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Research Article

Synthesis and evaluation of anti-cancer activity of some 6-Aminoflavones

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Abstract

A series of 6-Aminoflavones (**5a-o**) was synthesized and characterized by spectral techniques. Their cytotoxic effects have been evaluated *in vitro* in relation to colon HCT116 and breast MCF7 cancer cell lines, using MTT assay. The findings revealed that compound **5n** had significant cytotoxic effects against HCT116 and MCF7 with IC_{50} values of 30.02 µM and 40 µM respectively. The compound **5o** showed lowest IC_{50} value of 29.5 µM against HCT116 cell line. Acridine orange/ethidium bromide staining was used to assess the morphonuclear changes in HCT-116 treated with the test compound **5o**. **5o** showed 38% apoptotic nuclei. Flow cytometric studies on HCT-116 cell line treated with **5o** showed the arrest of cell cycle in G_0/G_1 phase.
Keywords: 6-Aminoflavones, MTT assay, acridine orange/ ethidium bromide, flow cytometry

1. Introduction

Cytotoxic drugs have the potential to be very harmful to the body unless they are very specific to cancer cells. The anticancer drugs which are currently used lack selectivity. Hence there is a great need to discover new classes of drugs with more selectivity and to increase the selectivity of the available anticancer drugs.

Flavonoids possess a common phenylbenzopyrone structure (C6-C3-C6).They are classified mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols[1,2]. They show remarkable biological activities which include anti-allergic, anti-inflammatory, antioxidant, anti-mutagenic, anti-carcinogenic, and modulation of enzymatic activities [3-6]. Natural flavone diosmetin showed inhibition of proliferation of breast adenocarcinoma MDA-MB 468 and normal breast MCF-7 cells and was found that this compound is selective for the cancer cells with slight toxicity in the normal breast cells [7]. Quercetin-3-O-amino acid-esters, show higher selectivity as inhibitors against Src tyrosine kinase than against EGFR tyrosine kinase [8].Myricetin-3-O-(L-rhamnopyranoside and quercetin-3-O-lactopyranoside isolated from *Byrsonima crassa*, *Davilla elliptica*, and *Mouriri pusa* showed antitumor and anti-inflammatory activities [9].

Aminoflavones have been studied as tyrosine kinase inhibitors and as antimitotic agents [10.11]. It has been reported that 6-amino-5,7-dihydroxyflavone demonstrates potent and specific rat intestinal alpha glucosidase inhibitory activity[12]. The anti-proliferative and CDK2-Cyclin A inhibitory activities of 8-amino-7-hydroxy flavone was also evaluated as remarkable by cytotoxic studies in MCF-7 and MDA-MB-435 breast cancer cell lines[13,14]. Very few reports are available on the anticancer activity of 6-Aminoflavones [15].

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2. Materials and methods

2.1 Chemistry

The chemicals required for the synthesis were purchased from Sigma Aldrich, Hi-Media, Loba Chemicals and Nice Fine Chemical India. Melting points were determined by open capillary method and are uncorrected. Purity of synthesized compounds was checked by thin layer chromatography. R_f values were recorded by using pre-coated silica gel aluminium backed plates Kieselgel 60 F254 Merck (Germany). The IR spectra in KBr pellets were recorded using Schimadzu FTIR 8400S spectrophotometer.1H NMR spectra were recorded in deuterated dimethylsulphoxide in Bruker AV400(400MHz) spectrometer using tetramethylsilane as internal standard. Mass spectra were scanned on a Shimadzu LCMS (ESI) 2010A spectrometer.

2.2 Synthesis of 6-Aminoflavones

The synthesis of 6-Aminoflavones was done by a four step process.Fifteen 6-Aminoflavones were synthesized.

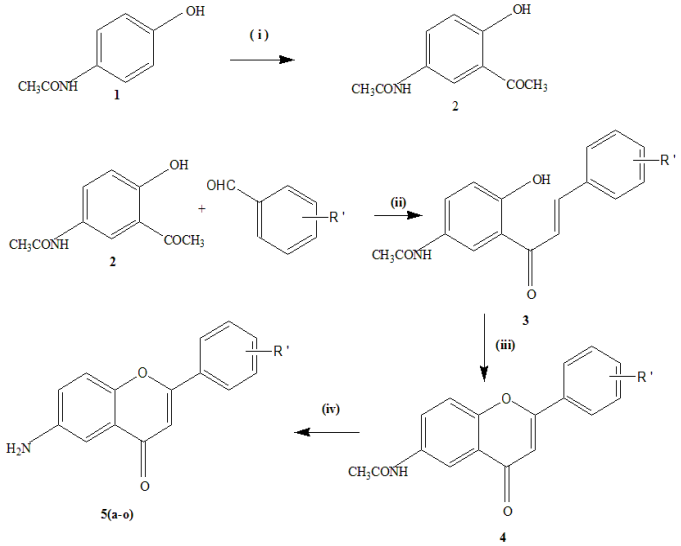
2.2.1 Synthesis of 5'-acetamido-2'-Hydroxyacetophenone: To a suspension of paracetamol(0.066 mol) and anhydrous aluminium chloride (0.016 mol) in nitrobenzene (50mL) was gradually added acetyl chloride (0.066 mol) over a period of 0.5 hr. The temperature was gradually raised to 130ºC over a period of 0.5 hr and then maintained for 2.5 hr. It was cooled to 40 ºC within 0.5 hr, poured into a mixture of crushed ice and 30 mL conc. hydrochloric acid with vigorous stirring and filtered. The crude product thus obtained was washed with water till free from acid followed by toluene and crystallized from isopropanol to yield light brown needle shaped crystals.

2.2.2 Synthesisof 5'-Acetamido-2'-Hydroxychalcones: A mixture of 5'-acetamido-2'-Hydroxyacetophenone (0.01mol) and aryl aldehyde (0.01 mol) was dissolved in ethanol (30 mL). To this, aqueous potassium hydroxide solution (0.03 mol) was added slowly and stirred for 24 h, at room temperature. After completion of the reaction, the reaction mixture was poured into crushed ice and acidified with 5N hydrochloric acid. The solid separated was filtered and crystallized from ethanol.

2.2.3 Synthesis of 6-Acetamidoflavones: Catalytic amount of iodine was dissolved in Dimethyl sulfoxide (10mL).To this was added 5'-Acetamido -2'-Hydroxychalcone and the mixture refluxed for 30 minutes to 1 hour. The reaction mixture was then cooled, poured on ice cold water and 10% sodium thiosulfate solution added to remove excess iodine. The product obtained was purified by recrystallization from methanol.

2.2.4 Synthesis of 6-Aminoflavones: A mixture of concentrated HCl and water (1:1) was added to 5'-Acetamido-2'-Hydroxychalcone, and boiled. The boiling time varied from 1h-2h for different derivatives. The reaction progress was monitored using TLC (pet ether:ethyl acetate 2:3). When the reaction was complete, ice cold water was added and the reaction mixture was cooled to room temperature. The solution was basified by adding 10% sodium carbonate solution to obtain brown colored precipitate of 6-aminoflavone.The crude product obtained was washed with water, dried and recrystallized from ethanol.

Scheme-1: Synthetic route for 6 -Amino flavone



Com. Code	R'	Com. Code	R'
5a	H	5i	3,4-dimethoxy
5b	4-Cl	5j	2-OH
5c	4-F	5k	4-OCH_3
5d	3-NO_2	5l	3,4-dichloro
5e	4-OC_2H_5	5m	methylene dioxy
5f	3-OCH_3	5n	3,4,5-trimethoxy
5g	4-OH,3-OCH_3	5o	O-CH_2-C_6H_5
5h	3-OH,4-OCH_3		

Reagents and conditions: (i) Acetyl chloride, nitro benzene,anhyrous$AlCl_3$,130°C,3h (ii)KOH/ EtOH stir for 18-24 h (iii) I_2/DMSO,reflux,1h (iv) Conc. HCl: Water 1:1,boiled for 1-2 h

2.3 Anti-cancer activity by MTT Assay

All the aminoflavones were screened for *in vitro* anticancer activity against MCF-7(human breast adenocarcinoma), HCT116 (human colon cancer) cell lines by MTT assay [16]. The test compounds were also screened for *in vitro* anticancer activity against the normal cell Vero (Monkey kidney epithelial) cell line. Doxorubicin was used as the standard drug. MTT assay was carried out using the standard procedure and the percentage cytotoxicity of each compound was calculated.

2.4 Acridine orange and Ethidium bromide double staining using fluorescent microscopy

DNA-binding dyes Acridine orange (AO) and Ethidium bromide (EB) (Sigma, USA) were used for the morphological detection of apoptotic cells. Acridine orange and Ethidium bromide double staining was done using standard procedure. The apoptotic index (AI) was calculated as % of apoptotic cells from randomly counted 100 cells in each treatment group.

2.5 Flow cytometric analysis

Flow cytometric analysis offers a precise technique to check the effect of test compounds on cell cycle progression and check points. 1 x 10^6 cells were seeded in 25 cm^2 flasks and after overnight adherence, incubated with test compounds. Then cells were detached by trypsinization and mixed with floating cells, centrifuged and washed with PBS. The cell pellets were fixed in 70% ice-cold methanol and stored at -20°C for 24 h. After that cell pellets were washed with PBS and isotonic PI solution [25 µM propidium iodide, 0.03% NP-40 and 40 µg /ml RNase A] was added. The stained cells were analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using excitation at 488 nm and emission at 575/40 nm. A minimum of 10,000 events were acquired for each sample and data analysis was done by using BD Accuri™ C6 software.

3. Results

3.1 Chemistry

The yields, melting points and retention factors of the test compounds are shown in Table 1. The synthesized compounds were characterized by infrared, nuclear magnetic resonance and mass spectroscopy. The spectral data were in accordance with the structures of the compounds.

Table 1: Physical Data of synthesized compounds (5a-o)

Compound Code	Yield (%)	MP(°C)	R_f
5a	75	174	0.31
5b	70	158-159	0.41
5c	68	162	0.40
5d	50	181-182	0.38
5e	68	176	0.43
5f	79	153	0.41
5g	61	232-235	0.45
5h	66	221	0.38
5i	73	151-153	0.37
5j	68	274	0.43
5k	70	188	0.48
5l	65	168	0.46
5m	70	188	0.44
5n	71	169	0.46
5o	71	254	0.49
Solvent system for TLC : petroleum ether: ethyl acetate 2:3			

6-Amino-2-phenyl-4H-chromen-4-one (5a)

IR (KBr) (cm^{-1}):1614(C=O str. flavones), 3338(NH_2symstr), 3402(NH_2asymstr), 1134 (C-N str), 3100(CH strAr);^{1}H NMR (400 MHz, $CDCl_3$) δ ppm:5.5(s,2H,NH_2 at C6), 6.86 (s,1H,H3),),7.10 (m, 3H, H 3,'4',5'),7.48(d,1H, H5,J = 8.8 Hz,),7.57 (m,2H,H 2',6') 8.03 (m, 2H,H7); mass spectrum *m/z* 237 (M^+).

6-Amino-2-(4'-chlorophenyl)-4H-chromen-4-one (5b)

IR(KBr) (cm^{-1}):1612(C=O str. flavones), 3330(NH_2symstr), 3402(NH_2 asymstr), 1138 (C-N str), 3070(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 5.50(s,2H,NH_2 at C6), 6.99 (s, H3), 7.08 (dd, 1H, J = 2.56Hz,8.9Hz

H7), 7.12 (d, 1H, J = 2.56Hz, H8), 7.46 (d, 1H,J = 8.8Hz, H5), 7.64 (d, 2H, J = 8.6Hz, H3'5'), 8.04 (d, 2H, J = 8.6Hz, H2',6'); mass spectrum *m/z* 271 (M^+).

6-Amino-2-(4'-fluorophenyl)-4H-chromen-4-one(5c)

IR (KBr) (cm^{-1}):1619(C=O str. flavones), 3338(NH_2symstr), 3404(NH_2asymstr), 1140 (C-N str), 3070(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 5.21(s,2H,NH_2 at C6), 7.08 (s, H3),7.45 (d, 2H, J = 8.8Hz, H3'5'),7.63 (m, 1H,H7), 7.82 (d, 1H, J = 2.6Hz, H8), 7.87 (d, 1H,J = 9.0Hz, H5), 8.20 (d, 2H, J = 8.8, 5.4 Hz, H2',6'); mass spectrum *m/z* 255 (M^+).

6-Amino-2-(3'-nitrophenyl)-4H-chromen-4-one(5d)

IR(KBr) (cm^{-1}):1608(C=O str. flavones), 3332(NH_2symstr), 3402(NH_2asymstr), 1132 (C-Nstr), 3069(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 5.50(s,2H,NH_2 at C6), 7.0 (s, 1H,H3), 7.08 (dd, 1H, J = 2.56Hz,8.9Hz H7), 7.12 (d, 1H, J = 2.56Hz, H8), 7.46 (d, 1H,J = 8.8Hz, H5), 7.64 (m,1H, H5'),7.82(d,1H, J = 2.56Hz, H4'), 8.01 (s, 1H,H2'),8.04 (d, 2H, J = 8.6Hz, H6'); mass spectrum *m/z* 298 (M^+).

6-Amino-2-(4'-ethoxy)-4H-chromen-4-one(5e)

IR (KBr) (cm^{-1}):1614(C=O str. flavones), 3338(NH_2symstr), 3402(NH_2asymstr), 1134 (C-N str), 3100(CH strAr);^{1}H NMR (400 MHz, DMSO-d6) δ ppm:1.40 (t,3H,CH_3), 4.23 (q,2H,OCH_2),5.43(s,2H,NH_2), 7.08 (s, H3),7.45 (d, 2H, J = 8.8Hz, H3'5'),7.63 (m, 1H,H7), 7.82 (d, 1H, J = 2.6Hz, H8), 7.87 (d, 1H,J = 9.0Hz, H5), 8.20 (d, 2H, J = 8.8, 5.4 Hz, H2',6'); mass spectrum *m/z* 281 (M^+).

6-Amino-2-(3'-methoxy)-4H-chromen-4-one(5f)

IR(KBr) (cm^{-1}):1608(C=O str. flavones), 3332(NH_2symstr), 3402(NH_2asymstr), 1132 (C-N str), 3069(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 3.81 (s, OCH_3), 5.43 (s,2H,NH_2),), 7.01 (s, H3), 7.08 (dd, 1H, J = 2.56Hz,8.9Hz H7), 7.12 (d, 1H, J = 2.56Hz, H8), 7.46 (d, 1H,J = 8.8Hz, H5), 7.64 (d, 2H, J = 8.6Hz, H3'5'), 8.04 (d, 2H, J = 8.6Hz, H2',6'); mass spectrum *m/z* 267 (M^+).

6-Amino-2-(4'-hydroxy-3'-methoxyphenyl)-4H-chromen-4-one (5g)

IR (KBr) (cm^{-1}):1623(C=O str. flavones), 3338(NH_2symstr), 3399(NH_2asymstr), 1134 (C-Nstr),3100(CH strAr);^{1}H NMR(400MHz,DMSO-d6)δppm:3.89(s,3H,3' CH_3),5.47(s,2H,NH_2 at C6), 6.80(s,1H,H3), 6.93-7.69 (6Ar-H),9.85(s,1H,OH at C4'); mass spectrum *m/z* 283 (M^+).

6-Amino-2-(3'- hydroxyl-4'-methoxyphenyl)-4H-chromen-4-one (5h)

IR (KBr) (cm^{-1}):1623(C=O str. flavones), 3338(NH_2symstr), 3399(NH_2asymstr), 1134 (C-Nstr),3070(CH strAr);^{1}H NMR(400MHz,DMSO-d6)δppm:3.85(s,3H,3' CH_3),5.40(s,2H,NH_2 at C6), 6.80(s,1H,H3), 7.01-7.78 (6Ar-H),9.80(s,1H,OH at C3'); mass spectrum *m/z* 283 (M^+).

6-Amino-2-(3',4'-dimethoxyphenyl)-4H-chromen-4-one (5i)

IR (KBr) (cm^{-1}):1607(C=O str. flavones),3331(NH_2symstr), 3405(NH_2asymstr), 1141 (C-N str), 3073(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm:3.86(s,6H,3',4'OCH_3), 5.50(s,2H,NH_2 at C6),6.81 (s, 1H,H3),7.03 (d, 1H,J = 2.7 Hz,H8),7.09(dd,1H,J = 8.8, 2.7 Hz, H7),7.15(d,2H,J = 8.9Hz, H3',5'),7.37 (d, 1H, J = 8.8 Hz,H5)7.42 (d, 2H, J = 8.9 Hz, H2',6'); mass spectrum *m/z* 297 (M^+).

6-Amino-2-(4'-methoxyphenyl)-4H-chromen-4-one (5k).

IR (KBr) (cm^{-1}):1610(C=O str. flavones), 3331(NH_2symstr), 3400(NH_2asymstr), 1141 (C-N str), 3076(CH strAr);1H NMR (400 MHz,DMSO-d6) δ ppm: 3.85 (s, OCH_3),5.49(s,2H,NH_2 at C6),6.78 (s, 1H,H3), 7.06 (dd, 1H, J = 8.8, 2.7 Hz, H7), 7.08 (d, 1H, J = 2.7 Hz,H7), 7.10 (d, 2H, J = 8.9 Hz, H3'5'),7.54 (d, 1H, J = 8.8 Hz, H5)8.00 (d, 2H, J = 8.9 Hz, H2',6'); mass spectrum m/z 267 (M^+).

6-Amino-2-(3',4'-dichlorophenyl)-4H-chromen-4-one (5l)

IR(KBr) (cm^{-1}):1610(C=O str. flavones), 3330(NH_2symstr), 3402(NH_2asymstr), 1140 (C-N str), 3070(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 5.48(s,2H,NH_2 at C6), 6.95 (s, H3), 7.05 (dd,1H, J = 2.56Hz,8.9Hz H7), 7.12 (d, 1H, J = 2.56Hz, H8), 7.40 (d, 1H,J = 8.8Hz, H5), 7.44 (d, 1H, J = 8.6Hz, H5'), 7.51 (d, 2H, J = 8.6Hz, H2',6'); mass spectrum *m/z* 306 (M^+).

6-Amino-2-(3',4',5'-trimethoxyphenyl)-4H-chromen-4-one (5n)

IR(KBr)(cm^{-1}):1610(C=Ostr.flavones),3336(NH_2symstr),3410(NH_2asymstr),1148(C-Nstr),3081(CHstrAr);1HNMR(400MHz.DMSO-6)δppm:3.75(s,3H,4'OCH3),3.86(s,6H,3',5'OCH3),5.60(s,2H,NH_2 at C6),6.93(s,1H,H3),7.03(dd,1H,J=8.9,2.7Hz,H7),7.13(d,1H,J=2.6 Hz, H5), 7.34(s,2H,H2',6'), 7.51 (d, 1H, J = 8.80 Hz,H8); mass spectrum m/z 327 (M^+).

6-Amino-2-(4'-benzyloxyphenyl)-4H-chromen-4-one (5o)

IR (KBr) (cm^{-1}):1600(C=O str. flavones),3375(NH_2symstr), 1168 (C-N str), 3001(CH strAr); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm:4.4(s,2H,CH_2) 5.37(s,2H,NH_2),6.89 (dd,1H, J =2.5Hz, 8.9 Hz), 7.03 (s,1H),7.19 (dd, 2H, J =2.5Hz, 8.9 Hz), 7.26 (d,2H, J = 2.5 Hz), 7.39 (d, 2H, J = 2.5 Hz), 7.41(m 2H), 7.43(m 2H), 7.84 (d,1H, J = 2.5 Hz), 7.85(d,1H, J = 2.5 Hz); mass spectrum *m/z* 343 (M^+).

3.2 Anti-cancer activity

Table 2: Cytotoxic activity of 6-Aminoflavones

Compound Code	IC_{50} (µM)		
	HCT116	MCF 7	Vero
5a	>200	>200	>200
5b	>200	>200	>200
5c	>200	>200	>200
5d	>200	>200	>200
5e	>200	>200	>200
5f	>200	>200	>200
5g	100.5	>200	>200
5h	>200	>200	>200
5i	33.11	>200	>200
5j	>200	>200	>200
5k	>200	>200	>200
5l	>200	>200	>200
5m	133.5	>200	>200
5n	30.02	40	>200
5o	29.5	>200	>200
Doxorubicin	1.6	0.9	2.9

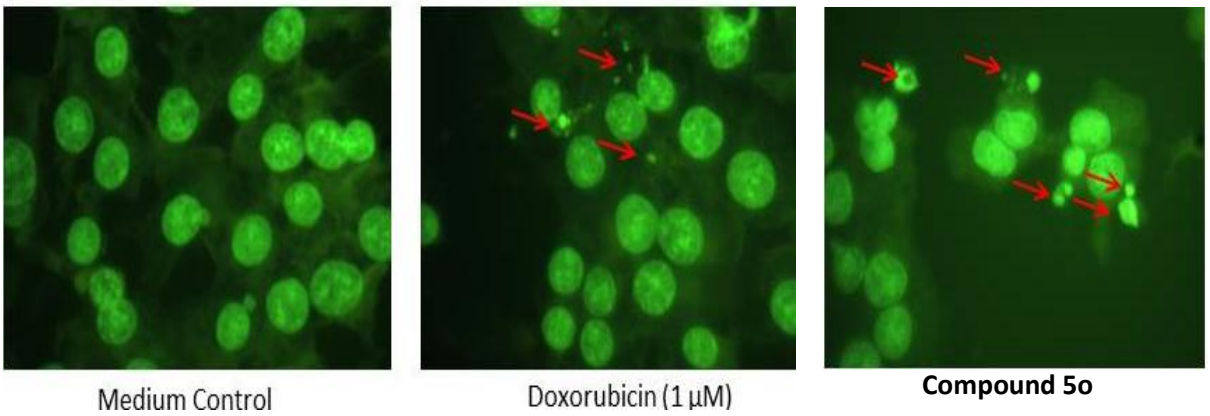
3.3 Acridine orange and Ethidium bromide double staining using fluorescent microscopy

The normal control (DMSO) showed the apoptotic index about 6.3 whereas the test compound **5o** at 50 µM concentration showed six-fold increase (36) in the apoptotic index. The results are shown in Table 3.

Table 3:Apoptotic index of test compound 5o

AO/EB	Apoptotic nuclei count				SEM
Medium control	1	2	3	Average	
	6	11	2	6.3	2.60
Doxorubicin	48	57	41	48.7	4.63
5o	44	26	38	36.0	5.29

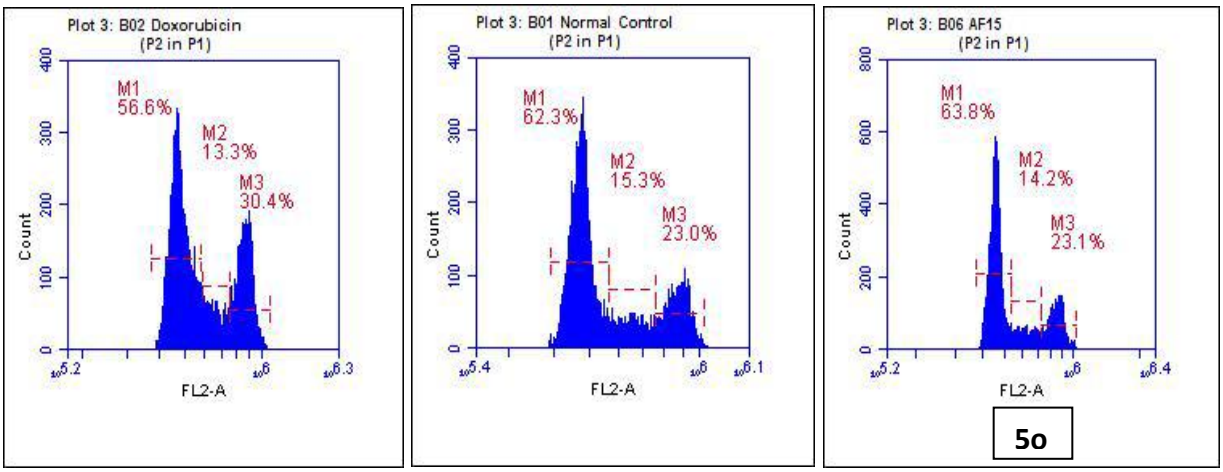
Figure1: The representative images for induction of apoptosis by 5o for 48 h in HCT- 116 cells.



3.4 Flow Cytometric Analysis

The effects of the compound 5o on cell cycle were assessed using flow cytometry and the result of the same was shown as % cells in G_0/G_1, S and G_2/M phase. The normal control showed the distribution of cells in G_0/G_1, S and G_2/M phase as 62.3%, 15.3% and 23.0% cells, respectively while **5(o)** at 50 µM showed the distribution of cells in G_0/G_1, S and G_2/M phase as 63.8%, 14.2% and 23.1% cells, respectively.

Figure 2: Effect of test compound 5o on the cell cycle of HCT-116 after 48 h treatment



4. Discussion

The compound **5n** showed significant anticancer activity against both HCT 116 and MCF 7 cell lines with IC_{50} 30.02 µM and 40 µM respectively.**5i** showed good cytotoxic activity against HCT 116 with IC_{50} 33.11 µM.**5o** showed maximum cytotoxicity with an IC_{50} (29.5 µM) in HCT-116 cells after 48 hours of incubation. It can be inferred that methoxy group at 3'and 4' positions of ring B (**5i**), methoxy group at 3',4'and 5', positions of ring B(**5n**) and benzyloxy group at position 4' of ring B(**5o**) increase the anticancer activity of 6-aminoflavones. The test compounds **5i, 5n**and **5o** are selective in their activity towards cancerous cells whereas the standard drug Doxorubicin is highly toxic to normal cells with IC_{50} 2.9 µM (Table 2).

5o caused accumulation of cells (63.8%), in G_0/G_1, phase which indicated the arrest of cell cycle in this phase.

5. Conclusion

In this study, a series of fifteen 6-aminoflavones were synthesized and characterized. The compound**5o** exhibited maximum cytotoxicity in HCT-116.**5n** exhibited significant cytotoxicity in both MCF-7 and HCT-116.**5o** at 50 µM concentrations showed six-fold increase in the apoptotic index. **5o** arrested G_0/G_1phase of the cell cycle at 50µM concentration.

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