

BONDUCELLIN SUPPRESSES COLORECTAL CANCER CELL PROLIFERATION AND STEMNESS THROUGH METHYLTRANSFERASE-LIKE 3

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ABSTRACT

Colorectal cancer (CRC) remains one of the most prevalent and lethal malignancies worldwide, necessitating the development of novel therapeutic strategies. Bonducellin, a naturally occurring flavonoid derived from *Caesalpinia bonducella*, has demonstrated promising anticancer properties. However, its molecular mechanism of action in CRC remains poorly understood. In this study, we investigated the potential of Bonducellin as an inhibitor of methyltransferase-like 3 (METTL3), a key RNA methyltransferase implicated in CRC progression. Computational analyses, including molecular docking and molecular dynamics simulations, revealed a strong binding affinity of Bonducellin toward the S-adenosylmethionine (SAM)-binding pocket of METTL3, stabilized by hydrogen bonding and hydrophobic interactions. Conceptual density functional theory (C-DFT) further supported the favorable electronic stability and reactivity profile of the molecule. In vitro assays demonstrated that Bonducellin significantly inhibited proliferation colon cancer cell line HCT116, with an IC₅₀ value of 45 μ M. In addition, Bonducellin inhibited the colony formation, and migration of HCT116 cells. Acridine orange/ethidium bromide staining confirmed apoptosis induction, while immunofluorescence analysis revealed downregulation of METTL3 expression following treatment. These findings demonstrate that Bonducellin suppresses CRC cell growth and migration through METTL3 inhibition and apoptosis induction, highlighting its potential as a natural small-molecule lead for CRC therapy.

KEYWORDS: Bonducellin, METTL3, Colorectal cancer, Phytochemicals, Epigenetics

INTRODUCTION

Cancer has remained to be one of the leading health challenges, accounting for significant morbidity and mortality across populations worldwide. Despite significant advancements in diagnostic precision and therapeutic strategies, cancer accounted for twenty-one million cases in 2024, a number projected to rise to at least thirty-four million by 2040. Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and remains one of the leading causes of cancer-related mortality worldwide^{1,2}. Its rising incidence, particularly in developing countries undergoing dietary and lifestyle transitions, underscores the urgent need for novel therapeutic approaches and the identification of new molecular targets³.

Natural products have been a valuable source of pharmacologically active compounds in the past, and in this area, flavonoids, which are polyphenolic metabolites that are ubiquitously found in plants, have received growing attention due to their versatile biological activity^{4,5}. These molecules regulate many of the signaling cascades that are regulated in carcinogenesis such as oxidative stress regulation, cell cycle control, induction of apoptosis, modulation of autophagy, and inhibition of invasion and angiogenesis. It has been reported that Bonducellin, a bioactive flavonoid compound that is isolated using *Caesalpinia bonducella* (Fever nut) has been shown to possess potent antioxidants,⁶ anti-inflammatory, and anti-tumor effects^{7,8}. The previous research proved its capability of causing cytotoxicity and apoptosis in ovarian cancer cells^{6,9}. The molecular mechanism that it may exert against colorectal cancer, however, is not well delineated.

The most essential N⁶-methyladenosine (m⁶A) methyltransferase is the N⁶-methyladenosine (m⁶A) methyltransferase complex, where methyltransferase-like 3 (METTL3) is the main catalytic subunit in the complex, and it is a critical regulator of RNA metabolism, affecting the turnover, transcript processing. Abnormal expression or functioning of METTL3 has been progressively associated with oncogenic changeover, metastatic growth and treatment resistance in numerous malignancies, such as colorectal carcinoma^{6,10}. Therefore, METTL3 pharmacological modulation will be an interesting approach to reducing tumor cell growth and survival¹¹.

We investigated the possibility of Bonducellin to serve as a small-molecule METTL3 inhibitor in this study applying an integrated computational and experimental model. Recent research indicates that epigenetics, especially the RNA methylation is very critical in cancer. The METTL3-Methyltransferase mediates the mRNA methylation process with METTL3 having the catalytic and SAM-binding domains necessary to carry out the methyltransferase. In this work, METTL3 was chosen as the particular target with the objective to find some natural small-molecule inhibitors. The METTL3-METTL14 complex has two binding pockets namely the RNA-binding pocket and SAM-binding pocket. The size of the RNA-binding pocket is greater and thus unsuitable to the small molecules so we concentrated on the SAM-binding pocket in order to successfully inhibit the methylation.

Molecular docking and conceptual density functional theory (C-DFT) tests were utilized to describe the binding affinity, electronic reactivity as well as physicochemical properties of Bonducellin in relation to structurally related natural products. The conformational stability and the dynamics of the interaction of Bonducellin-METTL3 complex were further explained by molecular dynamics (MD) simulations. In vitro studies of HCT116 colorectal cancer cells were complemented by evaluating the effect the compound had on the proliferation, migration, and apoptosis.

2. MATERIALS AND METHODS

2.1 Protein Preparation

The protein data bank (PDB) was used to get the crystallographic structure of the target protein, METTL3 (PDB ID: 5IL1) (<http://www.rcsb.org/>). Structural analysis and visualization were done with PyMOL and all crystallographic water molecules and non-essential co-crystallized elements like Zn, Ca and glycerol (GOL) were removed (<http://www.pymol.org>). The protein model was then optimized and ready to undergo molecular docking with AutoDock Vina which was a part of the PyRx platform. Assigning of Gasteiger and Kollman partial charges to the receptor was performed with energy minimization being performed to stabilize the structure followed by the conversion to PDBQT format to perform docking simulations^{12,13}.

2.2 Preparation of Ligands

The structures of the three-dimensional of the chosen ligands were collected at the IMPPAT (Indian Medicinal Plants, Phytochemistry, and Therapeutics) database. To achieve the best pharmacokinetic characteristics, compounds were filtered using a sophisticated search with the use of known parameters of drug-likeness, such as Lipinski Rule of Five, Veber, and Ghose filters. The files of SDF format were changed to PDB format in Open Babel¹². The further refinement of the ligands included correction of rotatable bonds, torsional parameters, assignment of partial charges as well as optimization of geometry through the Universal Force Field (UFF). The optimal structures were further exported in PDBQT format to be used in docking simulations.

2.3 Molecular Docking

Molecular docking was applied to find out the most energetically suitable orientation of the smallest molecules in the active site of the target receptor. This is a computational method, which is part of lead discovery and optimization because it enables one to virtually screen large collections of compounds, prioritize ligand affinities and clarify possible binding conformations that mediate the interactions between receptors. The main aims of docking are the determination of plausible binding poses, the estimation of the ligand-receptor affinities and structure-guided drug design^{12,14}.

The simulation of docking was conducted in this study with the use of the AutoDock Vina module of AutoDock Tools 1.5.7 that is based on the combination of empirical scoring functions and knowledge-based potentials to estimate the binding energies. To ensure the validation of the protocols, the co-crystallized ligand was re-docked in the natural binding pocket and the pose obtained was compared with the experimental pose to ascertain that the methodology was reliable. After the validation, all candidate compounds were docked in METTL3 active site. The optimal binding orientations were determined using the docking scores, the hydrogen-bonding interactions and similarity to the binding mode of the reference ligand. Reduced docking energy was a sign of stronger association of the receptor and the ligand¹².

Out of the original library of 1,803 compounds, ten best molecules with the best binding energies were shortlisted to be taken further. BIOVIA Discovery Studio Visualizer 2024 and PyMOL were used to examine the patterns of hydrogen bonding, hydrophobic contacts, and structural complementarity in the METTL3 active pocket using the interaction patterns of these high-affinity ligands and the reference compound abrocitinib.

2.4 Conceptual Density Functional Theory (C-DFT)

Density functional theory (DFT) is based on the Hohenberg-Kohn theorem and is a quantum mechanical theory, which can be used to explain the chemical behaviour of molecules using electron density as opposed to a wavefunction representation. This theory has been extended to conceptual DFT (C-DFT) which allows the calculation of the frontier molecular orbital energies and global reactivity descriptors which are important inferences into the structure activity relationship^{15–17}.

In this paper, the global reactivity descriptors of ten descriptors and their derivatives were calculated: total energy, highest occupied (EHOMO) and lowest unoccupied (ELUMO) molecular orbitals, energy gap (ΔE), global softness (S), absolute hardness (η), chemical potential (μ), electronegativity (χ), and electrophilicity index (ω). These descriptors were calculated on the basis of the theory of molecular orbital described by Fukui which has a correlation between EHOMO and ELUMO which can be assumed to be the electron-giving and electron-receiving tendencies respectively. The polarity of the

molecules is also indicated by the dipole moment, and the larger the value is, the more likely the molecule is to be reactive, whereas the HOMO-LUMO energy gap (ΔE) can be used to understand the electronic stability and accessibility of the transitions in the molecule. The rest of the descriptors were obtained in a systematic manner based on EHOMO and ELUMO values.

Geometry-optimization of all lead molecules was done using the B3LYP functional and 6-31G (2d, p) basis set, which was applied in Gaussian 16. The resulting calculated descriptors were then used in order to explain the electronic structure and reactivity profile of Bonducellin and other related compounds.

2.5 Toxicity Profile

The PubChem database was searched to find the SMILES (Simplified Molecular-Input Line-Entry System) representations of the chosen compounds to provide the possibility of in silico toxicity assessment. A toxicological analysis was done through the ProTox-II web server that gave the predicted median lethal dose (LD50) of the toxicological analysis in mg/kg¹⁸. Simultaneously, the pkCSM system was used to predict a variety of toxicity-related data, such as the Ames mutagenicity, hERG I and II channel inhibition, maximum tolerated human dose, oral rat chronic toxicity (LOAEL), oral rat acute toxicity (LD 50), hepatotoxicity, Tetrahymena pyriformis toxicity, skin sensitization, and minnow. Such computational predictions were to estimate the entire safety and pharmacological appropriateness of the candidate molecules.

2.6 Molecular Dynamic Simulation

Since the processes of ligand-protein interactions in biology are not static, the stationary form of the problem of molecular docking is only a snapshot of the complex dynamics. It is not always possible to accurately reflect the complex stability in the physiological environment. Molecular dynamics (MD) simulations were used to investigate the dynamic behavior and stability of the docked complexes and performed in 100-ns time to examine the behavior of the complexes with the help of the GROenen Machine of Chemical Simulations (GROMACS, version 2016.3) on a high-performance computing environment based on CentOS 7.3. The docking studies provided the initial coordinates of the docked complexes and ligand topologies were created using the PRODRG server. GROMOS96 54a7 force field was used to set the parameters of the system with a simple point charge (SPC) water model employed in solvation.

Boundary conditions on all the systems were periodic and the systems were equilibrated at a constant temperature of 300 K and pressure of 1.05 bar through conventional coupling algorithms. Structural stability and flexibility were measured by the trajectories in root mean square deviation (RMSD), root mean square fluctuation (RMSF) and analysis of hydrogen bonds. All the dynamic simulations were run on a high-performance computing (HPC) server, to guarantee the efficiency of computing and reproducibility^{19,20}.

2.7 Cell culture

Human colorectal carcinoma cells (HCT116) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; BioWest) and 1% penicillin-streptomycin antibiotic solution. The culture medium was filter-sterilized before use to ensure aseptic conditions. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ to promote optimal growth and viability.

2.8 MTT Assay

The cytotoxic potential of Bonducellin on colorectal cancer cells was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay. MTT and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. HCT116 cells were seeded in 96-well culture plates at a density of 5,000 cells per well and allowed to adhere and proliferate for 24 hours under standard culture conditions. Subsequently, the cells were exposed to varying concentrations of Bonducellin and incubated for an additional 48 hours to determine the half-maximal inhibitory concentration (IC₅₀)²¹.

Following treatment, the culture medium was replaced with freshly prepared MTT solution and incubated for 4 hours at room temperature to facilitate the formation of formazan crystals. All steps were performed in the dark to prevent photodegradation of the reagents. The resulting formazan product was solubilized in DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability and proliferation rates were calculated based on the optical density (OD) values relative to untreated control wells.

2.9 Hemolysis Assay

One popular technique for figuring out the cytotoxic effects of different chemicals is to measure the rate of hemolysis. In short, EDTA was used as an anticoagulant to stabilize fresh human blood²². To separate red blood cells (RBCs), 2 mL of whole blood was combined with 4 mL of phosphate-buffered saline (PBS) and centrifuged for 5 minutes at 10,000× g. Following five rounds of washing with 10 mL of PBS, the RBCs were finally diluted with PBS to a volume of 20 mL. While the positive control group was subjected to distilled water and the negative control group to PBS, the experimental group was treated to IC₅₀ doses of bonducellin in 0.1 mL of the diluted RBC solution. Three tubes from each group were placed in a special incubator and kept at 37 °C for 24 and 48 hours. The materials were centrifuged once more at 10,000× g for five minutes after the incubation time was over. After transferring 100 µL of each sample's supernatant to a 96-well plate, the absorbance was measured at 590 nm using a microplate reader.

$$\text{Hemolysis ratio} = \frac{\text{OD}(\text{test}) - \text{OD}(\text{negative control})}{\text{OD}(\text{postive control}) - \text{OD}(\text{negative control})} \times 100$$

This formula allowed for the determination of the hemolytic degree in relation to the controls^{23–25}

2.10 Clonogenic Assay

The survival of clonogenic cells was determined in order to determine the long-term proliferative potential of HCT116 cells. The cells were cultured in 6-well culture dishes and the cells were plated at a density of 1,000 cells per well to facilitate the emergence of individual colonies by single cells. The treatment group exposure to Bonducellin at its IC 50 concentration was done after 48 hours, and fresh, drug-free growth medium was added after that. The untreated control

group was kept in the same condition without the exposure to the compound. All plates were then incubated further in 5-7 days after treatment to allow the growth of visible colonies. The medium was then discarded at the expiry of the incubation period after which the wells were washed gently in phosphate-buffered saline (PBS). To fix the colonies, 10 percent formalin was used during 10 minutes and followed by the use of 1 percent crystal violet solution. To estimate the fraction of cells that survived and were able to continue their growth following the enacted treatments, the number of stained colonies was counted^{26,27}.

2.11 Scratch Assay

Cell migration was evaluated using the two-dimensional (2D) scratch assay. HCT116 cells were seeded in six-well tissue culture plates and cultured in complete DMEM supplemented with FBS and antibiotics until reaching approximately 70-75% confluence. Once the desired confluency was achieved, a uniform linear wound was created across the cell monolayer using a sterile 200 μ L micropipette tip. Detached cells and debris were carefully removed by washing with phosphate-buffered saline (PBS). HCT116 cells were exposed to Bonducellin at its IC₅₀ concentration in serum-free DMEM, which promotes migration while minimizing proliferation. In parallel, control wells cells were maintained under identical conditions in serum-free medium without the compound. The wound area was imaged at predetermined time intervals using an inverted phase-contrast microscope to monitor and compare the extent of wound closure between treated and control groups²⁶.

2.12 Colonospheroid Assay

Three-dimensional cell culture models, as opposed to two-dimensional cultures, are used to attempt to replicate tumor conditions. They allow the observation of interactions between cells and the extracellular matrix, giving a gradient of feeding and oxygenation that leads to a physiology more like the in vivo environment, even in the absence of other components of the tumor microenvironment. This could enhance the comprehension of the pharmacological response and the assessment of the efficacy and toxicity of drugs. Treatments with bonducellin were found to decrease colonosphere morphology. Three-dimensional spheroid formation was assessed using tissue culture plates coated with 1.2% poly (2-hydroxyethyl methacrylate) (Poly-HEMA; P3932) to inhibit cell attachment and promote suspension growth. The spheroid culture medium comprised a 1:1 mixture of DMEM and F-12, supplemented with B-27 and epidermal growth factor (EGF) at a final concentration of 20 ng/mL. HCT116 cells were enzymatically dissociated using trypsin, counted, and seeded into six-well plates at a density of 1,000 cells per well. During seeding, these cells were exposed to Bonducellin at its IC₅₀ concentration. Parallel control HCT116 cells were maintained under identical serum-free culture conditions without compound treatment. The defined medium composition provided a growth environment favoring the survival and self-renewal of cancer stem cell (CSC)-like subpopulations capable of forming spheroids under non-adherent, three-dimensional conditions. The emergence of well-defined spheroidal aggregates was considered indicative of the presence of CSC-like cells and effective spheroid formation^{26,27}.

2.13 Anchorage-Independent Growth Assay

The proliferation of HCT116 cells independent of anchorage was determined by soft agar colony formation assay in low melting agarose (LMA). A stock solution of LMA 1.6% was prepared in distilled water and autoclaved at 100 °C and kept in a water bath at 40-45 °C. To prepare the base layer (0.8% agar) the following volumes of 1.6% LMA and prewarmed complete DMEM with 10% FBS and 1% penicillin-streptomycin were mixed in equal proportions. About 2 mL of this blend was transferred into every well of a six well plate and left to harden at room temperature in 20-30 minutes. In the case of the upper layer, the cells were trypsinized, counted and resuspended in full-fledged DMEM. Prewarmed 0.6 percent LMA was added to the cell suspension to produce a final cell concentration of 0.3 percent agar in the well that contained 5 x 10³ cells. The treatment was done using Bonducellin at a concentration of IC₅₀ (45 μ M) and the untreated kept under the same conditions without the compound. The mixture on the top was carefully poured over the solid base layer in 2 mL, and left to dry at room temperature in 15-20 minutes. Plates were incubated at 37 °C and 5 percent CO₂ in 5-7 days to allow the development of colonies. The colonies appearing spherical were observed under an inverted phase-contrast microscope and representative images were taken at various random fields per well. ImageJ software was used to count and measure the diameter of the colonies. All the experiments were conducted thrice and the data are given in the form of mean and SEM²⁸.

2.14 Acridine Orange/Ethidium Bromide (AO/EtBr) Dual Staining Assay

The analysis of apoptotic cell death was done through acridine orange/ethidium bromide (AO/EtBr) dual staining. The HCT116 cells were cultured in 24 well culture plates at a density of 1 x 10⁵ cells per well and left to adhere overnight at standard growth conditions. On day two, the HCT116 cells were incubated with 48 hours of Bonducellin at its IC₀ concentration in parallel control groups, and HCT116 cells- were cultured under the same conditions without the compound. The cells were then rinsed in phosphate-buffered saline (PBS) gently after the treatment period to eliminate the remaining media and reagents. Each well was then directly stained by the use of a solution with acridine orange and ethidium bromide (100 μ g/mL of each). After staining the cells, they were immediately analyzed with the inverted fluorescence microscope, and the representative fields were photographed on the EVOS Fluid Imaging System (Thermo Fisher Scientific). Live, apoptotic, and necrotic cells were differentiated through the use of the differential fluorescence patterns depending on the membrane integrity and chromatin morphology²⁹.

2.15 Immunofluorescence Analysis

Sterile glass coverslips were placed in six-well culture plates, and non-transfected HCT116 cells were seeded onto them under aseptic conditions. The cells were allowed to adhere overnight under standard incubation parameters. The next day, the experimental group was treated with Bonducellin at its IC₅₀ concentration for 24 hours, whereas untreated control cells were maintained under identical conditions without the compound. Following treatment, cells were fixed using 10% neutral-buffered formalin and permeabilized with 0.5% Triton X-100 prepared in PBS. Non-specific antibody binding sites were blocked by incubating the cells for 30 minutes in 5% bovine serum albumin (BSA). Immunostaining was then

carried out using an anti-METTL3 primary antibody (Abclonal) diluted 1:50, followed by incubation with Alexa Fluor 633-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Thermo Fisher Scientific, A21070). Fluorescent signals were visualized and captured using the EVOS FLoid Imaging System (Thermo Fisher Scientific)³⁰.

3. RESULTS

3.1 Identification of Top Ten Natural Compounds Using Molecular Docking

Ten drug-like phytocompounds were shortlisted out of the IMPPAT database according to the Rule of five by Lipinski as well as other pharmacokinetic filters of ADME. Such compounds were then docked to the binding site of S-adenosyl methionine (SAM) of the target protein to determine their binding affinities and binding profiles. The molecules with the highest binding affinity that were screened included compound IMPHY001665 with the docking score of -9.2 kcal/mol. This ligand was found to have several hydrophobic contacts with Glu481, Thr510, Trp398, Thr408, Asp399, and Ser511, and hydrogen bond with Ile400, Val432 and His482, which means that it was a stable and specific association in the active pocket of METTL3. IMPHY013305 and IMPHY003529 were the next docking compounds with docking energy of -8.09 and -8.07 kcal/mol, respectively. The complex was well-stabilized as shown by the interaction of IMPHY013305 with Lys513, Glu481, Tyr406 and a large number of hydrophobic contacts. On the same note, IMPHY003529 interacted with a general hydrophobic network comprising Val507, Thr460 and His478, and some important residues such as Lys459, Lys480 and Ile467. Other compounds, IMPHY008016, IMPHY004968 and IMPHY011716 had docking scores of between -8.6 to -8.4 kcal/mol in a group of hydrogen and hydrophobic interactions with key residues, such as Glu506, Thr433, Glu481 and Gln550 (Table 1 and Figure 2). Together, these findings indicate that the most favorable compounds are energetic and structurally consistent interactions between the SAM-binding site and the top-scoring compounds, which implies that they could be used as lead compounds to be further validated by computational and experimental methods.

Table 1. Screening Top 10 compounds from IMPAAT database

S.No.	Compounds	Binding Affinity (kcal/mol)	H – bond interaction	Hydrophobic interactions
1.	IMPHY001665	-9.2	Ile400, Val432, His482	Glue481, Thr510, Trp398, Thr408, Asp399, Ser511
2.	IMPHY013305	-8.9	Lys513, Glu48, Tyr406	Gln500, Pro397, Leu404, Ile400, Thr433, Trp398, Pro396, Asp395, Trp431, Val432
3.	IMPHY003529	-8.7	Lys459, Lys480, Ile467	Val507, Asn461, Thr460, Ile466, Ile311, Gly479, Thr472, Thr433, His478
4.	IMPHY008016	-8.6	Arg536, Gln550	Ser511, Lys513, Glu481, Tyr406
5.	IMPHY004515	-8.5	-	Ile311, Gly479, His478, Thr472, Thr460, Asn461, Glu506, Arg508, Ser509
6.	IMPHY004968	-8.5	Glu506, Val507, Lys459, Ile467	Arg508, Gly462, Asn461, Thr460, Ile466, Ile311, Gly479, His470
7.	IMPHY012446	-8.4	Gln550	Ser511, Arg536, Tyr406 Pro397, Pro405, Thr510
8.	IMPHY011716	-8.4	Gly513, Thr433	Asp395, Ty406, Leu404, Thr472, His478, Lys480, Lys459, Glu481
9.	IMPHY003000	-8.3	Ser511, Lys513	Glu481, Thr510, Gln550, Pro397, Pro396, Thr433, Val432, Trp431, Glu481
10.	IMPHY003432	-8.2	Ser511, Trp398	Thr510, Gln550, Pro397, Pro396, Thr433, Val432, Trp431, Lys513, Glu481

Table 1 Indicates the affinity of binding (kcal/mol) and the interaction profile of the top 10 compounds of the IMPAAT database to the METTL3 protein. Hydrogen bond and hydrophobic interactions are used to emphasize the important residues that lead to the stability of the ligand protein.

3.2 C-DFT Analysis Shows that Bonducellin has the best chemical reactivity as one of the best candidates.

Quantum chemical studies showed that there were clear differences in electronic descriptors of the tested compounds indicating that the reactivity patterns of the compounds and their interactions with biological macromolecules are different. Table 2 is a summary of the calculated parameters of the shortlisted FDA-compliant molecules, which are electronegativity, chemical hardness, and softness, chemical potential and electrophilicity index. The smaller the HOMO-LUMO energy gap (ΔE) the lower the excitation energy and the more reactive the chemical system because it increases

the electron transfer between the frontier orbitals. Equally, a moderate drop on electronegativity tends to increase the inhibition power of a ligand as it increases its ability to bind with the active site of the target proteins. The best ten molecules that were screened exhibited the greatest dipole moments of IMPHY003305, IMPHY003529, and IMPHY012446, which are more polar, and thus could interact with charged or polar amino acid residues. The total energy of IMPHY001665 (Bonducellin) was found to be -957.12 eV and the dipole moment of this atom was 1.8665 D, which indicates mediocre polarity and equal probability of interaction. It has got frontier orbital energies of HOMO = -0.2213 eV and LUMO = -0.0605 eV, which gives it a HOMO-LUMO gap (ΔE) of 0.16 eV, which is in agreement with moderate electronic stability and reactivity. The values of ΔE were both negative (e.g. -0.62 eV in case of IMPHY012446) and small positive (e.g. 0.03 eV in case of IMPHY003529), which indicates that narrower or even negative gaps are associated with the higher reactivity and, possibly, with the lower thermodynamic stability. These observations are also affirmed by the global reactivity descriptors. The softness (IMPHY003529- 26.78) was the highest as it was expected, since this compound was highly chemically reactive, but electronegativity values were in a relatively small range (0.130.52). Expectedly, the values of all the calculated chemical potential (μ) of all were negative thus indicating spontaneous donation of electrons. In the case of Bonducellin, 0.14 and -0.14 are the values of 0 and 0.5 respectively, which proves its moderate donor nature. Its index of electrophilicity (= 0.12) was relatively lower than IMPHY003529 (0.65) and IMPHY012446 (0.43) that showed higher tendencies of being electrophilic. All these results (Figure 1) suggest that IMPHY001665 (Bonducellin) is a stable lead compound that is chemically balanced and has an intermediate reactivity, therefore, making it a promising electronic configuration. In comparison, IMPHY003529, IMPHY012446, and IMPHY003305 are more reactive and electrophilic, which implies they have an increased possibility of being an electron acceptor and should be validated in terms of biology further.

Table 2. Molecular descriptors from Conceptual Density Functional Theory (C-DFT) study of top compounds

S.No.	Compound	Total Energy (Er) (in eV)	Molecular Dipole Moment (Debye)	EHOMO (in eV)	ELUMO (in eV)	(ΔE)	(η)	(σ)	(χ)	(μ)	(ω)
1.	IMPHY001665	-957.12	186.65	-0.2213	-0.0605	-0.28	0.08	6.21	0.14	-0.14	0.12
2.	IMPHY003305	-1373.03	100.05	-0.20366	-0.5986	-0.39	-0.19	-2.53	0.40	-0.40	0.40
3.	IMPHY003529	-1219.32	76.762	-0.17511	-0.13778	0.03	0.01	26.78	0.15	-0.15	0.65
4.	IMPHY008016	-122.459	177.89	-0.32932	-0.0456	0.28	0.14	3.52	0.18	-0.18	0.12
5.	IMPHY004515	-1298.08	20.298	-0.24388	-0.12611	0.11	0.05	8.49	0.18	-0.18	0.29
6.	IMPHY004968	-1298.26	147.50	-0.2119	-0.05656	0.15	0.07	6.43	0.13	-0.13	0.11
7.	IMPHY012446	-1351.04	183.64	-0.21018	-0.8392	-0.62	-0.31	-1.58	0.52	-0.52	0.43
8.	IMPHY011716	-1286.79	175.21	-0.34057	-0.13258	0.20	0.10	4.80	0.23	-0.23	0.26
9.	IMPHY003000	-1334.03	113.87	-0.20917	-0.07735	0.13	0.06	7.58	0.14	-0.14	0.15
10.	IMPHY003432	-1297.21	121.46	-0.22815	-0.0657	0.16	0.08	6.15	0.14	-0.14	0.13

Table 2 Shows the quantum chemical descriptors of ten compounds, including total energy, dipole moment, HOMO–LUMO energies, and derived parameters (ΔE , η , σ , χ , μ , ω), highlighting their stability, electronic properties, and potential for molecular interactions.

3.3 ADMET and Toxicity Evaluation

A preliminary evaluation of the pharmacological safety of the five most promising compounds, IMPHY001665, IMPHY008016, IMPHY004968, IMPHY003432, and IMPHY003000, in this case, was conducted using an in-silico analysis of the absorption, distribution, metabolism, excretion, and toxicity. Analysis of Ames mutagenicity indicated that all the molecules with the exception of IMPHY003000 were positive on mutagenic potential which presents the possibility of genotoxic liability. Only one non-mutagenic compound was identified in the set which was IMPHY003000. The prediction of cardiotoxicity showed no hERG I inhibition in all compounds, although IMPHY001665 was the only compound that lacked hERG II inhibition and therefore may be considered a comparatively safer cardiac compound. The estimated maximum tolerated dose (MTD) of IMPHY00801655 and IMPHY00801656 in human beings was 0.339 to 0.723 mg/kg with the highest and lowest tolerances, respectively. In rats, the highest tolerance was recorded at 2.853 log mol/kg and the lowest tolerance at 1.712 log mol/kg, which is acute oral toxicity (LD 50) of the two drugs, indicating that IMPHY004968 was more tolerable than IMPHY001665. The estimates of chronic toxicity (LOAEL) of all compounds

(1.89- 2.014) indicated that the same compound has a similar long-term safety margin. Profiling of organ-specific toxicity showed that only IMPHY004968 had the potential of hepatotoxicity, whereas none of the compounds had the potential of skin-sensitizing effects. T. pyriformis toxicity values under ecological toxicity assessment were found to be between 0.283 and 0.467 and IMPHY001665 was the least toxic. Minnow toxicity values were lowest for IMPHY003000 (0.924) and highest for IMPHY001665 (2.775). The candidates were predicted to be toxicity classes 3-5 with IMPHY00655 having the highest LD 0 -5800 mg/kg (Class 5) which means no high risk of acute toxicity, and IMPHY008016 being the most toxic (1000 mg/kg, Class 3). The rest of the compounds (IMPHY004968, IMPHY003432, and IMPHY003000) were grouped as Class 4 since they showed moderate concentration of toxicity (LD 50 = 1190-2000mg/kg). All in all, IMPHY001665 (Bonducellin) was the best in terms of the safety profile, which does not include hERG II inhibition, LD 50, or hepatotoxicity, although it is Ames positive. In their turn, IMPHY008016 had the most acute toxicological threats, and IMPHY004968 and IMPHY003432 had moderate safety indices that should be additionally confirmed with the help of experimental toxicology (Table 3).

Table 3. Toxicity profiling of Top 10 compounds

S.No	Model Name	IMPHY Y 001665	IMPHY Y 008016	IMPHY Y 004968	IMPHY Y 003432	IMPHY Y 003000	IMPHY Y 003305	IMPHY Y 003529	IMPHY Y 004515	IMPHY Y 012446	IMPHY Y 011716
1.	AMES toxicity	No	No	No	Yes	No	Yes	Yes	No	No	No
2.	Max. tolerated dose (human)	0.723	0.339	0.414	0.475	0.435	0.485	0.276	0.247	0.192	-0.151
3.	hERG I inhibitor	No	No	No	No	No	No	No	No	No	No
4.	hERG II inhibitor	No	Yes	Yes	Yes	Yes	No	No	No	No	No
5.	Oral Acute Toxicity (LD50) Rat	1.712	2.524	2.853	2.316	2.519	2.094	2.236	2.572	2.774	3.084
6.	Oral Chronic Toxicity (LOAEL) Rat	1.948	1.916	2.014	1.945	1.89	2.099	1.696	1.835	1.36	1.17
7.	Hepatotoxicity	No	No	Yes	No	No	Yes	No	No	No	Yes
8.	Skin Sensitization	No	No	No	No	No	No	No	No	No	No
9.	T.Pyriformis toxicity	0.283	0.374	0.458	0.467	0.443	0.668	0.33	0.314	0.288	0.291
10.	Minnow toxicity	2.775	1.014	1.693	1.208	0.924	0.881	1.757	-0.163	2.114	1.385
11.	LD50 values (mg/kg)	3800	1000	1190	2000	1190	3600	2000	1800	2400	1900
12.	Toxicity class	5	3	4	4	4	5	4	4	4	4

Table 3 Provides a summary of predicted toxicity of IMPHY compounds namely mutagenicity, cardiotoxicity, hepatotoxicity, oral toxicity (LD50/LOAEL), skin sensitization and environmental toxicity and the values represent the level of toxicity and risk.

3.4 Bonducellin and its binding to METTL3 is maintained throughout a 200 ns simulation.

Molecular dynamics (MD) simulations were performed to evaluate the dynamic behavior and stability of the protein–ligand complexes under physiological conditions. Key parameters including RMSD, RMSF, and hydrogen bonding interactions were analyzed to assess the structural integrity and binding stability of the systems.

The root mean square deviation (RMSD) analysis was performed for 200 ns to assess the structural stability of the protein in complex with the novel compound (Bonducellin) and the UZH1A, along with the apo form of the protein. The apo protein had very minimal change with RMSD hardly going beyond ~0.50.8 nm signifying that the protein has an inherent structural stability even when no ligand is bound. On the other hand, the Bonducellin-complex displayed more variations but it fluctuated within the range of ~2.02.8 nm for most of the time. Interestingly, the Bonducellin RMSD trace did not show a significant drift indicating a strong binding to the pocket. By contrast, the drug-bound complex showed slightly higher RMSD values (~2.53.5 nm) with larger fluctuations, especially after ~60 ns. The larger changes represent more conformational flexibility of the protein- ligand system. In general, the RMSD data imply that Bonducellin produces a more stable complex as compared to an UZH1A as it preserves the structural features during the entire simulation time (Figure 3. a).

To further investigate residue-level dynamics, RMSF analysis was performed for all systems. The majority of residues in both ligand-bound complexes exhibited comparable fluctuation patterns, indicating that ligand binding does not significantly disrupt the global protein structure.

However, distinct differences were observed in specific regions. The UZH1A complex displayed higher fluctuations at key residues (notably around residues ~400–410 and ~490–500), reaching values above ~4.0 nm. In contrast, the Bonducellin complex showed relatively reduced fluctuations in these regions, indicating enhanced stabilization of flexible loops or binding-site residues. Importantly, residues involved in ligand binding exhibited lower RMSF values in the Bonducellin complex compared to the UZH1A, suggesting stronger local interactions and reduced mobility within the binding pocket. The apo form showed moderate fluctuations, confirming that ligand binding contributes to localized stabilization (Figure 3. b).

Hydrogen bonding interactions were analyzed to evaluate the strength and persistence of ligand binding. Both the Bonducellin and UZH1A complexes formed hydrogen bonds throughout the simulation, with counts ranging between 0 and 4 bonds over time.

The Bonducellin complex exhibited consistent hydrogen bond formation, frequently maintaining 1–3 hydrogen bonds across the trajectory, with intermittent peaks reaching up to 4 bonds. This indicates stable and sustained interactions with key residues in the binding site. Similarly, the UZH1A formed hydrogen bonds; however, the pattern was comparatively more intermittent, with frequent transitions between bonded and non-bonded states. Although occasional peaks of up to 4 hydrogen bonds were observed, the overall consistency was lower than that of Bonducellin. Figure 3. (a) Root Mean Square Deviation (RMSD) Figure 3. (b) Root Mean Square Fluctuation (RMSF) and hydrogen bond (H-bond) Figure 3. (c) profiles to describe the behavior of each of the complexes in the entire trajectory.

3.5 Bonducellin Suppresses Cell Proliferation, Hemolysis, Colony Formation, Migration, and Spheroid Growth in HCT116 Cells

The MTT assay was used to systematically assess the anticancer activity of Bonducellin at various concentrations (10, 25, 50, 75, 100, and 200 μ M) using the HCT116 colon cells, showed a 20% inhibition rate and increased cell viability after 48 hours of treatment at a dosage of 10 μ g/mL. 50% of the cells are viable at a concentration of 45 μ M. After 48 hours of treatment, cell viability was lower at concentrations of 50 and 75 μ M compared to 10 and 25 μ M. Bonducellin showed enhanced activity against cancer at the highest tested levels of 100 and 200 μ M, resulting in 10% cell survival after 48 hours. Cell viability decreased in a dose-dependent manner, resulting in a half-minimal inhibitory concentration IC₅₀ of 45 μ M (Figure 4). Bonducellin-treated cells showed strong anti-cancer activity against the HCT116 colon cancer cells, and their effectiveness increased with increasing concentrations and longer exposure times. These results demonstrate the therapeutic potential of Bonducellin in the management of colorectal cancer.

The hemolysis assay indicates that Bonducellin, treated with an IC₅₀ of 45 μ M, exhibits good hemocompatibility, as treated samples show intact red blood cell pellets with minimal hemoglobin release compared to the positive control (Figure 5). This is supported by quantitative data demonstrating ~100% cell viability at both 24 and 48 hours, with no significant time-dependent toxicity. Overall, these results suggest that Bonducellin does not induce erythrocyte membrane damage and is safe for further therapeutic evaluation.

A clonogenic assay was used to see if Bonducellin hinders long-term proliferation. A significant number of HCT116 cell colonies (428 ± 10 colonies) were seen in the control, as (Figure 6) illustrates. In contrast, the ability to form colonies and the quantity of colonies decreased significantly after 48 hours of treatment with an IC₅₀ concentration of Bonducellin. Colony formation was suppressed by the IC₅₀ dosage of bonducellin.

The migration experiment was used to determine whether Bonducellin reduces the HCT116 cells' ability to migrate. According to the cell migration experiment, HCT116 cells capacity to migrate was considerably reduced after 48 hours of bonducellin treatment (Figure 7). HCT116 cells were exposed to a 45 μ M Bonducellin IC₅₀ concentration for 48 hours. The migration inhibition rate was reduced by $7.8 \pm 1.2\%$ ($p > 0.001$) when the inhibition rate was measured for 0, 24, and 48 hours, suggesting that the medium concentration's inhibitory effect on migration was restricted.

HCT116 treated with the IC₅₀ (45 μ M) bonducellin seemed smaller and grew less than the control. The spheroid's outer layer, which is in charge of cell growth due to greater interaction with oxygen and nutrients, appears to be more damaged than in the control spheroids, and a decrease in size is observed ($p < 0.001$) (Figure 8). This finding suggests that Bonducellin inhibits the formation of CSC-like spheroids.

Bonducellin treatment on anchorage-independent proliferation was further examined using the soft agar assay. Bonducellin-treated HCT116 cells formed significantly fewer and smaller colonies than untreated controls ($p < 0.001$) (Figure 9). Together, these findings establish that Bonducellin effectively inhibits multiple hallmarks of tumorigenicity, including proliferation, colony formation, migration, and spheroid development.

3.6 Bonducellin Induces Apoptosis and Downregulates METTL3 Expression in HCT116 Cells

The pro-apoptotic effect of Bonducellin was assessed in non-transfected HCT116 cells using acridine orange/ethidium bromide (AO/EB) dual staining. Following treatment with Bonducellin at its IC₅₀ concentration, distinct morphological changes characteristic of apoptosis was observed under fluorescence microscopy. The treated cells exhibited bright orange-red nuclear staining, indicative of chromatin condensation and membrane compromise associated with late apoptotic stages. In contrast, untreated control cells displayed uniform green fluorescence, consistent with intact and viable nuclei (Figure 10). Quantitative analysis across ten randomly selected microscopic fields revealed a significant increase in early and late apoptotic populations in Bonducellin-treated cells compared with controls, confirming that Bonducellin triggers apoptosis in HCT116 cells.

In order to further determine the effect of Bonducellin on the expression of METTL3, the immunofluorescence technique was done in two groups, one control and the other using Bonducellin on HCT116 cells. The cells treated with Bonducellin showed a significant decrease in the intensity of the METTL3 fluorescence as compared to the non-treated controls and

indicated that the expression of METTL3 on the cellular level was inhibited by Bonducellin exposure (Figure 11). All these findings suggest that, in addition to causing apoptosis in colorectal cancer cells, Bonducellin has a down-regulatory effect on METTL3, which is one of the important regulators in tumor progression. This two-fold effect highlights the possible potential of Bonducellin as a therapeutic candidate that impacts on survival as well as epigenetic regulatory pathways in colorectal cancer.

4. DISCUSSION

Colorectal cancer (CRC) is the third most common cancer of its kind that is diagnosed globally and it has been a major therapeutic issue especially in the late and metastatic stages^{1,31}. Despite the fact that chemotherapy and targeted therapeutic advances have increased survival rates in patients, there are still patients who develop systemic toxicity, resistance to drugs, and recurrence of tumors. Therefore, a significant field of current research has been the identification of naturally occurring compounds with high potency as anticancer agents and good safety profiles. One of the bioactive flavonoids that are isolated in *Caesalpinia bonducella* is Bonducellin, which is reported to possess the antioxidant, anti-inflammatory, and anti-proliferative properties. Nonetheless, little has been done in defining the molecular targets and mechanistic basis of its action in CRC.

Hemolysis test proves that Bonducellin is hemocompatible and the erythrocytes were intact and little release of hemoglobin occurred in comparison to the positive control. The quantitative data indicates that the cell viability is high at 24 and 48 h, and there is no significant hemolysis ($p > 0.05$), which proves that this solution is safe to the red blood cells. We combined both *in silico* and *in vitro* in the current study to clarify the therapeutic value of Bonducellin in CRC with a focus on its binding with the RNA methyltransferase METTL3, one of the enzymes controlling m⁶A RNA modification. Virtual screening of phytochemicals filtered using Lipinski criteria with the help of docking predicted Bonducellin (IMPHY001665) as a leading candidate with high binding affinity (-9.2 kcal/mol) to S-adenosyl methionine (SAM) binding pocket of METTL3. The analysis of the interaction showed that Bonducellin interacts with several stabilizing hydrophobic and hydrogen-bonding contacts with the key catalytic residues, Glu481, Thr510, Trp398 and His482 implying a highly compatible and energetically favorable binding conformation.

This was further characterized with Conceptual Density Functional Theory (C-DFT) showing that Bonducellin has an intermediate HOMO-LUMO gap (0.16 eV) indicating that it has a good balance between stability and reactivity. Its polarity is sufficient because the dipole moment (1.8665 D) allows the drug to engage with proteins, and it is not excessively electronically reactive and therefore promotes off-target reactions. All these quantum characteristics justify Bonducellin as a suitable and unique METTL3-binding molecule.

The molecular dynamics of the 200ns simulation of the Bonducellin-METTL3 complex was also studied in dynamic behavior. There was a consistent value of the RMSD profile of the complex (not exceeding 0.8 nm), which means that the complex was stable in its conformation throughout the simulation. RMSF analysis showed that there was less flexibility in residues around the SAM-binding domain, and the hydrogen-bond analysis of the process showed the maintenance of the most important interactions in the course of the trajectory. All these results support the notion that Bonducellin is structurally stable and highly binding to METTL3, which is the behavior of a small-molecule inhibitor.

These computational insights were given experimental support using biological validation in HCT116 colorectal carcinoma cells. The treatment with Bonducellin resulted in a dose-dependent decrease in cell viability with an IC₅₀ of 45 μ M and significant decrease in colony-forming efficiency. The treatment of Bonducellin had a significant delay in wound healing, which indicates the lack of motility probably caused by METTL3 inhibition. These results are in line with the existing data that links METTL3 to epithelial mesenchymal transition and invasive phenotypes in CRC.

Bonducellin considerably decreased the diameter of the spheroid and the size of the colony in three-dimensional spheroid and soft agar assays, which suggests that the protein could inhibit the CSC-related self-renewal and reverse the effects of METTL3, an oncogenic protein. This result is in line with previous studies that METTL3 helps tumor progression, tumor stemness, and therapeutic resistance by means of m⁶a -dependent post-transcriptional regulation.

Pro-apoptotic effects of Bonducellin were verified through AO/EB dual staining, which showed the morphological features of the apoptotic cell which includes nuclear condensation and disintegration of the membrane. Assessment in a quantitative manner showed that there was a significant rise in the populations of apoptotic after treatment. In addition, immunofluorescence studies revealed that the exposure to Bonducellin significantly decreased the fluorescence levels of METTL3 as compared to the untreated controls indicating that Bonducellin not only suppresses METTL3 activity but also decreases its expression.

5. CONCLUSION

These results indicate that Bonducellin has a variety of complementary antitumor actions direct binding and METTL3 inhibition, inhibition of cell proliferation and colony-formation, inhibition of migratory and invasive behavior, inhibition of CSC-like sphere formation, and CSC-like induction of apoptosis. The fact that METTL3 expression is downregulated also indicates the likelihood of feedback in which Bonducellin alters the oncogenic signaling pathways via METTL3.

To sum up, our multidisciplinary computational and experimental research was able to discover Bonducellin as a potential multidimensional natural inhibitor of METTL3 with anticancer effects in colorectal carcinoma. Bonducellin suggests a two-pronged approach to reducing tumor growth and progression by attacking both proliferative and CSC-like subpopulations. The future studies with transcriptomic, proteomic, and *in vivo* studies will be required to determine the downstream signaling pathways governed by METTL3 inhibition and confirm Bonducellin as a lead scaffold in developing next-generation anti-METTL3 medications.

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Figure 1. Schematic representation of HOMO, LUMO, and molecular electron cloud of top compounds

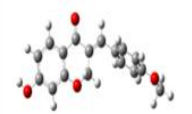
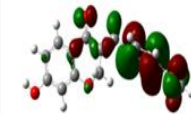
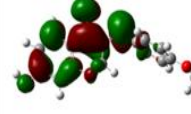
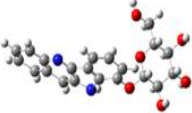
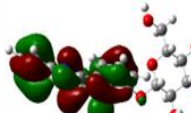
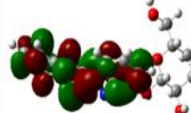
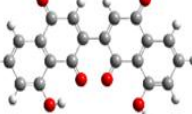
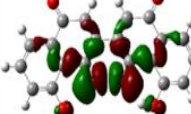
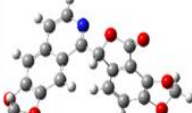
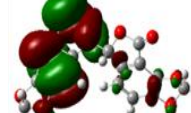
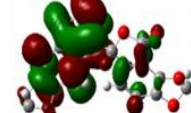
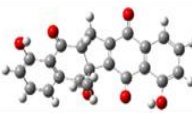
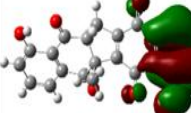
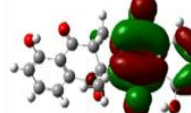
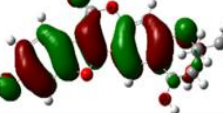
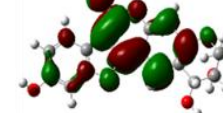
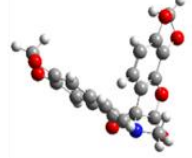
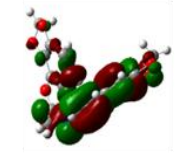
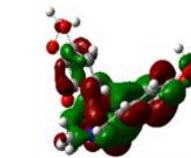
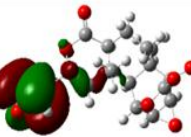
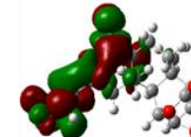
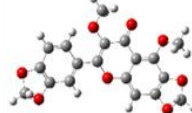
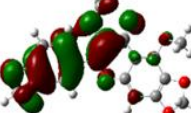
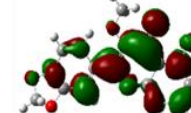
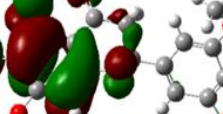
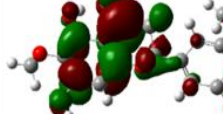
Compound	Optimized	HOMO	LUMO
IMPHY001665			
IMPHY003305			
IMPHY003529			
IMPHY008016			
IMPHY004515			
IMPHY004968			
IMPHY012446			
IMPHY011716			
IMPHY003000			
IMPHY003432			

Figure 1. This figure presents Frontier molecular orbital analysis of ten compounds with optimized molecular structures and HOMO and LUMO distributions of the same. The following representative images of five compounds indicate the electronic properties and reactive sites of molecules with green and red surfaces representing HOMO and LUMO electron density range, respectively.

Figure 2. Identification of Top Ten Natural Compounds Using Molecular Docking

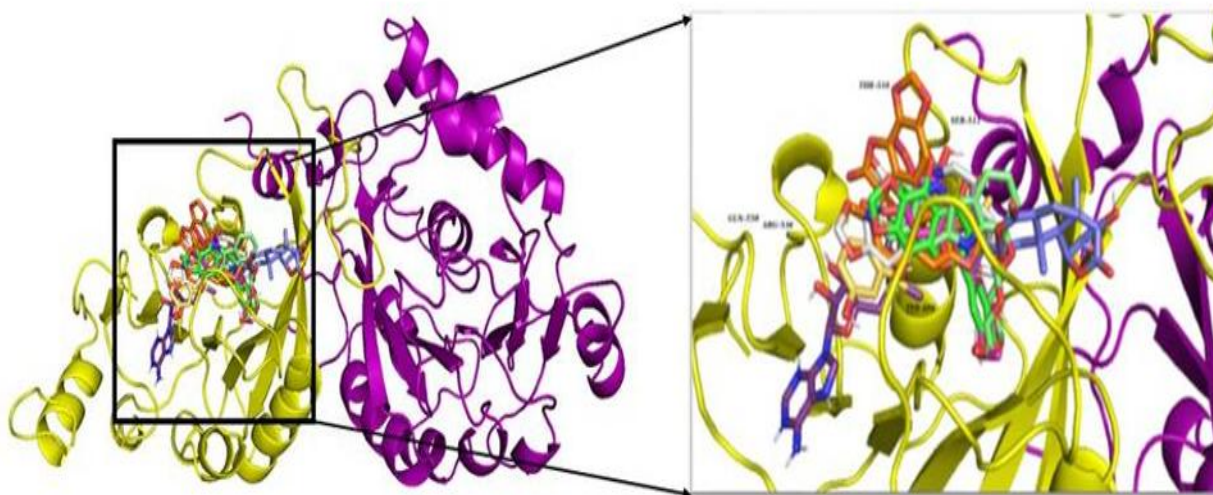
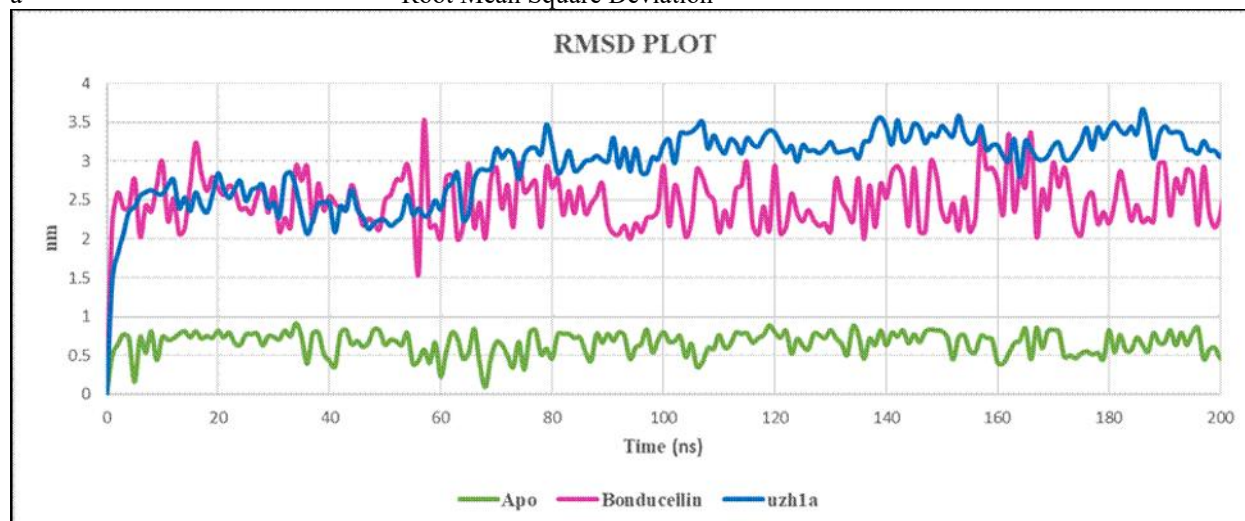


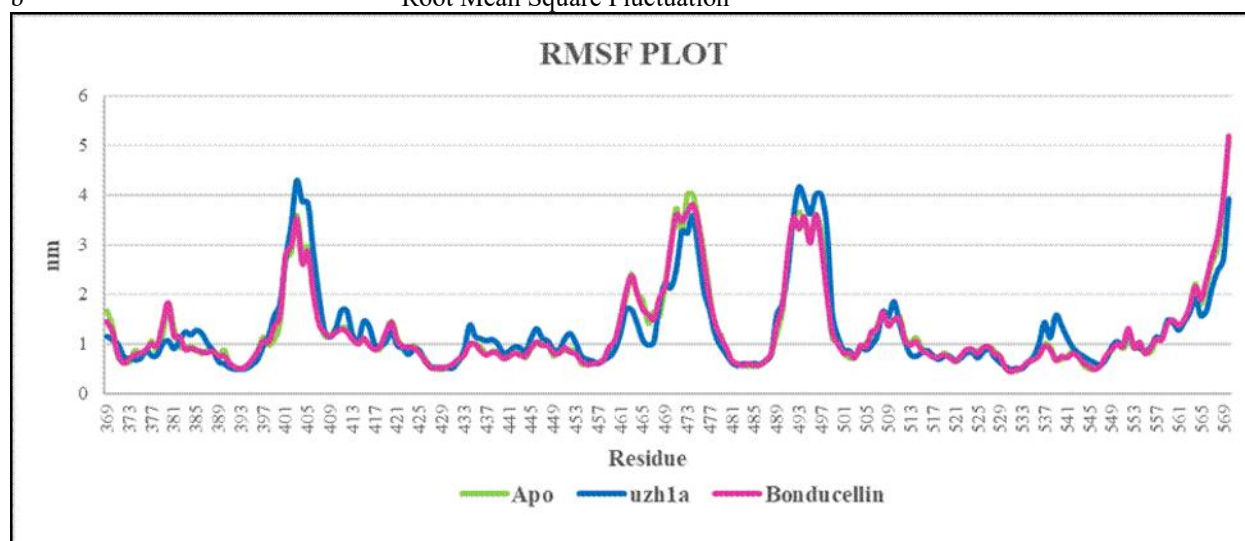
Figure 2. Structural representation of the protein METTL3–ligands complexes highlighting the binding pocket. The overall protein structure is shown in cartoon representation, with different domains colored distinctly yellow (METTL3) and magenta (METTL14).

Figure 3. Bonducellin and its binding to METTL3 is maintained throughout a 200 ns simulation.

a Root Mean Square Deviation



b Root Mean Square Fluctuation



c Hydrogen Bond

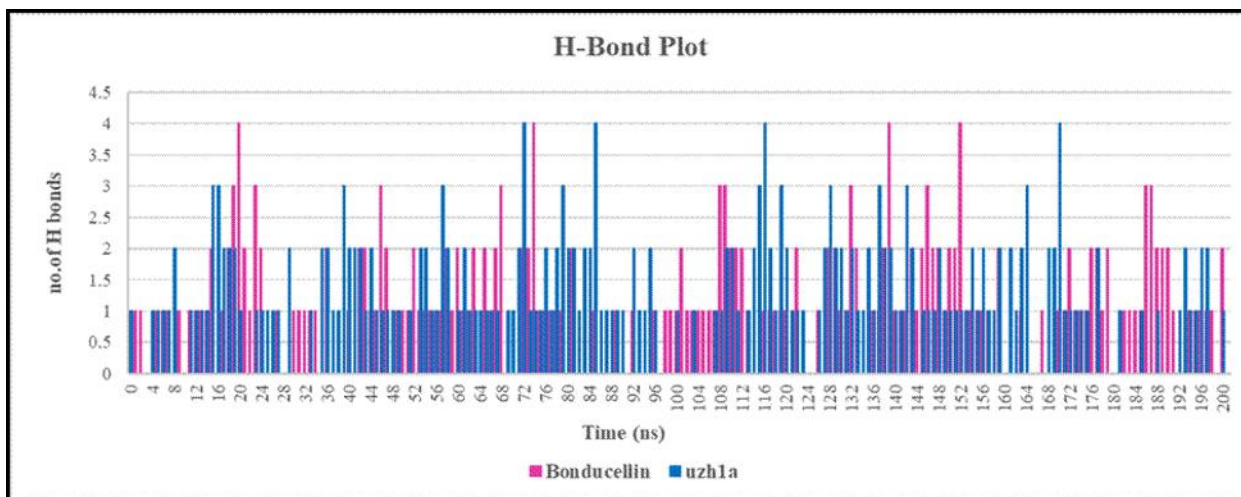


Figure 3. (a) RMSD Plot: RMSD profiles of apo, Bonducellin-bound, and uzhl1a-bound complexes over 200 ns indicating overall structural stability and conformational deviations. (b) RMSF Plot: Residue-wise RMSF analysis showing flexibility variations and localized stabilization upon ligand binding. (c) H-Bond Plot: Time evolution of hydrogen bonds highlighting interaction stability of Bonducellin and approved drug complexes.

Figure 4. Cell Proliferation Assay

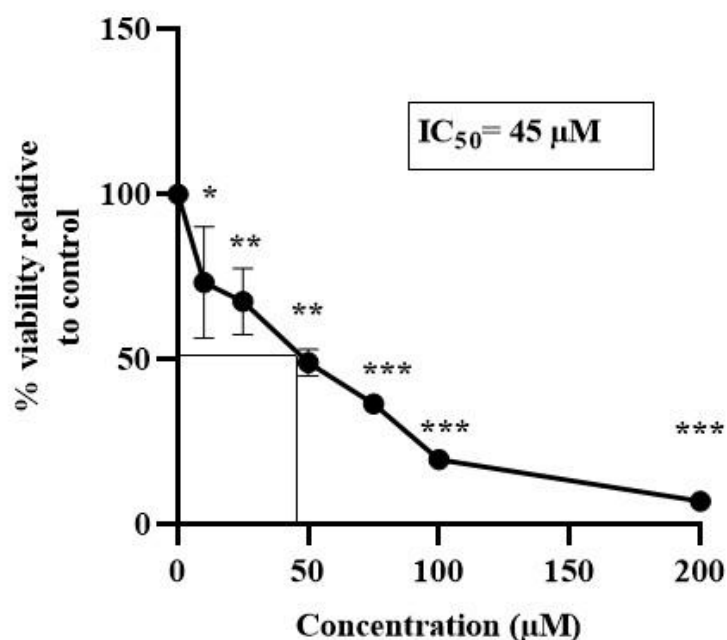


Figure 4. Cell proliferation assay using MTT, a reduction in colon cancer cell proliferation was observed. IC₅₀ values were calculated and found to be 45 μM for HCT116 colon cancer cell line. The graph represents mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5. Hemolysis

a

b

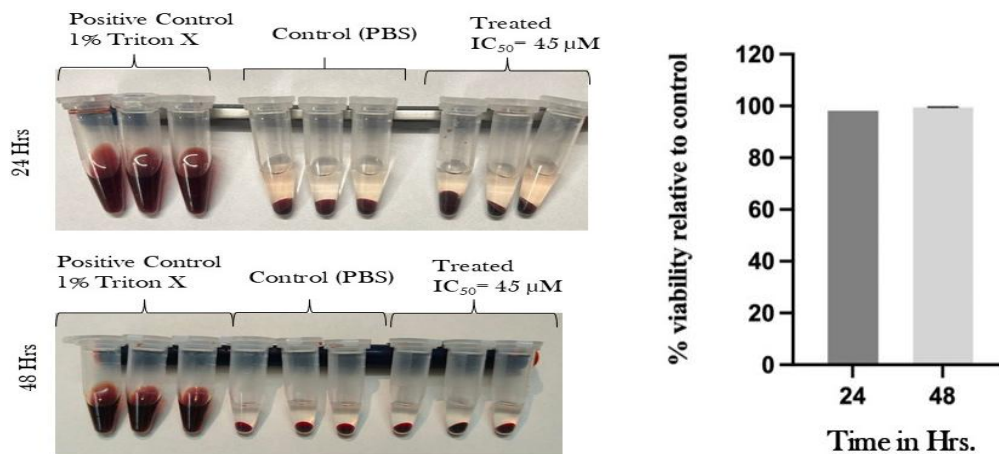


Figure 5. Hemolysis assay evaluating the hemocompatibility of Bonducellin. (a) Representative images of erythrocytes after treatment showing intact cell pellets with minimal hemoglobin release compared to the positive control (complete hemolysis). (b) Quantitative analysis of % cell viability at 24 and 48 h relative to control. Data are presented as mean $\pm$ SD. No significant hemolysis was observed (ns, $p > 0.05$).

Figure 6. Colony Formation Assay

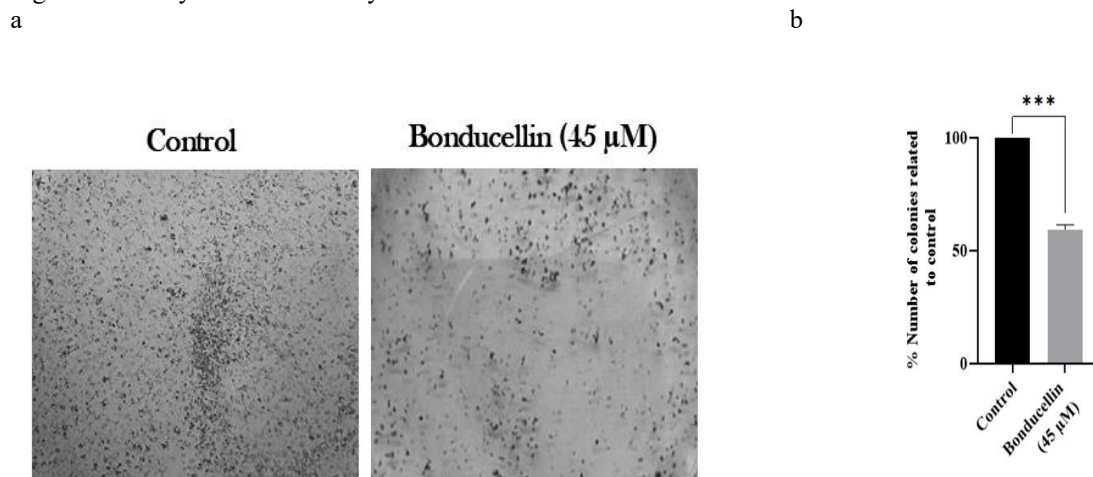
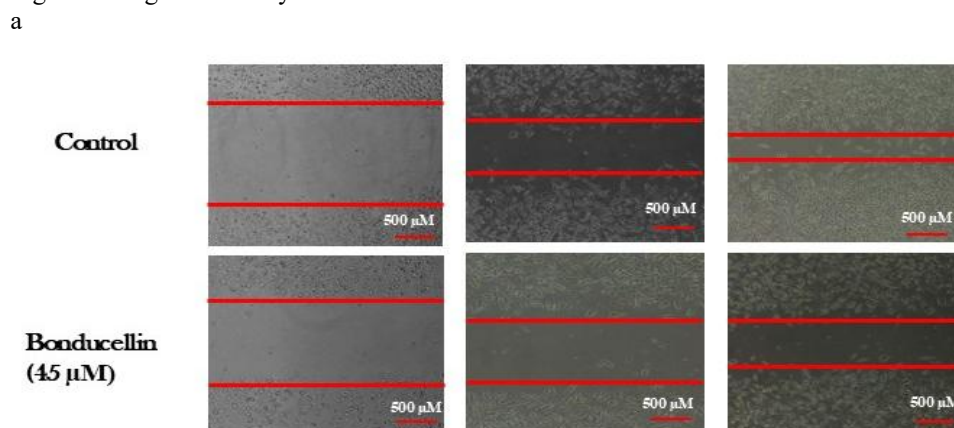


Figure 6. Colony formation assay. (a) Wells treated with bonducellin showed a reduction in the number of colonies compared to control wells. (b) Graphical representation of the effect of bonducellin on the percentage of colonies relative to control; graph shows mean $\pm$ SEM, *** $p < 0.001$.

Figure 7. Migration Assay



b

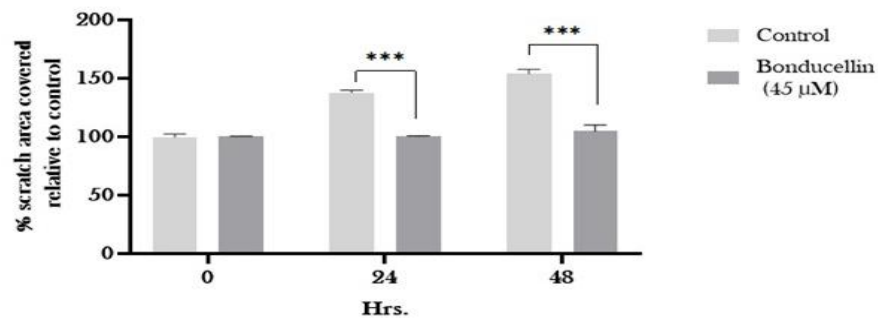
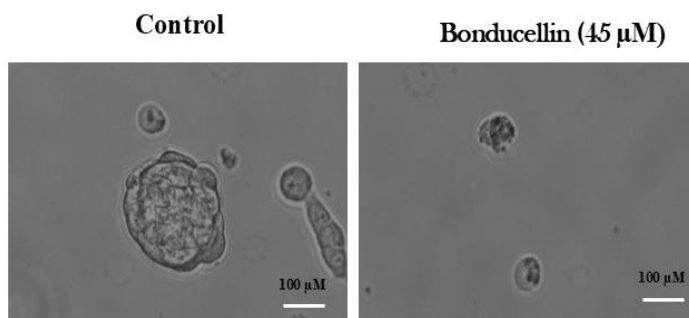


Figure 7. 2D Scratch

Wound closure of HCT116 cells was observed at 0 h, 24 h, and 48 h in the control wells, whereas it was notably reduced in bonducellin treated cells. (b) Graphical representation of the effect of bonducellin on the percentage of Scratch/wound area covered relative to control; graph shows mean $\pm$ SEM, ***P < 0.001.

Figure 8. Colonospheroid Assay

a



b

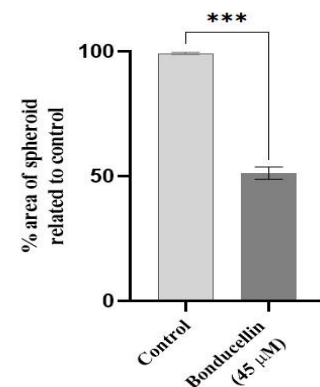
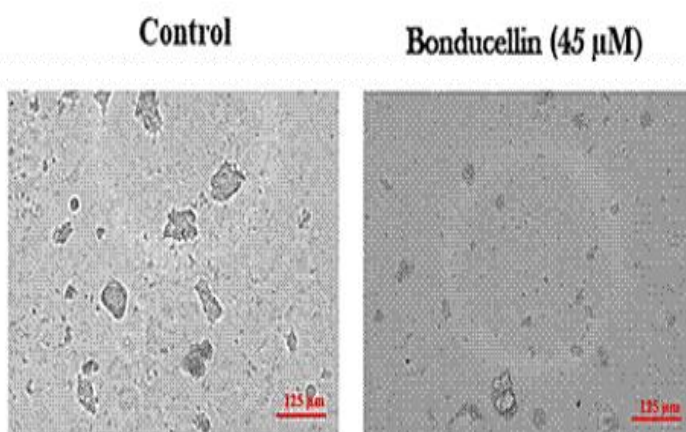


Figure 8. Colonospheroid assay. (a) HCT116 cells treated with bonducellin have shown reduced colon sphere growth when compared with the control. (b) Graphical representation of the effect of bonducellin on the percentage of colon spheroids; graph shows mean $\pm$ SEM, ** p < 0.01, *** p < 0.001.

Figure 9. Anchorage Independent Assay

a



b

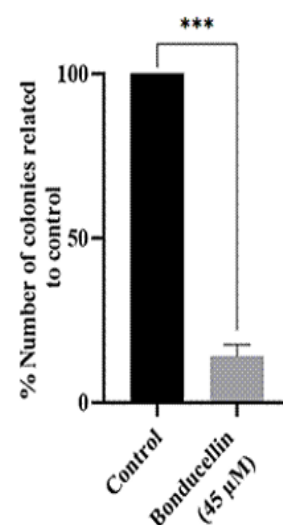


Figure 9. Anchorage-independent growth assay showing the effect of Bonducellin (45 μM) on colony formation. (a) Representative microscopic images of control and treated cells demonstrating reduced number and size of colonies upon

treatment (scale bar: 125 μ m). (b) Quantification of colonies expressed as % relative to control. Data are presented as mean $\pm$ SD. Bonducellin significantly reduced colony formation (** $p < 0.001$).

Figure 10. AO/EB Assay

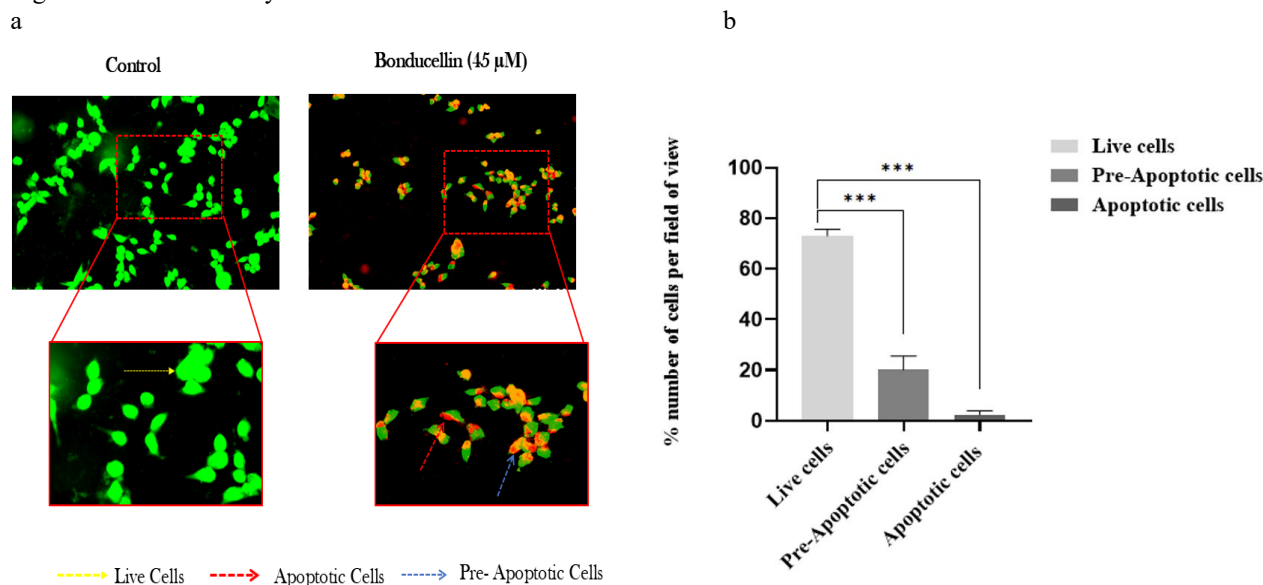


Figure 10. Dual AO/EB fluorescent staining. (A) HCT116 cells treated with bonducellin, the nucleus showed yellow-green fluorescence by acridine orange and orange fluorescence by ethidium bromide staining. The apoptotic cells are observed as orange-red fluorescence. The fluorescence image confirms cells undergo apoptosis once treated with bonducellin and stained with AO/EB. (B) Graphical representation showing the effect of bonducellin in inducing apoptosis in HCT116 cells compared to controls. Data are presented as mean $\pm$ SEM; ** $p < 0.01$, *** $p < 0.001$.

Figure 11. Immunofluorescence Assay

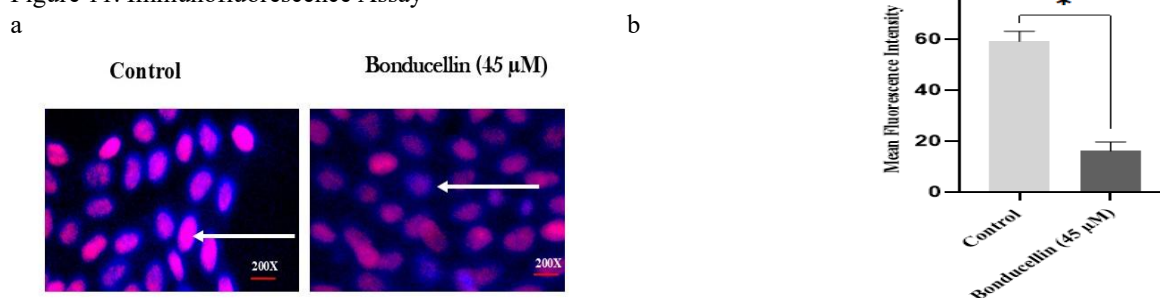


Figure 11. METTL3 expression in immunofluorescence and quantitative intensity analysis. (a&b) 200x immunofluorescence images of the expression of METTL3 (red) and nuclear counterstaining with DAPI (blue). (a) Control cells have moderate METTL3 fluorescence with homogenous nuclear morphology and Treated cells have lower signal intensity of METTL3, which means that METTL3 is down-regulated after treatment (arrows). (b) The mean fluorescence intensity quantification reveals that the levels of METTL3 are significantly reduced in the treated group as compared to control ($p < 0.05$) and they are expressed in mean plus SEM.