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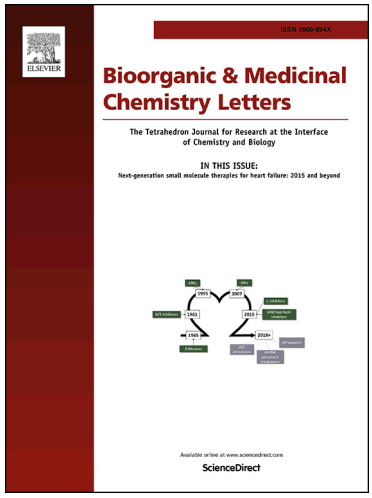

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PII: S0960-894X(19)30294-X
DOI: https://doi.org/10.1016/j.bmcl.2019.05.008
Reference: BMCL 26428

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 4 February 2019
Revised Date: 3 May 2019
Accepted Date: 5 May 2019

Please cite this article as: Govindaiah, P., Dumala, N., Grover, P., Jaya Prakash, M., Synthesis and biological evaluation of novel 4,7-dihydroxycoumarin derivatives as anticancer agents, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.05.008

Synthesis and biological evaluation of novel 4,7-dihydroxycoumarin derivatives as anticancer agents

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Abstarct:

A series of novel 4,7-dihydroxycoumarin based acryloylcyanohydazone derivatives were synthesized and evaluated for antiproliferative activity against four different cancer cell lines (**A549, HeLa, SKNSH, and MCF7**). Most of the compounds displayed potent cytotoxicity with IC_{50} values ranging from 3.42 to 31.28 µM against all the tested cancer cell lines. The most active compound, **8h** was evaluated for pharmacological mechanistic studies on cell cycle progression and tubulin polymerization inhibition assay. The results revealed that the compound **8h** induced the cell cycle arrest at G2/M phase and inhibited tubulin polymerization with IC_{50} = 6.19 µM. Experimental data of the tubulin polymerization inhibition assay was validated by molecular docking technique and the results exhibited strong hydrogen bonding interactions with amino acids (ASN-101, TYR-224, ASN-228, LYS-254) of tubulin.

Key Words: 4,7-dihydoxycoumarin, acryloylcyanohydarzone, anticancer, cell cycle, tubulin, molecular docking.

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Cancer is a group of diseases resulting in the uncontrolled division and involving in the frequent spatiotemporal changes in the cell physiology. The main reasons for the manifestation of cancer are an unhealthy diet, environmental pollution, overuse of drugs, hormonal imbalance and some genetic mutations [1]. No single therapeutic agent has been available for the 100% successful treatment of cancer. The incomplete understanding of cancer causing mutations and random errors in DNA replication renders difficulties to design novel anticancer agents for treating cancer[2]. Hence, the need of novel anticancer drugs is still persistent. Researchers are trying hard to identify potential drug molecules that would successfully pass the preclinical studies in the new drug development protocol [3] and pave the way for treating cancer in a better method. Recently, few novel organic compounds (coumarins, hydazones, benzthiazoles, etc) have gained much attention in this regards [4–9].

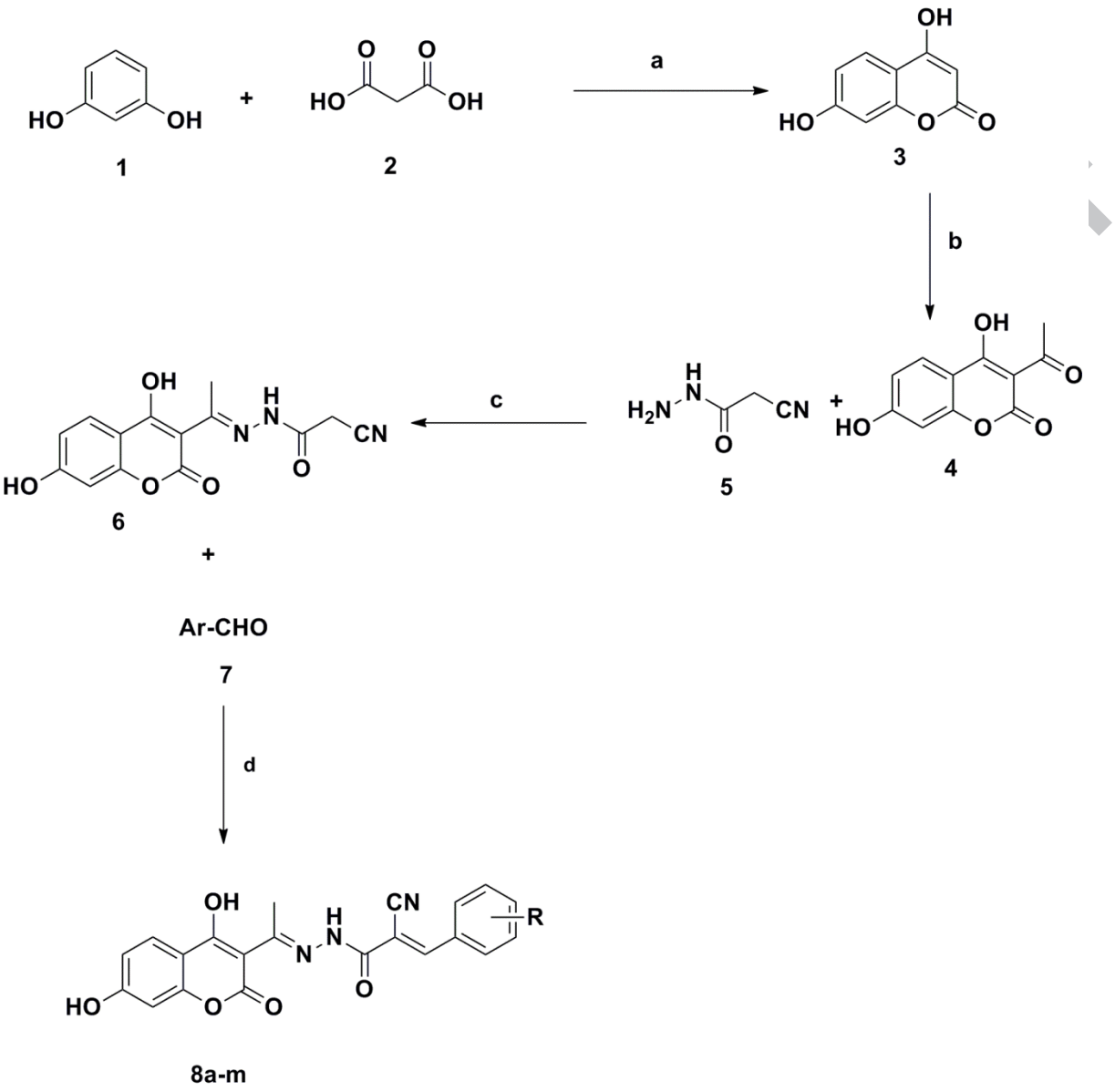
4,7-Dihydroxycoumarins are one of the important class of benzopyrones having several medicinal properties like antimicrobial[10], anti-tubercular[11], antileishmanial[12], antioxidant[13], antidiabetic[14] and anticancer[15]. These 4,7-dihydroxycoumarin can be prepared from the reaction of resorcinol by reacting with malonic acid [16]. The hydroxycoumarin based compounds could exert good cytotoxic activity against different cancer cell lines like promyelocytelukemia-derived (HL-60), urinary bladder carcinoma-derived (EJ) [17], MCF-7 [18] and Hela cell lines [19]. The coumarin-based chalcones, acryloylcyano hydrazide and pyridine derivatives effectively inhibited liver cancer (Hep G2) and leukemia (K562) [6]. Comarin based acryloylcyanohydrazones were evaluated for cytotoxic activity against HaCaT (human keratinocytes) cells [20]. These compounds exhibit anticancer activity by several mechanisms:1) Inhibition of non-structural 5B protein (NS5B) [21], 2) Inhibition of the enzyme Protein kinase CK2 [22]. 3)Target action with estrogen [23] and COX-2 [24] receptors. From through literature study we found that the major targets for anticancer agents are tubulins, metabolites, DNA, topoisomerase etc.

Tubulin is a prominent target for the anticancer drugs, due to it's involvement in wide range of cellular processes *i.e.* cell proliferation, migration, signaling, and trafficking in eukaryotic cells [25]. The clinically available drugs such as vincristine, vinblastine, colchicine and paclitaxel exhibit their anticancer activity by inhibiting the mitosis through interaction with tubulin. These drugs alters the tubulin activity in different ways [26] for producing the activity. The development of resistance and toxicity are major problems associated with the existing

antitubulin drugs. The discovery of antimitotic agents has led to new approaches in cancer chemotherapy. The mitosis is a vital phase in the cell cycle for many critical proteins. Tubulin, kinetochore and some other essential proteins are synthesized in this phase [27,28]. These proteins are involved in the formation of a chromatid during cell division which assists in the attachment with the spindle fiber on chromosome. There are few reports on novel class of coumarin derivatives being tested an antimitotic drugs with G2/M phase arrest [29–31].

Based on the above reported studies we understood the potential of hydroxycoumarin derivatives in treatment of cancer. Some reasearchers reported the cyotoxic properties of cyanohydarazone moiety. To the best of our knowledge, no literature is available on 4'7-dihydroxycoumarin based cyanohydrazone moieties with cytotoxic property. All these factors encouraged us to design coumarin based acryloylcyanohydrazone derivatives (**8a-m**) as anticancer agents. The target coumarin based acryloylcyanohydrazone derivatives were synthesized from the starting material, resorcinol in a sequence of chemical reactions, depicted in scheme 1. The compounds were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR and ESI-MS spectral techniques. The newly synthesized compounds were evaluated for their biological activity by both computational as well as experimental works. The most active compound (**8h**) was subjected to cell cycle analysis and tubulin polymerization inhibitory activity to identify the pharmacological mechanism studies. To find out the type of binding interactions between compounds and tubulin protein, simulation was performed using molecular docking technique.

All the target compounds (**8a-m**) were synthesized according to Scheme-1. The coumarin based acryloylcyanohydrazones (**8a-m**) were synthesized from resorcinol (**1**) in a sequence of reactions. First, resorcinol reacted with malonic acid (**2**) in presence of $POCl_3$ that gives 4,7–dihydroxycoumarin (**3**).



Scheme 1: Synthesis of coumarin based acryloylcyanohydrazones (**8a-m**). **Reagents and conditions:** a) $POCl_3$, $ZnCl_2$, 60 ^oC, 12h b) CH_3COOH, $POCl_3$, reflux, 3h; c) ethanol, reflux, 4h; d) ethanol, piperidine, reflux, 3-4h.

Acetylation of compound **3** with glacial acetic acid in the presence of phosphorus oxychloride yields compound **4** [32,33]. The compound **4** on condensation with the cyanoacetohydrazide (**5**) produced the key intermediate **6** finally, the compound **6** condensed with appropriate aromatic aldehydes (**7**) in presence of piperidine base. The reaction the mixture was poured into ice cold dilute HCl which afforded the title compounds (**8a-m**) in good yield. The newly synthesized

compounds were purified by recrystallization method with DMF/ethanol (1:5) solvent mixture. The purity was determined by Thin Layer chromatography (TLC) method. Further, devoid of additional absorption band/devoid of additional signal in FT-IR/^{1}H NMR, ^{13}C NMR reflects the good purity of the synthesized compounds. The structures of the compounds were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR, and ESI-MS spectral techniques.

All the synthesized coumarin based acryloylcyanohydrazone derivatives were evaluated for antiproliferatve activity using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay against four human cancer cell lines (**A549, HeLa, SK-N-SH, MCF7**) and normal rat kidney cell line (**NRK-49F**) using doxorubicin as reference drug. The results are summarised in Table-1. Thes synthesized compounds were showed good to excellent cytotoxicity with IC_{50} values ranging from 3.42 to 31.28 µM against all the tested cancer cell lines. The potency of the compounds varies with respect to substitution on the simple phenyl ring. Hydrazide-hydrazone backbone is more potent when cyano group is attached to hydrazone moiety, which tends to increases the activity of compounds. From the figure 3B it was noticed that cyano group was involved in the formation of hydrogen bond with the target. The same was performed with the compound without cyano group that exhibited less interactions (Figure 4B) with the target compared to compound having cyano group. The *para*-substituted compounds (**8b, 8e, 8g, 8h and 8j**) and *ortho*-substituted compounds (**8k** and **8m**) exhibits high potency compared to *meta*-substituted compounds (**8i** and **8l**). Among the *para*-substituted compounds, compounds (**8b**, **8e** and **8h**) with an electron releasing group have more potency than the compounds (**8g** and **8j**) with an electron withdrawing group. Introduction of additional electron releasing group(s) in *para*-substituted compounds gives compounds **8c**, **8d** and **8f**. These compounds retain the cytotoxic activity but less potent than monosubstituted compounds.

Table-1: *In vitro* cytotoxicity results of target compounds (**8a-m**) against human cancer cell line A549, HeLa, SKNSH, MCF7 along with normal rat kidney cell line NRK 49F.

Sample Code	R	IC_{50} (in µMol)				
		A549	**HeLa**	**SKNSH**	**MCF7**	**NRK 49F**
8a	**H**	14.82 ± 0.61	15.75 ± 0.86	13.52 ± 0.71	19.22 ± 0.48	55.45 ± 0.62
8b	**4-OH**	6.23 ± 0.18	6.02 ± 0.28	8.12 ± 0.35	7.32 ± 0.58	61.31 ± 0.55
8c	**3-OCH_3, 4-OH**	12.63 ± 0.23	10.46 ± 0.32	14.35 ± 0.56	13.65 ± 0.54	55.42 ± 0.14
8d	**3,4-OCH_3**	14.32 ± 0.65	13.52 ± 0.26	21.53 ± 0.45	14.35 ± 0.42	45.28 ± 0.36
8e	**4-OCH_3**	8.15 ± 0.48	7.85 ± 0.26	9.46 ± 0.39	8.94 ± 0.73	96.10± 0.89
8f	**3,4,5-OCH_3**	15.32 ± 0.35	18.46 ± 0.35	14.61 ± 0.18	11.34 ± 0.05	55.34 ± 0.36
8g	**4-Br**	11.43 ± 0.13	10.56 ± 0.26	12.05 ± 0.14	9.85 ± 0.06	49.64 ± 0.11
8h	**4-$N(CH_3)_2$**	4.31 ± 0.04	5.14 ± 0.16	6.09 ± 0.32	3.42 ± 0.52	53.41 ± 0.26
8i	**3-NO_2**	21.9 ± 0.41	24.64 ± 0.29	28.26 ± 0.09	16.35 ± 0.24	46.53 ± 0.09
8j	**4-NO_2**	9.64 ± 0.26	8.36 ± 0.05	10.26 ± 0. 15	8.20 ± 0.21	43.22 ± 0.10
8k	**2-NO_2**	11.35 ± 0.26	10.31 ± 0.36	12.26 ± 0.18	9.18 ± 0.39	68.59 ± 0.57
8l	**3-Br**	22.15± 0.54	24.28 ± 0.82	31.28 ± 0.36	19.64 ± 0.67	59.39 ± 0.38
8m	**2-Cl**	14.23 ± 0.18	11.78 ± 0.41	14.02 ± 0.34	8.88 ± 1.41	55.89 ± 0.17
Doxo		6. 22 ± 0.03	7.01 ± 0.015	6.42 ± 0.56	9.86 ± 0.12	24.22 ± 0.18

IC_{50} is the 50% inhibitory concentration of the samples, and the results were represented as average values ± standard deviation.

Doxo = Doxorubicin used as positive control (Standard)

From the screening results we are concluded that the compounds **8b**, **8e**, **8h and 8j** were showed excellent cytotoxicity against all cancer cell lines. The compound **8h** has exhibited highest cytotoxicity than standard drug doxorubicin and further pharmacological mechanistic studies were evaluated.

To uncover the pharmacological mechanism, the highly potent compound **8h** was selected against MCF-7 cell lines by flow cytometry method. After exposure of MCF-7 cells to compound **8h** at 4µM for 24 h, there was a significant increase in the percentage of cells at Sub G_0 phase as compared to control (Fig.1A). In addition, accumulation of cells was detected at

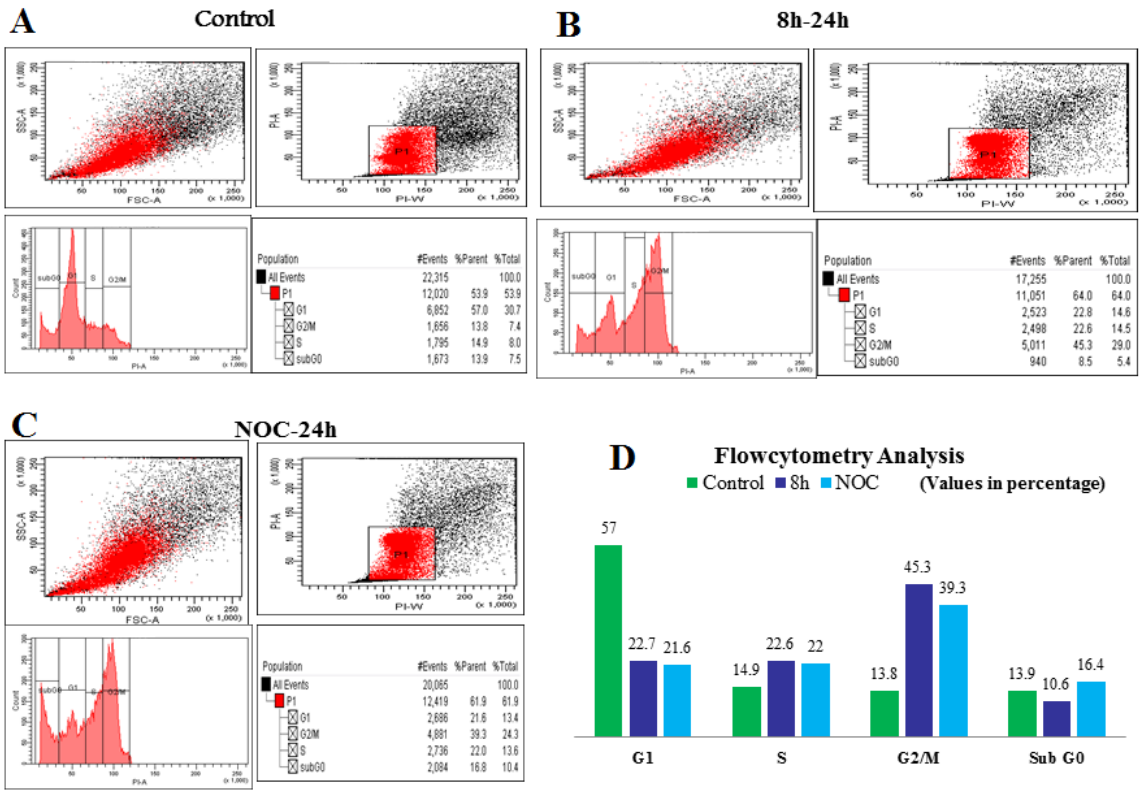


Figure 1: Flow cytometric analysis of MCF-7 cell lines treated with synthesized compound (**8h**) and standard drug nocodazole. A) Exposure with negative control DMSO B) Cell cycle disruption of compound **8h** for 24h treatment at indicated concentration analysis of MCF-7 cell line C) Cell cycle disruption of Reference drug nocodazole treatment at indicated concentration analysis of MCF-7 cell line D) Bar graph represents mean ± in SD at least three independent experiments.

G2/M phase by almost 4 folds compared to the control [from 13.9% in the vehicle group (Fig.1A) to 45.3% after 24h (Fig. 1B). On the other hand, the reference drug nocodazole showed 39.3% of G2/M arrest (Fig. 1C)] These results suggested that the compound **8h** inhibited MCF-7 cells proliferation through cell cycle arrest at G2/M phase followed by cell death. These results were in good agreement with the previously reported findings [34].

Tubulin is one of the major targets for chemotherapeutic agents. These agents are called as antimitotic agents since they act at the mitotic phase and stop the mitosis process by inhibiting tubulin action. These drugs attack the cancer cells at both primary and metastatic phase. These therapeutic agents are primarily bound to the *beta*-tubulin protein in the mitotic spindle.

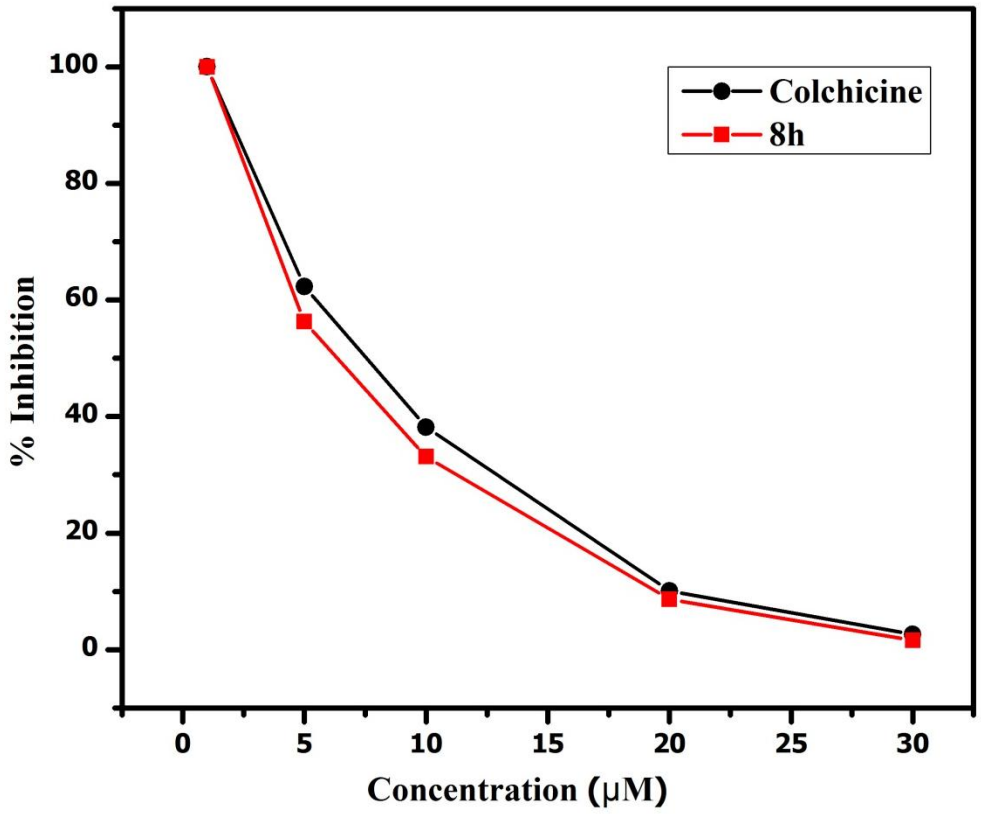
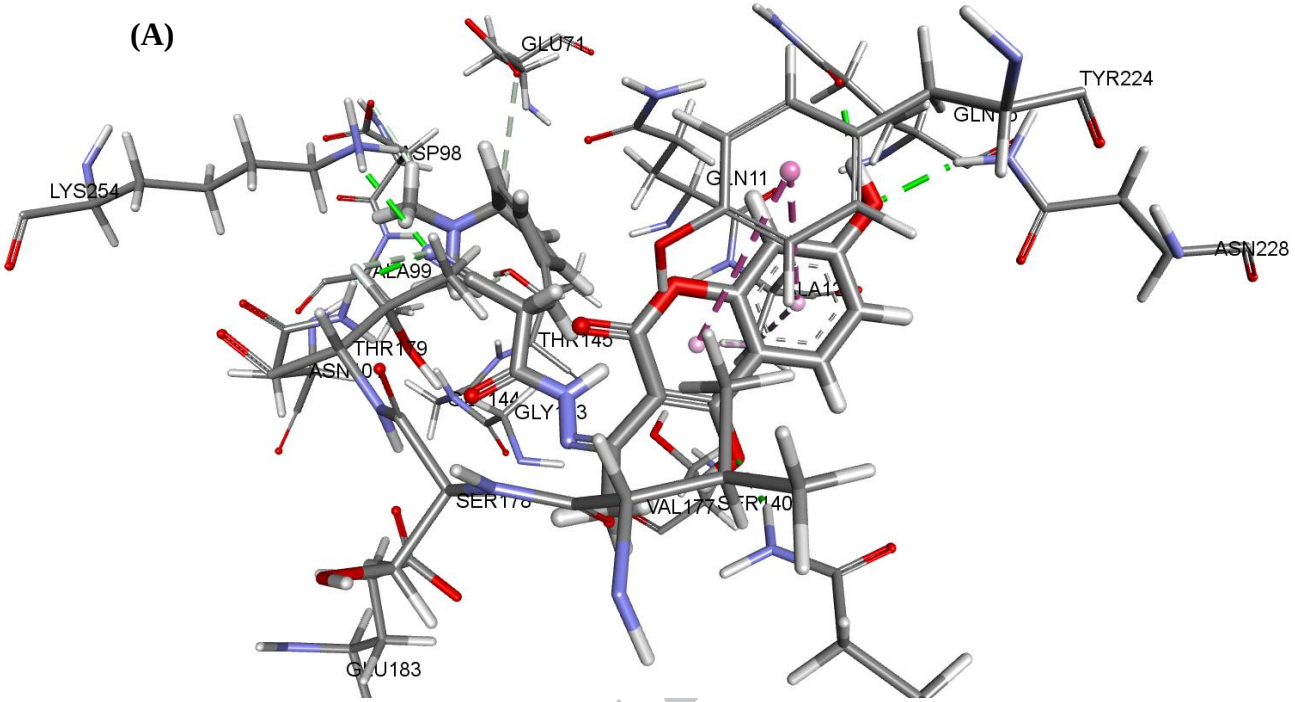


Figure 2: Effect of tubulin polymerization inhibition activity of compound (**8h**) and colchicine at different concentrations.

Since compound **8h** showed good G2/M arrest or mitotic arrest, we have tested this compound for tubulin polymerization inhibition property and compared with colchicine, a reference drug. The test compound **8h** exhibited significant tubulin polymerization inhibitory potency with IC_{50} 6.19 μM in comparison to the reference drug, colchicine, IC_{50} 7.60 μM (figure-2).

Docking studies were carried out in order to find plausible binding modes of the novel inhibitor with tubulin. From Figure 3 of docking picture it can be observed that deep green color

(A)



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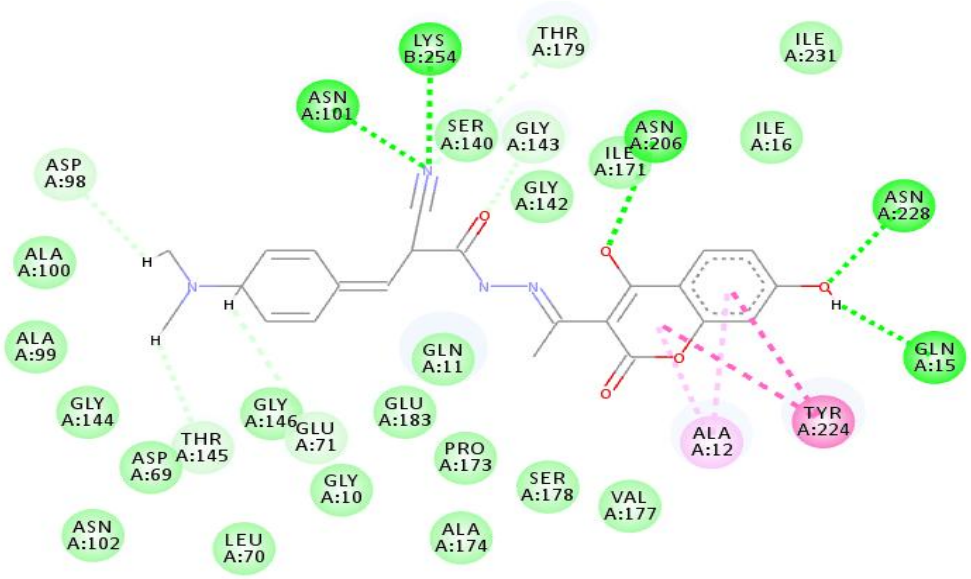
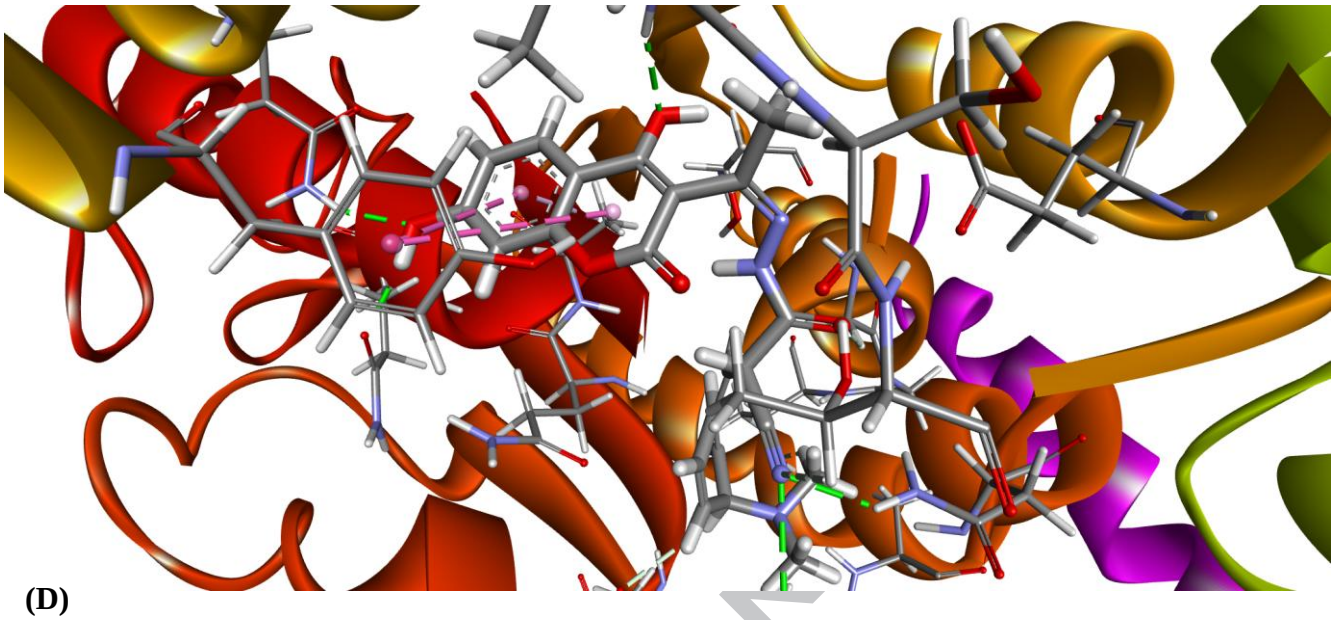


Interactions

- van der Waals
- Conventional Hydrogen Bond
- Carbon Hydrogen Bond
- Pi-Pi Stacked
- Pi-Alkyl

(C)



(D)

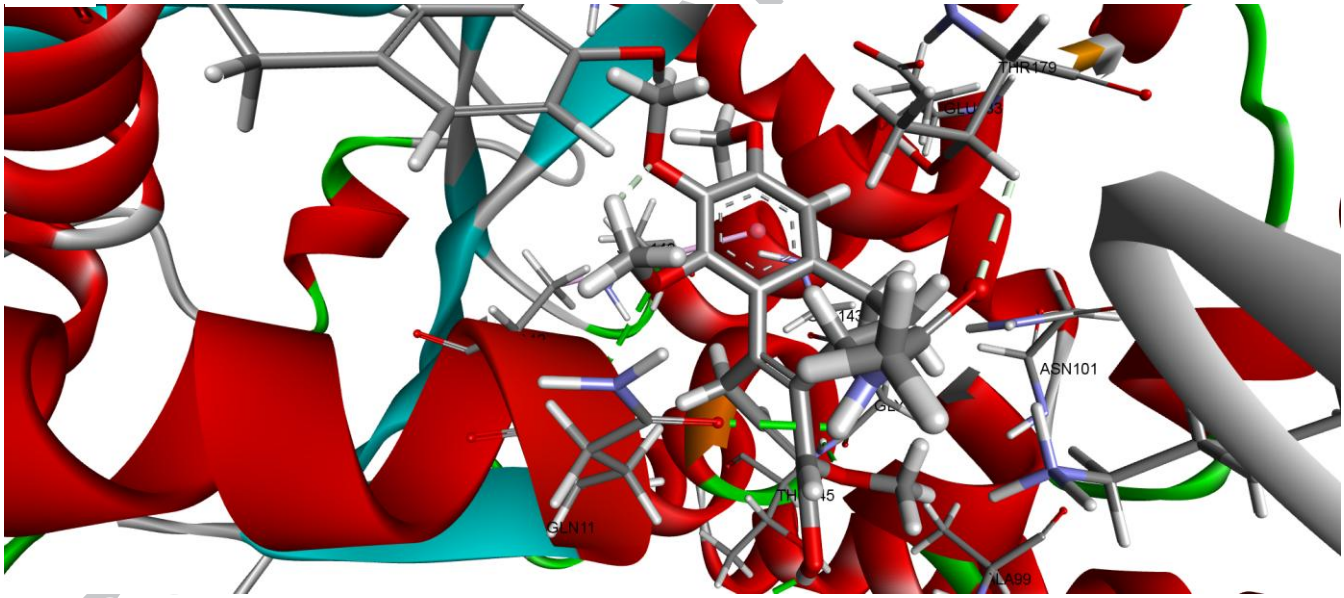
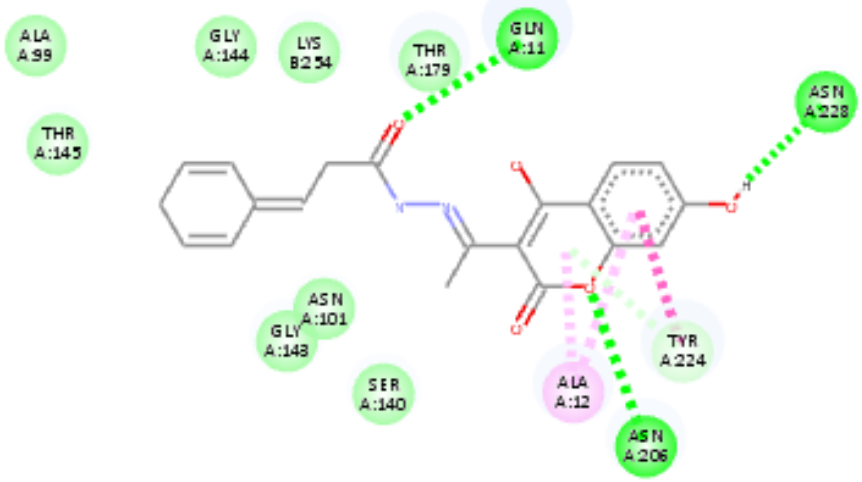
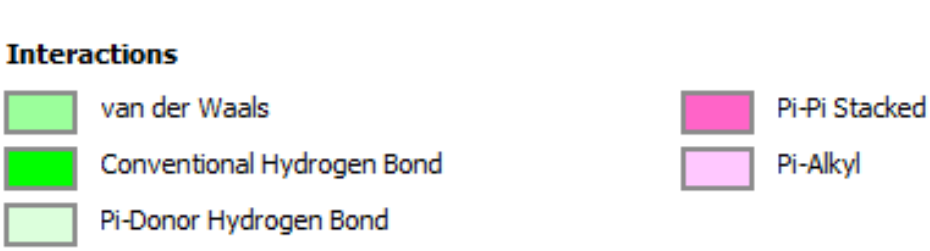


Figure 3: Plausible binding mode of the compound **8h** with tubulin protein (**PDB ID: 4YJ3**). A) Stick model of the proposed binding mode B) 2D representation of binding interactions the green colour indicates hydrogen bonding. C) 3D representation of binding interactions. D) 3D representation of binding interactions of colchicine with tubulin protein.

interactions designate the covalent hydrogen bonding interactions. The cyano group and two hydroxyl groups of the coumarin ring are involved in formation of hydrogen bonds with target tubulin protein. Figure 3, it can be observed that the compound **8h** was formed hydrogen bond interaction with amino acids with ASN-101, TYR-224, ASN-228, LYS-254, amino acids of tubulin protein with -63.66 CDOCKER interaction energy. To study the involvement of cyano group in the hydrogen bond formation, the docking result of compound 8h has compared the results with compound without cyano moiety. Further we have also compared with standard tubulin targeted anticancer drug colchicine (Figure 3D).

A)





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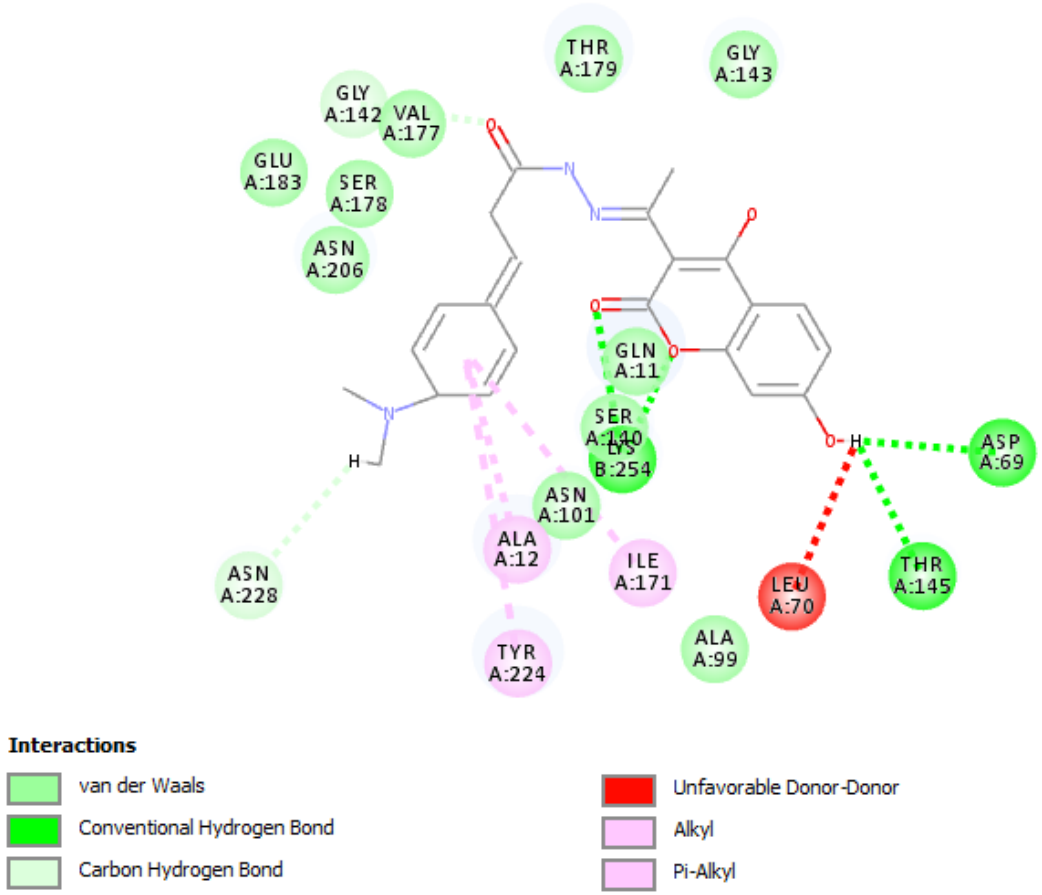

Figure 4: Docking results of the two examples without cyano group A) Docking result of the compound 8a without cyano group B) Docking result of the compound 8h without cyano group

Figure 4 depicted that docking interactions of compounds without cyano group shown less interactions with tubulin. The results pointed out to the fact that the cyano group has the ability to form hydrogen bonding interactions with the target. Further to validate these results we also performed docking study on the standard drug doxorubicin. The results revealed that it also formed strong hydrogen bonding interactions with the tubulin protein and same can be noticed in figure-5.

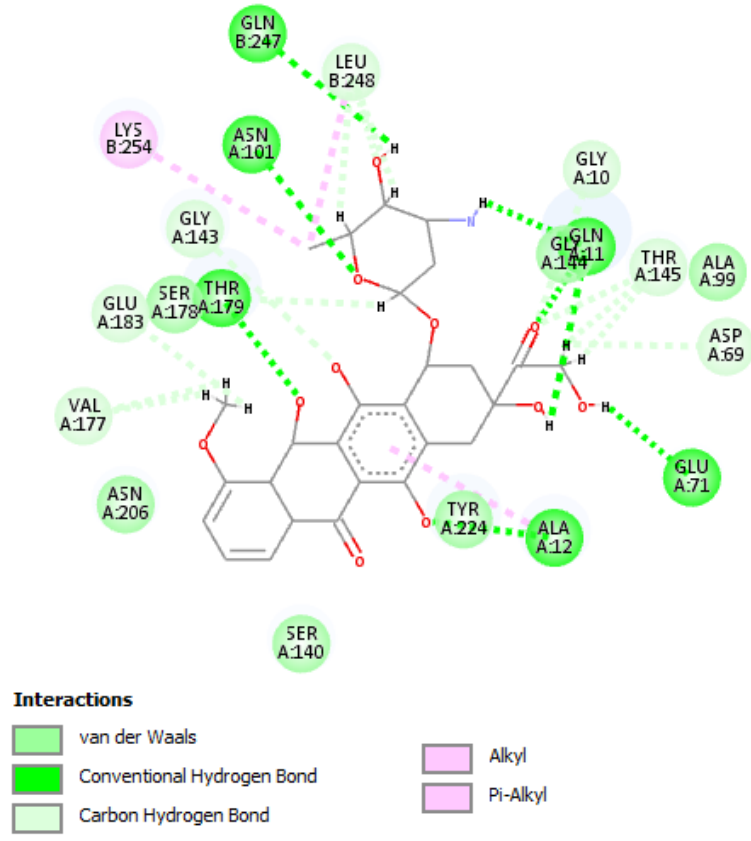

Figure 5: Docking result of the doxorubicin with the tubulin protein.

The hydrogen bonding interactions occurred with GLN-11, ALA-12, GLU-71, ASN-101 and GLN-247. The obtained results suggested that these compounds may show the activity against pancreatic, prostate, ovarian, and GBM cancers. The most active compound 8h can be taken up for cancer treatment drug delivery, labeling for imaging and act at the direct on the targeted cell specific site.

In summary, we have successfully synthesized novel coumarin-based acryloylcyanohydrazone compounds by a facile method. These chemical entities were well characterized by FT-IR, NMR and, ESI-MS spectroscopic methods. Furthermore, these compounds were screened for antiproliferative activity against A-549, Hela, SKNSH, MCF-7 human cancer cell lines and normal rat kidney cell line NRK 49F by using MTT assay. Among the all compounds **8b**, **8e**, **8h, and 8j** were showed good antiproliferative activity. The most active compound **8h** was subjected to further pharmacological evaluation studies; cell cycle analysis was carried out on MCF-7 cell lines which possess G2/M phase arrest. Further, **8h** was evaluated for the tubulin polymerization inhibition and the obtained result (IC_{50} 6.19 μM) were

promising than colchicine standard tubulin inhibitory drug. The molecular docking studies were done to validate the experimental results of the tubulin inhibition assay which exhibited strong hydrogen bonding interactions between compound 8h and aminoacids ASN-101, TYR-224, ASN-228 and LYS-254, amino acids of tubulin. In the present study, we found **8h** act as antiproliferative agent which arrest the cell cycle at G2/M phase and inhibits the tubulin polymerization. Hence, we can conclude that the molecule **8h** has the potential to act as antimitotic agent by targeting tubulin protein for treating cancer.

ACKNOWLEDGMENTS:

We would like to thank the National Institute of Technology, Rourkela for providing the necessary infrastructural and instrumentation facilities and TEQIP-II for partial funding. We would like to convey my gratitude towards Dr. Thangaraj Devadoss and Mr. Tuhin Subhra Dash for their valuable suggestions.

SUPPORTING INFORMATION:

^{1}H, and ^{13}C NMR of the compounds, antiproliferation assay (MTT assay), tubulin polymerization inhibition assay, molecular docking protocols were included in the supporting information.

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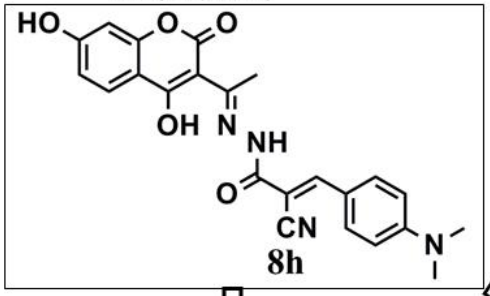
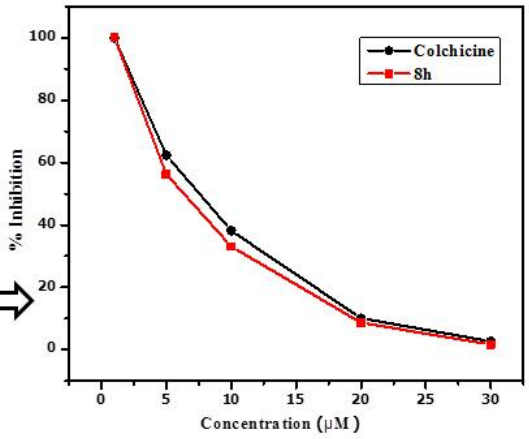
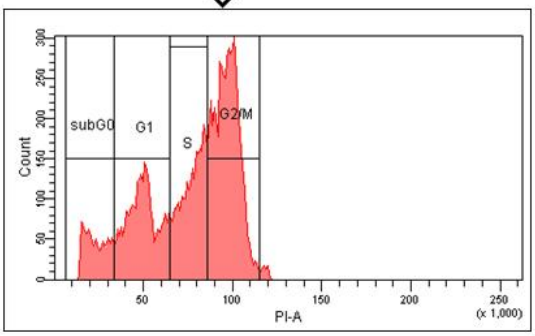
IC_{50}=4.31µM (A549)
=5.14µM (HeLa)
=6.09µM (SKNSH)
=3.42µM (MCF-7)

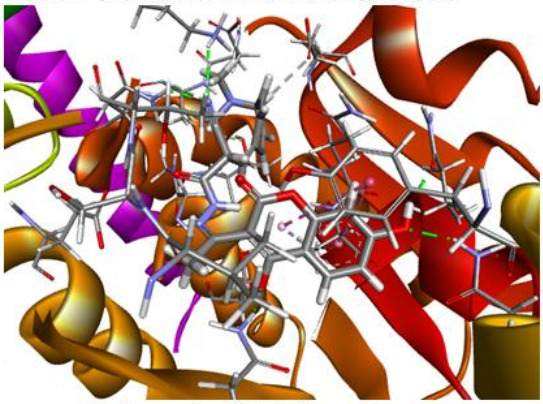




Tubulin polymerization inhibition (IC_{50}-6.19µM)



G2/M Phase arrest in MCF-7 cell lines



Binding mode with tubulin protein