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Research Article

IN-SILICO ANALYSIS OF THYMOQUINONE AS AN ANTI-CANCER AGENT AGAINST CHEMORESISTANCE-ASSOCIATED PROTEINS IN OVARIAN CANCER

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ABSTRACT
Thymoquinone (TQ), a polyphenol obtained from *Nigella sativa* plays a fundamental role in controlling homeostasis. It possesses anti-cancer, anti-oxidant, anti-inflammatory, and analgesic properties. Its anti-cancer properties extend to lymphoma, leukaemia, medulloblastoma, prostate, pancreatic, breast, and ovarian cancer (OvCa). OvCa is one of the most lethal malignancies worldwide having distinct symptoms and a poor prognosis. Resistance to Cisplatin (CDDP) and subsequent reoccurrence of tumors lead to treatment failure. To tackle this, combination therapies along with polyphenols are being studied and have been proven to enhance CDDP response. Certain proteins such as Bcl-2, Bax, STAT3, p53, and caspase-3/9 are involved in cancer progression and signaling of chemoresistance. In this study, Thymoquinone (TQ) interactions with these proteins have been observed through molecular docking. The 3D structure prediction was carried out using Phyre2 and SWISS-MODEL. The molecular docking of protein targets and TQ was done using the PatchDock server. The docked results were then visualized using Discovery Studio. This study helps us to understand the interactions between TQ and apoptotic proteins that help in the augmentation of conventional therapy. This knowledge will also help in developing a new approach and alternate therapeutic strategies using polyphenols like TQ, even in resistant cells.

Keywords: Thymoquinone, Ovarian cancer, Cisplatin, Bcl-2, Bax, STAT3, p53, Caspase-3, Caspase-9.

1. INTRODUCTION
Traditional medicines that include herbal materials or herbal formulations have become one of the most significant sources in health care. In many instances, herbal products have minimal side effects along with possessing incredible therapeutic outcomes. World Health Organization (WHO) has described integrated therapies involving natural products as a supplementary reserve in research as well as in health care [1]. Thymoquinone (TQ) is a phytoconstituent obtained from *Nigella Sativa* and is popularly known as black seed or cumin. The properties such as anti-proliferative, anti-oxidant, anti-oxidant, anti-hypertensive, gastro and neuroprotective, and analgesic are reported in TQ [2]. For decades studies have revealed the chemo-protective effect of TQ has been reported in various cancer types such as bladder, breast, colorectal, gastric, lung, prostate, pancreatic, and ovarian cancer [3]. TQ acts as a boon to healthy cells as it prevents the cells from oxidative stress while inducing apoptosis in cancer cells. It also inhibits cell migration and proliferation, alters epigenetic modifications, restricts tumorigenesis, and downregulates STAT3, Bcl-2, Wnt, MAPK, and PI3K signalling pathways [4].

Universally women are diagnosed with Ovarian cancer (OvCa) at advanced stages due to a lack of symptoms and experience resistance to cisplatin (CDDP). Despite deciphering several therapeutic strategies, understanding the molecular mechanisms and signalling pathways in OvCa treatment has nevertheless been a challenge. CDDP resistance has been one of the more common reasons for OvCa treatment failure [5]. Chemoresistance is also correlated with the interaction of the drugs with cellular/membranal proteins. There is a plethora of

proteins that are involved in causing cancer chemoresistance and phosphorylation or acetylation of these proteins can be a major drawback during therapy. The events such as proliferation, apoptosis, DNA repair mechanisms, DNA influx/efflux activation of a signalling pathway, autophagy, and vesicular transport are compromised after acquiring resistance [6]. Thymoquinone (TQ) has been predicted to have antiproliferative activity in lung cancer through the inactivation of MAPK signalling, activating p53 and down-regulating protein-protein interaction [7]. A similar study in pancreatic cancer shows different analogues of cancer having anti-cancer effects by sensitizing the cells that have acquired resistance [8].

In OvCa, TQ reduces the expression of B-cell lymphoma (Bcl-2) and increases Bax, increases mitochondrial damage, and ROS activation, prevents the formation of carcinogenesis, and suppresses the process of EMT [9]. Bcl-2 is an anti-apoptotic protein consisting of BH1-BH4 domains and its first group of proteins is Bax, Bim, and Bid. Bax being the pro-apoptotic protein is suppressed due to acquired chemoresistance whereas Bcl-2 is over-expressed. TQ has been reported to reduce the expression of Bcl-2 on the other hand increasing the Bax levels with permeabilization of mitochondria. Thus, representing the anti-apoptotic and pro-apoptotic activity role of TQ [10].

Cellular homeostasis is well-maintained by p53 through protein-protein interactions or transcription factors, however, the phosphorylation of p53 can lead to carcinogenesis in cancer subtypes [11]. p53 is a promising therapeutic agent that can enlarge clinical manifestations with the help of TQ, thereby triggering elevated levels of ROS and regaining the tumor suppressor function [12]. In OvCa, phosphorylation of STAT3 leads to cancer expansion for instance increasing the Bcl-2 expression and reducing Bax, inhibiting the function of IL-6, deactivating the caspases, and causing resistance to drugs [13]. The irregular functioning of caspases can activate certain genes that promote tumorigenesis. The promotion of metastasis in cancer is initiated by caspase-3 due to deficient expressions of N-cadherin, Snail, Slug, and zinc finger proteins [14]. Furthermore, caspase-9 is recognized to activate other caspases that trigger the initiation of the death domain and is important to preserve equilibrium TQ induces both extrinsic and intrinsic pathways thus reducing the cancer progression and acting as a promising therapeutic agent [15].

In this study, we have analyzed the interaction of TQ with Bax, Bcl-2, STAT3, p53, and Caspase-3/9. These proteins take part in the up-regulation/down-regulation of OvCa proliferation. Our team is currently investigating the *in-vitro* effects of TQ on the above-mentioned proteins in OvCa. Hence, we attempt to study the interaction of TQ with these proteins as done by others in different cancers, to supplement and give preliminary information on the study. We will report our *in-vitro* studies as soon as the investigations are completed, Phyre2 and SWISS-MODEL were used for the 3D structure prediction of the proteins. The PatchDock server was used to analyze the molecular docking of targeted proteins. Discovery Studio was used to visualize the docked results. In this study, the interaction between TQ and apoptotic proteins is noted and these changes can help overcome the resistance of CDDP. TQ can be a novel drug in the treatment of OvCa or can be used as an adjuvant along with chemotherapeutic agents.

2. MATERIAL AND METHODS

2.1. Material

The information on TQ and SMILES were taken from PubChem (PubChem ID: CID 10281) (National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 10281, Thymoquinone (Retrieved March 10, 2022, from https://pubchem.ncbi.nlm.nih.gov/compound/Thymoquinone) and the online SMILES translator (https://cactus.nci.nih.gov/translate/index.html#Form). The protein structures and information were collected from UniProt (The UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res. 49: D1 (2021)). Homology modelling software SwissModel [16] and Phyre2 [17]. Avogadro [18] and AutoDock Tools [19] were used to optimize the structures of TQ and the proteins, respectively. Finally, Patchdock [20, 21] was used for molecular docking and BIOVIA Discovery Studio Visualizer (BIOVIA, Dassault Systèmes, [Discovery Studio Visualizer], [4.5], San Diego: Dassault Systèmes, [2021]) was used for virtual screening.

2.2. Methods

2.2.1. Ligand datasets

TQ (ligand) was used as a therapeutic target and docked with Bax, Bcl-2, STAT3, p53, and Caspase3/9 proteins. The initial information on TQ such as structure and physical and chemical properties including the canonical smiles was collected from PubChem.

2.2.2. Receptor dataset (Protein template)

The receptor (Bax, Bcl-2, STAT3, p53, and Caspase3/9) details were retrieved from UniProt. The proteins were optimized by adding polar hydrogen atoms and removing the water molecules. The optimization of proteins was carried out using AutoDock tools.

2.2.3. Energy optimization

TQ was subjected to energy optimization by Avogadro's software. To build a computational model for TQ, we added hydrogen bonds to satisfy valency. The optimize geometry option which is located from the extension tab, allows the user to optimize the structure of a molecule into stable conformation. These molecules make several changes in their atom position through rotation and calculate energy position. From the extension tab, select molecular mechanism (that allows the user to select parameters required to calculate energy).

2.2.4. Structure generation

The collected canonical SMILES from PubChem were subjected to a structure generator. The canonical SMILES were uploaded in the translator and .pdb and 3D output were selected. The output generates the structure of the desired protein.

2.2.5. Validation of protein

The protein templates were validated with a Ramachandran plot using SWISS-MODEL and Discovery Studio. The Ramachandran plot shows four quadrants depending on the bond angle. The Ramachandran plot shows the geometry for "allowed regions" where the valency is full and new molecules can bind whereas "disallowed regions" where no new molecules can bind. The

2.2.6. Homology Modeling

The sequence was retrieved from UniProt and the protein template was saved in .pdb format. Homology modelling was carried out for the template using SWISS-MODEL and Phyre2.

2.2.7. Protein optimization

The proteins were optimized by AutoDocktools. The water molecules were removed and hydrogen molecules were added. The file was saved in .pdb format.

2.2.8. Molecular Docking

Molecular docking analyses of proteins with TQ (ligand) were carried out using PATCHDOCK. The receptor proteinswith .pdb were uploaded in PATCHDOCK. The further ligandwas uploaded in PATCHDOCK. The clustering RMSD was set at <1.5 A°. For the results, the email ID has be to add to submit the documents. The software exhibits major binding sites along with their atomic contact energy (ACE) values. The results will be received via email.

2.2.9. Ligand and protein visualization

The interaction between the receptor and ligand was visualized using Discovery Studio. The .pdf file was uploaded to Discovery Studio. The ligand interaction was observed and the images were saved for analysis.

3. RESULTS

3.1. TQ 3D Structure generation

The 3D structure of TQ was generated in the Online SMILES converter and generator and opened in Discovery Studio and Avogadro Software for optimization. After energy minimization, the calculated energy was obtained at 16.993 kJ/mol in Avogadro software (Fig. 1).

3.2. Protein homology modeling

Phyre2 Protein folds recognition server and Swiss-Model Interactive Workspace was the modelling software used to obtain 3D structures of the proteins and their specific regions. Bax whole protein structure was matched on Phyre2 with 1f16a template (100% confidence and % I. D) of PDB and also built by Swiss Model with the template 4s0o.1.An of PDB, with 99.48% sequence identity of Apoptosis regulator box. The Phyre2 match with 1f16a was done with the Bax isoform alpha and was also used for docking. Swiss-Model further gave built models for the domains BH1, 2, and 3 with inbuilt MolProbity validations for the structures along with Ramachandran Plots (Fig. 2). Similarly, structures for other proteins were built using Swiss-Model and Phyre2.

3.3. Molecular docking and Visualisation

The PatchDock solutions that showed conventional hydrogen bonds and Carbon-hydrogen bonds (some of which are shown in Fig. 3) were selected while hydrophobic interactions were ignored and the amino acids involved in the interactions with TQ were recorded as in Table 1, after screening with Discovery Studio. These interactions were observed to lie within certain domains like BH (in Bax and Bcl-2), CARD, SH2, and DNA binding domains that take part in apoptotic

activities and are often targeted by chemotherapeutic drugs as well. Ramachandran Plot validations of the protein structures after docking were also obtained from Discovery Studio (Fig.4).

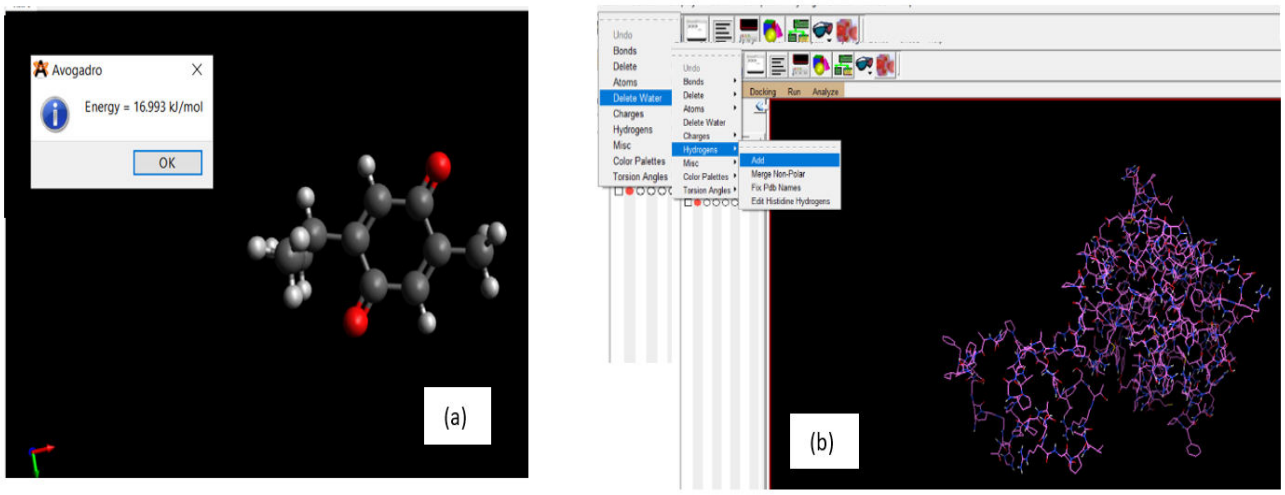

Fig. 1: Screenshot from (a) Avogadro software showing the energy calculated after minimization and (b) from AutoDock Tools showing optimization of protein (deletion of water molecules and addition of polar hydrogens)

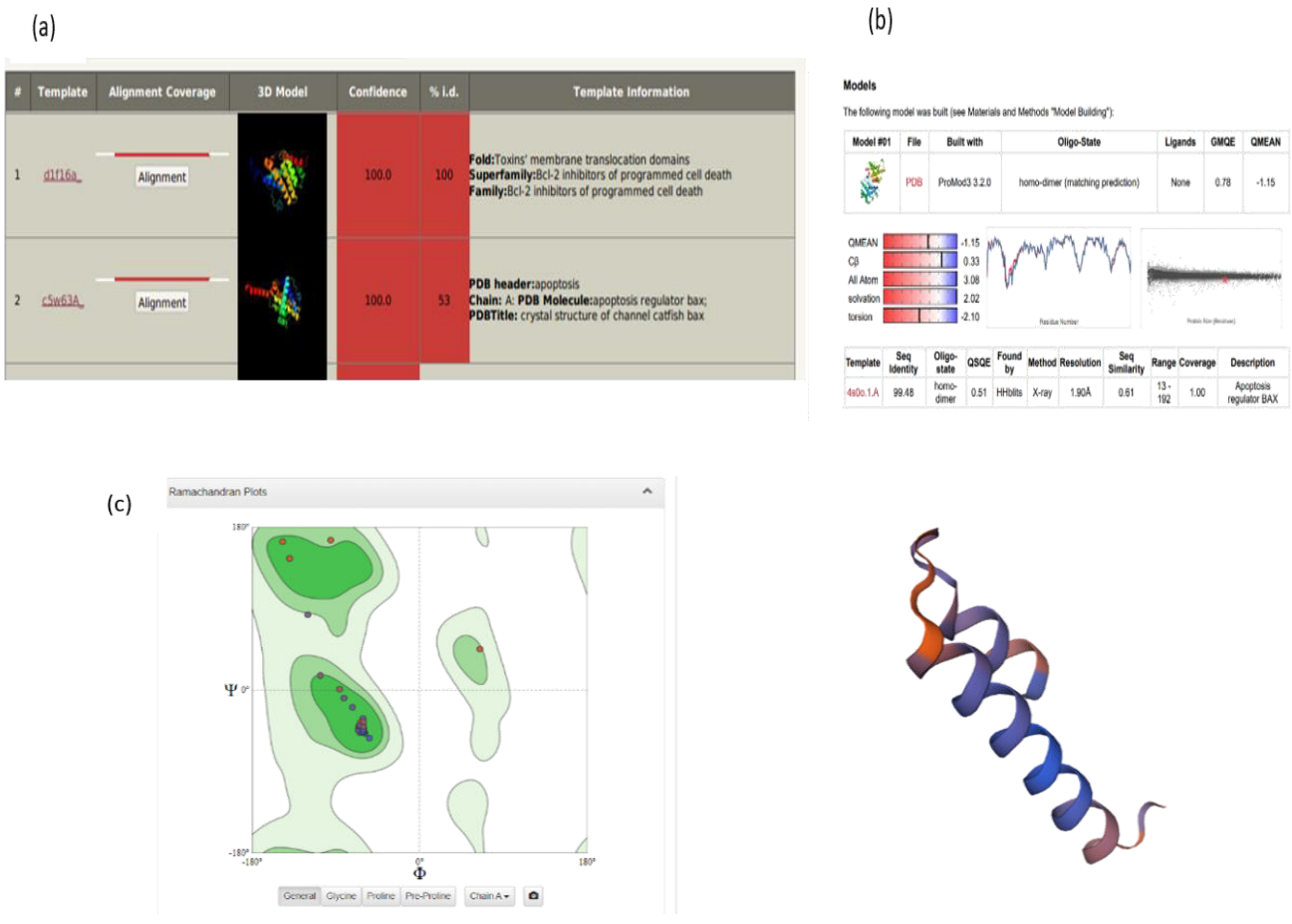

Fig. 2: (a) Bax structure prediction from Phyre2, (b) Swiss-Model and (c) Ramachandran Plot of Bax-BH1 with the built structure

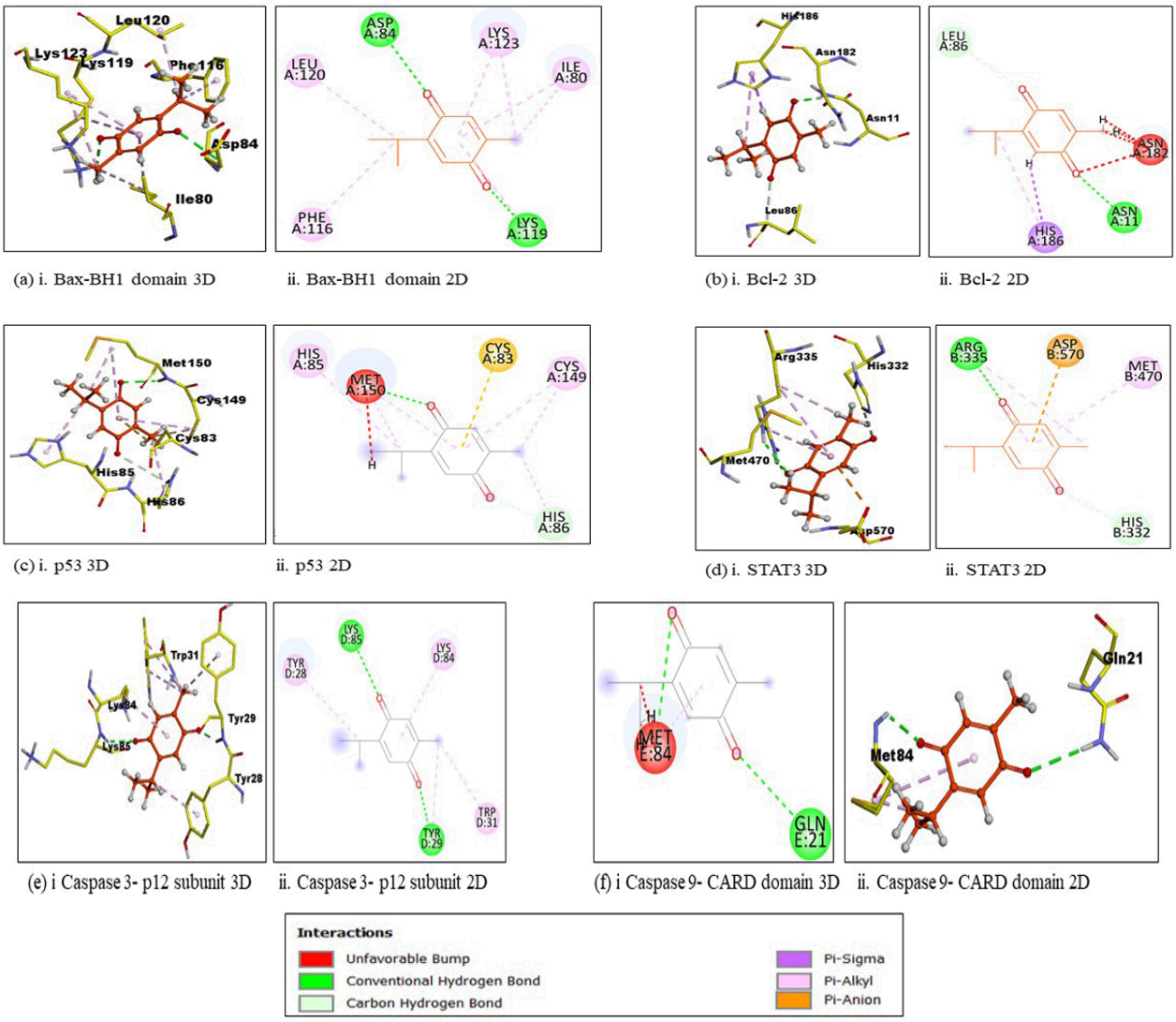

Fig. 3: Interactions between TQ and (a) i. Bax-BH1 domain in 3D and ii. 2D, (b) i. Bcl-2 in 3D and ii. 2D, (c) i. p53 in 3D and ii. 2D, (d) i. STAT3 in 3D and ii. 2D, (e) i. Caspase-3's p12 subunit in 3D and ii. 2D, (f) i. Caspase 9's CARD domain in 3D and ii. 2D, as visualized in Discovery Studio.

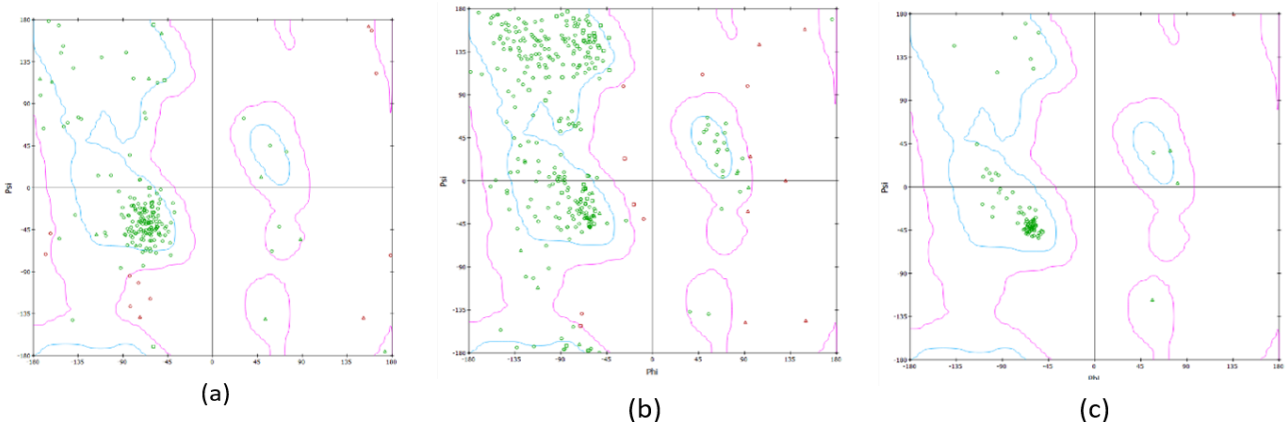

Fig. 4: Ramachandran Plots of (a) Bax, (b) p53 and (c) Caspase 9 after docking with TQ and visualization in Discovery Studio

Tables 1: Interactions of TQ with (a) Bax, (b)Bcl-2, (c) p53, (d) STAT3, (e) Caspase 3 and (f) Caspase 9 with their bond lengths (A°) and atomic contact energies (ACE) in kcal/mol.

(a) Bax

Residue	Bond length	ACE	
Asp84	2.783	-100.27	
Leu120	3.723	-114.27	
Lys123	2.896	-44.47	
Cys126	2.684	-53.99	
Ala97	3.319	-114.86	
Trp158	2.871	-87.32	BH3
Glu75	3.010	-50.42	
Lys57	2.983	-24.52	
Bax isoform alpha			
Asp84	2.806	-93.89	
Lys119	2.353		

(b) Bcl-2

Residue	Bond length	ACE	
His186	3.035	-126.92	
Asn11	2.622	-97.59	
Leu86	3.042	-106.66	
Gly33	1.724	-92.08	
Pro71	3.778	-92.08	
Ser87	2.844	-132.37	
Cys158	2.530	-134.35	
Arg127	3.661	-150.85	
Gly155	3.619	-114.03	BH1
His120	2.090	-71.38	BH3
Ser105	1.854	-88.38	
His20	2.98	-86.41	BH4

(c) p53

Residue	Bond length	ACE
Cys238	2.652	-194.90
Cys135	3.380	-207.54
Tyr234	1.697	-187.88
Met237	2.894	-196.94
Ala161	2.904	-186.72
Arg174	3.610	-57.170
Arg280	2.763	-28.70
Arg282	2.813	-34.71
Ser240	2.118	91.22
Ser106	2.437	-54.88

(d) STAT3

Residue	Bond length	ACE	
Leu438	2.410	-100.39	
Arg379	2.519	-59.37	
Leu312	2.960	-123.10	
His311	2.850	-140.02	
Leu252	2.491	-133.07	
Arg609	2.446	-104.86	
Pro669	3.150	-112.93	SH2
Gln644	2.771	-98.46	

(e) Caspase-3

Residue	Bond length	ACE	
Trp214	1.965	-115.37	
Tyr204	2.516	-113.17	
Phe250	1.784	-125.17	
Asn208	3.674	-115.37	
Arg64	2.572	-23.37	
Gln161	2.503	-23.37	
His121	1.908	-78.47	P17
Ser150	2.693	-130.52	
Arg144	2.572	-129.56	

(f) Caspase-9

Residue	Bond length	ACE	
Gln21	2.797	-96.40	CARD
Thr337	3.305	-89.46	
Tyr345	2.121	-106.85	
Gly350	2.350	-70.98	
Pro349	3.654	-70.98	

4. DISCUSSION

TQ has been studied in various cancers where it is reported to inhibit STAT3 and Bcl-2, and up-regulate Bax, p53, and Caspases-3/9 [3]. In OvCa too, it has been shown to regulate Bcl-2 and Bax [22], [23]. These chemo-modulatory effects of TQ have similar to the ones imposed by CDDP. CDDP has shown interactions with these proteins in other molecular docking studies

[24] even in the case of synergism with natural products for cancer treatment [25]. Hence their combination is more effective on these targets than the individual compounds. However, the effects of TQ are slightly different as it also imposes protective effects in CDDP-induced toxicity [26]. Since TQ is also indicated ineffective treatment of CDDP-resistant cancers, we are looking at these activities in OvCa. Since we are simply studying the general interactions of TQ with these cancer proteins, we have carried out docking with the PatchDock server, with default settings for grid parameters and flexibility within the server's algorithm.

It has been shown that the interaction of these pro-and anti-apoptotic proteins with chemical inhibitors, agonists, and antagonists leads to changes in the proteins that either activate or deactivate them [27]. Since their inactive form is available as a soluble form in the cytosol, some amino acids are hidden and inaccessible. Upon activation, these amino acids then become available for apoptotic activities [28]. Carrying out molecular docking of the protein targets expressed in OvCa and its resistant type cells will give a better understanding of their mechanisms as it focuses on the amino acids and regions involved in the interaction with the anti-cancer compounds. In this study, we carried out this molecular docking by using software like PatchDock, AutoDock Tools, Swiss Model, and Discovery Studio. PatchDock scores the docking solutions based on a geometric complementarity of the ligand and protein submitted as .pdb files [29]. The analysis parameters used from the docking and visualizing servers were the bonds involved in the interactions. The non-covalent bonds are designated as non-bonds in visualization software, which includes hydrophobic bonds, hydrogen bonds, alkyl bonds, etc. Of these, hydrogen bond (H-bond) defines a stronger interaction between ligands and proteins. This strength of the interaction is also determined by the number of H-bonds between a ligand and a protein. Conventionally, the complex with the lowest binding energy is considered the most stable one [29]. This is recorded as atomic contact energy (ACE) in this study. We also recorded all the interactions involving H-bonds obtained from the docking solutions (Table 1).

We observed interactions in Bcl-2, with the strongest binding energy at -150.85 kcal/mol and a conventional hydrogen bond between TQ and Ser87 of Bcl-2. This site is known to undergo phosphorylation when treated with drugs like paclitaxel. Although it is not clear if this has any significant effects on the function of Bcl-2, it is interesting to see TQ interact with this residue which leaves room for further investigation on such TQ-induced changes in the protein [30]. Apoptotic activities of Bcl-2 and Bax are regulated by their protein-protein homodimer (Bcl-2 with itself) and heterodimer (Bcl-2 with Bax) formation. Although an exact binding site and trigger of apoptotic activities are not clear, BH domains are responsible for the interactions and dimer formations that actively regulate the apoptosis [31]. Similarly, in STAT3, synthetic blockers and inhibitors act directly or indirectly by interacting with domains like DNA-binding, SH2, or N-terminal domains [32], and TQ has shown interactions with amino acid residues in this study having the least ACE at -140.02 kcal/mol. Since STAT3 signalling is indicated in chemoresistance through activation of anti-apoptotic factors [33], its interaction with TQ is relevant in these scenarios as well. TQ has shown significant interactions with Bax with the lowest ACE of -114.86 kcal/mol and involved BH domains, responsible for pro-apoptotic activities. Strong interactions having hydrogen bonds with the BH3 domain were observed while other domains interacted through hydrophobic bonds. This shows TQ's activity in activating the pro-apoptotic Bax proteins in the cancer [34, 35].

Interesting interactions were observed for p53 as well, with -207.54 kcal/mol as the least ACE. We observed an interaction of TQ with Cys135, which is indicated as a site for Zinc metal binding. This binding is significant in proper transcriptional activities, indicating the modulatory role of TQ in the p53 misfolding [36]. Moreover, other interactions lie between the positions 102-292 (DNA binding domain) responsible for its structural integrity and active transcription [37]. Therefore, TQ is significantly involved in the modulation of these proteins in cancer. Interactions with Caspase 3, having the least ACE at-130.52 kcal/mol involve an active site at His121. TQ also binds to caspase-3 at their active subunits p12 and p17, which have an enzyme active site [38, 39]. TQ shows interactions with amino acids including the CARD domain (caspase recruitment domain) of Caspase 9. These are significant for apoptotic activities, even in the absence of apoptosomes [40, 41].

Therefore, the molecular docking and virtual screening analysis of TQ with the proteins Bax, Bcl-2, p53, STAT3, and Caspase-3/9 show the anti-cancer activities of TQ through structural interventions. It also shows that TQ acts through the Caspase-3/9 pathway of apoptosis. Further confirmations on the role of TQ on

apoptotic proteins and their involvement in chemo resistance will be shown in our future publications throughout ongoing studies in the same.

5. CONCLUSION

TQ has been regarded as novel therapeutic as per reference to the docking studies and can modulate the pathways can prevent the triggering of anti-cancer properties. The predicted drugs can be further used for *in-vitro* and *in-vivo* studies for discovery of novel drug therapeutics.

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ST and AH have equally carried out the experiments, and analysis and drafted the manuscript. SD has conceptualised the experiment and compiled and edited the manuscript.

Conflict of Interests

The authors declare no competing interests.

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