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Original article

Synthesis, anticancer and antioxidant activities of 7-methoxyisoflavanone and 2,3-diarylchromanones

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ABSTRACT

A convenient, fast and high yielding method for the preparation of 7-methoxyisoflavanone and 2,3-diarylchromanones has been developed by the condensation of benzyl-2-hydroxy-4-alkoxyphenylketone with arylaldehyde/paraformaldehyde in presence of diethylamine, assisted by microwave activation. All the synthesized compounds were screened for anticancer as well as antioxidant activities. Among the nine compounds, 7-methoxyisoflavanone **7** and diarylchromanone **6c** shows potential anticancer activity and diarylchromanone **6b** has potential antioxidant activity. Compound **6h** possesses anticancer and antioxidant activity at the same concentration.

1. Introduction

Microwave assisted reactions offer a considerable advantages over conventional thermal reactions because it results in substantial rate enhancement in a wide range of organic reactions. Moreover, majority of the microwave assisted reactions are solvent-free reactions, hence they are considered as clean, efficient and economical technology. This methodology has been widely used in a variety of organic reactions [1]. However, the solvent-free synthesis of 2,3-diarylchromanones has not been reported so far.

Chromanones and flavones are integral part of human diet and have been reported to exhibit a wide range of biological effects [2]. They also demonstrate antioxidant [3], anti-inflammatory [4], antibacterial [5] and antitumor [6] properties. Several synthetic analogs of the compounds such as cromokalim, demiflin and flavaxate have been developed into useful drugs [7]. Therefore, many methods of synthesis of 7-methoxyisoflavanone and 2,3-diarylchromanones have been explored. Generally, these reactions were carried out in solution and have drawbacks of using large amount of volatile and poisonous solvents with high reaction time.

2. Chemistry

In order to find an environmentally benign procedure, a new technique of solvent-free, microwave activated synthesis of 2,3-diarylchromanones has been developed (Scheme 1). Using this technique, seven new compounds of 2,3-diarylchromanones (Table 1) and one reported 2,3-diphenylchromanone **6a** and 7-methoxyisoflavanone **7** have been synthesized in short reaction period. Equimolar amounts of benzyl-2-hydroxy-4-alkoxyphenylketone and arylaldehyde/paraformaldehyde were allowed to react in the presence of diethylamine under microwaves with good yield (Table 1). The time taken for the formation of 2,3-diphenylchromanone **6a** was found to be 16 min against 3 h in the conventional method [8]. The time taken for the 7-methoxyisoflavanone **7** was found to be 30 min against 3 h in the conventional method [9].

It is reported that the methylation of various compounds under microwave irradiation results in good yield in a short period of reaction [10]. In the present study, it was noted that the methylation/ethylation of benzyl-2,4-dihydroxyphenylketone to benzyl-2-hydroxy-4-methoxy/ethoxyphenylketone is found to be completed within 3 min against 4 h in the presence of large volume of solvent in the conventional method [11,12].

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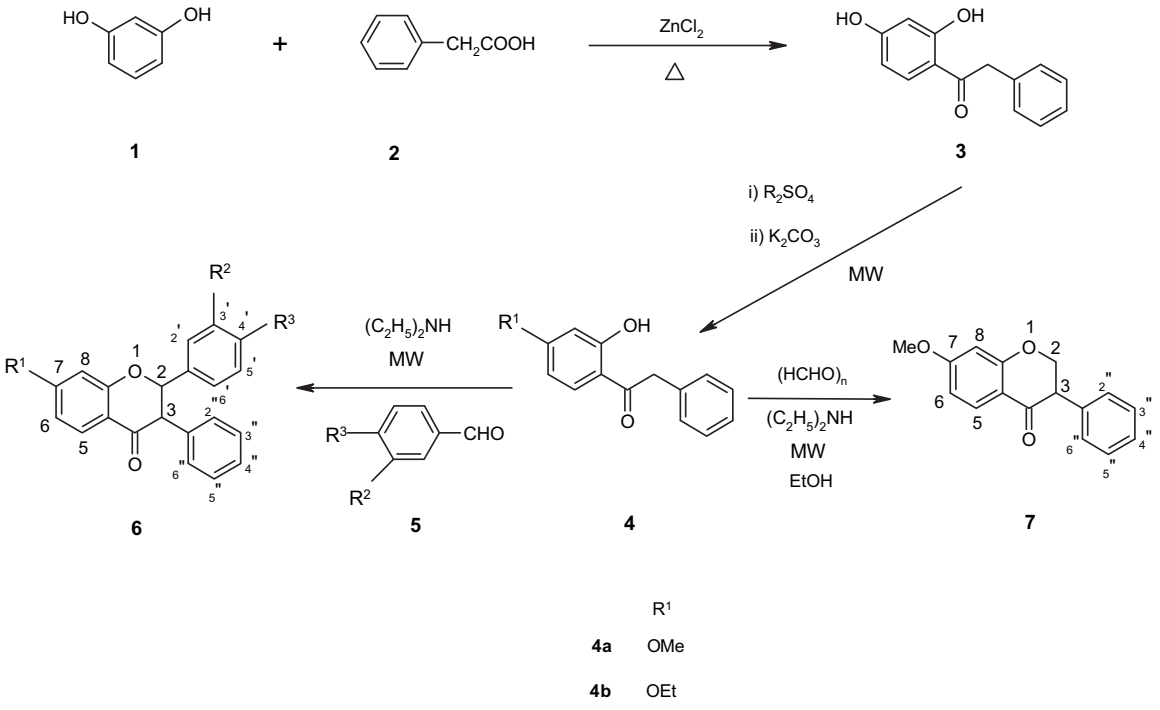

	6a	6b	6c	6d	6e	6f	6g	6h
R^1	OMe	OMe	OMe	OMe	OEt	OEt	OEt	OEt
R^2	H	NO_2	H	H	H	NO_2	H	H
R^3	H	H	NO_2	Cl	H	H	NO_2	Cl

Scheme 1. Synthesis of 7-methoxyisoflavanone and 2,3-diarylchromanones.

Table 1
Physical characterization data and elemental analysis of compounds.

Compound	Reaction period MWI (min.) without solvent	Melting point (°C)	Yield (%)	Molecular formula	Elemental analyses found % (Calcd)		
					C	H	N
6a	16 (3 h)[a]	179–180	70	$C_{22}H_{18}O_3$	79.91 (79.98	5.28 5.49)	
6b	30	124–126	80	$C_{22}H_{17}NO_5$	70.33 (70.39	4.28 4.56	3.73 3.73)
6c	30	135–137	60	$C_{22}H_{17}NO_5$	70.33 (70.39	4.28 4.56	3.73 3.73)
6d	30	168–171	75	$C_{22}H_{17}ClO_3$	72.33 (72.43	4.35 4.70)	
6e	30	129–131	85	$C_{23}H_{20}O_3$	80.18 (80.21	5.42 5.85)	
6f	26	148–151	85	$C_{23}H_{19}NO_5$	70.74 (70.94	5.49 4.92	3.56 3.60)
6g	30	139–142	70	$C_{23}H_{19}NO_5$	70.74 (70.94	5.49 4.92	3.56 3.60)
6h	26	134–137	80	$C_{23}H_{19}ClO_3$	72.87 (72.92	5.01 5.06)	
7	30 (3 h)[a]	72–73	80	$C_{16}H_{14}O_3$	75.48 (75.58	5.45 5.55)	

[a] The value inside the parenthesis indicates the time taken for the completion of the reaction in the conventional method.

3. Pharmacology

Synthesized compounds were evaluated for antioxidant activity and cytotoxicity using HL60 cells and PBMC (Peripheral blood mononuclear cells). Antioxidant activities of the compounds were determined using lipid peroxidation assay (Table 3). The cytotoxicity was assessed by the MTT assay (Table 4). In addition, treatment with HL60 also resulted in nuclear DNA fragmentation, as seen in agarose gel electrophoresis (Fig. 2). This is a hallmark of cells undergoing apoptosis. Confirmation of apoptosis was performed by staining the cells with Annexin V. Annexin V-positive cells were defined as apoptotic cells (Fig. 3).

4. Results and discussion

All the compounds were characterized by elemental analysis (Table 1), UV, FT-IR and ^{1}H NMR spectra (Table 2). The UV-spectra of all compounds showed two maxima one around 274–278 nm and the other at 298–317 nm. The infrared spectra of the chromanones **6a**–**h** showed carbonyl absorption in the region 1670–1677 cm^{-1}, aromatic C–H stretching band in the region 3031–3076 cm^{-1} and aliphatic C–H stretching band in the region 2839–2983 cm^{-1}. In the ^{1}H NMR spectra of the chromanones, apart from the expected aromatic protons in the region 6.47–8.16 δ, two doublets each integrating to one proton were

Table 2
Spectral data of compounds **4b**, **6a**–**h** and **7**.

Compound	UV (λ_{max})	IR (cm^{-1}) (KBr)	^1H NMR ($CDCl_3$) (δ ppm)
4b	321, 280	3446, 3060, 2983, 1637, 1587, 1290, 1197, 1035, 858, 813	1.35(t, CH_3, 3H); 4.0(q, OCH_2, 2H); 4.14(s, CH_2, 2H); 12.64(s, OH, 1H); 7.66(d, H-3, 1H); 6.33–6.37(m, H-4 & H-6, 2H); 7.19–7.27(m, phenyl ring, 5H)
6a	312, 278	3031, 2839, 1672, 1616, 1446, 1251, 1163, 1022, 831	3.8(s, OCH_3, 3H); 4.1(d, H-3, 1H); 5.6(d, H-2, 1H); 6.96(d, H-6, 1H); 6.52(s, H-8, 1H); 6.98–7.06 (m, C-3 phenyl-H, 5H); 7.93(d, H-5, 1H); 7.18–7.23(m, C-2 phenyl-H, 5H)
6b	307, 274	3064, 2940, 1677, 1616, 1537, 1440, 1352, 1251, 1161, 831	3.88(s, OCH_3, 3H); 4.0(d, H-3, 1H); 5.61(d, H-2, 1H); 6.71(d, H-6, 1H); 6.54(s, H-8, 1H); 6.95(m, H-3″, 5″, 2H); 7.22–7.35(m, 5′, 6′, 2″, 4″, 6″, 5H); 8.07(d,H-2′,4′,2H); 7.93(d, H-5, 1H)
6c	308, 277	3046, 2930, 1670, 1616, 1521, 1446, 1348, 1249, 1163, 1108, 833	3.88(s, OCH_3, 3H); 4.03(d, H-3, 1H); 5.61(d, H-2, 1H); 6.71(d, H-6, 1H); 6.54(s, H-8, 1H); 6.96(m, H-3″, 5″, 2H); 7.21(m, H-2″, 4″, 6″, 3H); 7.93 (d, H-5, 1H); 8.16(s, H-3′, 5′, 2H); 7.36(s, 2′, 6′, 2H)
6d	312, 276	3040, 2840, 1672, 1616, 1581, 1575, 1446, 1361, 1249, 1163, 1110, 831	3.85(s, OCH_3, 3H); 4.02(d, H-3, 1H); 5.49(d, H-2, 1H); 6.64(d, H-6, 1H); 6.51(s, H-8, 1H); 7.14(s, H-2′, 6′, 2H); 7.21(m, H-3′, 5′, 2″, 4″, 6″, 5H); 7.93(d, H-5, 1H); 6.96(m, H-3″, 5″, 2H)
6e	298, 276	3050, 2983, 1683, 1608, 1442, 1359, 1251, 1178, 1105, 840, 813	1.43 (3H, t, CH_3); 4.00 (2H, q, OCH_2); 4.05 (1H, d, H-3); 5.53 (1H, d, H-2); 6.49 (1H, d, H-8); 6.65 (1H, dd, H-6); 7.15–7.25(10H, m, C-2 & C-3 phenyl-H); 7.88 (1H, d, H-5)
6f	312, 277	3076, 2926, 1680, 1608, 1529, 1442, 1352, 1253, 1170, 1107, 1037, 846.	1.43 (3H, t, CH_3); 4.05(2H, q, OCH_2); 4.07 (1H, d, H-3); 5.6 (1H, d, H-2); 6.49 (1H, d, H-8); 6.64 (1H, dd, H-6); 8.08–8.14(2H, m, H-2′, 4′); 7.34–7.38 (2H, m, H-5′, 6′); 6.93–6.96 (2H, m, H-3″, 5″); 7.19–7.26(3H, m, H-2″, 4″, 6″); 7.92 (1H, d, H-5)
6g	317, 275	3050, 2929, 1670, 1616, 1521, 1446, 1348, 1249, 1108, 1004, 833	1.45 (3H, t, CH_3); 3.59 (2H, q, OCH_2); 4.1(1H, d, H-3); 5.6 (1H, d, H-2); 6.52 (1H, s, H-8); 6.9 (1H, dd, H-6); 7.2–7.4(5H, m, C-3 phenyl-H); 7.68 (2H, d, H-2′, 6′); 7.95 (2H, d, H-3′, 5′); 8.1 (1H, d, H-5)
6h	312, 277	3034, 2982, 1670, 1612, 1492, 1446, 1359, 1251, 1176, 1107, 1045, 823, 748	1.43 (3H, t, CH_3); 4.05 (2H, q, OCH_2); 4.10 (1H, d, H-3); 5.49 (1H, d, H-2); 6.47 (1H, s, H-8); 6.62 (1H, d, H-6); 6.94–7.26 (9H, m, C-2 & C-3 phenyl-H); 7.8 (1H, d, H-5)
7	275, 234	3050, 2964, 1681, 1614, 1577, 1242, 698	3.8 (s, OCH_3, 3H); 3.9 (t, H-3, 1H); 4.65 (d, H-2, 1H); 6.44 (s, H-8, 1H); 6.62 (d, H-6, 1H); 7.26–7.35 (m, C-3, phenyl-H, 5H); 7.88 (d, H-5, 1H)

observed in the regions 5.49–5.61 δ and 4.0–4.1 δ were assigned to C-2 and C-3 protons, respectively. These two doublets indicated the formation of the products **6a**–**h**. Further, the coupling constant value (11–12 Hz) suggested that the protons at C-2 and C-3 are *trans* oriented [13]. The formation of 7-methoxyisoflavanone **7** was confirmed by UV, IR and ^1H NMR spectra. In ^1H NMR spectra of the 7-methoxyisoflavanone **7**, apart from the expected aromatic protons, C-2 proton appeared as a doublet at 4.35 δ and C-3 proton appeared as a triplet at 3.9 δ.

4.1. Free radical scavenging activity

Testing the antioxidant activity of the 7-methoxyisoflavanone **7** and eight diarylchromanones **6a**–**h** were measured by the inhibition of lipid peroxidation. Table 3 shows the radical modulation activity of the compounds. It was found that the chromanone **6b** possesses highest activity with EC_{50} value of 129.86 μM followed by **6d**, **6f**, **6a** and **6 h** with EC_{50} value of 164.86, 193.29, 499.56 and 578.39 μM, respectively. The higher activity of the chromone is attributed to the conjugation of double bonds and the methoxy group. It can possibly contribute to the activity of this compound as a scavenger of free radicals. This is confirmed by the isolation of the 3-[2-(3,5-dimethoxyphenyl)ethenyl]-2methylchromone from *Erucaria microcarpa* and it was proved to be active as a scavenger [14]. The diarylchromanone derivative **6b** showed the highest radical modulation activity compared to other tested compounds due to the presence of nitro group in the *meta* position. Free radicals are known to induce cellular damage and may play a role in heart disease, rheumatoid arthritis, cancer, inflammatory disorders as well as aging processes [15]. The flavonoids are most common natural antioxidants [16]. These are not only the defense molecules in prevention of different pathological disorders but also commonly used in industry for the prevention of oxidative degradation of polymers, synthetic and natural pigments. In the present study, five diarylchromanones were found to have antioxidant activity.

4.2. Cytotoxicity assay

MTT assay was used to assess the cell viability based on its reduction by mitochondrial dehydrogenase enzyme of the viable cells to purple formazan product [17]. The cytotoxicity of the chromanone derivatives was studied in the acute myeloblastic leukemia HL60 cells and PBMC. The cytotoxicity of the compounds was determined after five days of exposure and its CC_{50} values were calculated. Among the tested compounds, 7-methoxyisoflavanone **7** and diarylchromanone **6c** exhibited highest cytotoxicity with CC_{50} of 82.58 μM and 73.16 μM respectively, while the two diarylchromanones **6g** and **6h** exhibited significant cytotoxicity (Table 4). Their cytotoxicity seems to be specific for tumor cells since normal human lymphocytes were not susceptible. It is realized, however, that normal and neoplastic cells have different rates of proliferation and it is not surprising that an active drug is ineffective on slow-growing normal cells.

Increase in lipid peroxidation denotes cytotoxicity and hepatocellular dysfunction on mice [18]. The EC_{50} and CC_{50} values were compared using one-way analysis of variance ANOVA (Fig. 1). This study showed that the compounds which possess high antioxidant

Table 3
Radical modulation assay of compounds **6a**–**h** and **7**.

Test compound	EC_{50} (μM)
6a	499.56 ± 123.07
6b	129.86 ± 025.58
6c	>2500
6d	164.86 ± 009.45
6e	876.81 ± 597.17
6f	193.29 ± 034.41
6g	>2500
6h	578.38 ± 037.64
7	964.71 ± 239.93
Ascorbic acid	754.14 ± 7.03

Table 4
MTT assay of compounds **6a**–**h** and **7**.

Test compound	CC_{50} (μM)	
	HL60	PBMC
6a	2331.25 ± 499.36	>2500
6b	>2500	>2500
6c	73.16 ± 007.84	594.81 ± 43.31
6d	2414.02 ± 176.49	>2500
6e	>2500	>2500
6f	>2500	>2500
6g	202.92 ± 015.36	1359.74 ± 123.81
6h	514.21 ± 040.58	>2500
7	82.58 ± 007.84	520.51 ± 59.66
5-Fluorouracil	0.07 ± 0.01	NT

Values represent the mean ± standard deviation of three separate experiments ($p < 0.05$).

activity have less cytotoxicity and the compounds which showed high cytotoxicity have less antioxidant activity.

The 7-methoxyisoflavanone **7** showed highest cytotoxicity than 2,3-diarylchromanones except diarylchromanone **6c** due to the absence of phenyl ring in C-2 position. Among the three diarylchromanones **6c** posses highest cytotoxicity than **6g** and **6h** due to the presence of methoxy group in C-7 position and the nitro group in the C-3′ position. Generally, in diarylchromanones presence of methoxy group and nitro group will enhance the activity and the presence of ethoxy group will decrease the activity. Thus, the 7-methoxyisoflavanone **7** and the three diarylchromanones **6c**, **6g** and **6h** have been selected for further studies.

4.3. DNA fragmentation assay

The DNA profile of the cells treated with 7-methoxyisoflavanone **7** and diarylchromanone **6a**–**h** along with control cells was analyzed. In control cell the DNA was not fragmented whereas when the cells are treated with diarylchromanones **6a**–**h** and 7-methoxyisoflavanone **7** have shown apoptosis fragmentation of DNA (Fig. 2). Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments is regarded as a biochemical manifestation of apoptosis [19].

4.4. Apoptosis detection

For further confirmation of apoptosis, FACS analysis was carried out. Apoptosis is an active, genetically regulated disassembly of the cell form within. Disassembly creates changes in the phospholipid content of the cytoplasmic membrane outer leaflet. Phosphatidylserine (PS) is translocated from the inner to the outer surface of the cell for phagocytic cell recognition. The human anticoagulant, Annexin V, is a Ca^{2+}-dependent phospholipid protein with a high affinity for PS. Annexin V labeled with fluorescein can identify apoptotic cells in the population (Fig. 2). It is a confirmatory test for apoptosis. It was also reported that the flavonoid Berberine exhibits the ability to induce apoptosis in HL60 cells [20].

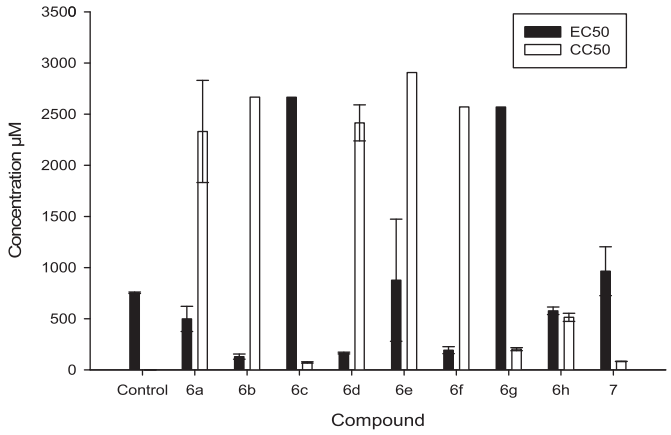

Fig. 1. Comparison between EC_{50} and CC_{50} values. Each bar represents the mean ± SD for three separate experiments. Statistical analysis was performed by ANOVA ($p < 0.05$).

5. Conclusion

The present study, is a quick and simple method for the synthesis of 2,3-diarylchromanones in a solvent-free environment by microwave irradiation with good yield. Further, it is demonstrated that the compounds are potential anticancer and antioxidant agents. Among the nine compounds prepared, diarylchromanone **6b** possesses highest antioxidant activity and 7-methoxyisoflavanone **7** has highest anticancer activity. The diarylchromanone **6h** shows antioxidant activity with EC_{50} value of 578.38 μM and cytotoxicity with CC_{50} value of 514.21 μM. Since it shows both antioxidant activity and cytotoxicity at almost the same concentration, it can prevent the free radical induced cancer as prophylactic agent and kill the cancer cells by apoptotic process as a chemotherapeutic agent. It will be the candidate compound for further studies to be developed as a viable anticancer drug.

6. Experimental protocols

6.1. Chemistry

Melting points were determined in open capillaries and are uncorrected. The IR spectra were recorded on a 8400S SHIMADZU spectrophotometer and the UV-spectra on a SHIMADZU UV-1700 UV–vis spectrophotometer. The ^1H NMR spectra were obtained on a Bruker 200 MHz spectrometer in $CDCl_3$ (Chemical shifts in δ, ppm

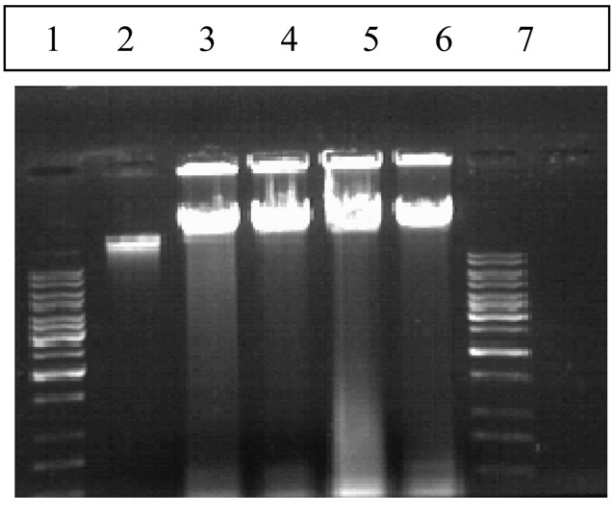

Fig. 2. DNA fragmentation assay of 7-methoxyisoflavanone and 2,3-diarylchromanones.

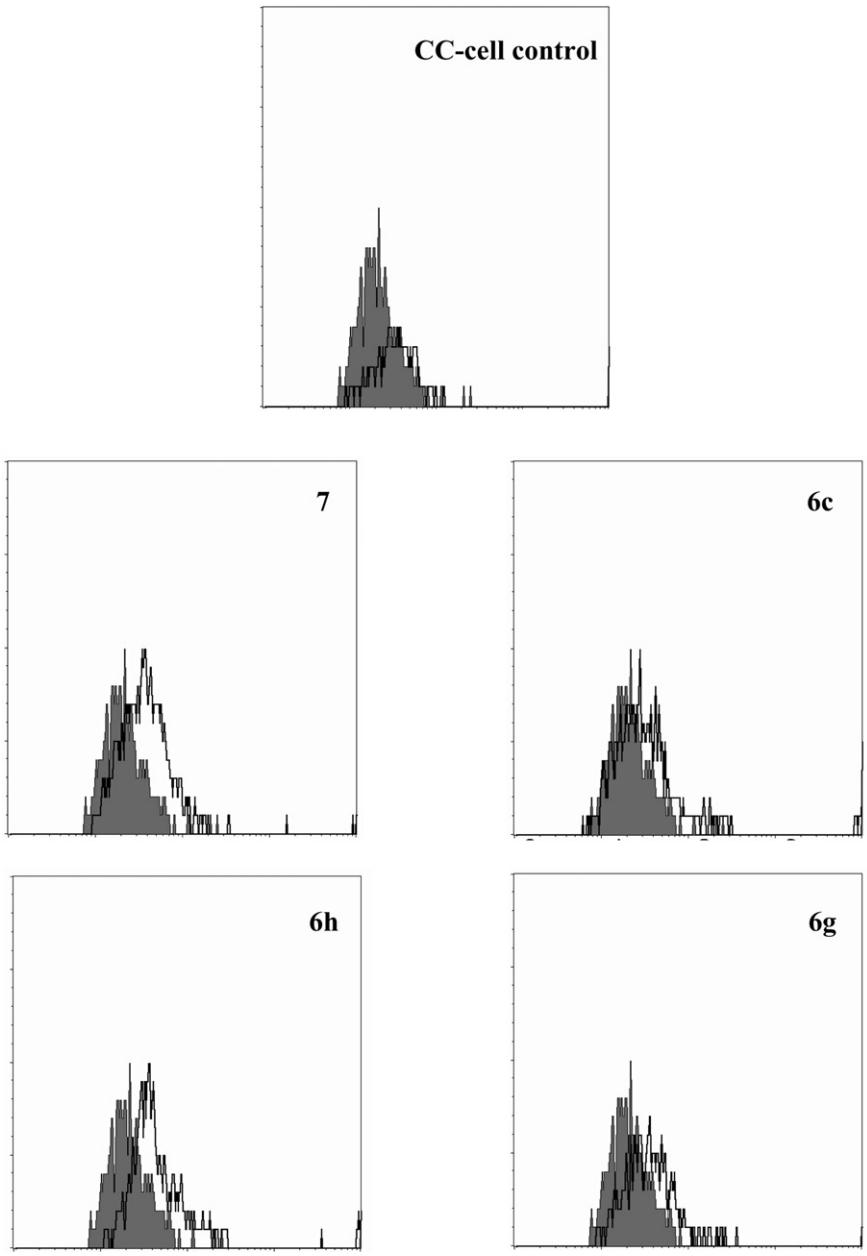

Fig. 3. FACS analysis for apoptosis induction. HL60 cells treated with test compound and then stained with FITC-Annexin V. Shaded histograms represent cellular fluorescence of the control cells and open histograms represent the cellular fluorescence resulting from the specific binding of FITC-Annexin V to apoptotic cells.

relative to TMS as an internal standard). Elemental analyses were done on Elementar Vario EL III.

*6.1.1. Synthesis of benzyl-2-hydroxy-4-methoxy/ethoxyphenyl-ketone (**4a** – methoxy; **4b** – ethoxy)*

Benzyl-2,4-dihydroxyphenylketone (4.56 g) was dissolved in dry acetone (15 mL) in a 100 mL conical flask. To this, dimethyl/ethylsulphate (2 mL) and anhydrous potassium carbonate (5 g) were added. A funnel was kept in the conical flask over which a small round bottom flask filled with water was kept which acted as a condenser. The suspension was irradiated under microwave for 3 min with the time interval of 5 s at 300 W. After the completion of the reaction (monitored with TLC), the reaction mixture was filtered and potassium salt was washed with several portions of acetone. The combined filtrates were evaporated. The residue was treated with ice cold water and neutralized with concentrated hydrochloric acid, when benzyl-2-hydroxy-4-methoxy/ethoxyphenylketone separated as a solid. It was crystallized from ethanol as white crystals. It gave deep red colour with neutral ferric chloride. [**4a**: m.p. 88 °C (lit. [11,12] m.p. 88 °C); 4b: m.p. 77 °C].

6.1.2. General procedure for the synthesis of 2,3-diarylchromanones

To a mixture of benzyl-2-hydroxy-4-methoxy/ethoxyphenyl ketone (0.005 mmol), arylaldehyde (0.005 mmol) and diethylamine (0.001 mmol) were taken in a 50 mL conical flask. A funnel was kept in the conical flask over which a small round bottom flask filled with ice-water was kept which acted as a condenser. The mixture was irradiated in a microwave oven [SAMSUNG M 197 DL] at 100 W for 16 min with the time interval of 30 s. The reaction mixture was treated with ice-water and then neutralized with

concentrated hydrochloric acid. The solid obtained was filtered and crystallized from ethanol as colorless crystals. It was negative towards neutral ferric chloride solution.

6.1.3. Synthesis of 7-methoxy-3-phenylchroman-4-one

An ethanolic solution of benzyl-2-hydroxy-4-methoxyphenylketone (1.21 g, 0.005 mmol), paraformaldehyde (0.3 g, 0.005 mmol) and diethylamine (1.05 mL, 0.001 mmol) were taken in a 50 mL conical flask. A funnel was kept in the conical flask over which a small round bottom flask filled with ice-water was kept which acted as a condenser. The mixture was irradiated in a microwave oven [SAMSUNG M 197 DL] at 100 W for 30 min with the time interval of 30 s. The reaction mixture was treated with ice-water and then neutralized with concentrated hydrochloric acid. The solid obtained was filtered and crystallized from ethanol as colorless crystals. It was negative towards neutral ferric chloride solution. [m.p. 72–73 °C (lit. [9] m.p. 73 °C)].

6.2. Bioassays

6.2.1. Free radical scavenging activity

The free radical scavenging activity of different compounds was determined using lipid peroxidation assay [21]. Briefly, Lipid peroxidation was induced in liposome prepared from egg lecithin by adding 5 μL of 400 mM $FeCl_3$ and 5 μl of 200 mM L-ascorbic acid. To this, different concentrations of the test compound were added. The control was prepared which contained no compound. The samples were incubated at 37 °C for 60 min. The reaction was inhibited by adding 1 mL of stopping solution which contains 0.25 N HCl, 1.5% Trichloroacetic acid, 0.375% Thiobarbutric acid. These reaction mixtures were kept in boiling water bath for 15 min, cooled and centrifuged. The absorbance of the resulting solution was measured at 532 nm. The activity was calculated by using the formula: 50% inhibition (EC_{50}) = [(control OD − sample OD)/control OD] × 100.

6.2.2. Cytotoxicity assay

HL60 cell obtained from the NCCS (National Centre for Cell Sciences), Pune, India were grown in RPMI1640 medium supplemented with 2 mg/mL sodium bicarbonate, 4.5 mg/mL glucose, 100 μg/mL streptomycin sulphate, 40 μg/mL gentamycin, 100 U/mL penicillin as well as 10% heat inactivated fetal calf serum. An environment of humidified air containing 5% CO_2 was maintained at 37 °C. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and cultured as described [22]. Cytotoxicity was determined by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, the cells were suspended at 3×10^5 cell/mL. The cells were placed in 96 well microtiter plates (200 μL/well) and incubated at 37 °C in a CO_2 incubator in the presence of the test compound. After 5 days, cell viability was measured by MTT assay [17], from which 50% cytotoxic concentration (CC_{50}) was calculated.

6.2.3. DNA fragmentation study

HL60 cells were incubated with appropriate concentration of the test compound with their CC_{50} value. After 48 h, DNA was extracted using DNA isolation kit (Genei, Bangalore, India), evaluated on 0.8% agarose gel using ethidium bromide and DNA pattern was documented by gel documentation system (Vilber Lourmet, France).

6.2.4. Apoptosis detection by Annexin V marker

HL60 cells were incubated with appropriate concentration of the test compound with their CC_{50} value. After 24 h, apoptosis induction was analyzed using the apoptotic, necrotic, and healthy cell quantification kit (Biotium inc., USA) following the manufacturer's protocol for flow cytometry (FACS caliber, BD Biosciences, USA) assay [23].

6.2.5. Statistical analysis

Data were analyzed using the software SigmaPlot for Windows (Version 11.0). Values were expressed as mean ± standard deviation of the mean values of three independent experiments followed by student *t*-test. EC_{50} and CC_{50} values were compared using one-way analysis of variance ANOVA. Statistical significance was acceptable to a level of $p < 0.05$.

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