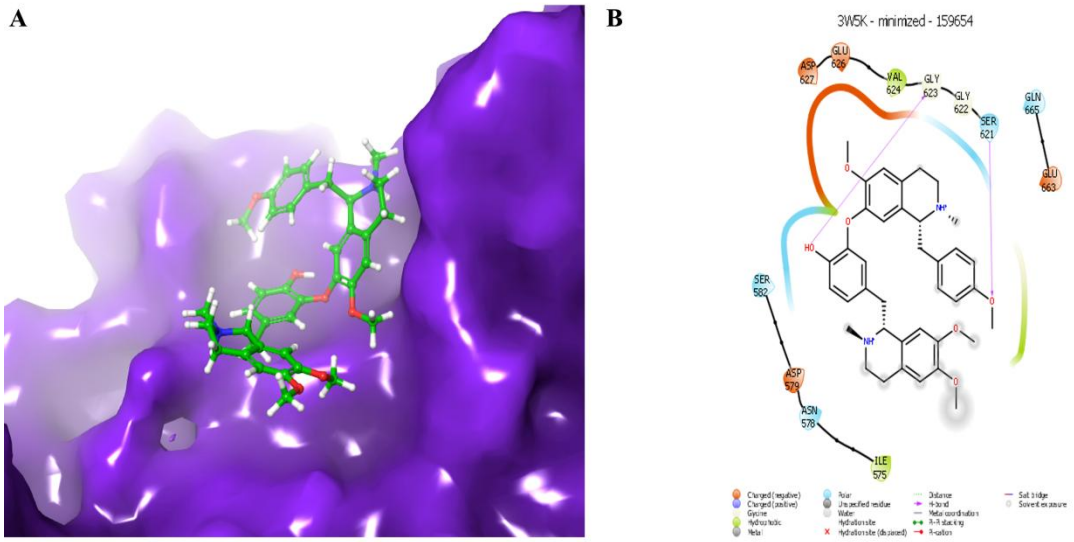

Fig.5: Docking studies of Neferine with SNAIL1 (PDB id: 3W5K). (A) Neferine within the active site **(B)** Hydrogen bonding residues Gly623, Ser621 with the ligand.

Protein STAT3 (PDB ID = 6QHD) and TGFβR1 (PDB ID = 3KCF) are the next two transcription factor proteins targeted by the compound when sorted according to the docking score, scoring -6.216 and -6.068 respectively (Figure - 6). The former protein formed four hydrogen bonds with residues Gln247, Ile258, Glu324, Arg325 while the later formed a single bond with Phe216. Thus,

from our docking studies we could suggest that neferine is most active for cell surface proteins IGF1R receptor, FGFR2 and TGFβR1 including transcription factors such as SNAIL1 and STAT3 when sorted according to their docking score.

Figure – 6:

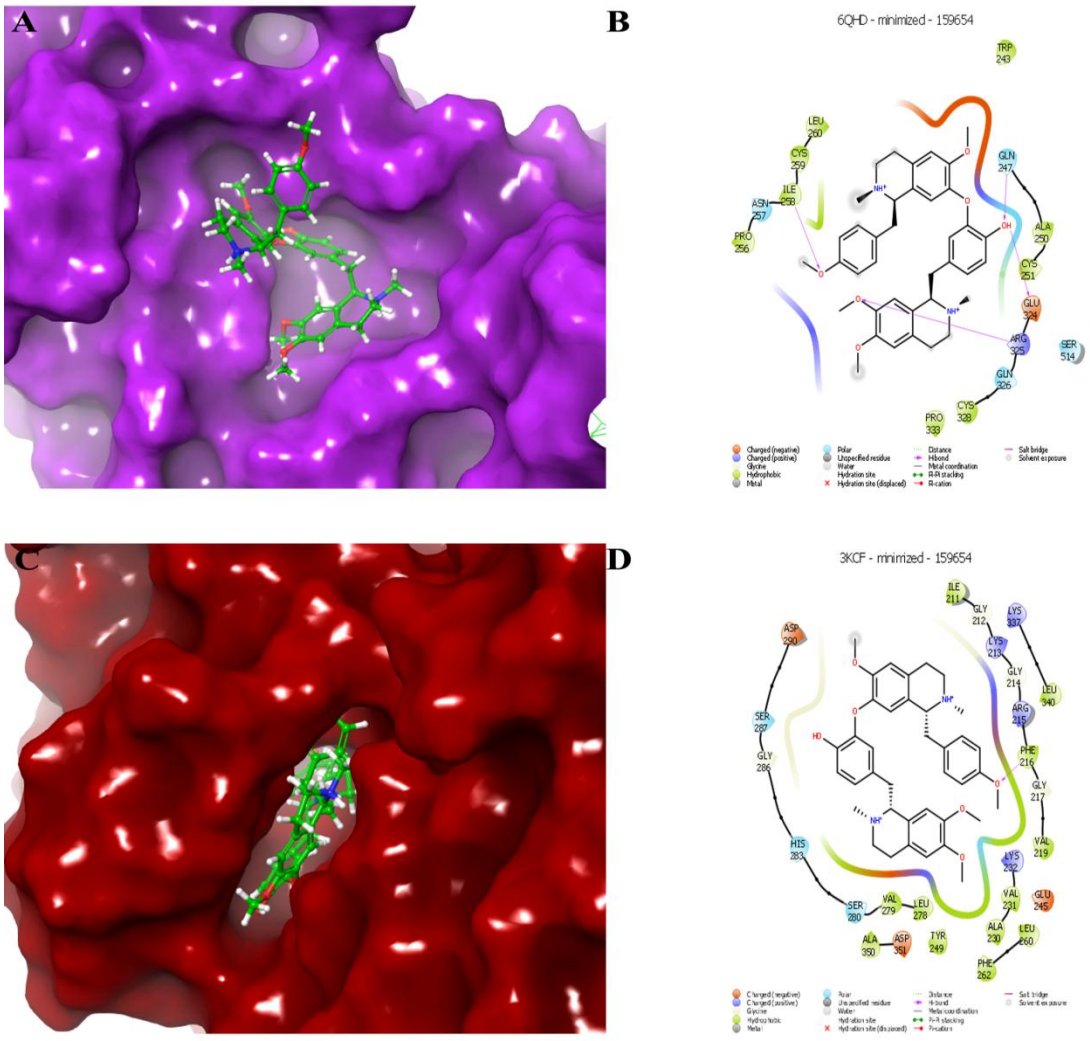

Fig.6: Docking studies of Neferine with STAT3 (PDB id: 6QHD) and TGFβR1 (PDB id: 3KCF). (A & C) Neferine within the active site **(B)** Hydrogen bonding residues Gln247, Ile258, Glu324, Arg325 with the ligand **(D)** Hydrogen bonding residues Phe216 with the ligand.

4. CONCLUSION

The present study evaluated the cytotoxic effect of neferine on cisplatin-resistant CRCs. Further, molecular docking study was carried out to assess the ability of neferine to interact specifically with various target proteins such as IGF-1R, FGFR2, SNAIL1, STAT3, TGFβR1, α5β6, TGFB1, SMAD, α5β3, TGFβR2, GSK3B, FOXO3, Gamma-Secretase, MDR1/ABCB1 and ABCG2 based on the protein – ligand (neferine) interaction. Neferine interacted with proteins related to

cisplatin – resistance with low energy. The binding interaction with target proteins is in the following order, IGF1R > FGFR2 > SNAIL1 > STAT3 > TGFβR1 > α5β6 > TGFβ1 > SMAD3/4 > α5β3 > TGFβR2 > GSK3β > FOXO3 > gamma-secretase > MDR/ABCB1 > ABCG2. Further, from the docking studies it is evident that Neferine interaction with insulin-like growth factor 1-receptor is strongest compared to all the proteins considered for the study. This shows that neferine may have the ability to target these proteins which have abnormal expression in CRCs which requires validation through and *in-vitro* and *in-vivo* studies.

CONSENT

It is not applicable

ETHICAL APPROVAL

It is not applicable

COMPETING INTERESTS

Authors have declared that no competing interests exist.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

AUTHOR'S CONTRIBUTIONS

Author SB designed the study, carried out the *In-vitro* assay and molecular docking study, and wrote the first draft of the manuscript. **Author SG** interpreted the molecular docking data and performed statistical analysis. **Author SS** carried out the revision of manuscript and managed the literature searches. **The corresponding author VVP** supervised the whole research progress, given the constructive suggestion to complete the research work and revised, corrected and proof-read the manuscript. All authors read and approved the final version of the manuscript.

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