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Research Article

IN VITRO CYTOTOXICITY AND ANTIOXIDANT EVALUATION OF 7-AMINO-2-STYRYLCHROMONE DERIVATIVES

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ABSTRACT

Objective: The objective of this study was to synthesize 7-amino-2-styrylchromone derivatives and evaluate their *in vitro* cytotoxic and antioxidant potential.

Methods: 7-amino-2-styrylchromones were synthesized from 7-amino-2-methylchromone by condensing it with various substituted aromatic aldehydes. The cytotoxicity of the synthesized molecules was assessed against two cell lines, MCF-7 and HCT-116 by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cell cycle analysis of the most potent molecule ASC-7 was carried out. The antioxidant studies were conducted by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide methods.

Result: (*E*)-7-amino-2-(3,4-methylenedioxystyryl)-4*H*-chromen-4-one (ASC-7) with inhibitory concentration 50% (IC_{50}) 56.0 μM was found to be the most potent molecule against MCF-7. ASC-7 induced G_0/G_1 phase arrest of MCF-7. Furthermore, (*E*)-7-amino-2-(3,4-methylenedioxystyryl)-4*H*-chromen-4-one(ASC-7) showed good DPPH scavenging activity (IC_{50} 54.6 μM). However, none of the tested compounds exhibited nitric oxide scavenging property.

Conclusion: This study reports the synthesis of 7-amino-2-styrylchromones. Some of the synthesized compounds showed moderate cytotoxicity against the tested cell lines MCF-7 and HCT-116. (*E*)-7-amino-2-(3,4-methylenedioxystyryl)-4*H*-chromen-4-one (ASC-7) was found to be the best cytotoxic and antioxidant agent.

Keywords: 7-akmino-2-styrylchromone, Cytotoxicity, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, Flow cytometry, Antioxidant activity.

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INTRODUCTION

Cancer is a class of diseases characterized by out of control cell growth, and there are over 100 different types of cancer. Normal cells in the body follow an orderly path of growth, division, and death. Programmed cell death is called apoptosis, and when this process breaks down, cancer begins to form. Unlike regular cells, cancer cells do not experience programmatic death and instead continue to grow and divide. This leads to a mass of abnormal cells that grow out of control. Cytotoxic drugs are primarily used to treat cancer, frequently as part of a chemotherapy regime. The most common forms of cytotoxic drugs are known as antineoplastic. Cytotoxic drugs can prevent the rapid growth and division of cancer cells. Cytotoxic agents cannot distinguish between normal and malignant cells. They act by interfering with cell division which is not specific to tumor cells, hence normal cells may also be damaged.

Chromones serve as ligands for diverse receptors involved in anticancer, anti-HIV, antibacterial, and anti-inflammatory activities [1-8]. 2-styryl chromones are a small group of chromones with only two members of natural occurrence, (i) hormothamnione and (ii) 6-desmethoxyhormothamnione. Hormothamnione demonstrated potential cytotoxicity against P-388 lymphocytic leukemia and HL-60 human promyelocytic leukemia cell lines [9,10], while 6-desmethoxyhormothamnione exhibited antitumor activity against 9 kb and Colon 38 tumor cells, respectively [11]. 2-styrylchromones have a structure analogous to the flavonoids, with an extra two-carbon olefinic bond between the chromone and the phenyl rings. Due to the double bond system in the structure, 2-styrylchromones are reactive molecules. A number of synthesized 2-styryl chromone derivatives exhibited anti-inflammatory, antiviral, antitumor, anticancer, and antioxidant activities [12-14]. Many simple 2-styryl chromones exhibited moderate activity against PC-3 cells [15]. Several fluorinated 2-styrylchromones were synthesized and screened for their antibacterial activity. The fluorinated styrylchromones were most active against *Bacillus subtilis* followed by *Staphylococcus aureus*. The styrylchromones with two fluorine substitutions showed activity against Gram-negative bacteria also. The 3',5'-difluorostyrylchromone showed the best activity indicating that more fluorine substitutions on the styrylchromone could lead to enhanced activity [16]. However, the cytotoxicity and antioxidant effects of 2-styrylchromones having an amino function on the ring A of 2-styrylchromones have not been reported.

METHODS

7-amino-2-methylchromone required for the synthesis was purchased from Sigma-Aldrich, India, and the other chemicals from Loba Chemicals, India. The cell lines, human colon cancer (HCT-116) cells, human breast cancer (MCF-7) cells, and normal kidney epithelial cells (Vero) were procured from National Centre for Cell Science, Pune, India. Melting points were recorded by open capillary method and purity was assessed using Rf value in thin layer chromatography (TLC) on pre-coated silica gel aluminum backed plates (Kieselgel 60 F254 Merck, Germany) and visualization of spots was done in ultraviolet chamber. The infrared (IR) spectra in KBr pellets were recorded using Shimadzu Fourier Transform IR (FTIR) 8400S spectrophotometer. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded by Bruker

AV500 (400 MHz) spectrometer in deuterated dimethyl sulfoxide (DMSO)/$CdCl_3$ using tetramethylsilane as internal standard. Mass spectra were scanned on a Shimadzu liquid chromatography–mass spectrometry (ESI) 2010A spectrometer.

Synthesis of 7-amino-2-styrylchromones (ASC 1-12)

The synthesis of 7-amino-2-styrylchromone derivatives ASC1-9 was reported in our recent paper [17]. The new compounds ASC 10-12 were synthesized by the same procedure. To a solution of sodium methoxide (0.02 mol) in 20 mL of methanol was added 7-amino-2-methylchromone (0.01 mol) and 0.012 mol of aromatic aldehyde. The solution was stirred for 12-18 hrs. The reaction progress was monitored using TLC (dichloromethane:ethyl acetate 4:1). After the reaction was complete, the reaction mixture was cooled and poured into water containing ice and filtered. The product was washed with water and recrystallized from methanol to obtain 7-amino-2-styrylchromones.The IUPAC names of ASC1-12 are given in Table 1 and Fig. 1.

***In vitro* cytotoxicity studies**

3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT cell proliferation and viability assay are a safe, sensitive, *in vitro* assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. The principle involved is the cleavage of the tetrazolium salt MTT into a blue colored product, formazan by mitochondrial enzyme succinate dehydrogenase. The number of viable cells was found to be proportional to the extent of formazan production by the cells used [18].

Exponentially growing cells were harvested from 25 cm^2 tissue culture flasks, and a stock cell suspension (1×10^5 cell/mL) was prepared. A 96 well flat bottom tissue culture plate was seeded with 1×10^4 cells in 0.1 mL of MEM and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and allowed to attach for 24 hrs. Test compounds were prepared and serially diluted with medium to get a stock solution of 200, 100, 50, and 25 µM. After 24 hrs of incubation, the cells were treated with 20 µL of test solution from respective stocks, 80 µL of fresh medium was added, and the cells were incubated for 48 hrs. Each treatment was performed in triplicate. After the treatment, drug containing media was removed and washed with 200 µL of phosphate-buffered saline (PBS). To each well of 96 well plate, 100 µL of MTT reagent (stock: 1 mg/mL in PBS) was added and incubated for 4 hrs at 37°C. After 4 hrs of incubation, the MTT reagent was removed and to solubilize formazan crystals in the well, 100 µL of 100% DMSO was added to each well. The optical density was measured by an enzyme-linked immunosorbent assay plate reader at 540 nm. The percentage growth inhibition was calculated using the formula below:

$$\%\text{Growth inhibition} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

From the data obtained, the inhibitory concentration 50% (IC_{50}) was calculated for all the compounds against the cell lines MCF-7 and HCT-116 by plotting the dose response curve.

Flow cytometry

Flow cytometric analysis offers a precise technique to check the effect of test compounds on cell cycle progression and check points. 1×10^6 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 hrs. 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 was acquired for each sample, and data analysis was performed using BD Accuri™ C6 software.

***In vitro* antioxidant studies**

1,1-diphenyl-2-picrylhydrazyl (DPPH) method

DPPH, a stable radical and investigated as reactive hydrogen acceptor, has been widely used for studying antioxidant properties of bioactive compounds isolated from the plant extracts. The DPPH free radical is reduced to corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor's changes to yellow color. The discoloration is directly proportional to the amount of antioxidant. To 100 µL of various concentrations of test solution in different wells, 100 µL of DPPH solution was added; control was prepared by adding 100 µL of methanol and 100 µL of DPPH solution and kept for 20 minutes in a dark cupboard for incubation. After 20 minutes, the decrease in the absorbance of the test solution (due to quenching of DPPH free radicals) was read at 540 nm.

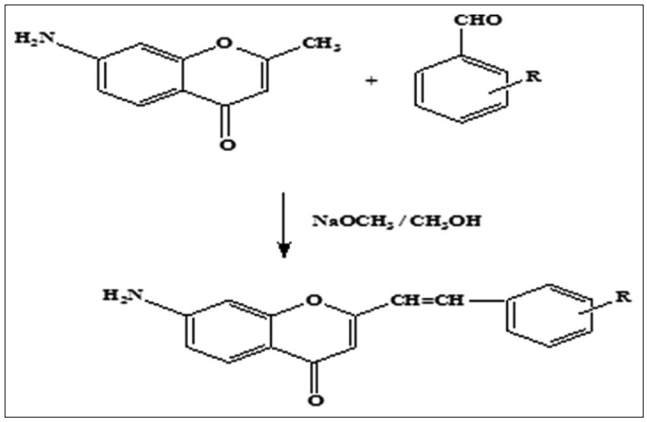

Fig. 1: Scheme for the synthesis of 7-amino-2-styrylchromones

Table 1: IUPAC names for the synthesized 7-amino-2-styrylchromones

Compound code	R	IUPAC name
ASC-1	H	(*E*)-7-amino-2-styryl-4*H*-chromen-4-one
ASC-2	4-Cl	(*E*)-7-amino-2-(4-chlorostyryl)-4*H*-chromen-4-one
ASC-3	4-NO_2	(*E*)-7-amino-2-(3-nitrostyryl)-4*H*-chromen-4-one
ASC-4	3,4-OCH_3	(*E*)-7-amino-2-(3,4-dimethoxystyryl)-4*H*-chromen-4-one
ASC-5	2-OH	(*E*)-7-amino-2-(2-hydroxystyryl)-4*H*-chromen-4-one
ASC-6	3,4-Cl	(*E*)-7-amino-2-(3,4-dichlorostyryl)-4*H*-chromen-4-one
ASC-7	3,4 O-CH_2-O	(*E*)-7-amino-2-(3,4-methylenedioxystyryl)-4*H*-chromen-4-one
ASC-8	4-$C_6H_5CH_2O$	(*E*)-7-amino-2-(4-benzyloxystyryl)-4*H*-chromen-4-one
ASC-9	4-CH_3	(*E*)-7-amino-2-(4-methylstyryl)-4*H*-chromen-4-one
ASC-10	4-F	(*E*)-7-amino-2-(4-fluorostyryl)-4*H*-chromen-4-one
ASC-11	4-OH,3-OCH_3	(*E*)-7-amino-2-(4-hydroxy-3-methoxystyryl)-4*H*-chromen-4-one
ASC-12	4-OCH_3	(*E*)-7-amino-2-(4-methoxystyryl)-4*H*-chromen-4-one

Nitric oxide radical scavenging method

Nitric oxide scavenging activity can be estimated by the use of Griess Illosvoy reaction [19]. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO^+. Under aerobic conditions, NO^+ reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations (5-200 µg/mL) of the compound dissolved in methanol and incubated at 30°C for 2 hrs. The same reaction mixture without the compound but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 550 nm. The % scavenging in both the methods was calculated using the formula:

% Scavenging=(Control absorbance−Test absorbance/Control absorbance) × 100

RESULTS AND DISCUSSION

The molecular weight, % yield, melting point and R_f value of the synthesized compounds are listed in Table 2. The spectral data of compounds ASC1-9 was reported in our previous paper [14]. Here, the spectral data of the newly synthesized compounds ASC10-12 and the ^{1}H NMR, ^{13}C-NMR, mass and IR spectra of a representative molecule ASC-10 are presented (Figs. 2-5).

The FTIR spectra of styrylchromones displayed a strong absorption band due to α,β-unsaturated C=O stretching at 1643-1680 cm^{-1}, 3220-3330 cm^{-1} (NH_2). Aromatic and vinylic protons appeared in the down field region of δ 5.96-8.01. The singlet proton signal observed at δ 6.29-6.40 is due to the presence of H-3 cyclic unsaturated ketone system. Amino protons' signal appeared as singlet at δ 5.00-5.20. The OCH_3 protons resonated at 3.82-3.84 ppm and were observed as a singlet. Carbonyl carbon (C-4) was assigned δ 177.85 in ASC-10. The mass spectra of styrylchromones showed molecular ion peaks, which were in accordance with their respective molecular masses.

Solvent system for TLC-dichloromethane:ethyl acetate 4:1.

(*E*)-7-Amino-2-(4-fluorostyryl)-4*H*-chromen-4-one (ASC-10)

IR (KBr) (cm^{-1}): 1643(C=O), 3220 (NH_2), 3001 (CH str Ar); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 5.00 (s, 2H, NH_2), 6.36 (s, H-3), 5.96-7.78 (9 H, Ar-H and vinylic H); ^{13}C-NMR (DMSO-d_6)δ: 100.05 (C-3), 110.52, 113.51, 114.15, 114.34, 114.44, 115.99, 116.21, 120.36, 127.32, 129.12, 129.27, 129.35, 132.03, 134.58, 151.96, 158.08, 160.70 (15-C), 177.85 (C=O); GC-MS*m/z* 281 (M^+).

Table 2: Physical data of 7-amino-2-styrylchromones

Compound code	R	Yield %	MP (°C)	Rf*
ASC-1	H	81	146-148	0.54
ASC-2	4-Cl	75	158-160	0.57
ASC-3	4-NO_2	71	150-152	0.51
ASC-4	3,4-OCH_3	80	168-170	0.49
ASC-5	2-OH	76	174-176	0.60
ASC-6	3,4-Cl	81	156-158	0.59
ASC-7	3,4 O-CH_2-O	80	212-214	0.61
ASC-8	4-$C_6H_5CH_2O$	75	218-220	0.54
ASC-9	4-CH_3	79	165-167	0.48
ASC-10	4-F	71	156-158	0.50
ASC-11	4-OH,3-OCH_3	74	180-182	0.54
ASC-12	4-OCH_3	78	142-144	0.44

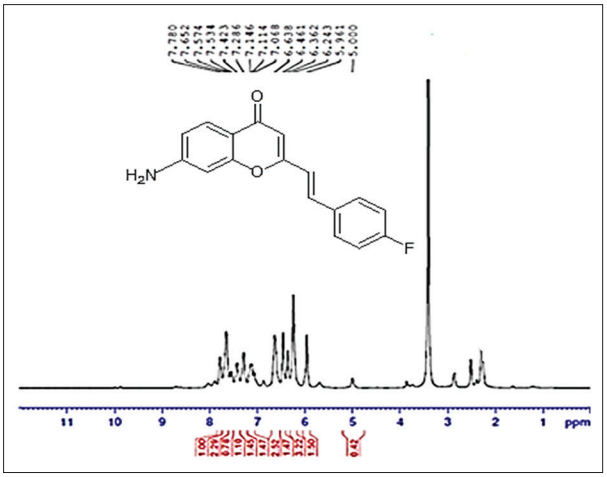

Fig. 2: ^{1}H nuclear magnetic resonance spectrum of (*E*)-7-amino-2-(4-fluorostyryl)-4*H*-chromen-4-one (ASC-10)

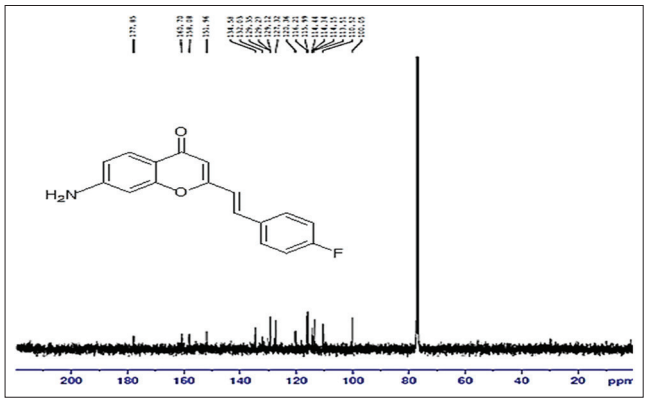

Fig. 3: ^{13}C nuclear magnetic resonance spectrum of (*E*)-7-amino-2-(4-fluorostyryl)-4*H*-chromen-4-one (ASC-10)

(*E*)-7-Amino-2-(4-hydroxy-3-methoxystyryl)-4*H*-chromen-4-one (ASC-11)

IR (KBr) (cm^{-1}): 1684 (C=O), 3330 (NH_2), 3021 (CH str Ar); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 3.82 (s, 3H, OCH_3), 5.02 (s, 2H, NH_2), 6.29 (s, H-3), 6.10-7.70 (8 H, Ar-H and vinylic H), 9.50 (s, 1H, OH); LC-MS *m/z* 309 (M).

(*E*)-7-Amino-2-(4-methoxystyryl)-4*H*-chromen-4-one (ASC-12)

IR (KBr) (cm^{-1}): 1670 (C=O), 3330 (NH_2), 3004 (CH str Ar); ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 3.84 (s, 3H, OCH_3), 5.20 (s, 2H, NH_2), 6.40 (s, H-3), 6.34-7.88 (9H, Ar-H and vinylic H); LC-MS*m/z* 294.17 (M+1).

***In vitro* cytotoxicity screening by MTT assay**

The cytotoxicity screening of all synthesized compounds was done against cell lines MCF-7, HCT-116 and Vero using MTT assay. The results are listed in Table 3. The compounds were more cytotoxic to breast cancer cells than colon cancer cells. (*E*)-7-amino-2-(3,4-methylenedioxystyryl)-4*H*-chromen-4-one (ASC-7) exhibited maximum cytotoxicity (IC_{50} 56.0 µM) followed by (*E*)-7-amino-2-(4-benzyloxystyryl)-4*H*-chromen-4-one (ASC-8) with 67.4 µM against MCF-7. Methylenedioxy grouping, which increases the electron density of the phenyl ring system from two neighboring positions, could impart best cytotoxicity. The benzyloxy substitution, which is another electron releasing function could impart better activity to styrylchromones. ASC-2 having one chloro substituent at the 4th position displayed better activity against MCF-7 than ASC-6 having two chloro groups at adjacent carbons-C3 and C4.The loss of activity in ASC-6 may be due to steric effects of the bulky chlorine atoms. The test compounds were found to be toxic toward Vero cells only at a concentration above 200 µM thus showing good safety profile.

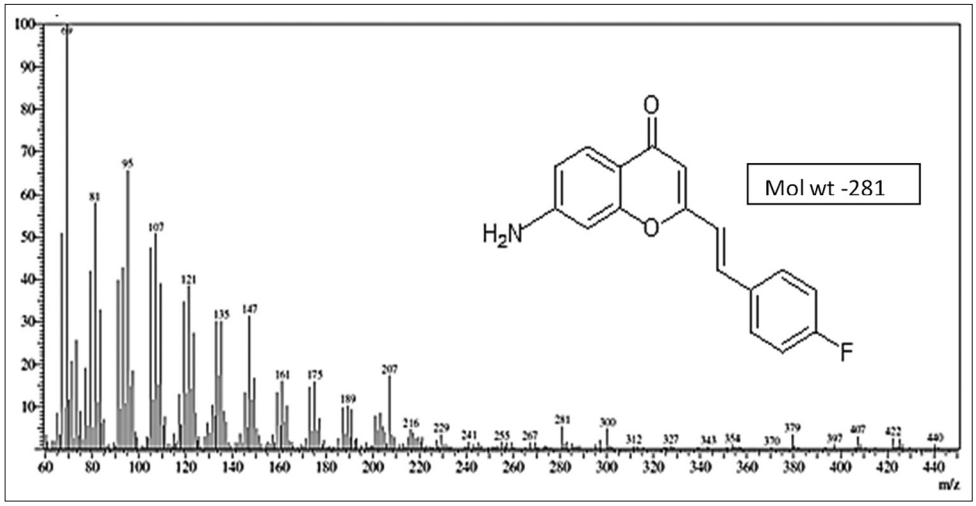

Fig. 4: Mass spectrum of (*E*)-7-amino-2-(4-fluorostyryl)-4*H*-chromen-4-one- (ASC-10)

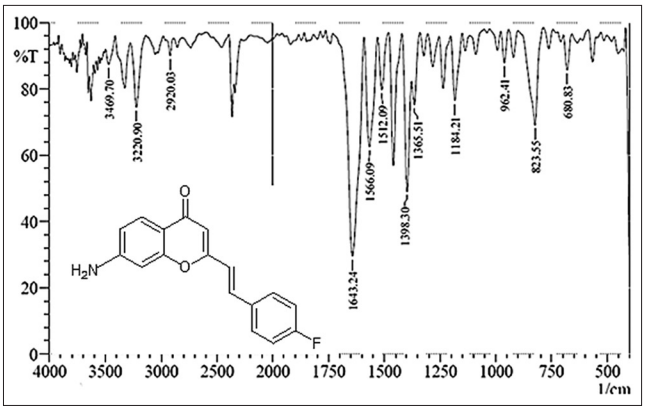

Fig. 5: Infrared spectrum of (*E*)-7-amino-2-(4-fluorostyryl)-4*H*-chromen-4-one (ASC-10)

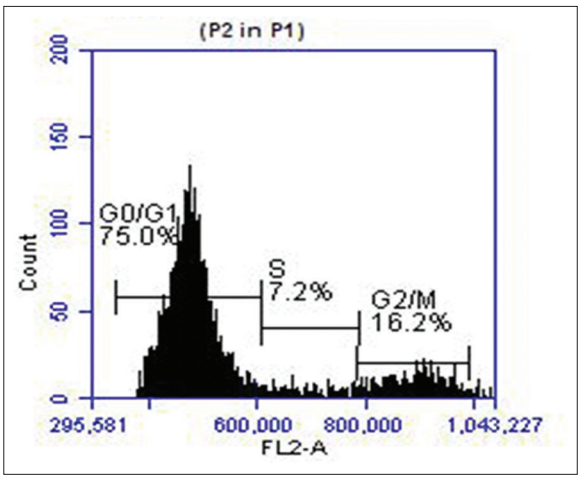

Fig. 6: Effect on the cell cycle of MCF-7 after 48 hrs treatment with ASC-7

Table 3: Cytotoxicity of 7-amino-2-styrylchromones by MTT assay after 48 hrs incubation

Compound code	IC_{50} (µM)		
	HCT-116	MCF-7	Vero
ASC-1	>200	>200	-
ASC-2	153.2	71.6	>200
ASC-3	>200	>200	-
ASC-4	>200	>200	-
ASC-5	>200	97.9	>200
ASC-6	>148.6	>200	>200
ASC-7	>198.0	56.0	>200
ASC-8	>183.1	67.4	>200
ASC-9	>200	>200	-
ASC-10	>200	>200	-
ASC-11	>200	98.6	>200
ASC-12	>200	>200	-
Doxorubicin	0.5	0.5	4.3

-: Not determined, IC_{50}: Inhibitory concentration 50%, MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide

Table 4: Antioxidant activity of 7-amino-2-styrylchromones by DPPH assay

Compound code	IC_{50} (µM)
ASC-1	No activity
ASC-2	231.4
ASC-3	No activity
ASC-4	No activity
ASC-5	123.2
ASC-6	No activity
ASC-7	54.6
ASC-8	71.4
ASC-9	No activity
ASC-10	201.1
ASC-11	No activity
ASC-12	143.5
Ascorbic acid	20.8

IC_{50}: Inhibitory concentration 50%, DPPH: 2,2-diphenyl-1-picrylhydrazyl

Flow cytometric analysis

The cell cycle involves different phases such as G_0-G_1, S, G_2, and M with check point at G1 (restriction point), G2 check point and M (metaphase) check point. These check points play an important role, working as sensors to assess the extent of DNA damage caused by the external factors and facilitate the cell's need to undergo proliferation or apoptosis. If the arrest in the cell cycle continues, it could either repair the damaged DNA or induce apoptosis if the repair of damaged DNA does not happen.

The effect of the compound ASC-7 on the cell cycle of MCF-7 after 48 hrs treatment showed 75.0% cells in G_0/G_1, 7.2% in S, and 16.2% in G_2/M phase (Fig. 6).

Antioxidant activity

The synthesized test compounds were evaluated for their antioxidant activity by DPPH radical scavenging method. Ascorbic acid was used as a standard which had an IC_{50} value of 20.81 µM. The results are displayed in Table 4. Interestingly, the molecules which displayed better cytotoxicity profile, ASC-7 and ASC-8 were found to be better scavengers of DPPH radical. The molecules bearing chloro and fluoro functions (ASC-2 and ASC-10) exhibited activity in the same range, IC_{50} 231.4 and 201.1 µM, respectively. However, ASC-6 having two chloro substituents at 3rd and 4th positions of ring B was inactive. Similarly, ASC-4 having two methoxy groups at 3rd and 4th positions of ring B was inactive whereas ASC-12 is having one methoxy group was found to be reasonably active with IC_{50} 143.5 µM. This may be due to the steric constraints when two bulky groups occupy adjacent positions on the benzene ring.

Nitric oxide radical scavenging assay

None of the tested compounds exhibited nitric oxide scavenging property.

CONCLUSION

In this study, the cytotoxicity and the antioxidant potential of twelve 7-amino-2-styrylchromones were evaluated. The most cytotoxic compound in ASC series are (E)-7-Amino-2-(3,4-methylenedioxystyryl)-4H-chromen-4-one,ASC-7(IC_{50}=56.0µM) and (*E*)-7-Amino-2-(4-benzyloxystyryl)-4*H*-chromen-4-one,ASC-8 (IC_{50}=67.4µM).ASC-7 (IC_{50}=54.6) is the most active antioxidant. To rule out toxicity on normal cells, screening of compounds on Vero cells was also conducted. No indication of toxicity was observed as IC_{50} values in these cases were all above 200 µM. The cell cycle analysis showed ASC-7 caused accumulation of cells in G_0/G_1 phase which indicated the arrest of cells in this phase.

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