

## SYNTHESIS AND EVALUATION OF ANALOGUES INDOLE-BASED AGENTS AS TUBULIN INHIBITORS USING CELL LINES

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### ABSTRACT

Combretastatin A-4 (CA-4) is a natural vasodilating agent (VDA) derived from the bark of *Combretum caffrum* and has demonstrated selective activation of tumor vasculature, leading to vessel rupture. It effectively inhibits tubulin polymerization by binding to colchicine sites, inducing mitotic arrest and cytotoxicity at low nanomolar concentrations. Structural activity relationships (SARs) have identified essential modifications, including the 3'-hydroxyl group of the B ring and various substitutions at this position to enhance antitumor activity. CA-4 phosphate (CA-4P), a water-soluble prodrug, has entered clinical trials for its vascular effects in combination with cytotoxic drugs. Inspired by the tubulin-inhibitory properties of vincristine and bromoindirubin, researchers have explored the integration of an indole moiety into CA-4 derivatives to improve efficacy. Given the challenges of drug resistance in microtubule-targeting agents, this study aims to develop novel tubulin inhibitors incorporating the indole ring structure.

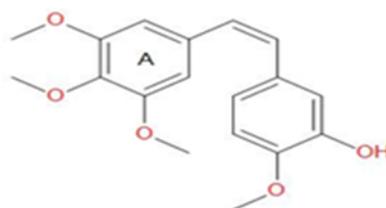
**KEYWORDS:** Combretastatin A-4, tubulin inhibitor, vasodilating agent, antitumor activity, drug resistance.

### INTRODUCTION

Combretastatin A-4 (CA-4, Figure 1) was first described by Pettit et al. It is a natural product defined by. From the bark of the *Combretum caffrum* tree in South Africa. In 1989.<sup>[1]</sup> As a potential vasodilating agent (VDA), CA-4 can distinguish between normal and tumor blood vessels and selectively activate the tumor vasculature, leading to vessel rupture.<sup>[24]</sup> Additionally, CA-4 is effective in preventing cancer from affecting cells. Regarding tubulin dynamics, CA-4 inhibits tubulin polymerization by binding to colchicine sites and causes mitotic arrest.<sup>[5]</sup> However, the trans-isomer of CA-4 is no better than CA-4 (Figure 1). For example, CA-4 exhibits cytotoxicity at low nanomolar concentrations,

while the trans-isomer inhibits cell growth in the micromolar concentration range.<sup>[6]</sup> CA-4 has recently become the focus of clinical attention, 4 and various CA-4 analogs and derivatives have been developed and evaluated for antitumor activity in vitro. Based on these derivatives, several specific interactions (SARs) of CA-4 were identified and shown in Figure 2. These SARs contain the A ring substituted with 3,4,5-trimethoxy and stilbene-linked A and B rings. Both cis configurations are required for the protective activity of the compounds.<sup>[7-9]</sup> The 3'-hydroxyl group of the B ring has been shown to be used in the interaction of CA-4 with tubulin, and this particular moiety can be replaced by other suitable modifications.<sup>[10]</sup> Various CA-4 derivatives with different properties coexist at the 3' position of the B ring, such as halogen atoms (such as fluorine or bromine), amines, boric acids, nitro groups, amides, alkoxy groups or acyl groups. and evaluated for antitumor activity.<sup>[11-13]</sup> Among these derivatives, CA-4 phosphate (CA-4P, Figure 1) is a water-soluble CA-4 prodrug that has been identified and entered clinical trials for its potential to induce vascular effects. It will be used together with cytotoxic drugs used in cancer treatment.<sup>[14]</sup> Vincristine and vinblastine are anti-inflammatory drugs extracted from *Catharanthus roseus* and used in cancer treatment, and this effect is due to the interaction of these drugs with tubulin.<sup>[15]</sup>

For the design of combretastatin, it was found that it was necessary to combine another ring with an indole, replacing one ring with an indole moiety. Because vincristine, rosette<sup>[16]</sup> and bromindirubin have indole moiety and good tubulin polymerization inhibitory properties.<sup>[17]</sup> As with many chemotherapeutic drugs, drug resistance through changes in microtubule dynamics is still a significant problem in paclitaxel treatment.<sup>[18]</sup> Our goal is to develop tubulin inhibitors on the indole ring.



**Fig. 1: Structure of Combretastatin.**

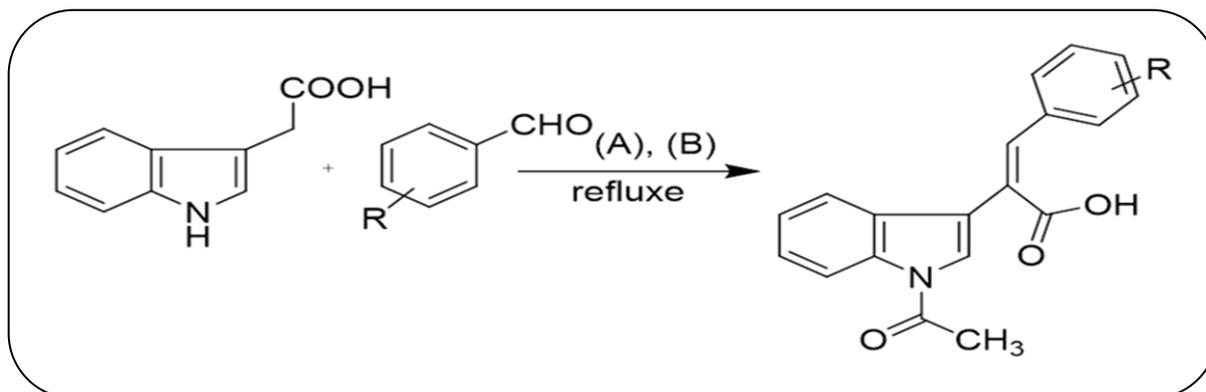
## METHODS

Combretastatin 2-(1-acetyl-1H-indol-3-yl)-3-(phenyl) propene analogues (2a to 2y) were synthesized by condensation of indole-3-acetic acid and different products. Tri ethylamine and acetic anhydride (shown in Scheme 1). For prevention, seven types of cancer such as lung cancer (A-549), ovarian (IGROV-1), prostate (DU-145), breast cancer (HEP-2), leukemia (THP-1) and breast cancer (using MCF- 7).

## Materials and Methods for Immunofluorescence Confocal Microscopy

THP1 cells ( $8 \times 10^4$  cells/well) were seeded on 18 mm square coverslips in six plates. Cells were allowed to adhere as desired for 24 h and 2 days before injection. Use 1  $\mu$ M paclitaxel as a positive control. After the fixation period, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton-X (Sigma-Aldrich, MO, USA) in PBS for 5 min. Cells are closed. 10% goat serum is left at room temperature for 20 minutes. The microbiome was detected using a monoclonal  $\alpha$ -tubulin antibody (Sigma Corporation, Cream Ridge, USA) diluted 1:100 in 0.1% Triton X-100 and Alexa in PBS at 37°C. Tube 1 hour Fluorine 488-labeled secondary antibody (Invitrogen, Carlsbad, USA), diluted 1:1,000 in PBS, 1 hour at room temperature. Cells were washed three times in PBS and stained with DAPI diluted 1:1,000 in PBS.<sup>[19]</sup> A coverslip was mounted on a glass slide, and cells were

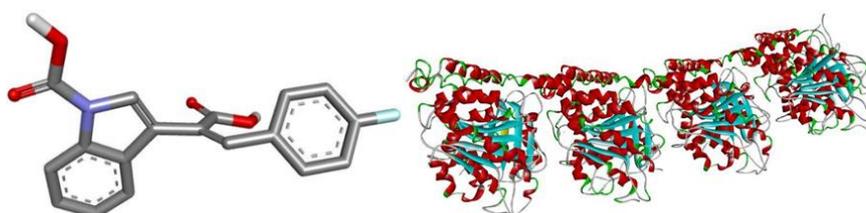
observed using a confocal microscope and an Olympus Fluoview FV1000 laser scanning microscope (Olympus Inc., Center Valley, USA).



**Scheme 1: Reagents and conditions. (A) Triethylamine; (B) acetic anhydride, refluxed for 6 to 10 h.**

### Material and methods for molecular docking

**(a) Ligand preparation:** Tubulin regulation can be found in the Protein Data Bank (PDB ID: 1SA0).<sup>[18,19]</sup> The selected ligands were validated before preparing for analysis, and the second assignment required slight modification. 2D models of the ligands were created using Chem Draw Pro 12.0 and converted to 3D models. All 3D models were reduced using the MMF94 force field method and then the open babel 3.1.1 program was used to generate the data required for fitting.



**Fig. 2: (a) Structure of ligand (b) Binding pocket.**

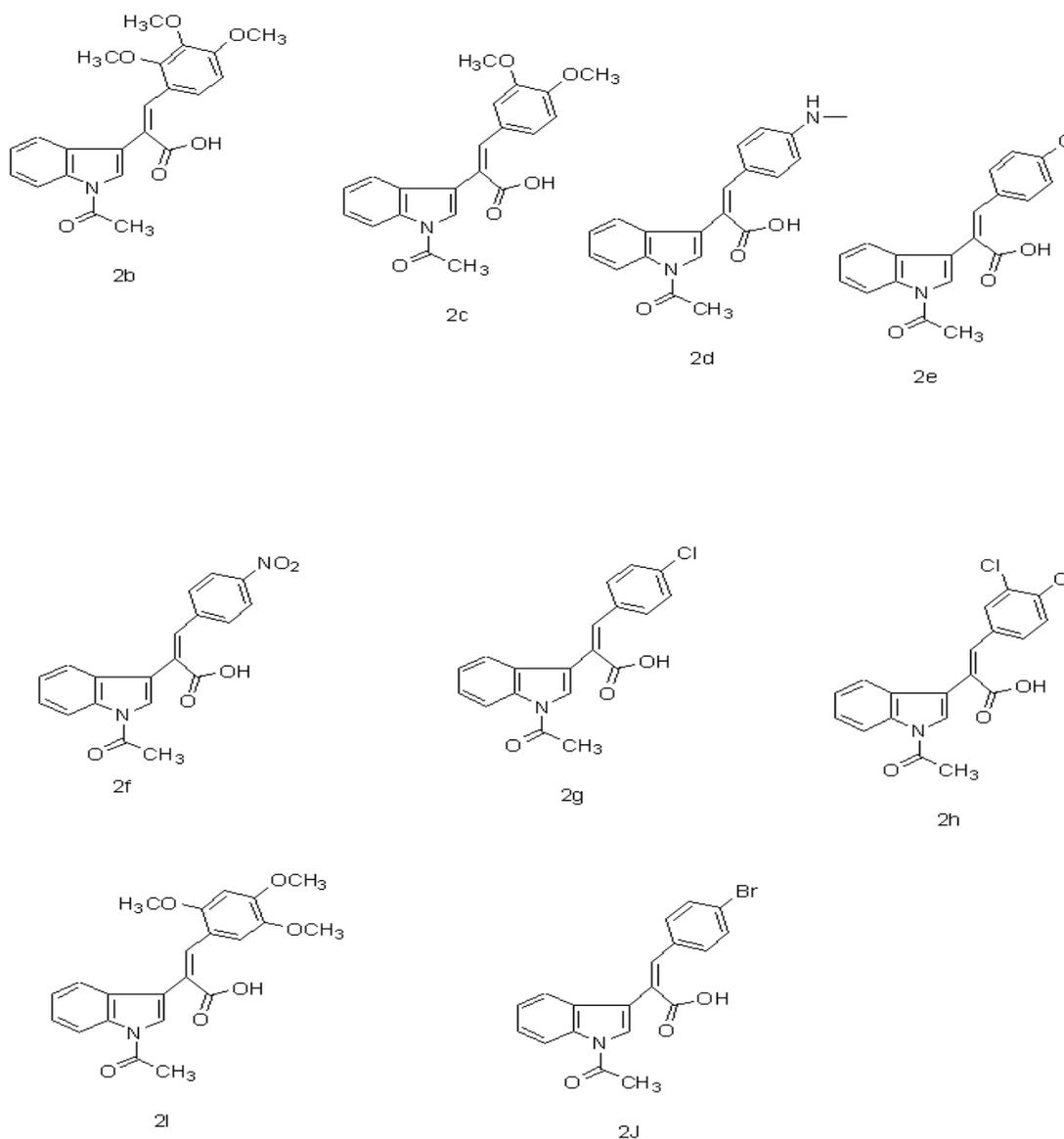
**(b) Protein Preparation:** After all the different structures were considered, targets were selected for the protein database [PDB:1SA0] using the RCSB database. Remove normal ligands, water, and heteroatoms from the protein crystal structure in preparation for docking. The program used is "BIOVIA Discover Visualizer 2021".

### RESULTS AND DISCUSSION

**Chemistry:** The current work involves the synthesis of combretastatin analogues specifically having free carboxyl functionality at the bridgehead as part of the target compound. This change should provide a rotation constraint that could help increase synthetic power for the need to combat disease. Scheme 1 shows the strategy for preparing target molecules by condensing phenylacetic acid with various substituted benzaldehyde. Ten combretastatin analogs (2a to 2j) were prepared via indole condensation. 3-acetic acid (1) was prepared from various substituted aldehydes using triethylamine and acetic anhydride as previously described (scheme 1). [twenty three]. Monitor the reaction by TLC.

Compounds were purified by column chromatography using 60 to 120 pore size silica gel (48 to 63% yield). All synthesized compounds (Figure 3) were purified. Characterization was performed by spectral methods such as infrared

spectroscopy (IR), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR) ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, NMR) (Figure 4 and Table 1). N-acetyl group protecting the cone. H-3 is not protected due to its proximity to the N-acetyl carbonyl group. H-3 is not protected due to its carbonyl position at the  $\beta$  position, but is no less protected than H-7 because it is not affected by the magnetic anisotropy of the carbonyl group. group. No change in the incorporation of H-3 was observed when H-2' was subjected to double irradiation. These results show that H-2' and H-3' are not very close. This is only possible if the two rings are relative to each other. If the two rings are in the cis configuration, two light beams of H-2 will cause the H-3 signal to be higher due to the nuclear Overhauser effect (nOe). It has also been confirmed that free OH ( $2''$ ) when combined with a  $2''$  hydroxyl group is used to form lactone because the geometry is as follows: The proximity of OH and C=O facilitates lactone formation. H-3 is less protected than H-7 because the guard cone of the carbonyl group and the guard cone of the benzene ring plane are within the guard cone. Due to lactone formation, the IR value of the carbonyl group changes from 1.710 to 1.763. Combining phenylacetic acid with an aldehyde successfully completed the PSchorr addition reaction<sup>[24]</sup>, producing combretastatin in the cis configuration. Based on the above analysis, it can be obtained that the composite material has two bell trans configurations.



**Fig 3: Chemical structure of all synthesized compounds (2a- 2j).**

**Anticancer activity:** All synthesized compounds were evaluated for anti-inflammatory effects (Table 2): lung (A-549), ovarian (IGROV-1), prostate (PC-3), and breast (MCF-7) cancer cells. Compounds 2j, 2k and 2r showed good activity against THP-1, DU-145 and MCF-7 cell lines. However, compounds 2d (IC<sub>50</sub> = 0.80 and 0.37  $\mu$ M) and 2y (IC<sub>50</sub> = 3.6  $\mu$ M) showed significant inhibitory activity. When the synthesized compound has a simple indole moiety, it binds to the colchicine binding site. The vinblastine binding site opens a space between the binding sites, as both vincristine and vinblastine molecules are very large molecules, and additional binding material is incorporated, as the actual small, vinblastine rolls into place. The corresponding IC<sub>50</sub> values were calculated and listed in Table 1.

**Table 1: In vitro cytotoxicities against four different tumor cell lines.**

Cell line	PC-3	IGROV-1	A-549	MCF-7
TISSUE	Prostate	Ovary	Lungs	Breast
Compound code				
2a	12.29	8.00	7.68	693
2b	7.26	8.38	14.78	486
2c	7.77	7.59	14.88	476
2d	2.30	1.32	<2.50	180
2e	7.94	10.04	14.90	309
2f	7.94	11.29	8.76	330
2g	6.68	10.39	9.42	435
2h	6.40	12.46	12.28	316
2i	4.39	7.09	8.85	265
2j	7.27	10.85	9.65	735
Standard	6.27	10.49	5.75	428

As shown in Table 1, all of the synthesized compounds showed good in vitro cytotoxicity against all four tumor cell lines tested; IC<sub>50</sub> values were below 800 nM in all cases except the compound. Compound (2d) shows all compounds tested. It is the most cytotoxic against all four cell lines, with IC<sub>50</sub> values ranging from 1 to 180 nM. Importantly, 2d showed lower cytotoxicity than the standard. Compared to the product, compound 2a had strong cytotoxicity only on MCF-7 cells. Compounds 2a, 2b, 2c, 2e, 2f, 2g and 2i showed good cytotoxicity against parent model MDA-MB-231, MCF-7 and A549 cells. However, compounds 2a and 2b, especially 2e, showed lower cytotoxicity than CA-4, and this lower cytotoxicity was generally attributed to the large 3'-O-substituted carbon ether moiety due to long side chains that prevent binding. Tubulin is the active part of the colchicine domain. These results therefore indicate that mutation of CA-4 with a large mutation at the C-3' position may lead to decreased activity.

### Molecular Docking of 2d

The highest binding energy shown by compound 2d which is having  $\Delta G = -8.8$  kcal/mol and forming a two hydrogen bond with ARG946 amino acid which is having bond distance of 2.30 and 2.37 Å. enlisted in Table 2



Fig. 4: (a) 2d interaction of 2d (b) 2d interaction of standard.

Table 2: Drug likeness, ADME and Toxicity properties of synthesized compound.

Comp code	MW	RB	HBA	HBD	LogP	GI Abs	BBB permeability	P-gp Substrate	Log K	LD 50
2a	341.17	3	4	1	1.84	High	No	No	-5.49	260
2b	317.28	4	6	1	1.63	Low	No	No	-5.96	250
2c	430.07	3	4	1	2.10	High	No	No	-5.94	240
2d	263.27	2	4	1	2.41	High	Yes	Yes	-5.64	260
2e	317.24	2	7	1	2.39	High	No	Yes	-5.76	245
2f	329.24	3	5	1	2.92	High	No	Yes	-5.12	260
2g	329.73	3	5	1	1.53	High	No	No	-6.68	275
2h	272.28	2	2	1	1.72	High	Yes	Yes	-5.10	245
2i	296.18	2	2	1	2.71	High	Yes	Yes	-5.10	230
2j	296.19	2	2	1	2.95	High	Yes	Yes	-5.10	230

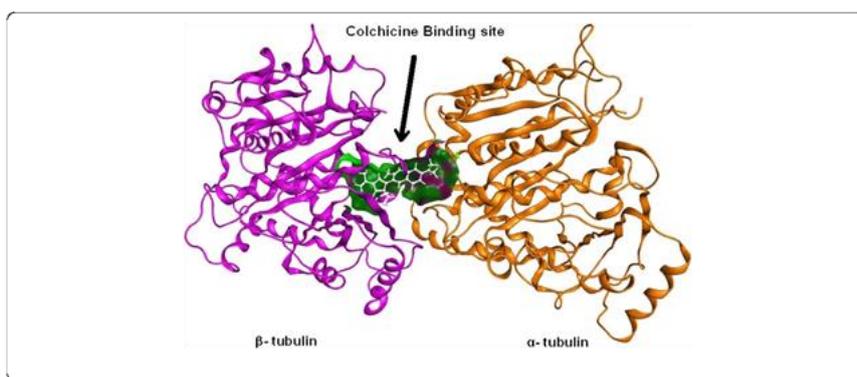


Fig 5: Colchicine binding site at the interface between  $\alpha$  and  $\beta$  subunits of tubulin. The inhibitor 2d is shown in silver color.

**Experimental:** Reagents were purchased from Sigma-Aldrich (Missouri, USA), Loba Chemie (Mumbai, India) and Central Drug House (New Delhi, India) and used without further purification. All yields refer to the product isolated after purification. These products are characterized by spectral data (IR,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra).

Spectra measured in DMSO- $d_6$  by TMS (0.00 ppm). Infrared (KBr tray) spectra were recorded on a Fourier transform infrared thermal spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA).

**Typical experimental procedure for synthesis (2a to 2j):** Add 2 millilitres of triethylamine and 4 millilitres of acetic anhydride to a combination of substituted benzaldehyde and indole acetic acid. After six to ten hours of reflux, chill the mixture and add thirty-five percent of the six millilitre HCL. Let it sit for the whole evening. When the ice cubes are added, mix them well to create crystals. After that, sift them and store them till they dry. **Scheme 1**

**Characterization data:** As mentioned in the previous scheme total synthesized compound from 2a to 2j But 2d showed positive results as compared to other compounds so we are showing only interpretation of 2d compound.

**IR Spectra of 2d:** FTIR range: 3113.78  $\text{cm}^{-1}$  (Ar. C-H str), 3105  $\text{cm}^{-1}$  (Ali. C-H str), 1552.35  $\text{cm}^{-1}$  (C=N str), 1508.96  $\text{cm}^{-1}$  (C=C), 1416.37  $\text{cm}^{-1}$  (N-H), 1262.06  $\text{cm}^{-1}$  (C-N str), 716.19  $\text{cm}^{-1}$  (C-S-C str), 574.41  $\text{cm}^{-1}$  (C-Clstr). Fig 6

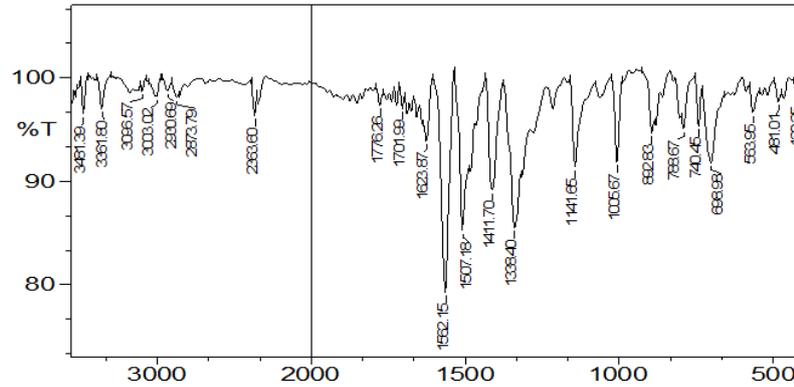


Fig. 6: IR Spectra of 2d.

### NMR Spectra of 2d

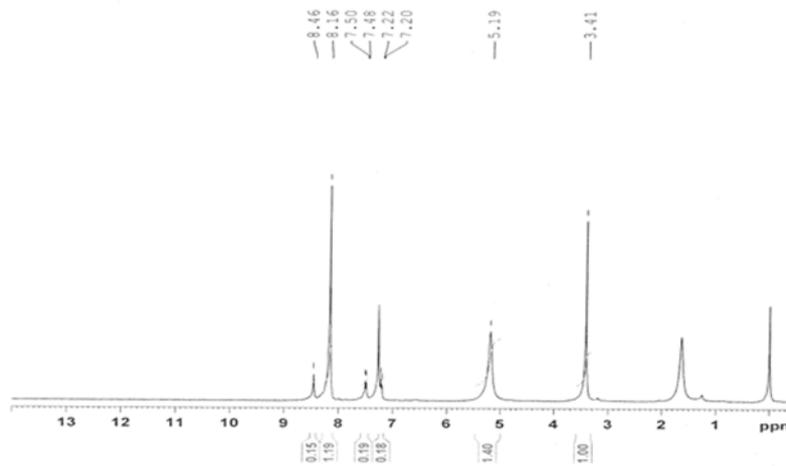


Fig. 7: NMR spectra of 2d.

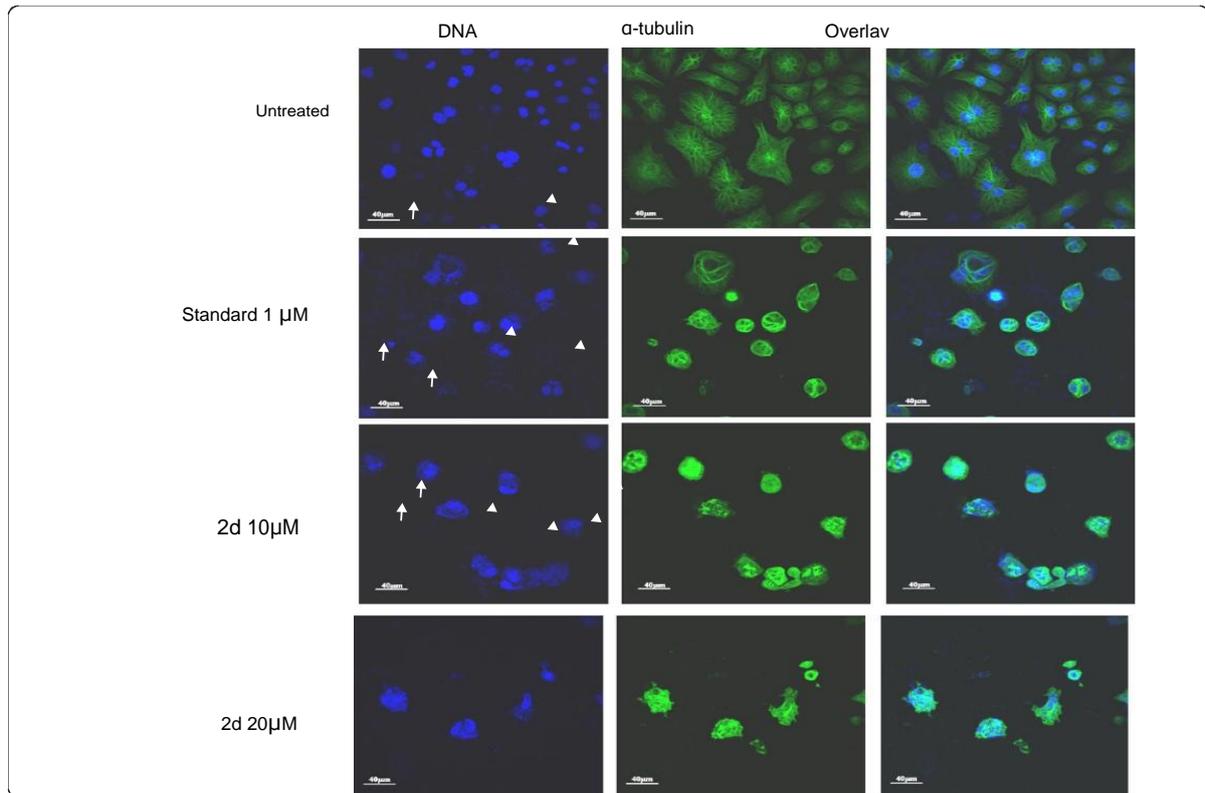
### Interpretation of 2d

$^1\text{H-NMR}$  (500MHz,DMSO,TMS=0): 2.5 (3H, s, OAc), 4.69(1H,d,J=12.5Hz, $\beta$  H-7), 5.83 (1H,d,J=6.0 Hz, H-2), 3.76 (1H,dd,J=4.3, 3.6Hz,H-4), 4.1(1H,d,J=4.5 Hz,H-6),4.27 (1H,d,J=12.5Hz,  $\alpha$ H-7), 7.57 (2H, d, J=7.0, 7.3, aromatic),4.16 (1H,dd, J=4.3, 4.5Hz, H-5), 7.9 (2H, d, j=7.0, aromatic), 4.98 (1H,dd,J=3.6, 3.7Hz, H-3), 3.2, 3.4, 5.2,5.3, 5.5 (5H, OH, D2O exchangeable), 7.7 (1H, d, J=7.3,aromatic).

### Biological evaluation

#### Cytotoxicity by MTT assay

It is now well established that tetrazolium salt reduction is a reliable method to assess cell proliferation. Metabolically active cells reduce the amount of yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide), thereby decreasing the balance of NADPH and NADH, due in part to the dehydrogenase action. Plasma formazan obtained using spectrophotometric methods can be solubilized and measured. This measurement evaluates the rate of cell division and, on the other hand, the decrease in cell viability due to metabolic processes leading to apoptosis or necrosis.



**Fig. 8: Effect of 2d on the microtubules of A-549 cells.**

**Cell culture media:** 10% fetal bovine serum (FBS), 1X Dulbecco phosphate buffered saline (DPBS), Germany, DMEM medium supplemented with MTT reagents, MP Biomedicals, MP Biomedicals, 0.25% trypsin-EDTA solution, Germany Germany DMSO, Thermo Fisher Scientific' It was purchased from., USA, Germany Cell culture treatment T-25 flask, cell culture grade, from Biolite, Merck, Thermo Fisher Scientific Inc., USA, 10 mL serological pipette and 96-well plates, 1.5 mL, from Sunny Instruments, Nunc, 2 mL and 5 mL tubes, Tarsons, India.

**Procedure:** Trypsinization and aspiration of cells grown in T-25 flasks into a 5 mL centrifuge tube were performed. A cell pellet weighing 300 x g was produced after centrifugation. With DMEM-HG media, the cell count was modified to ensure 200 $\mu$ l of suspension contained around 10,000 cells. 200 $\mu$ l of the cell suspension was added to each well of the 96-well microlitre plate. The plate was then incubated at 37 $^{\circ}$ C with five percent carbon dioxide in the air for a full twenty-four hours. The used medium was aspirated after a 24-hour period. 200 $\mu$ l of various test drug concentrations were introduced into their corresponding wells. After that, the plate was left to incubate for twenty-four hours at 37  $^{\circ}$ C in an environment 5% CO<sub>2</sub>. The moment the plate was removed from the incubator, the drug-containing media was aspirated. Following the addition of 200  $\mu$ l of media comprising 10% MTT reagent to each well to achieve the final concentration of 0.5 mg/mL, the plate was incubated for three hours at 37  $^{\circ}$ C in an environment of 5 % CO<sub>2</sub>. Without causing any disruption to the crystals that had grown, the culture media was fully withdrawn. After adding 100  $\mu$ l of DMSO solubilization solution, the plate was mildly agitated in a gyratory shaker to dissolve the formazan that had formed. A device called a microplate reader was used to determine the absorbance at 630 and 570 nm wavelengths. Upon subtracting the background and blank, the growth inhibition % was calculated. The amount of test drug needed to impede cell growth by fifty percent was found using the dose-response graph for the cell line (IC<sub>50</sub>).

## CONCLUSIONS

A series of new combretastatin analogues were synthesized from the condensation of indole-3-acetic acid and differently substituted aldehydes, and their structures were determined by various spectroscopic techniques such as infrared, mass spectrometry, and nuclear magnetic resonance. [<sup>1</sup>H], [<sup>13</sup>C], HMBC and HSQC). When a hydroxyl group is present at the second position of the aldehyde, the configuration is formed, forming a lactone. Moreover, this situation is confirmed by nOe (double irradiation of H-3, H-4' density increases). The antibiotics of the compounds were evaluated. Effects on microtubule structure were determined using confocal microscopy dose-dependent disruption, and microtubule loss indicated apoptosis after 2 days. The binding of the compound at the colchicine binding site is determined by molecular docking studies. This helps rationalize the preventive effect of inhibitors. The carbonyl oxygen (carbonyl group of indole) forms a hydrogen bond with the sulfhydryl group of Cys241β. This is similar to the interaction of colchicine with tubulin. Another hydrogen bond forms between the hydroxyl group of the carboxylic acid moiety of 2d and the nitrogen of Leu255β. These two hydrogen bonds play an important role in stabilizing the 2D conformation of the colchicine binding site. The effects of the synthesized compounds indicate that they could be promising anticancer drugs. To confirm its effectiveness, additional in vivo experiments are needed to address the anti-inflammatory properties in cancer treatment.

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