*R*esearch Article

Virtual screening of anticancer efficacy of phloretin against apoptotic targets – An *In Silico* molecular docking study

**Thangavelu Ranjanamala[1], Krishanan Vanmathiselvi[1]*,
Ramanathan Shreemaya[2], Sundarajan Nagarajan Rajeswari[3],
Casimeer C Sangeetha[4], Alaa Yousef Ghidan[5], Fatima Yousel Ghidan[6]**

[1]PG and Research Department of Microbiology, Sri Akilandeswari Womens Arts and Science College, Wandiwash-604408, Tamil Nadu, India, [2]Department of Biotechnology, DY Patil Deemed to be University, Navi Mumbai-400614, India, [3]Department of Botany, Seethalakshmiachi College for Women, Pallathur, Karaikudi, Tamilnadu 630107, India, [4]Department of Physics, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati,Andhra Pradesh 517502, India, [5]Nanotechnology and Microbiology, Research and Development Center, The Higher Council for Science and Technology, Amman, 11941, Jordan, [6]Head of Quality and Medication Saftey Department, King Abdullah Medical City, Al Mashair, Makkah 24246, Saudi Arabia

ABSTRACT

Recent advances demonstrate phytochemicals to be a potent anticancer therapeutic agent as various anti-cancer targets. This study depicts the anti-cancer potential against certain crucial common cancer targets leading to cancer cell proliferation and survival. The main objective of this study is to study the anti-cancer potential of phloretin against certain cancer targets. Ligand analysis was performed and Phloretin was chosen as the experimental ligand and Bcl-2, NF Kappa B, Carbonic anhydrase I (CA-1), Inducible Nitric Oxide Synthase (iNOS), Endothelial Nitric oxide synthase (eNOS), Caspase 3, and Caspase 9 proteins were chosen as targets. Induced fit molecular docking was performed by the use of Glide 6.5 software (Schrodinger-2015). The docked poses were further evaluated based on binding energy, Conformational changes, and the amino acid residues involved in the protein-ligand interaction. The docking results depicted that phloretin showed notable binding affinity especially with carbonic anhydrase I, ENOS, and INOS. It also showcased significant potential against Caspase 3 and NF Kappa, thereby showing its potential as an effective anti-cancer therapeutics. During this study, the Inhibitory potential of Phloretin was studied as a result of this molecular docking study. This Insilico study revealed the binding efficiency of phloretin against the aforementioned targets. In vitro analysis is required for further validation of this data.

KEYWORDS: Computer-aided drug discovery; Phloretin; Molecular docking, Anti-cancer activity.

INTRODUCTION

Worldwide, cancer is the primary cause of death and it presents itself as a hindrance and leads to a significant decrease in life expectancy. Female breast cancer is the most frequently diagnosed cancer, followed by lung, colorectal, prostate, and stomach cancer (Sung *et al.*, 2021). Regardless of recent advancements in the development of modern medicine, Cancer is still the root cause of mortality worldwide. Presently, the major treatment options for cancer are chemotherapy, radiotherapy, and surgery (Pratheeshkumar *et al.*, 2015). There has been a constant development of novel anti-cancer drugs. However, the use of synthetic drugs hasn't ameliorated the treatment

efficiency and there has been no overall improvement in the survival rate of the patients (Choudhari *et al.*, 2019). The major disadvantages of chemically synthesized drugs are high toxicity, drug resistance, and fatal effect on the non-targeted tissues. Due to this, there is a need for the development of novel therapeutics for a safer and effective treatment approach.

Initially, traditional Drug designing approaches took around 7 to 10 years via trial and error methods for screening of potential compounds. This methodology was labor-intensive and time-consuming. After, finding potential compounds, It took around 10 years to perform clinical trials to develop a novel drug for a particular disease. For this purpose, a Computer-aided drug

designing method was developed using Molecular mechanics to understand the composition of the ligands. Multiple ligands can be chosen and around 200 – 500 compounds can be screened simultaneously using virtual screening. Molecular docking can be performed in silico, wherein the interaction between small molecules and proteins is analyzed at an atomic level. It is used in the characterization of the binding site of target proteins as well as to understand the inner workings of human diseases at the molecular level (Mariadoss *et al.*, 2018). Compounds with higher efficacy can be chosen by performing Blind docking. Potential compounds can be further tested during clinical trials. Nowadays, Target - Specific inhibitors can be designed by targeting specific domains which correspond to certain functions implicated by that protein.

In this study, various targets are analyzed for understanding its potential as a biomarker/anti-cancer target for developing a novel drug for various types of cancers. The BCL-2 family comprises pro-apoptotic factors (BAX, BAK, BIM, BID, and BAD), which induce apoptosis, and anti-apoptotic factors (BCL-2, BCL-xL), which inhibits apoptosis. BCL-2 is an attractive target to investigate and develop novel anti-cancer therapeutic for various types of cancers. BCL-2 protein is an antiapoptotic protein that impairs apoptosis, thereby promoting cancer progression. It consists of 4 domains BH1-4. The BH1, BH2, and BH3 domains interact to configure the hydrophobic groove and it accommodates the BH3 of pro-apoptotic proteins like BAX, thereby inhibiting apoptosis. BCL-2 protein is generally overexpressed in various cancers, thereby inhibiting the release of cytochrome C into the cytosol and leads to subsequent caspase activation (Giam *et al.*, 2008).

NFκB (nuclear factor kappa beta) is an inducible transcription factor and it is known to escalate cell proliferation, angiogenesis, and metastasis thereby making the cell resistant to anticancer drugs and chemotherapy treatment in breast cancer, colon cancer, and lymphoma (Xia *et al.*, 2018). BRCA1 silencing in breast cancer triggers phosphorylation of the Ser536 site of p65 and processing of p100/p52, resulting in constitutive activation of the canonical NFκB pathway (p65/p50) and noncanonical NFκB pathway (p100/p52), along with nuclear translocation and accumulation of p52/RelB, which can enhance MCF1 cell proliferation (Sau *et al.*, 2016). It leads to suppression of apoptosis via inhibition of apoptotic machinery in normal and cancerous cells (Baud & Karin, 2009). Activation of NFκB is shown to influence inflammation-associated cancers. Through binding of specific dimers of activated NFκB to promoters of pro-inflammatory genes, activation of canonical and noncanonical NFκB pathways accelerates tumour development and enhances the synthesis of proinflammatory proteins (Xia *et al.*, 2018).

Carbonic anhydrases are known to mediate the pH in tumor cells by modulating the proton and bicarbonate concentrations, thereby inducing cellular survival and proliferation (Mboge *et al.*, 2018). It is highly expressed in lung cancer. Lung cancer is difficult to detect and diagnose at early stages. CT scans are employed for the detection of lung cancer in high-risk patients, but it lacks sensitivity and specificity and it leads in false-positive results.

Carbonic anhydrases-1 was significantly upregulated and differentially expressed in lung cancer samples (Wang *et al.*, 2016). Thus, Carbonic anhydrases -1 demonstrates to be a potent biomarker for the early diagnosis of lung cancer.

Caspases are proteases and play a crucial aspect in inducing apoptosis. There are two major pathways involved regarding the activation of caspases-intrinsic and extrinsic pathways. The intrinsic pathway is characterized by the release of cytochrome C and interacts with adapter protein Apaf-1(Apoptotic protease activating factor-1) and forms apoptosome complex and induces activation of caspase 9 and ultimately activating caspase 3 protein (Parrish *et al.*, 2013). Downregulation of caspase 3/caspase 9 is generally associated with overexpression of the anti-apoptotic protein like BCL-2, which inhibits mitochondria from releasing cytochrome c in the cytosol and thus prevents the caspase activation, thereby inhibiting apoptosis and induces drug resistance in cancer cells. High Caspase-3 expression was significantly related to the PR and HER-2 status of patients (Yang *et al.*, 2018). It is associated with prognosis for patients with breast cancer (Yang *et al.*, 2018). Studies show that overexpression of caspase 9 induces its activation and leads to aberrant apoptosis in pituitary cells and HeLa cells (Druskovic *et al.*, 2006). So, a specific inhibitor of caspase 9 is required to control its expression *in vitro*.

iNOS (Inducible nitric oxide synthase) is an enzyme that is proficient in NO (nitric oxide) production in response to various stimuli especially in response to certain endotoxins and endogenous pro-inflammatory cytokines (Lechner *et al.*, 2005). Upregulated iNOS expression is mediated via NFκB and many transcription factors. The elevated expression of iNOS was significantly associated with angiogenesis, chemotherapeutic resistance, metastasis, and immune resistance in some malignant tumors (Liao *et al.*, 2019), e.g., targeting angiogenesis pathway in colorectal cancer (Gao *et al.*, 2019), breast cancer (Fahey & Girotti, 2017; Garrido *et al.*, 2017), pancreatic cancer (Wang *et al.*, 2016), gastric cancer (Chen *et al.*, 2006), and melanoma (Lopez-Rivera *et al.*, 2014). It is a promising biomarker for anti-cancer therapy. Endothelial nitric oxide synthase (eNOS) is proficient in the endothelial synthesis of nitric oxide (NO) and has been observed to depict inflammation, angiogenesis, apoptosis, and metastasis. Increased expression of eNOS and iNOSis was observed in malignant tissues of breast cancer (Loibl *et al.*, 2002). eNOS induces lymphatic metastasis in many human cancers (Lahdenranta *et al.*, 2009).

Phytochemicals, natural compounds obtained from plants depict great potential as anti-cancer agents. They depict low toxicity, target specific mechanism of action, and bioavailability of the drug and can be utilised to treat a variety of cancers. Potent phytochemicals inhibit cancer-related signalling pathways, but they also work in well with chemotherapy and radiotherapy (Pratheeshkumar *et al.*, 2015). Phytochemicals work by modulating molecular pathways that have been linked to cancer growth and progression. Increased antioxidant status, carcinogen inactivation, inhibition of proliferation, induction of cell cycle arrest and apoptosis; and immune system control are

some of the specific mechnanisam for phytochemical mediated cancer treatment method (Choudhari *et al.*, 2019).

Phloretin (2′,4′,6′-Trihydroxy-3-(4-hydroxyphenyl)-propiophenone), a natural polyphenolic compound, is found in apples, pears, and the Rosaceae family species (Lu & Foo, 1997; Mariadoss *et al.*, 2019a). Phloretin has demonstrated various pharmacological effects, especially anti-oxidant, anti-inflammatory (Huang *et al.*, 2015), and anti-tumor properties (Mariadoss *et al.*, 2019b). Phloretin depicts anti-cancer activity in vitro in human esophageal cancer cells via mitochondria-dependent pathway by inducing downregulation of BCL-2 and upregulation of p53 protein thereby promoting apoptosis in cancer cells (Duan *et al.*, 2017). It also induces apoptosis in human liver cancer cells (Wu *et al.*, 2009). It is observed that phloretin inhibits cell growth and induces apoptosis by increases mitochondrial permeability thereby activating caspase 9 leading to caspase 3 activation (Park *et al.*, 2007). Phloretin proves to be a potent anti-cancer therapeutic agent (Mariadoss *et al.*, 2019c). During this research study, phloretin is chosen as an experimental ligand and it is docked with BCL-2, NFκB, carbonic anhydrase 1 (CA-1), iNOS, eNOS, caspase 3, and caspase 9 which are crucial anti-cancer targets to assess the binding efficiency of phloretin and analyze its anti-cancer efficacy against these targets.

MATERIALS AND METHODS

Protein Preparation

3D structure of BAX, Bcl-2, NF Kappa B, Carbonic anhydrase I, Inducible Nitric Oxide Synthase, Endothelial Nitric oxide synthase, Caspase 3, and Caspase 9 were retrieved from the Protein Data Bank (PDB). It is a repository with the collection of protein 3D structures generated by NMR spectroscopy and X-ray crystallography techniques. Protein preparation was conducted by deletion of water molecules and addition of missing hydrogen bonds and correct bond orders were assigned to non-standard residues. Following this, the Optimization of hydroxyl group and amine groups of the amino acids and optimization of H-bonds on the target proteins were carried out. OPLS3e force field was used for the energy minimization process. The non-hydrogen atoms were minimized until the average root mean square deviation reached the default value of 0.3 Å.

Active Site Prediction

The active sites of the proteins were predicted using the Uniprot database, Interpro scan server, and the Sitemap module of Schrodinger. Uniprot is a crucial database to perceive and analyze diverse information of target proteins. Interpro scan is a sequence analysis application server that provides information on the functional domain, active sites, and putative sites. The sitemap module of Schrodinger is another source to predict the binding sites of the protein.vIn this research study, the active site residues of Carbonic anhydrase 1, INOX, ENOX, Caspase 3, and Caspase 9 were predicted by Uniprot and Interpro scan

web server. Whereas the active site residues of BCl-2 and NF Kappa B were predicted by Sitemap.

Ligand Preparation

The 3D structure of compound Phloretin (PubChem ID: 4788) was downloaded from the PubChem database. The physical-chemical properties of the compound were analyzed using the Swiss-ADME server. The LigPrep program provides a versatile and robust procedure for the preparation of the ligands (Repasky *et al.*, 2007). The optimization of the ligand was achieved by deploying OPLS3e as the force field.

Domain Analysis

Domains are capable to fold their structure alone and the domain is the functional part of the protein. From the concept of structural biology, the domains are the conserved region of the protein. The protein domain analysis was carried out using the Pfam database to predict the functional domain region of the selected protein.

Receptor Grid Generation

The position and size of the active site are represented by the formation of receptor grids. For this, a receptor grid generation module was used for the docking process where compounds have to be docked using a centroid of selecting residues of the proteins. It is a crucial step to perform before undertaking molecular docking.

Induced Fit docking using XP Glide Algorithm

Induced Fit docking is incorporated in this research study via the use of the XP(Extra Precision) scoring algorithm. The ligand docking module was incorporated for the docking of compound Phloretin with the target proteins using the XP GLIDE module of Schrödinger. XP Glide involves an extensive sampling of poses and a scoring function based on a rigorous assessment of protein-ligand interactions (Repasky *et al.*, 2007). To comprehensively sample ligand degrees of freedom, an anchor-and-grow technique has been used to refine the predicted docking modes. Anchor fragments of the docked ligand, typically ring or other rigid fragments, are chosen from the set of poses output from an initial docking with SP Glide designed to obtain a large diversity of docked structures. Ligand postures are regrown one side chain at a time from these anchor positions at very high resolutions. Combining high-scoring individual conformations at each side chain yields a list of potential compounds. The candidate molecules' energy is minimized, and ligand poses are rated using the Emodel pose-selection function (Repasky *et al.*, 2007). The default scaling factors for the van der Waal's radii in Glide (1.0 for receptor and 0.8 for ligands) have been selected to strike a balance between accurate docking/scoring and compensation for the lack of receptor flexibility (Repasky *et al.*, 2007). The top-scoring structures are subjected to grid-based water scoring methodology, and the entire XP GlideScore scoring function and identification of structural motifs are computed.

RESULTS AND DISCUSSION

Protein preparation and Active Site Prediction

The targets were chosen based on molecular function and biological processes of the proteins in cancer progression. 3D structures of all seven proteins were downloaded in PDB format from PDB (Protein Bank Database) for further analysis were shown in Figure 1.
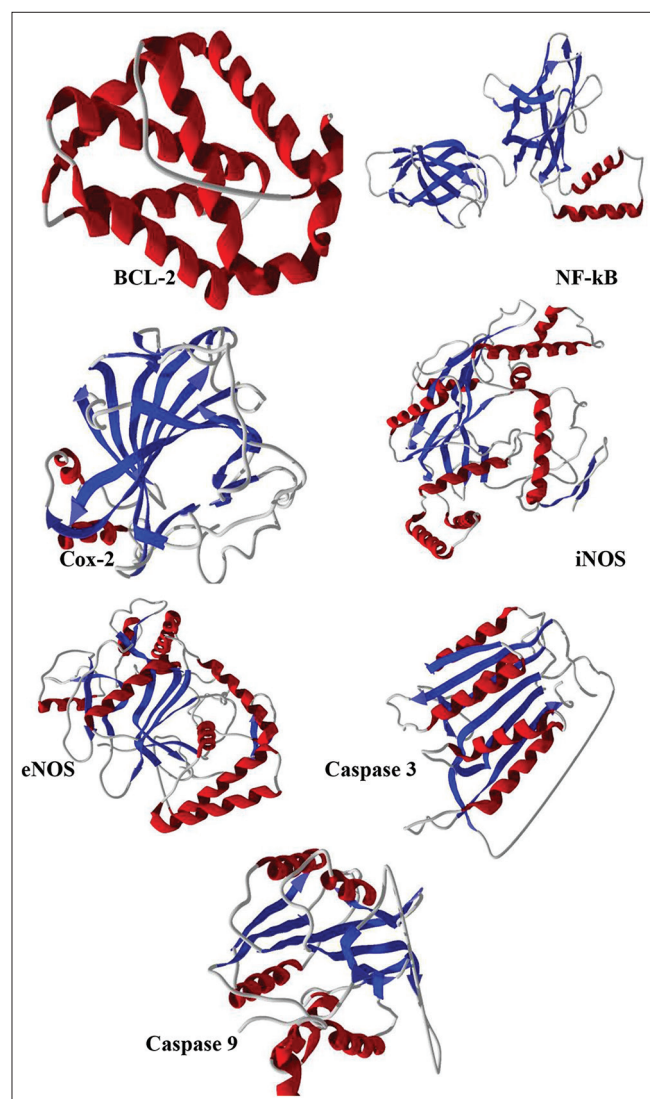

Figure 1: 3D structures of target proteins

Domain Analysis

The functional domain analysis of the target proteins was performed using the Pfam database and this information is shown in Table 1. These details were crucial in determining the active sites and their amino acid residues in each of our target proteins.

Active site Prediction of Selected Proteins

The amino acid residues of the active sites of target proteins were predicted using Uniprot, Interproscan, and Sitemap. The active site of Carbonic anhydrase 1, INOX, ENOX, Caspase 3, and Caspase 9 were predicted by Uniprot and Interproscan. And remaining proteins BAX, BCl-2, and NF Kappa B binding sites were predicted by Sitemap (Table 2).

Ligand Preparation

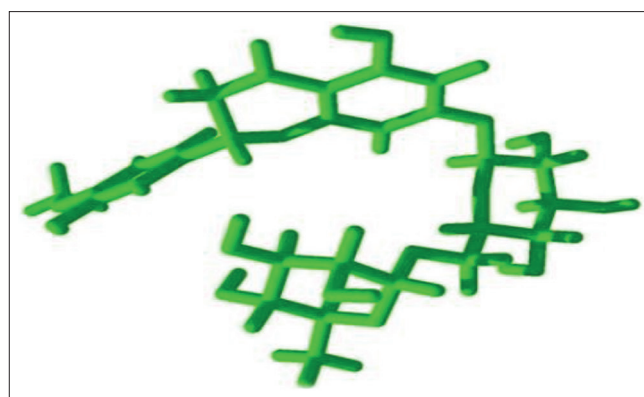The ligand molecule was downloaded from the PubChem database (Figure 2).

Figure 2: 3D structure of phloretin

Table 1: Domain analysis for the given anti-cancer targets

Sr.No	Protein Name	Domain Name	Domain Region
1.	Bcl-2 protein (Q07817)	Bcl-2	90-188
2.	NF Kappa B (Q00653)	RHD_DNA_bind	40-220
3.	Human carbonic anhydrase 1 (P00915)	Carb_anhydrase	12-261
4.	INOS (P35228)	NO_synthase	82-508
5.	ENOS (P29474)	NO_synthase	119-481
6.	Caspase 3 (P42574)	Peptidase_C14	43-167
7.	Caspase 9 (P55211)	Peptidase_C14	160-412

Table 2: Prediction of the active site and its amino acid residues

| Sr.No	Protein Name	PDB ID	Active site/Binding site residues
1.	Bcl-2 protein (Q07817)	4IEH	55, 58, 59, 62, 63, 66, 67, 103, 104, 105, 107, 108, 157, 161-163.
2.	NF Kappa B (Q00653)	1A3Q	94, 95, 103-105, 107-109, 117-119, 153, 154, 156, 157, 160, 193
3.	Human carbonic anhydrase-1 (P00915)	5GMM	62, 64, 65, 67, 91, 92, 94, 96 ,119, 121, 122, 131, 135, 141, 143, 198-200, 207, 209
4.	INOS (P35228)	4NOS	118, 200, 347, 370, 372, 381, 382, 463, 491
5.	ENOS (P29474)	1M9M	184, 247, 353, 354, 356, 357, 368, 366, 447, 475
6.	Caspase-3 (P42574)	1NMS	64, 121, 122, 161, 163, 168, 204-209, 213, 214
7.	Caspase-9 (P55211)	1JXQ	180, 237, 238, 285, 287, 292, 352-357, 361, 362
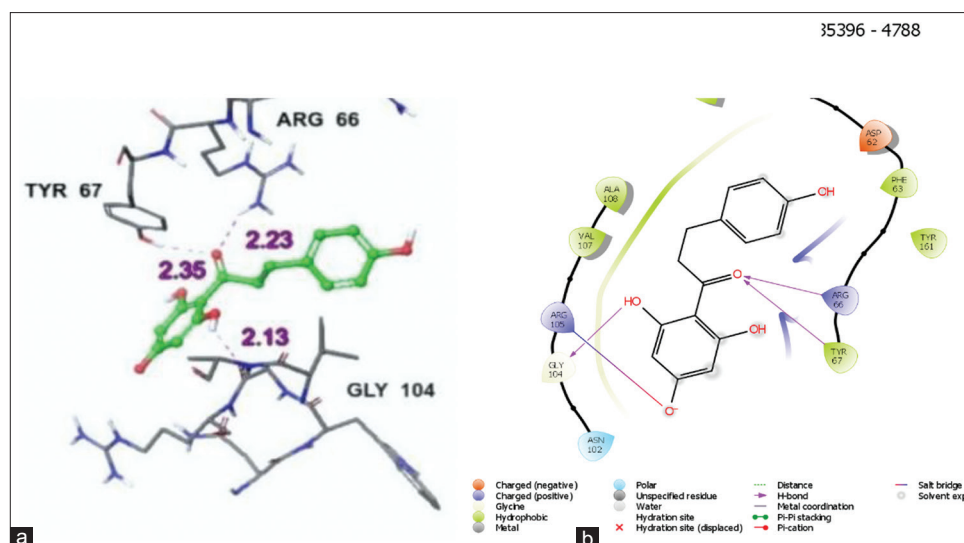
Figure 3: Interaction of Phloretin with BCL-2 protein (4IEH) (a) Visualization of Interaction of phloretin with BCL-2 protein. (b) Visualization of various non-covalent molecular interactions
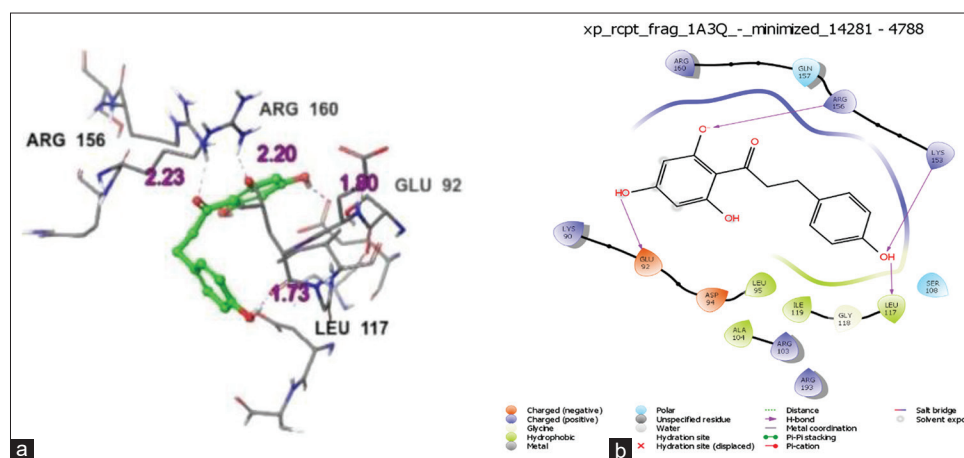

Figure 4: Interaction of Phloretin with NFκB protein (1A3Q). (a) Visualization of Interaction of phloretin with NFκB protein. (b) Visualization of various non-covalent molecular interactions

Table 3: Prediction of ADMETox properties of Phloretin

Properties	Specifications
Ligand Name	Phloretin
Molecular Weight	274.27
LogP0/w	2.63
H bond Donor	4
H bond Acceptor	5
Polar surface Area	97.99
R05	Yes
Log S	-3.38
GI Absorption	High
Lead likeness	Yes

Prediction of ADMETox properties

The ADMETox properties were predicted using the SwissADME server. The predicted values (Table 3) suggest phloretin as an appropriate compound to be considered for docking based on

its physical-chemical properties, oral bioavailability, and lead likeness.

Molecular Docking

In this research study, crucial anti-cancer targets like B cell lymphoma-2 (BCL-2), Nuclear factor kappa beta (NFκB), carbonic anhydrase 1(CA-1), iNOS (inducible nitric oxide synthase), eNOS (endothelial nitric oxide synthase), caspase-3 and Caspase-9 was docked against phloretin to analyze its anti-cancer potential. The induced-fit molecular docking process was carried out using the XP (Extra precision) method by the GLIDE module of Schrödinger. Phloretin depicts good binding affinity against all anti-cancer targets. Various interactions of Phloretin like h-bond, pi-pi with various proteins, bond lengths of interactions, and dock score values of dock score and interacting residues are shown in Table 4. The
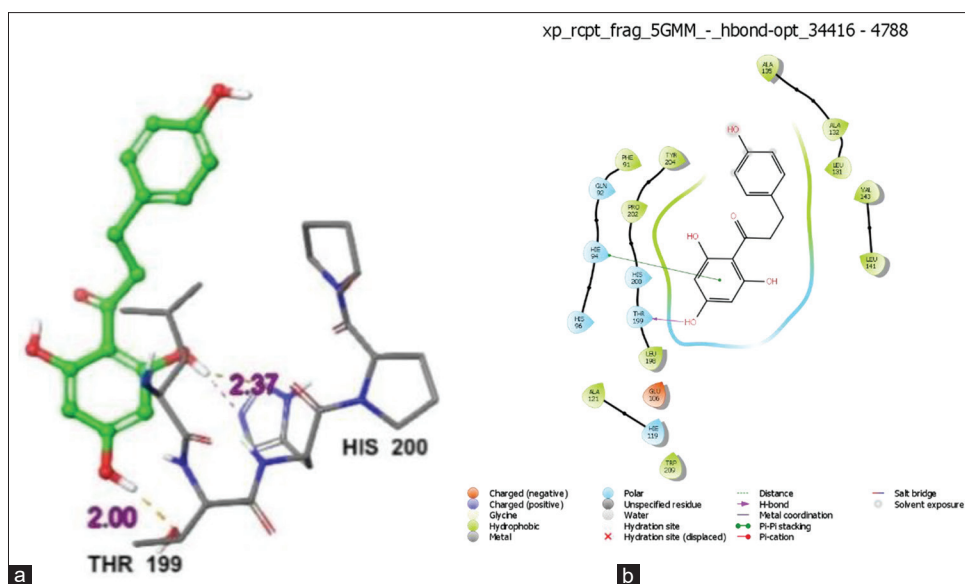
Figure 5: Interaction of Phloretin with Carbonic anhydrase 1 protein (a) Visualization of Interaction of phloretin with carbonic anhydrase 1 protein. (b)Visualization of various non-covalent molecular interactions
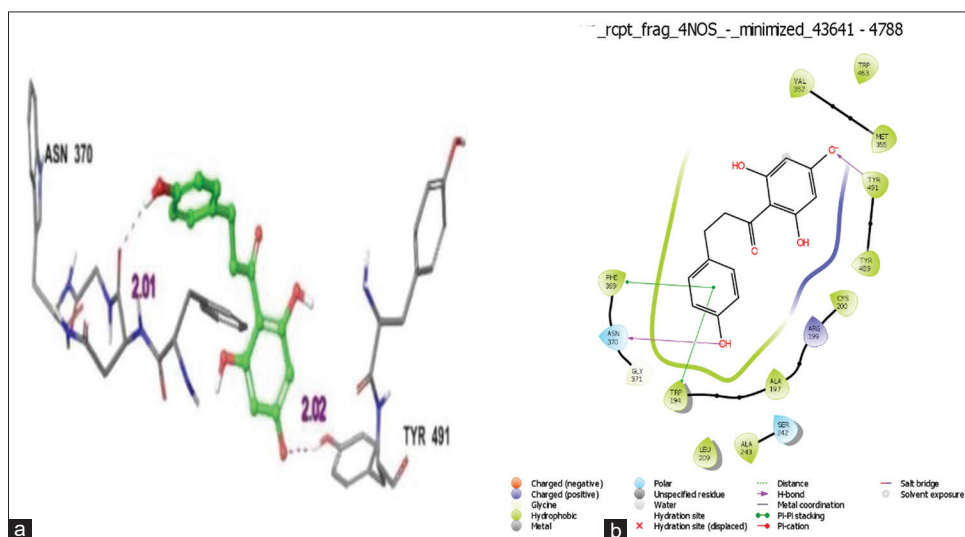

Figure 6: Interaction of Phloretin with Inducible nitric oxide synthase protein (a) Visualization of Interaction of phloretin with iNOS protein. (b) Visualization of various non-covalent molecular interactions

Table 4: Phloretin docked with selected proteins associated with cancer

Protein Name	Dock Score (Kcal/mol)	Interacting Residues	Bond length (Å)
Bcl-2 protein	−4.3	Arg 66, Tyr 67, Gly 104	2.23, 2.35, 2.13
NF Kappa B	−5.3	Glu 92, Leu 117, Lys 153, Arg 156	1.80, 1.73, 1.86, 2.20
Carbonic anhydrase 1	−6.2	His 94 (Pi-Pi), Thr 199	4.93, 2.00
INOS	−7.2	Trp 194, Phe 369, Asn 370, Tyr 491	3.46, 3.98, 2.01, 2.02
ENOS	−7.3	Trp 178 (2), Ser 354, Tyr 475 (2)	3.46, 4.25, 1.96, 2.03
Caspase 3	−5.4	Trp 206 (pi-pi), Arg 207, Asn 208, Asp 211, Trp 214, Phe 250	5.30, 2.03, 2.71, 1.59, 1.81, 2.09
Caspase 9	-3.0	Trp 348, Glu 351, Asp 355, Lys 380	2.26, 1.66, 1.72, 2.18

docking analysis showed that phloretin interacted with BCL-2 protein through a carbonyl group, wherein C=O interacted with amino acid residue ARG 66 of hydrogen bond interactions and the hydroxyl group of ligand interacted with GLY 104 are similar to the residues observed on the interaction of inhibitor N-heteroaryl sulfonamide against BCL-2 (Toure *et al.*, 2013). Phloretin also interacted with another binding site residue Tyr 67 with an overall docking score of 4.3 Kcal/mol (Figure 3). The docking analysis showed that phloretin interacted with NFκB protein through the hydroxyl group interacted with amino acid residues LEU 117, LYS 153, and GLY 92, which depicted hydrogen bond interaction. The overall docking score is -5.3 Kcal/mol (Figure 4).

The docking analysis showed that phloretin interacted with Carbonic anhydrase I, wherein the hydroxyl group interacted
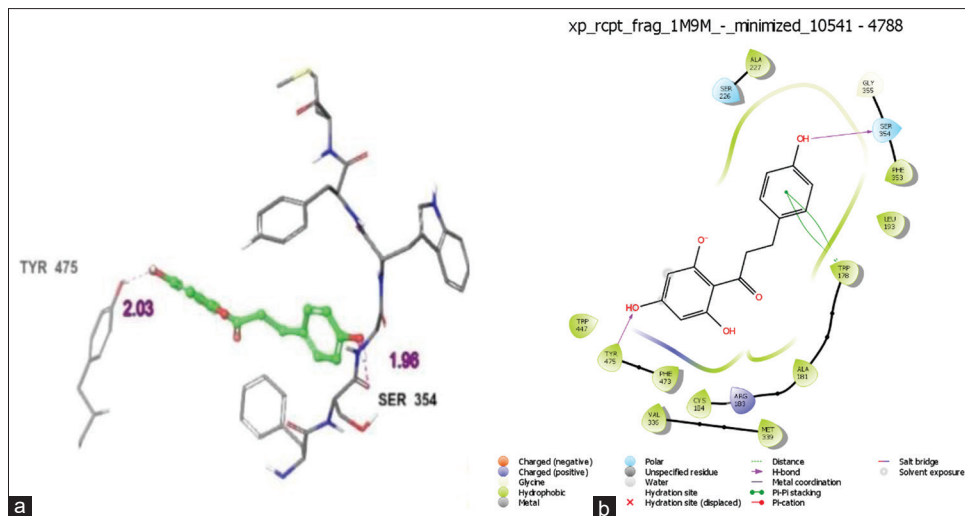
Figure 7: Interaction of Phloretin with Endothelial nitric oxide synthase protein (a) Visualization of Interaction of phloretin with eNOS protein. (b) Visualization of various non-covalent molecular interactions
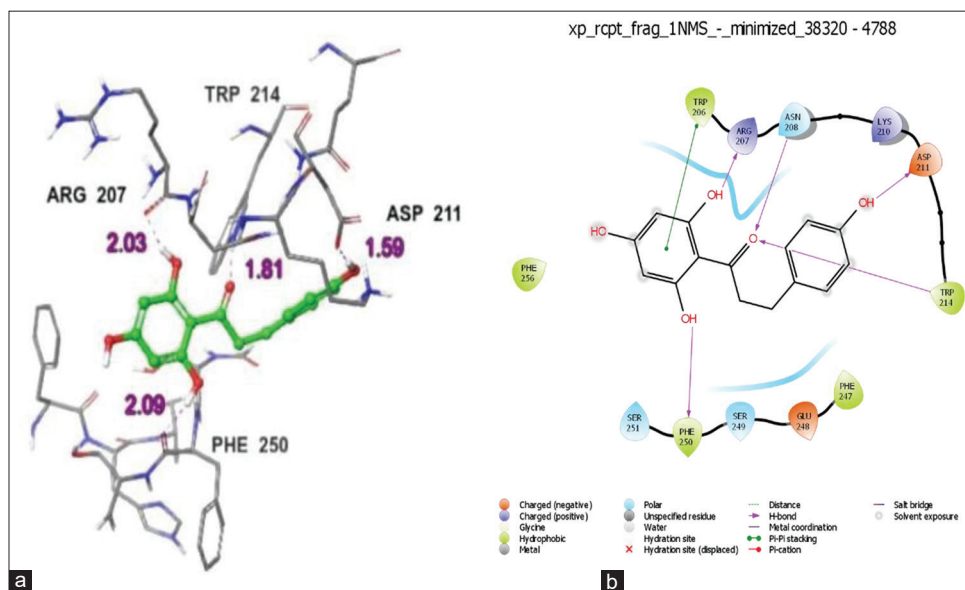

Figure 8: Interaction of Phloretin with Caspase 3 protein. (a) Visualization of Interaction of phloretin with caspase 3 protein. (b) Visualization of various non-covalent molecular interactions

with amino acid residues THR 199 which depicted hydrogen bond interaction and benzene ring of phloretin interacts with HIE 94 (Histidine with hydrogen on the epsilon nitrogen) which depicted pi-pi interaction. The crucial interactions are similarly depicted by binding inhibitor Polmacoxib against carbonic anhydrase 1 (Kim *et al.*, 2016). It resulted in an overall docking score of -6.2 Kcal/mol (Figure 5).

The docking analysis showed that phloretin interacted with Inducible nitric oxide synthase (iNOS), wherein the carbon-oxygen bond interacted with TYR 491 and the hydroxyl group interacted with amino acid residues ASN 370, the benzene ring of phloretin interacts with TRP 194, PHE 369 and depicted pi-pi interaction. It resulted in an overall docking score of -7.2 Kcal/mol (Figure 6).

The docking analysis showed that phloretin interacted with the endothelial nitric oxide synthase (eNOS), wherein hydroxyl group interacted with amino acid residues TYR 475 and SER 354 which depicted hydrogen bond interaction, and benzene ring of phloretin interacted with TRP 178 which depicted pi-pi interaction. It resulted in an overall docking score of -7.3 Kcal/mol (Figure 7).

The docking analysis showed that phloretin with Caspase 3 protein, wherein hydroxyl group interacted with amino acid residues ARG 207, ASP 211, and PHE 250 and carbonyl group C=O interacted with ASN 208 and TRP 214, which depicted hydrogen bond interaction. It resulted in an overall docking score of -5.4 Kcal/mol (Figure. 8).
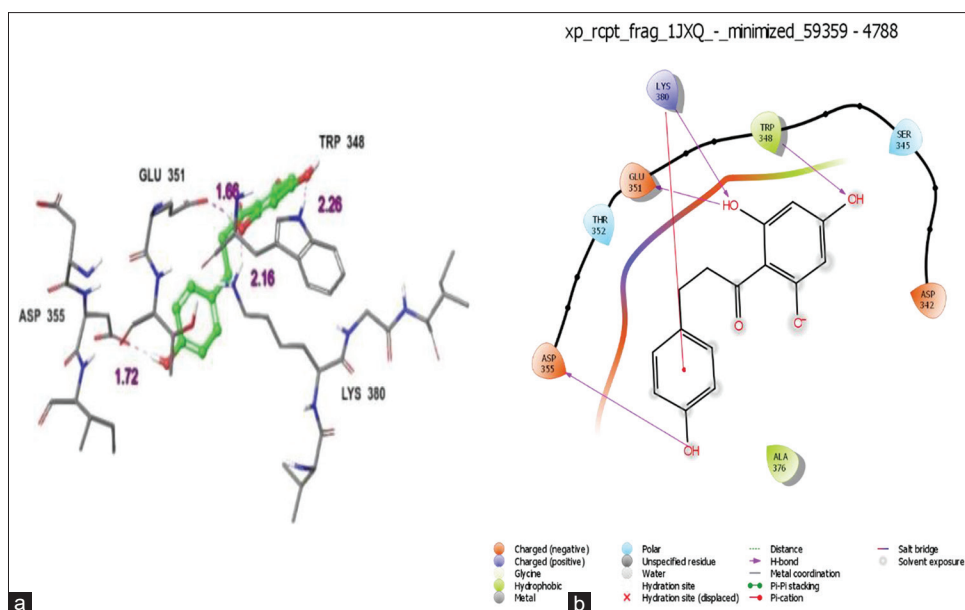
Figure 9: Interaction of Phloretin with Caspase 9 protein (a) Visualization of Interaction of phloretin with caspase 3 protein. (b) Visualization of various non-covalent molecular interactions

The docking analysis showed that phloretin with Caspase 9 protein, wherein hydroxyl group interacted with amino acid residues TRP 348, LYS 380, GLU 351, and ASP 355, which depicted hydrogen bond interaction. It resulted in an overall docking score of -3.0 Kcal/mol (Figure 9). Overall, Phloretin showed good binding affinity and showed good interaction with the active site residues with the respective target proteins.

CONCLUSION

The results from the present research study suggest that phloretin proves to be a potent anti-cancer therapeutic and depicts significant binding efficacy and affinity against these crucial anti-cancer targets. The anti-cancer mechanism of phloretin against these targets cannot be fully understood by an in silico study. Further analysis via *in-vitro* techniques are required to understand the anti-cancer potential of phloretin. Phloretin showed good binding values and can be considered and further developed as a potent anti cancer therapeutic to aid in the treatment of various cancers.

AUTHOR'S CONTRIBUTION

All authors contributed equality in carrying out the research study and the development of this paper.

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