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SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL 1, 2, 3-TRIAZOLE-CHALCONE HYBRIDS AS ANTI-PROLIFERATIVE AGENTS AGAINST HT29 COLON CANCER CELLS

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ABSTRACT: Independent studies have shown that chalcones and 123 triazole exhibited cytotoxic effects on human cancer cell lines. In the present study, we prepared a series of novel 1,2,3-triazole-chalcone hybrid compounds and evaluated the cytotoxic effect and probable mode of action in HT29 colon cancer cell line. Among the 10 evaluated compounds, compounds 4 (3-nitro) and 8 (3-chloro) were the only compounds that elicited a dose-dependent decrease in cell viability (IC_{50} values 9.7 and 33 M, respectively) of HT 29 cells. The same compounds exhibited a significant inhibitory effect on the cell proliferation and colony-forming capability of HT29 cells. Co-administration of compounds 4 and 8 with electron transport chain inhibitors and mitochondrial K+-ATP channel blockers in H9C2 enhanced the anti-proliferative capacity of the compound. Furthermore, Insilco molecular docking studies revealed that the anti-proliferative effect of these compounds could be attributed to interactions with the colchicine-binding site of tubulin. In conclusion, our results demonstrated that novel 1,2,3-triazole-chalcone hybrid can be a potent therapeutic agent against HT-29 cells, and its anti proliferating capacity against the cancer cell can be augmented in the presence of mitochondrial complex-I or mitochondrial KATP channel inhibitor.

INTRODUCTION: Colon cancer is the second most common reason for tumor mortality in developed countries and continues to increase rapidly [1, 2]. Chalcone (1,3-diaryl-2-propen-1-ones) and its derivatives exhibit a broad spectrum of biological activities which include anti-oxidant, anti-inflammatory [3] anti-fungal [4] anti-bacterial [5] and anti-cancer activity [6], suitable for structural modifications with promising anticancer activity. As anticancer agents, chalcones are reported to have other advantages like poor interactions with DNA and low risk of mutagenicity [7, 8].

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1,2,3-Triazole and its derivatives are common structural scaffolds reported to have a similarly broad spectrum of biological activities9. Recently, our group synthesized novel 1,2,3-triazole-chalcone hybrid molecules using green synthesis10. In the present study, we have evaluated the anti-proliferative effects of the synthesized 1,2,3-triazole-chalcone derivatives as an effective chemical agent to combat colon cancer.

MATERIALS AND METHODS:

Chemicals: The cell culture media Dulbeccos Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Waltham, MA, USA). Crystal violet and (3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) MTT were procured from HiMedia. Sulphorhodamine B, DL-Propargylglycine (PAG), (Aminooxy) acetic acid (AOA), Rotenone, Azide,

Glibenclamide, Diazoxide, 4-Aminobenzamide, and other chemicals used in the study were procured from Sigma Aldrich.

Chemical Synthesis: 1,2,3-triazole-chalcone hybrids were synthesized under green aqueous conditions and characterized by elemental analysis, IR, 1H NMR, 13C NMR and Mass spectral analysis as reported recently by our group (Hari *et al.,* 2016). The synthesized 1,2,3-triazole-chalcone hybrids include: Compound 1) E-4-(5-(3-(4-nitrophenyl)acryloyl)-4-methyl-1H-1,2,3-triazol-1-yl)benzamide; Compound 2) E-4-(5-cinnamoyl-4-methyl-1H-1,2,3-triazol-1-yl) benzamide; Compound3) E-4-(5-(3-(4-N,N-dimethylphenyl) acryloyl) -4-methyl-1H-1, 2, 3-triazol-1-yl) benzamide; Compound4) E-4-(5-(3-(3-nitrophenyl) acryloyl) – 4 - methyl-1H-1, 2, 3 – triazol – 1 - yl) benzamide; Compound5) E-4-(5-(3-(4-methylphenyl) acryloyl) -1H-1, 2, 3 – triazol - 1-yl) benzamide; Compound6) E-4-(5-(3-(4-methoxyphenyl) acryloyl) – 4 – methyl - 1H-1, 2, 3-triazol-1-yl)benzamide; Compound7) E-4-(5-(3-(2-chlorophenyl) acryloyl)-4-methyl-1H-1,2,3-triazol-1-yl)benzamide; Compound8) E-4-(5-(3-(3-chlorophenyl)acryloyl)-4-methyl-1H-1,2,3-triazol-1-yl)benzamide; Compound9) E-4-(5-(3-(4-hydroxyphenyl) acryloyl)-4-methyl-1H-1, 2, 3-triazol-1-yl)benzamide; Compound10) E-4-(5-(3-(4-fluorophenyl) acryloyl)-4-methyl-1H-1, 2, 3-triazol-1-yl)benzamide.

Cultivation of HT29 Cells: The human colon adenocarcinoma cell line HT29 was authenticated and procured from National Center for Cell Science (NCCS, Pune, India). It was cultured in high-glucose DMEM supplemented with 10% Fetal Bovine Serum (FBS), 4 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The cells were maintained at 37 °C in a humidified chamber of 95% air and 5% CO_2 atmosphere. Culture media was changed for every two-three days and the cells were sub-cultured once they reached 70-80% confluency. The compounds and drugs were added to the cells only at 60% confluency. Cells between passages seven and thirteen were used for the experiments.

Cytotoxicity Assessment: The anti-proliferative effect of the synthesized compounds was assessed by incubating the HT29 cells with 10 µg, 20 µg and 50 µg for 24 h of synthesized compounds. After incubation, the cell viability was measured in each group by MTT assay11and compared to the control. All experiments were carried out in triplicates in a single plate and reported as

Cell viability (%) = (absorbance of cells treated with synthesized compounds) / (absorbance of control cells) × 100%

Inhibition of Electron Transport Chain, mitochondrial K+-ATP channel: The synergic effect of synthesized compounds with electron transport chain enzyme inhibitor and mitochondrial K+-ATP channel inhibitor was evaluated. The cells were pretreated with a complex-I inhibitor Rotenone (25 µM), and a complex-IV inhibitor, Azide (25 mM), to block the Electron Transport Chain (ETC) before treatment with compounds 4 and 8. Glibenclamide (25µM) was administered to the cells to block the mitochondrial K+-ATP channel prior to treatment with the compounds 4 and 8. Pre-treatment of the cells with Diazoxide (300 µM) was done to open the mitochondrial K+-ATP channel prior to treatment with the compounds 4 and 8.

Computational studies:

a. Protein Preparation: The protein with the PDB (Protein Data Bank) accession number 4O2B (tubulin-colchicine complex) with a resolution of 2.3 Å was downloaded from PDB. Downloaded protein structures were prepared using the protein preparation wizard of the Schrödinger Suite 2014-2. Protein preparation briefly includes adding missing hydrogen, removing co-crystallized water molecules, correcting metal ionization state, enumerating bond orders to HETATM, determining optimal protonation states, and restrained minimization [12].

b. Ligand Preparation: The structure of E-4-(5-(3-(3-chlorophenyl)acryloyl)-4-methyl-1H-1,2,3-triazol-1-yl)benzamide (compound 8) and E-4-(5-(3- (3-nitropheyl)acryloyl)-4-methyl-1H-1, 2, 3-triazol-1-yl)benzamide (compound 4) were built using Maestro of Schrödinger Suite 2014 (Maestro, Schrödinger, LLC, New York, NY, 2014). Ligand preparation was carried out using ligand preparation wizard of Schrödinger Suite (LigPrep, Schrödinger, LLC, New York, NY, 2014) [13]. The chirality of the molecules was retained.

All low energy conformations were generated and used for molecular docking.

c. Molecular Docking: The receptor grid was generated using receptor grid generation wizard of the Schrödinger Suite 2014-2. The grid box was centered on the colchicine-binding site located at the interface between α-tubulin and β-tubulin. The size of the grid box is set such that it can easily accommodate the synthesized chalcone-1,2,3-triazole derivatives.

Flexible molecular docking was performed using Schrödinger GLIDE extra precision (XP) algorithm (Glide, Schrödinger, LLC, New York, NY, 2014) [14]. No constraints were set for the ligand-receptor interactions. The docking results were written as a pose viewer file, and the protein-ligand complex interactions were studied using PyMOL.

d. Estimation of Ligand Binding Affinities by MM/GBSA: All docking poses were rescored with the MM/GBSA approach outlined in the Prime program in the Schrödinger Suite. The variable dielectric solvent model VSGB 2.0 was employed to perform optimized implicit solvent model as well as physics-based corrections for hydrogen bonding, π-π interactions, self-contact interactions, and hydrophobic interactions [15]. During MM minimization of the complex, residues within five Å of the ligand were allowed to relax while keeping the rest of the structure rigid and fixed.

Statistics: All the values were represented as mean ± SD. One-way ANOVA was used to calculate the significant differences in variables between groups. A probability value of $P < 0.05$ was considered to be statistically significant. Data analysis was done using GraphPad PRISM 7.0 software.

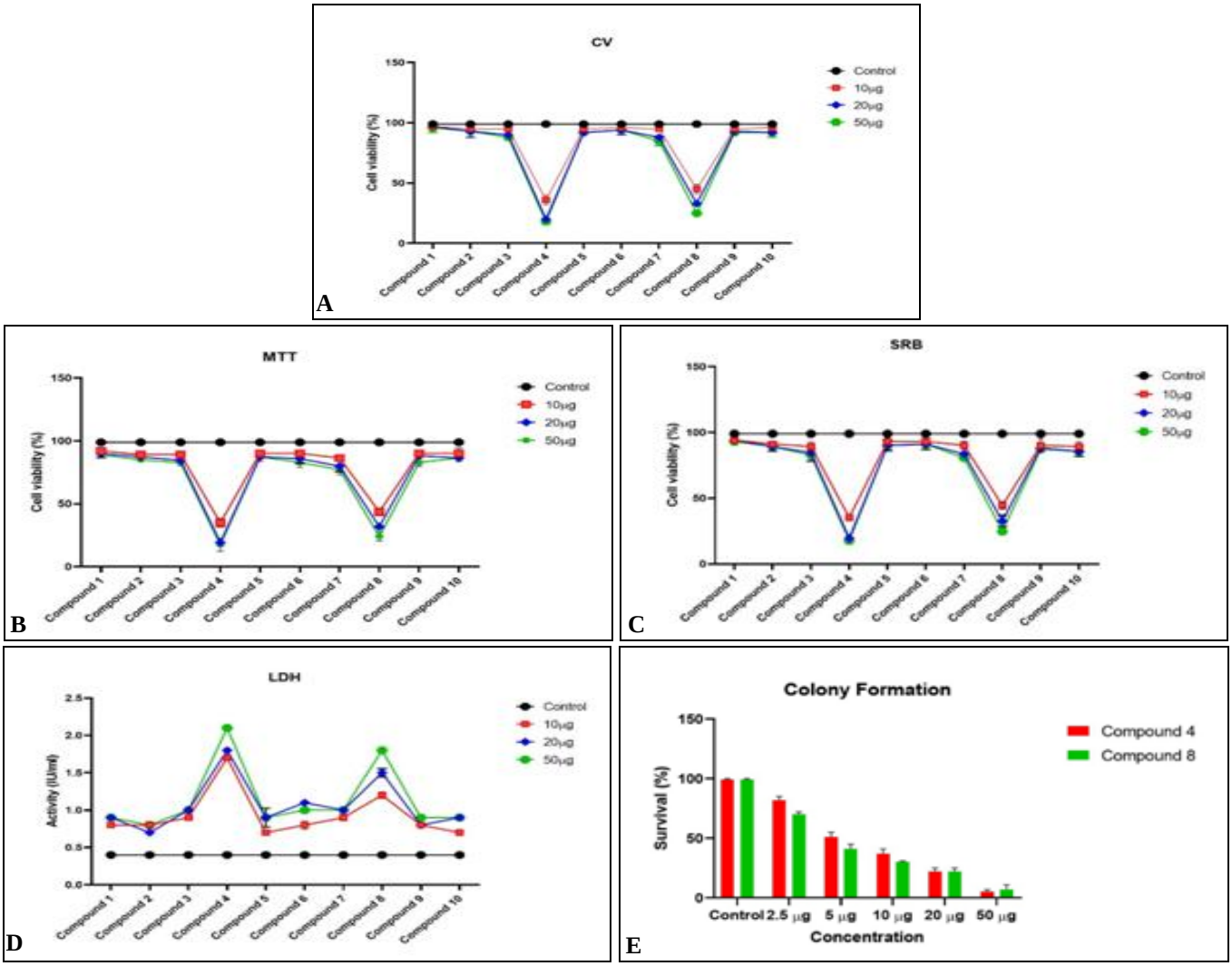

FIG. 1: CYTOTOXICITY ASSESSMENT OF TRIAZOLE-CHALCONE HYBRIDS: A) CV B) MTT C) SRB D) LACTATE DEHYDROGENASE (LDH) ASSAY E) CLONOGENIC ASSAY FOR COMPOUND 4 AND 8.* indicates significant change ($p < 0.05$) with respect to control group.

RESULTS:

Assessment of Anti-proliferative Activity: The anti-proliferative capacity of 1,2,3 triazole chalcone hybrid was evaluated in HT29 colon cancer cell line using MTT assay and the results are given in **Fig. 1B**. Accordingly, out of the ten synthesized derivatives, only two derivatives (compounds 4 and 8) showed a dose-dependent decrease in cell viability in the HT29 cell line, measured *via* MTT assay. The anti-proliferative capacity of the derivation (4 & 8) was further confirmed by measuring its potential to inhibit colony formation **Fig. 1E**.

Blocking Electron Transport Chain Enzyme Augments the Anti-proliferative Effect of Compounds 4 and 8: The potential role of mitochondrial electron transport chain enzymes in the treatment of cancer lies with its ability to produce reactive oxygen species (ROS).

Increasing the ROS production in cancer cells is considered one of the modes of action of chemotherapeutic agents where complex I, III, and IV are the therapeutic targets as they are the sources of ROS.

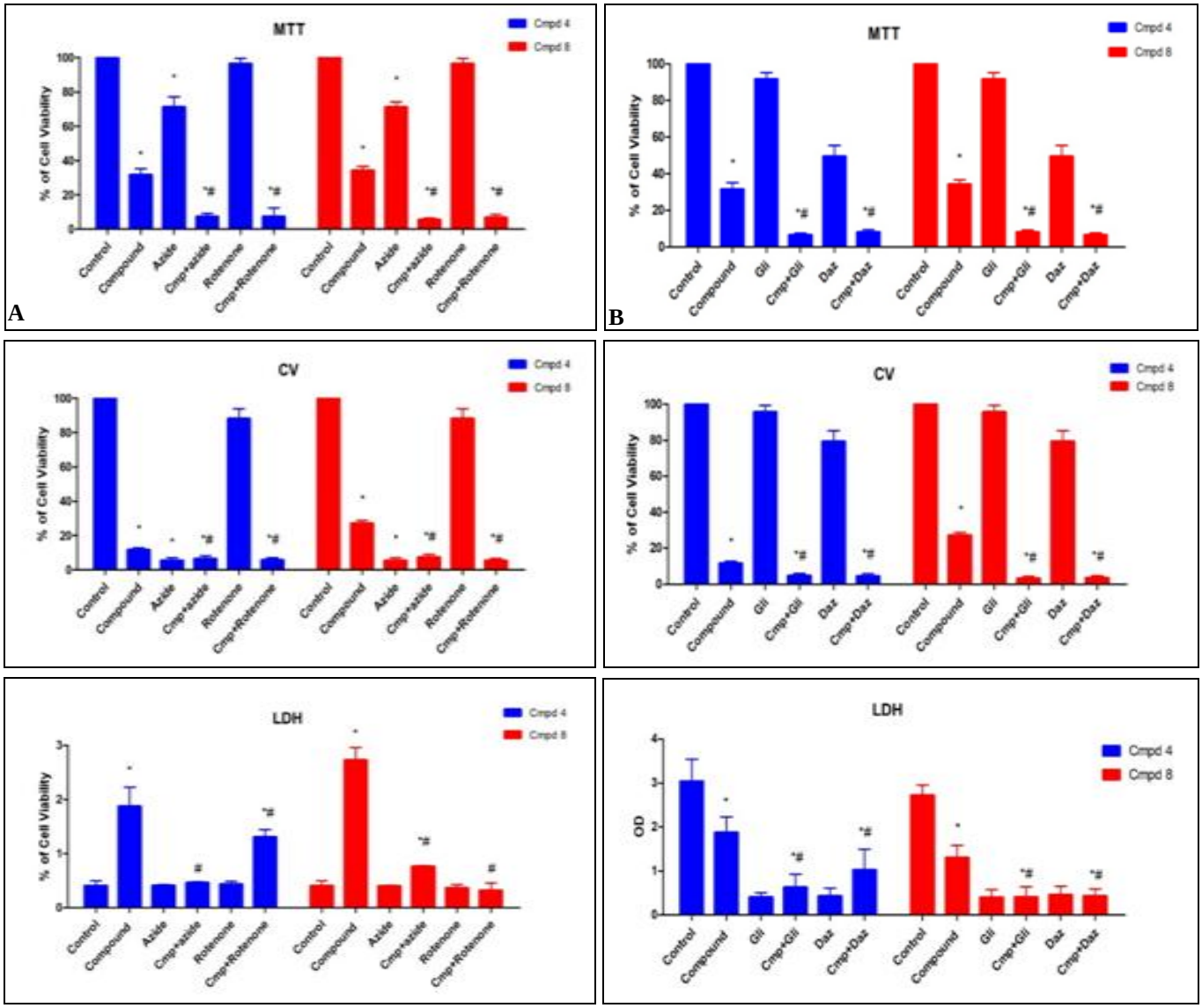

FIG. 2: A) IMPACT OF ETC INHIBITORS ON CHALCONE HYBRID-INDUCED TOXICITY: DECLINE IN CELL VIABILITY MEASURED USING MTT ASSAY WHEN ETC IS BLOCKED FOLLOWING ADMINISTRATION OF THE SYNTHESIZED COMPOUNDS. * indicates (P<0.05) against a control group, # indicates (P<0.05) against respective compound group **B) IMPACT OF MITOCHONDRIAL K+-ATP CHANNEL BLOCKERS ON CHALCONE HYBRID-INDUCED TOXICITY: CELL VIABILITY CHANGE MEASURED USING MTT ASSAY WHEN MITOCHONDRIAL K+-CHANNEL IS BLOCKED FOLLOWING ADMINISTRATION OF THE SYNTHESIZED COMPOUNDS.** * indicates (P<0.05) against a control group, # indicates (P<0.05) against a compound group

We, therefore, blocked the electron transport chain complexes I and IV with the corresponding inhibitors (Rotenone and Azide) and determined the effect of compound 4 and 8 treatment in HT29 cells. The results are given in **Fig. 2A**. Interestingly, we found the increased anti-proliferation effect by 13 & 14 folds with rotenone and 9 & 12 folds with azide in cells treated with compound 4 and 8, respectively.

Blocking Mitochondrial K+-ATP Channel Increases the Anti-proliferative Effect of Compounds 4 and 8: Mitochondrial K+-ATP channel plays a significant role in tumor growth of non-excitable cells through manipulating ROS production and maintenance of ATP levels.

We observed that the mitochondrial K+-ATP channel blocker glibenclamide inhibited the proliferation of HT29 cells. The results are visualized in **Fig. 2B**. A 14-fold increase in the anti-proliferative effect of glibenclamide when treated along with compounds 4 and 8 was observed.

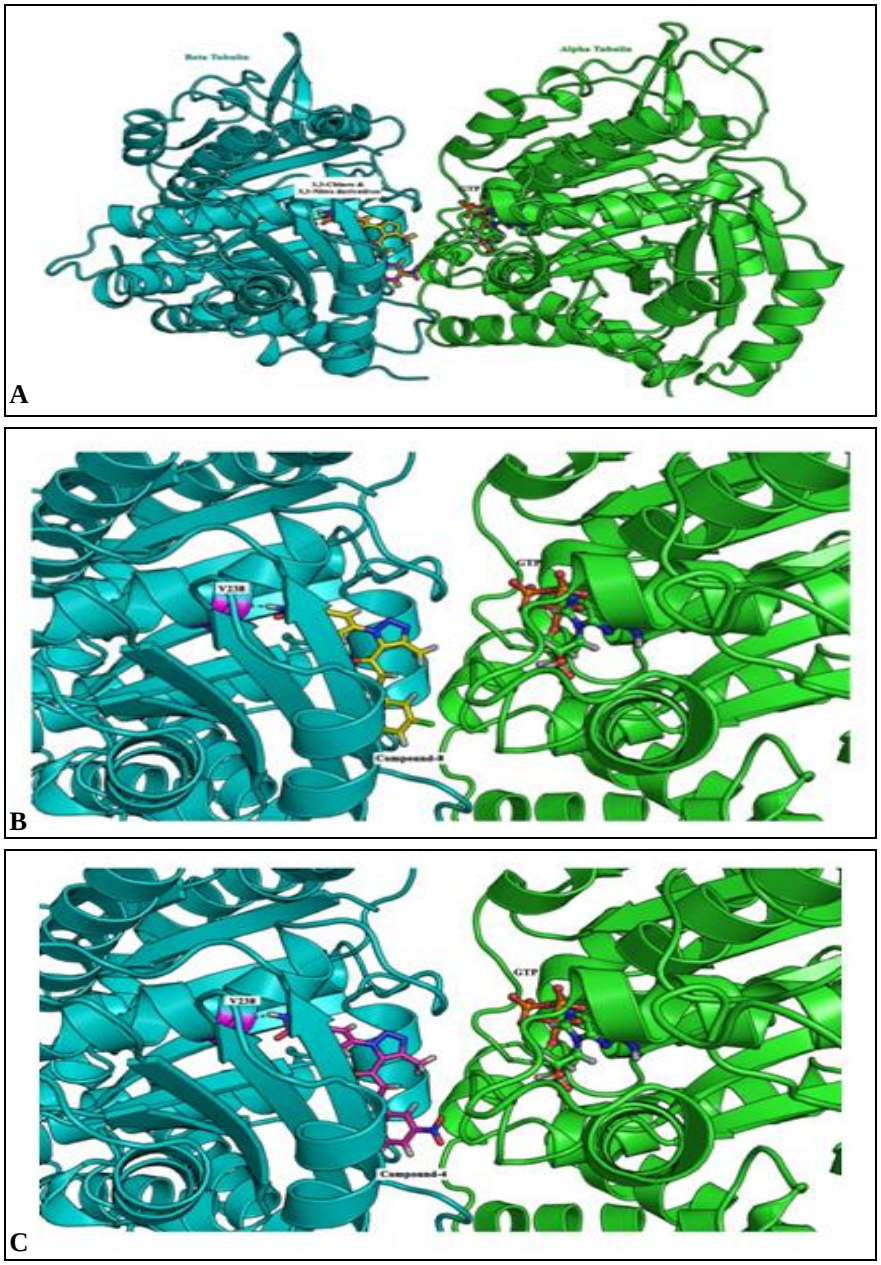

FIG. 3: BINDING MODES OF COMPOUNDS 4 AND 8: A) BINDING MODES OF COMPOUNDS 4 AND 8 WITH THE COLCHICINE-BINDING SITE IN TUBULIN; B) HYDROGEN-BONDING INTERACTION OF COMPOUND 4 WITH VAL238 IN THE COLCHICINE-BINDING SITE; C) HYDROGEN-BONDING INTERACTION OF COMPOUND 8 WITH VAL238 IN THE COLCHICINE-BINDING SITE

***In-silico* Evaluation of Anti-proliferative Effect of Compound 4 and 8:** Antitubulin polymerization effect of chalcone is well known and reported earlier. Hence we checked the effect of our newly prepared chalcone hybrid via in silico analysis, and it confirms the interaction of the hybrid with colchicine binding site of tubulin. In order to validate the docking algorithm, colchicine (5JVD) was re-docked into its binding site in tubulin, and the Glide XP score was calculated to be -9.608 with 0.23 Å RMSD. Docked poses of compounds 4 and 8 had a calculated Glide XP score of -8.317 and -7.446, respectively. The estimated MM/ GBSA ΔG bind is -61.892 and -58.623 Kcal /mol, respectively, for compounds 4 and 8. Binding analysis of the docked compound 4 and compound 8 **Fig. 3A** showed that they form hydrogen bonding interaction with amino acid Val 238 **Fig. 3B** & **3C**.

DISCUSSION: Chalcones or (E)-1,3-diphenyl-2-propene-1-one is a scaffold that is rapidly gaining therapeutic interest due to its wide range of biological activity and ease of synthesis. In this study, ten novel 1,2,3-triazole chalcone derivatives were screened for anti-proliferative activity. Compounds 4 and 8 were the only compounds that exhibited a dose-dependent decrease in cell viability in the HT29 cell line. A clonogenic survival assay was done to assess the effect of compounds 4 and 8 on the adaptability and survivability of HT29 cells, and it was found that these abilities of HT29 cells were severely inhibited. These two compounds were selected for further investigation into the mechanisms behind the observed anti-proliferative activity.

Chalcones have a well-documented anti-cancer activity that works by disrupting energy metabolism in the mitochondria by inhibiting the ETC. Therefore, mechanistic studies were carried out to determine if the mode of action of compounds 4 and 8 involved the mitochondria. One aspect scrutinized was the dependence of the compounds on ROS generation to kill HT29 cells. This was investigated by inhibiting complex I and complex IV of ETC, which are the prime sources for free radicals [16]. The results showed that blocking complex I and III had a negligible effect on the cytotoxicity of the two compounds, indicating that both the compounds possessed a mode of action independent of the ETC.

To further assess the dependence on the mitochondria, the role of the mitochondrial K+-ATP channel was investigated. The results showed that the mode of action of the compounds was independent of the opening or closing of the mitochondrial K+-ATP channel, as there was no significant difference observed in the anti-proliferative potential. Based on these observations, we concluded that the mode of action of compounds 4 and 8 was independent of the mitochondria.

Once the nature of the mode of action of compounds 4 and 8 was confirmed, we performed *in-silico* molecular docking studies to identify the targets of 4 and 8. Both compounds exhibited a high binding affinity with the colchicine-binding site of tubulin.

The mitochondrion has been steadily gaining prominence as a promising therapeutic target in cancer due to its key role in ROS generation, energy metabolism, and regulation of apoptosis. The present paradigm for mitochondrial-targeted chemotherapy in vogue involves either directly targeting the altered mitochondria and its functions using drugs like tamoxifen 17 rotenone or indirectly targeting the same using compounds like artemisinin which intrinsically initiate apoptosis [18].

While this method holds great promise, it is unable to eradicate the malignancy completely. This is because targeting the mitochondria is only a partial solution. The hallmarks of cancer cells have been elucidated at great length, and the scientific community has accepted ten hallmarks. It is imperative that we target all the hallmarks in order to implement a holistic method of treating cancer. This is possible only when we consider extra-mitochondrial targets, too, when deciding on therapy. Here, we suggest a combinatorial therapy of compounds 4 and 8 alongside a mitochondria-targeted drug.

Chalcone and its derivatives are promising lead compounds in the management of different cancerous conditions in the prostate, kidney, colon, lung, breast, bladder, and liver. The anti-cancer effects of these molecules are primarily due to their ability to target multiple sites that modulate the action of proliferation, invasion, and metastasis.

Similarly, heterocyclic compounds like 1,2,3-triazole is also known to be potent anticancer agents. However, the mode of action of chalcones and 1,2,3 triazole compounds are different.

The present study utilized the unique properties of chalcone and 1,2,3 triazole to design a new hybrid for the effective management of colon cancer that can act in a synergistic manner with drugs that therapeutically target the mitochondria. Based on the results, we found that compounds 4 and 8 have the ability to inhibit cell proliferation and induce cell death in the HT29 cell line.

Further computational studies suggest that these molecules could exhibit their anticancer effect by inhibiting tubulin by interacting with the colchicine-binding site. This study shows that 1,2,3-triazole-chalcone hybrids could be a potential lead molecule for developing anti-cancer drugs.

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