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Synthesis, *In vitro* Antiproliferative and Antibacterial Evaluation and Molecular Docking Studies of 6-Chloro-3,4-Dihydro-4-Oxo-2*H*-Chromen-3-yl Methylene-2-Cyano-3-Phenyl Acryloyl Hydrazide

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ABSTRACT

Chromene derivatives containing cyano moiety are heterocyclic compounds with interesting pharmacological activities. The present study aimed to synthesize and determine the pharmacological activity of 6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl acryloyl hydrazide (3a-o). Compounds 3a – 3o were synthesized following the Knoevenagel condensation and Wolff-Kishner reduction methods. The synthesized compounds were characterized by different spectroscopic techniques including proton and carbon-13 Nuclear Magnetic Resonance (^{1}H- and ^{13}C-NMR) Spectroscopy, Fourier Transform Infrared (FT-IR) Spectroscopy, and Mass Spectrometry (MS). The compounds were screened for their *in vitro* antiproliferative and antibacterial activities using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and agar well diffusion assay, respectively. *In silico* molecular docking analysis of the compounds were performed against four different proteins; 5C5S, 6XXN, 3K46, and 1MI7 using PyRx0.8 software. Furthermore, their pharmacokinetics (ADMET) properties were predicted using SWISS ADME software. Among the compounds tested, compounds 3b and 3c exhibited the most potent antiproliferative activity against all four cancer cell lines; MCF-7, PC-3, HT-29, SKVO3 with IC_{50} of 15.28, 13.28, 10.25, and 13.12 µM, respectively for compound 3b, 16.33, 14.16, 12.22, and 14.75 µM, respectively for compound 3c. The standard drug doxorubicin had IC_{50} of 15.29, 12.26, 9.06, and 13.01 µM, respectively, while 5-Fluorouracil had IC_{50} of 16.15, 13.73, 10.25, and 14.28 µM, respectively. Most of the compounds showed potent antibacterial activity with their inhibition zone diameter comparable to that of the standard antibiotic ciprofloxacin. Molecular docking analysis gave good binding affinity of the compounds with the target proteins. Physicochemical analysis showed that all the compounds obeyed the Lipinski's rule of five.

***Keywords*:** Hydrazine derivatives, Antiproliferative, Antibacterial, Molecular Docking, Cancer cell lines.

Introduction

According to World Health Organisation (WHO), cancer is the second most common cause of death globally. It is estimated that there are about 9.6 million deaths and 18.1 million new cases annually.[1-2] More than 90% of pharmacophoric moieties are reported to have one or more heteroatoms like oxygen, sulphur, nitrogen, and the halogens in their structure.[3] Heterocyclic compounds are of great significance to the medicinal chemists owing to their numerous biological activities. A great deal of heterocyclic compounds with significant biological activity have been developed by researchers.[4] Despite the millions of heterocyclic compounds that have been developed, researchers are still working on developing both natural and synthetic compounds with heterocyclic nucleus, which have potent pharmacological activity.[5]

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Chromene is an heterocyclic moiety consisting of a phenyl nucleus fused with an oxine ring.[6] Chromene ring is widely present in natural products like anthocyanins, tocopherols, polyphenols, alkaloids and flavonoids. Chromene derivatives have been found to possess nutritional benefits and have the ability to boost immunity.[7] Chromene derivatives have drawn the attention of scientists in recent times due to their high lipophilicity, and use in the treatment of neurodegenerative diseases, they have been found to possess numerous pharmacological activities, including antiproliferative, anti-inflammatory, antiprotozoal, antidiabetic, antiepileptic, antiparkinson, anti-alzheimer, antipsychotic, and anticoagulant activities.[8-14] Chromene nucleus fused with cyano (C≡N) or cyano acyl-hydrazide moiety are strongly electron-withdrawing due to the anionic group.[15-20] As a result of this electron-withdrawing nature, they bind effectively and destabilizes micro tubulin polymerization and inhibit tumor cell growth at the G2/M phase. They bind to various targets such as apoptosis inducing protein (AIP), tubulin, tumor necrosis factor alpha (TNF-α), epidermal growth factor receptor (EGFR), tyrosine kinase, and FabH gene and exhibits inhibitory, or agonist effect on the receptor, resulting in anticancer activity.[21-26] Cyano group fused with various heterocyclic nucleus have been reported to have potent antiproliferative effect on a variety of cancer cell lines. Some of these agents have been approved by USFDA as antiproliferative drugs (Figure 1), while others are in various stages of clinical trials.[27-28]

On the basis of the above, the present study was designed to synthesize a chromene and acryloyl-hydrazides condense with cyano group and other aldehydes derivatives and investigate their anti-proliferative effect on various cancer cell lines, the anti-bacterial

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activity investigation as well as *in silco* studies of the synthesized molecules were also done.

Materials and Methods

Chemicals

All chemical were of analytical grade and were purchased from SD Fine and AURA Laboratories. They include Ethyl cyanoacetate (98%), Hydrazine hydrate (80%), and Ethanol (92%).

General Experimental Procedures

Thin layer chromatography (TLC) analysis was done using silica gel TLC plates. Compounds were characterised by Infrared (IR) spectroscopy (BRUKER), ^{1}H-NMR spectroscopy (400 MHz AvanceCore), ^{13}C-NMR spectroscopy (Avance 400 MHz), and Mass spectrometry (Thermo fisher scientific Orbitrap Exploris 120). Antiproliferative activity was performed using MTT assay, antibacterial activity was performed using agar well diffusion method, and *in silico* studies was done using PyRx0.8 molecular docking and SWISS ADME softwares.

Synthesis of 6-Chloro-3,4-dihydro-4-oxo-2H-chromen-3-yl methylene-2-cyano-3-phenyl-acryloylhydrazide

Step 1: Synthesis of Ethyl 2-Cyano-acetate

Ethyl cyanoacetate (0.01 mole) and hydrazine hydrate (0.01 mole) were mixed in a round bottom flask at 0°C. Ethanol (30 mL) was added with continuous stirring for 30 min. The progress of the reaction was monitored by TLC.

Step 2: Synthesis of 6-Chloro-3,4-dihydro-4-oxo-2H-chromen-yl methylene-2-cyano-acetohydrazide

To the product of the step 1 reaction was added 0.5 g of 6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-Carbaldehyde in a reaction vessel, followed by 30 mL of ethanol. The reaction mixture was stirred continuously for 2 h. The reaction was monitored by TLC.

Step 3: Synthesis of 6-Chloro-3,4-dihydro-4-oxo-2H-chromen-3-yl methylene-2-cyano-3-phenyl-acryloylhydrazide

The product of step 2 reaction (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-yl methylene-2-cyano-acetohydrazide was reacted with various aldehydes (3a-o) in ethanol solvent. Piperidine was added as a catalyst and the reaction was refluxed for 5 h at 100^{o}C. The reaction mixture was monitored by TLC (Hexane:Ethylacetate 3:7). The products were purified by flash column chromatography and then characterized using different analytical techniques. The synthetic scheme is illustrated in Figure 2.

Biological activity evaluation

In-vitro Antiproliferative activity screening

In-vitro Antiproliferative activity of the synthesized compounds (3a-o) was evaluated against four cell lines; breast cancer (MCF-7), prostate cancer (PC-3), human colon cancer (HT-29) and human ovarian cancer (SKVO3) cell lines using the MTT assay. MTT assay was performed according to the method described by Grela *et al*., 2018.[29] Trypsinization of the cells and trypan blue assay were performed to determine the viability of the cells in suspension. The cells were then counted using a hemocytometer (Improved B.S.748 Neubauer, Rohem, india),the cells were seeded at a density of 5.0 X 10^{3} cells/well in a 96 well plate containing 100 µL culture medium/well. The cells were incubated at 37^{o}C for 12 h in a 5% CO_2 incubator (Thermo scientific Model 370). The existing culture media were removed and each well was filled with new media containing MTT salt. The plates were further incubated for 3 h at 37^{o}C. at the end of the incubation period, precipitations were produced as a result of the reduction of MTT salt to formazan crystals by metabolically active cells. The chromophoric formazan crystals were dissolved in DMSO. The optical density was measured at 570 nm using a microplate reader (FlexStation3 plate reader). Cell toxicity was calculated using the formula;

(Average OD*100/positive control OD)-100

The 50% inhibitory concentration (IC_{50}) was calculated.

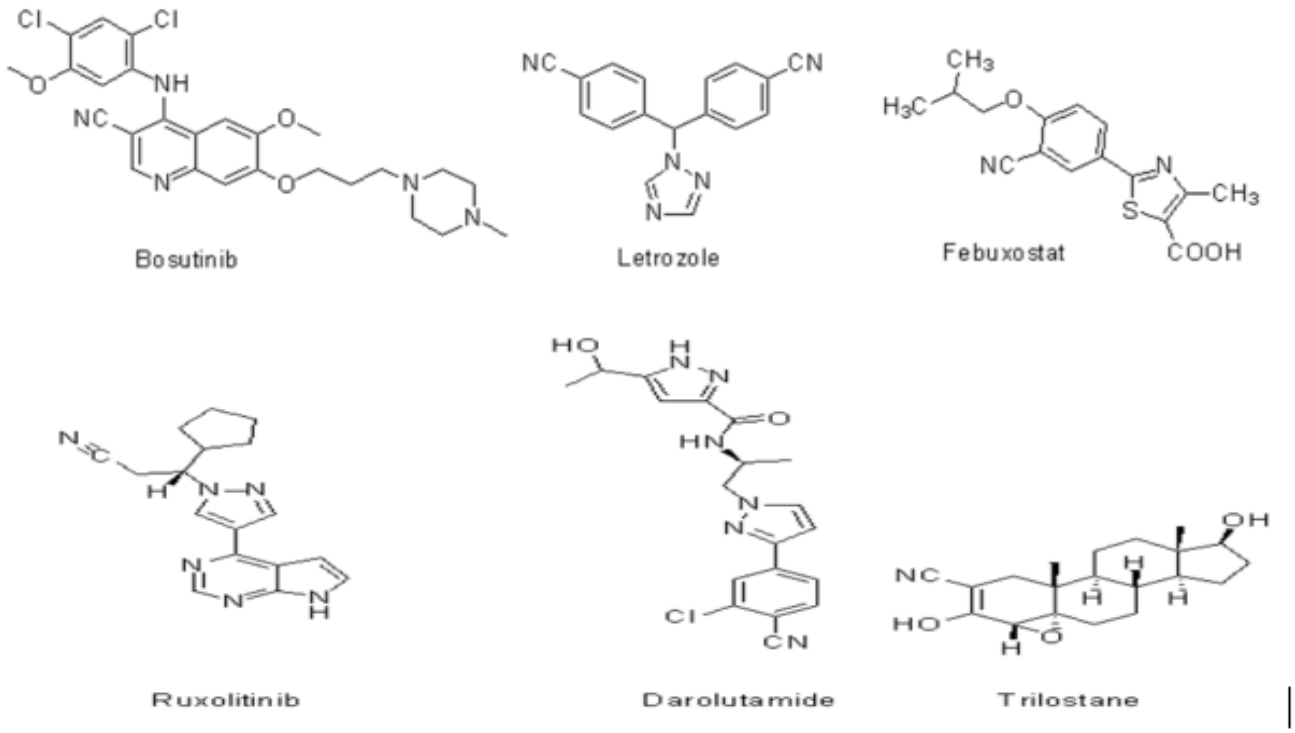

Figure 1: Cyano group-contain antiproliferative drugs approved by USFDA

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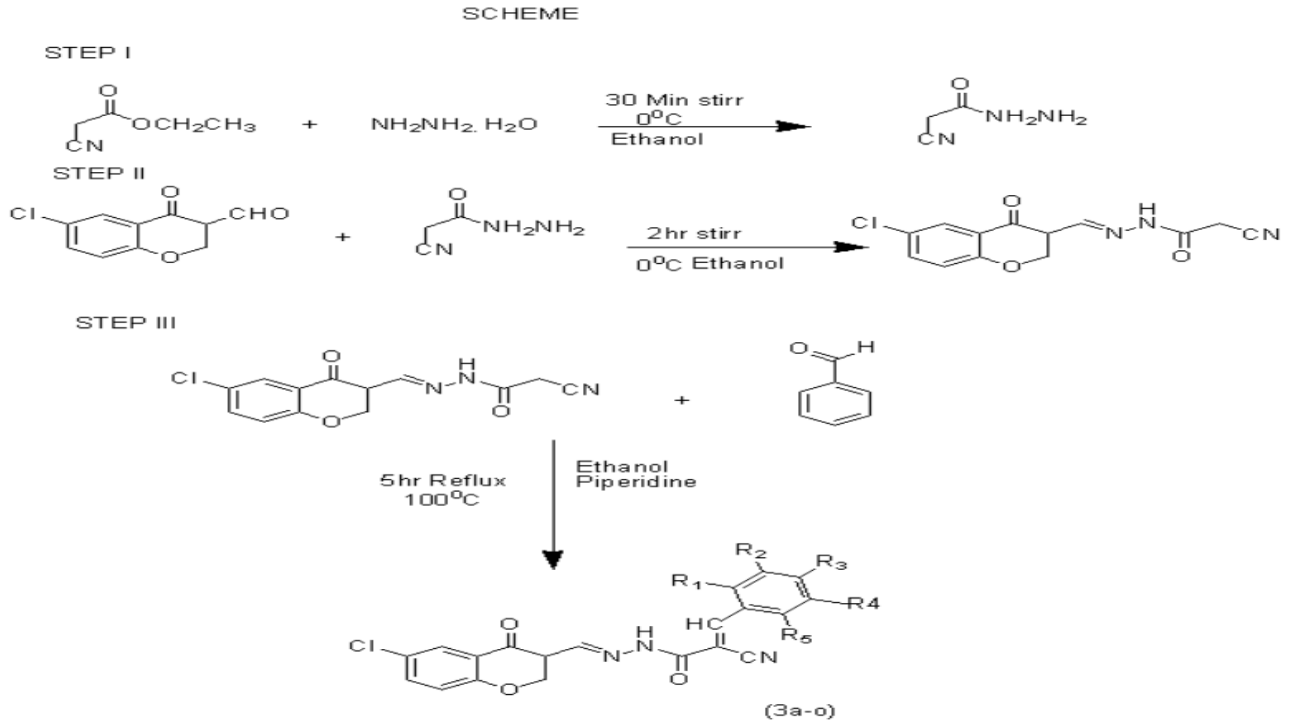

Figure 2: Synthesis scheme of 6-chloro-3,4-dihydro-4-oxo-2*H*-Chromen-3-yl methylene-2-cyano-3-phenyl acrylo hydrazide and derivatives.

In-vitro Antibacterial activity screening

In-vitro Antibacterial activity of the compounds (3a-o) at concentrations of 50 µg/mL and 100 µg/mL were evaluated against two gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using the agar well diffusion assay.[30] Ciprofloxacin was used as standard.

In silico molecular docking analysis

Molecular docking of the compounds was done using the PyRx0.8 software.[31-32] the target proteins with the following IDs PDBID: 5C5S (human-myosin 9b RhoGAP), 6XXN (sting CTD complex), 3K46 (E. coli beta glucurinadase), and 1MI7 (Swapped trp Aporepressor) were downloaded from www.rcsbpdb.com protein data base (PDB). Ligands interactions with various receptor amino acids were visualized and their binding affinities predicted in discovery studio and Molegro Molecular Viewer, followed by docking analysis.

Determination of ADMET Properties

All the synthesized compounds (3a-o) had their ADME properties analyzed with the SWISS ADME software.[33]

Spectroscopic analysis of the synthesized compounds

All the synthesized compounds were characterized by a combination of spectroscopic techniques including proton and carbon-13 Nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy and mass spectrometry (MS).

Results and Discussion

Synthesis and characterisation of compounds 3a-3o

A novel series of compounds; 6-chloro-3,4-dihydro-4-oxo-2H-Chromen-3-yl methylene - 2-cyano-3-phenyl acryloyl hydrazide (3a-o) were synthesized. The structures of the synthesized compounds were confirmed by different spectroscopic techniques including ^{1}H-NMR, ^{13}C-NMR, FT-IR and Mass spectrometry. In the synthetic step 1, ethyl cyano-acetate and hydrazine hydrate were reacted to obtain ethyl-2-cyano-acetate, the reaction proceeded via Knoevenagel condensation mechanism. Step II of the reaction involved the Wolff-Kishner reduction of aldehydes and convertion to hydrazides. Step III involved the reaction of the product obtained from the previous step (step II) with various substituted benzaldehyde in ethanol woth piperidine as a catalyst. The synthetic scheme is shown in Figure 2 and physical properties of the products are presented in Table 1.

In the FT-IR spectra of the synthesized compounds, the carbonyl group (>C=O) absorption bands were observe between 1700-1775 cm^{-1}, the absorption band due to hydrazide group (NH=NH$_2$) were observe between 3200-3350 cm^{-1}, the cyanide (C≡N) absorption band were observed between 1600-1650 cm^{-1}, while Ar-Cl group showed strong absorption at 700-800 cm^{-1}. The ^{1}H-NMR spectra of all the compounds showed doublet signals between δ 6.8-7.5 ppm corresponding to the aromatic protons of chromene and substituted aldehyde, the hydrazide protons showed singlet peaks between δ 7.0-7.2 ppm, the alkyl chain protons and substituted aldehydes produced a multiplet signals between δ 8.2-8.5 ppm. In the ^{13}C-NMR spectra, all the compounds displayed peaks that corresponded with their structures. The peaks due to the cyanide (C≡N) carbon were observed between δ 110-140 ppm, the carbons of the alkyl halide (C-Cl) displayed signal at δ 20-60 ppm, signals due to alkene (C=C) carbons were displayed between δ 110-140 ppm, the aromatic carbon peaks were observed between δ 120-180 ppm. The Mass spectra of all the compounds (3a-o) showed the m/z for the molecular ions.

Compound 3a (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-acryloylhydrazide):

IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH$_3$ Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O Str, amide), 1640 (C≡N Str), 809 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 380 (M+1, 100%).

Compound 3b (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-p-nitro acryloyl hydrazide):

IR cm^{-1} (KBr): 3068 (-NH Str), 3046 (-CH Str, benzene), 2983 (-CH$_3$ Str, aliphatic), 1702 (C=O Str), 1684 (C=O amide), 1643 (C≡N Str), 1320 (NO$_2$), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, amide),

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6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 1.8 (s, CH_2); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 425 (M+1, 100%).

Compound 3c (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-P-chloro acryloyl hydrazide):

IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 743 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 415 (M+1, 100%).

Compound 3d (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-P-hydroxy acryloyl hydrazide):

IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3400 (OH-Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 396 (M+1, 100%).

Table 1: Physical Properties of Synthesized Compounds 3a - 3o

Compound	R1	R2	R3	R4	R5	M.F	M.Wt	R.F	%YIELD
3a	H	H	H	H	H	$C_{20}H_{14}ClN_3O_3$	379.07	0.8	78
3b	H	H	NO_2	H	H	$C_{20}H_{13}ClN_4O_5$	424.79	0.9	75
3c	H	H	Cl	H	H	$C_{20}H_{13}Cl_2N_3O_3$	414.24	0.7	83
3d	H	H	OH	H	H	$C_{20}H_{14}ClN_3O_4$	395.8	06	82
3e	H	H	F	H	H	$C_{20}H_{13}ClFN_3O_3$	397.79	0.8	79
3f	H	H	Br	H	H	$C_{20}H_{13}BrClN_3O_3$	458.69	0.9	80
3g	H	H	CN	H	H	$C_{21}H_{13}ClN_4O_3$	404.81	0.5	75
3h	H	NO_2	H	Cl	H	$C_{20}H_{12}Cl_2N_4O_5$	458.24	0.7	77
3i	H	H	OHC_6H_5	H	H	$C_{26}H_{18}ClN_3O_4$	471.89	0.6	81
3j	OCH_3	OCH_3	OCH_3	H	H	$C_{23}H_{20}ClN_3O_6$	469.87	0.8	83
3k	H	H	$CH(CH_3)_2$	H	H	$C_{23}H_{20}ClN_3O_3$	421.88	0.9	73
3l	H	OC_2H_5	OH	H	H	$C_{22}H_{18}ClN_3O_5$	439.85	0.7	76
3m	CH_3	H	H	H	CH_3	$C_{22}H_{18}ClN_3O_3$	407.85	0.7	75
3n	OCH_3	H	H	H	OCH_3	$C_{22}H_{18}ClN_3O_5$	439.85	0.8	80
3o	Cl	OCH_3	OCH_3	H	H	$C_{22}H_{17}Cl_2N_3O_5$	474.29	0.6	77

Table 2: Antiproliferative Activity of Synthesized Compounds 3a - 3o

Compound	IC_{50} (μM)			
	MCF-7	PC-3	HT-29	SKVO3
3a	50.26 ± 0.18	49.26 ± 0.23	52.36 ± 0.59	56.35 ± 0.25
3b	15.28 ± 0.32	13.28 ± 0.30	10.25 ± 0.19	13.12 ± 0.45
3c	16.33 ± 0.41	14.16 ± 0.50	12.22 ± 0.25	14.75 ± 0.30
3d	55.18 ± 0.05	50.24 ± 0.44	60.15 ± 0.32	60.36 ± 0.16
3e	48.32 ± 0.18	46.26 ± 0.72	52.31 ± 0.68	50.16 ± 0.50
3f	90.19 ± 0.26	90.69 ± 0.58	82.68 ± 0.32	92.12 ± 0.32
3g	23.38 ± 0.52	22.30 ± 0.32	20.35 ± 0.52	19.62 ± 0.26
3h	18.05 ± 0.19	17.36 ± 0.21	17.55 ± 0.18	16.32 ± 0.50
3i	37.12 ± 0.25	46.54 ± 0.24	43.19 ± 0.65	50.23 ± 0.80
3j	52.24 ± 0.21	52.24 ± 0.21	54.28 ± 0.69	58.77 ± 0.30
3k	80.25 ± 0.32	82.29 ± 0.65	87.15 ± 0.42	90.12 ± 0.58
3l	85.45 ± 0.58	87.21 ± 0.69	83.24 ± 0.22	80.23 ± 0.32
3m	55.26 ± 0.29	58.32 ± 0.23	57.27 ± 0.68	65.80 ± 0.09
3n	78.23 ± 0.32	75.16 ± 0.33	77.35 ± 0.72	80.23 ± 0.28
3o	20.58 ± 0.06	19.15 ± 0.02	20.16 ± 0.18	22.28 ± 0.32
Doxorubicin	15.29 ± 0.03	12.26 ± 0.04	9.06 ± 0.03	13.01 ± 0.89
5-Flurouracil	16.15 ± 0.02	13.73 ± 0.025	10.25 ± 0.018	14.28 ± 0.07

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Compound 3e (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2 cyano-3-phenyl-P-fluoro acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 1050 (C-F), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161, 19.9, 170.3, 44.5, 95.3, 61, 134, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 398 (M+1, 100%).
Compound 3f (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-P-bromo acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 724 (C-Cl), 640 (C-Br). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm; MS (EI-MS): 459 (M+1, 100%).
Compound 3g (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl -p-cyano acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103(-CH Str, benzene), 2956(-CH_3Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 2230 (C-CN), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 406 (M+1, 100%).
Compound 3h (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl -3-chloro-5-nitro acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103(-CH Str, benzene), 2956(-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic),1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str) 13560 (NO_2), 754 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 459 (M+1, 100%).
Compound 3i (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenoxy-phenyl acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH $_3$Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86(d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 138.0, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 473 (M+1, 100%).
Compound 3j (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-2,3,4, trimethoxy acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 13560 (NO_2), 754 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 3.73 (t, methoxy); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 471 (M+1, 100%).

Table 3: Antibacterial activity of Synthesized Compounds 3a - 3o

Compound	**Inhibition Zone Diameter (mm)**							
	Gram positive bacteria				**Gram negative bacteria**			
	Staphylococcus aureus		***Bacillus subtilis***		***Escherichia coli***		***Pseudomonas aeruginosa***	
	50 µg/mL	100 µg/mL	50 µg/mL	100 µg/mL	50 µg/mL	100 µg/mL	50 µg/mL	100 µg/mL
3a	20	26	18	21	17	20	20	22
3b	30	37	29	33	30	35	34	37
3c	28	34	29	31	30	33	32	36
3d	24	28	21	25	21	29	25	26
3e	22	25	22	27	23	25	26	27
3f	23	25	22	26	24	26	25	28
3g	26	30	24	27	25	30	28	30
3h	28	33	27	30	26	29	30	32
3i	21	25	20	22	20	25	24	25
3j	22	27	19	24	23	22	25	25
3k	23	31	20	23	21	22	24	24
3l	20	25	21	23	22	25	25	26
3m	21	29	22	22	20	24	23	25
3n	21	30	22	20	21	23	23	25
3o	24	31	23	26	24	28	27	31
Ciprofloxacin	28	33	25	29	27	32	30	34

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Table 4: Binding affinities of synthesized compounds (3a-3o) docked with selected protein targets

Compound	Binding Affinity (kcal/mol)			
	5C5S	3k47	6XXN	1MI7
3a	-8.0	-8.4	-7.8	-5.8
3b	-8.5	-9.1	-8.1	-6.1
3c	-8.4	-8.8	-8.3	-6.6
3d	-8.6	-8.4	-8.3	-6.4
3e	-8.9	-8.6	-8.2	-7.1
3f	-9.0	-8.5	-8.0	-6.7
3g	-9.1	-8.5	-8.0	-6.6
3h	-8.8	-9.0	-8.2	-6.2
3i	-10.2	-9.5	-9.1	-7.5
3j	-8.1	-7.8	-7.5	-6.0
3k	-9.1	-9.2	-8.5	-6.8
3l	-8.5	-7.7	-8.1	-6.1
3m	-9.4	-9.0	-7.9	-6.5
3n	-8.0	-6.8	-7.9	-6.5
3o	-8.2	-7.3	-8.5	-5.9

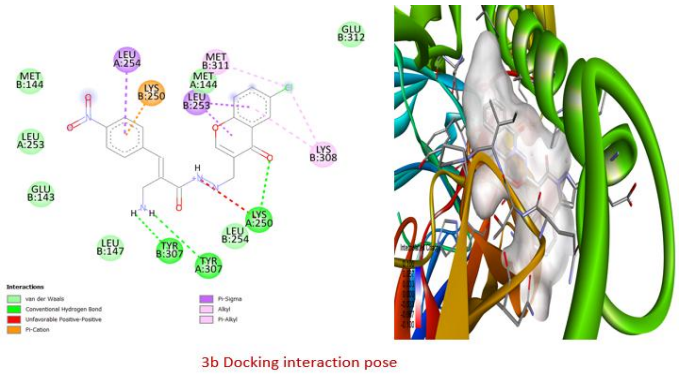

Figure 2: Docking pose of the interaction of the most active ligand 3b with target protein 5C5S

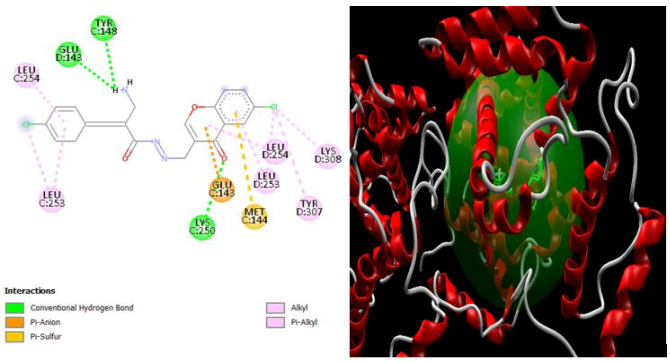

Figure 3: Docking pose of the interactions of the most active ligands (3b and 3c) with target protein

Compound 3k (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-p-propyl- acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956(-CH_3Str, aliphatic), 2876(-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 13560 (NO_2), 754 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 1.29 (t-CH_3), 3.12 (S-propyl); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. (EI-MS): 471 (M+1, 100%).
Compound 3l (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-3-ethoxy-4-hydroxy acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3275 (str-hydroxy) 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 13560 (NO_2), 754 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d,2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 1.33 (t-CH_3), 3.00 (d, CH_3) 5.00 (S-hydroxyl); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 441 (M+1, 100%).
Compound 3m (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2,6-dimethyl-acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 13560 (NO_2), 754 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 1.20 (t-CH_3); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 409 (M+1, 100%).
Compound 3n (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-2,6-dimethoxy acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103 (-CH Str, benzene), 2956 (-CH_3 Str, aliphatic), 2876 (-CH Str, aliphatic), 1775 (C=O Str), 1684 (C=O amide), 1640 (C≡N Str), 13560 (NO_2), 754 (C-Cl), 724 (C-Cl). ^{1}H-NMR (DMSO δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 3.40 (d, methoxy); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 441 (M+1, 100%).
Compound 3o (6-Chloro-3,4-dihydro-4-oxo-2*H*-chromen-3-yl methylene-2-cyano-3-phenyl-2-chloro 3,4-dimethoxy acryloyl hydrazide):
IR cm^{-1} (KBr): 3320, 3275 (-NH Str), 3103(-CH Str, benzene), 2956(-CH_3Str, aliphatic), 2876(-CH Str, aliphatic), 1775 (C=O Str), 1684

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(C=O amide), 1640 (C≡N Str), 1356 (NO_2), 754 (C-Cl), 724 (C-Cl), 665 (C-Cl).^{1}H-NMR (DMSO-*d*6 δ ppm): 7.35 (d, 2H, ArH), 6.86 (d, 2H, ArH), 7.40–7.44 (m, 1H, ArH), 7.0 (1H, -NH, amide), 6.86 (d, 2H, ArH), 8.10 (d, ethylene), 7.30 (s, 1H, benzene), 3.9 (d, methoxy); ^{13}C-NMR δ 127.4, 133.5, 12.4, 147.1, 120.9, 128.8, 164.0, 161.0, 19.9, 170.3, 44.5, 95.3, 61.0, 134.0, 128.7, 128.0, 128.7, 126.4, 126.3, 24.3, 24.3 ppm. MS (EI-MS): 475 (M+1, 100%).

Biological Activity of compounds 3a-o

In vitro Antiproliferative Activity

All the newly synthesized compounds (3a-o) were screened for their antiproliferative activity *in vitro* against four cancer cell lines; MCF-7, PC-3, HT-29 and SKVO3 using the MTT assay. Compounds 3b and 3c exhibited potent antiproliferative activity against all the four cell lines with IC_{50} values of 15.28, 13.28, 10.25, and 13.12 µM, respectively for compound 3b, and 16.33, 14.16, 12.22, and 14.75 µM, respectively for compound 3c. The IC_{50} values for the standard drugs were as follows; doxorubicin (15.29, 12.26, 9.06, and 13.01 µM, respectively) and 5-Fluorouracil (16.15, 13.73, 10.25, and 14.28 µM, respectively). The other compounds showed moderate activity. The results of the antiproliferative activity screening are summarized in Table 2.

In vitro Antibacterial Activity

All the newly synthesized compounds (3a-o) were screened *in vitro* for their antibacterial activity against gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacterial strains using the agar well diffusion method. The results showed that most of the synthesized compounds have comparable antibacterial activity with the standard drug ciprofloxacin. Compounds with electron withdrawing groups like nitro, chlorine, bromine showed more promising activity compared to the alkyl chain. The summary of the results is presented in Table 3.

In silico molecular docking analysis

Molecular docking screening is used to find lead compounds with desired biological activity. The molecular docking was done using the PyRx 0.8 program which is open-source software that can run in major operating systems like Linux, Windows and Mac OS. The molecular docking was used to predict the antiproliferative and antibacterial activities of the synthesized compounds (Ligands) by their interaction with the amino acid residues in the active sites of selected protein targets. In this study, four different proteins including 3K46 (E. coli beta glucurinadase), 6XXN (sting CTD complex), 5C5S (human-myosin 9b RhoGAP), and 1MI7 (Swapped trp Aporepressor) were selected. The ligand-protein binding affinity values indicated good binding of the ligands to the target proteins. The ligand interacted with several amino acid residues including LysB 308, LysA 250, TyrA 307, TyrB 307, LeuB 147, LeuD 254, GluD 143, LysC 250, and TyrD 307 in the active sites of the proteins. Finally, the docking pose visualization analysis were done in Molegro Molecular viewer and Discovery Studio. Table 4 shows the binding affinities of the ligand-protein interaction, and Figure 3 shows the docking pose of the active ligands.

Physicochemical and Pharmacokinetics (PK) Properties

The SWISS ADME tool was used to predict the physicochemical and PK characteristics of the synthesized compounds. From the results obtained, all the synthesized compounds obeyed the Lipinski rule of five in terms of their molecular weight, octanol/water partition coefficient (MLogP) values, Hydrogen bond acceptor, Hydrogen bond donor, and NViolation (Table 5). The interaction of compounds with cytochrome p450 (CYP) isoenzymes in drug elimination through metabolic biotransformation is essential to the drug development process. All of the synthetic compounds displayed favorable pharmacokinetics characteristics (Table 6).

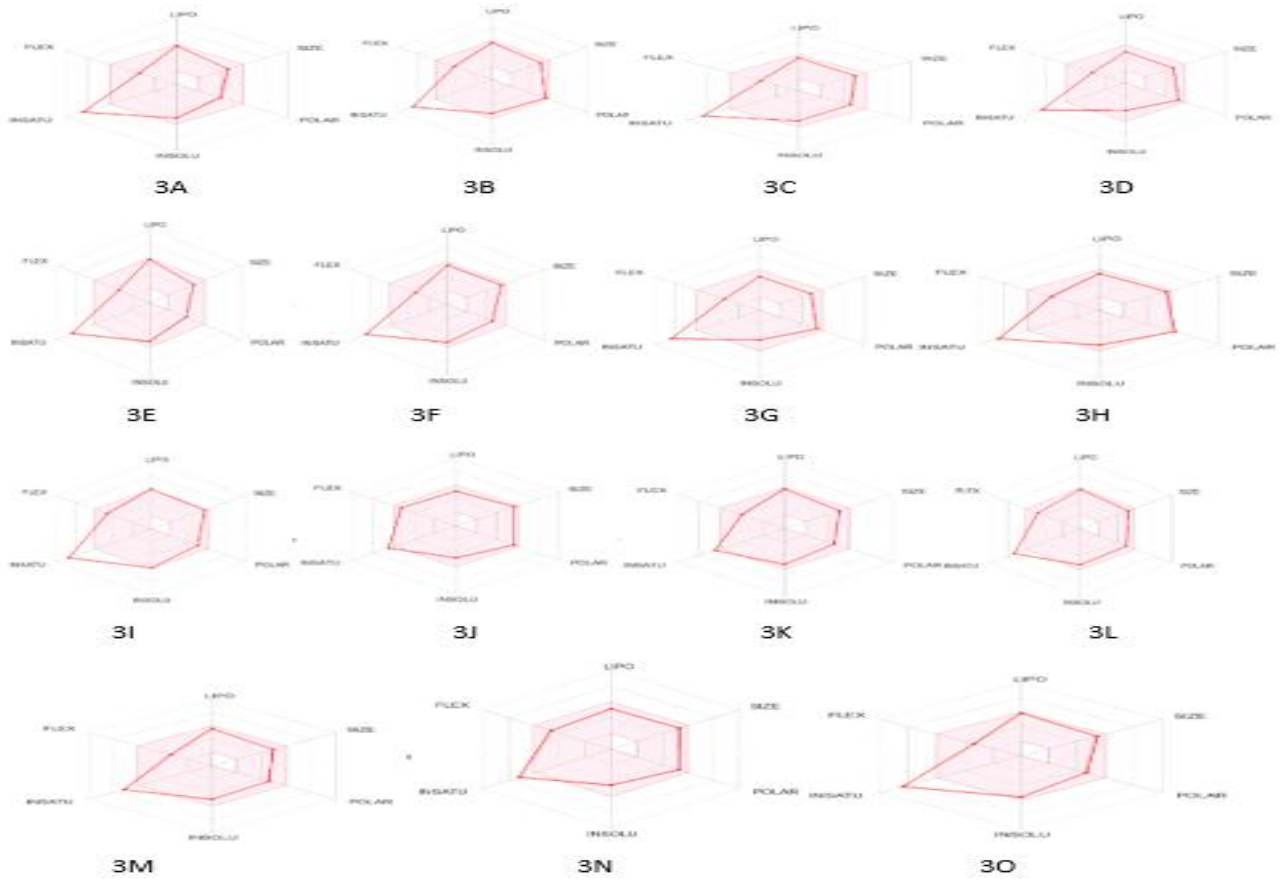

Figure 4: Physico-chemical properties of bioavailability RADER of compounds 3a - 3o

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Table 5: Physico-chemical properties relevant to Lipinski rule of 5 of synthesized compounds 3a - 3o

Compound	Mol.Wt (g/mol)	MLogP	No. of H-Bond acceptor	No. of H-Bond donor	NViolation
Optimum Range	< 500	<5	<10	<5	<1
3a	379.07	2.32	4	1	0
3b	424.79	2.21	6	1	0
3c	414.24	2.18	5	1	0
3d	395.8	1.16	6	2	0
3e	397.79	2.69	5	1	0
3f	458.69	2.29	5	1	0
3g	404.81	1.04	6	1	0
3h	458.24	2.11	7	1	0
3i	471.89	2.48	6	1	0
3j	469.87	0.77	8	1	0
3k	421.88	2.34	5	1	0
3l	439.85	1.67	6	2	0
3m	407.85	2.12	5	1	0
3n	439.85	1.07	7	1	0
3o	474.29	1.55	7	1	0

Table 6: Pharmacokinetics parameters of synthesized compounds 3a - 3o

COMpd	GIP Absorption	BBB Permeation	P-gp Substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Skin Permeation	Bioavailibility score
3a	HIGH	YES	NO	YES	YES	YES	NO	YES	-5.33ms/s	0.55
3b	HIGH	NO	NO	YES	YES	YES	NO	YES	-5.73ms/s	0.55
3c	HIGH	NO	NO	YES	YES	YES	NO	YES	-5.87ms/s	0.55
3d	HIGH	NO	NO	NO	NO	YES	NO	YES	-6.46ms/s	0.55
3e	HIGH	NO	NO	YES	YES	YES	NO	YES	-5.37ms/s	0.55
3f	HIGH	NO	NO	YES	YES	YES	NO	YES	-6.09ms/s	0.55
3g	HIGH	NO	NO	NO	YES	YES	NO	YES	-6.45ms/s	0.55
3h	LOW	NO	NO	YES	YES	YES	NO	YES	-6.27ms/s	0.55
3i	HIGH	NO	NO	NO	YES	YES	NO	YES	-5.58cm/s	0.55

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3j	HIGH	NO	NO	YES	YES	YES	NO	YES	-6.72cm/s	0.55
3k	HIGH	NO	NO	NO	YES	YES	NO	YES	-5.56cm/s	0.55
3l	HIGH	NO	NO	YES	YES	YES	NO	YES	-5.71ms/s	0.55
3m	HIGH	NO	NO	NO	YES	YES	NO	YES	-5.76 cm/s	0.55
3n	HIGH	NO	NO	YES	YES	YES	NO	YES	-6.51ms/s	0.55
3o	HIGH	NO	NO	YES	YES	YES	NO	YES	-5.61ms/s	0.55

Table 7: Physico-chemical properties of oral bioavailability of synthesized compounds 3a - 3o

Compound	**XlogP3**	**TSP(A^2)**	**Log S (ESOL)**	**Fraction of SP^3 carbon**	**No. of rotatable bonds**
Optimum Range	**-0.7 to 5.0**	**20-130**	**-6 to 0**	**0.25 to1**	**0 to 9**
3a	4.61	74.48 Å²	-5.09	0.10	5
3b	4.44	120.30 Å²	-5.16	0.10	6
3c	4.17	91.55 Å²	-5.02	0.10	5
3d	3.18	111.78 Å²	-4.28	0.10	5
3e	4.71	74.48 Å²	-5.25	0.10	5
3f	4.23	91.55 Å²	-5.34	0.10	5
3g	3.26	115.34 Å²	-4.38	0.10	5
3h	3.99	137.37 Å²	-5.09	0.10	6
3i	5.07	100.78 Å²	-5.89	0.08	7
3j	3.45	119.24 Å²	-4.67	0.22	8
3k	4.66	91.55 Å²	-5.29	0.22	6
3l	4.59	103.94 Å²	-5.27	0.17	7
3m	4.27	91.55 Å²	-5.03	0.18	5
3n	3.48	110.01 Å²	-4.58	0.18	7
3o	4.79	91.55 Å²	-5.62	0.10	5

Physico-chemical properties of oral bioavailability
The SWISS ADME program automatically generates a RADER graph for prediction of the oral bioavailability of the synthesized compounds. The colour zone for oral bioavailability was determined using the following physicochemical parameters: LIPO (Lipophilicity), SIZE (Molecular weight), POLAR (Polarity), INSOLU (Insolubility), INSATU and FLIX (Rotable Bond Flexibility). From the results obtained (Table 7 and Figure 4), all the synthesized compounds had acceptable physicochemical properties for oral bioavailability.

Conclusion

The present study involved the synthesized 6-chloro-3,4-dihydro-4-oxo-2*H*-Chromen-3-yl methylene-2-cyano-3-phenyl acryloyl hydrazide and its derivatives (3a - 3o) and obtain a good yield (75-85%) and investigated *in vitro* antiproliferative and antibacterial activities. Among the synthesized compounds, those with electron withdrawing (nitro and chlorine) groups (compounds 3b and 3c showed the most potent activities which were comparable to the standard drugs Doxorubicin and 5-Flourouracil in terms of antiproliferative activity and Ciprofloxacin in terms of antibacterial activity. *In silico* molecular docking study predicted the compounds as potential drug candidates are binds various amino acids and they assessed a good binding affinity with acceptable drug-likeness and pharmacokinetics properties.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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