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Research Article

In Vitro Enzyme Inhibition Potentials and Antioxidant Activity of Synthetic Flavone Derivatives

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Free radicals are produced by an important chemical process known as oxidation that in turn initiates chain reactions to damage the cells and originate oxidative stress. Flavones have got special position in research field of natural and synthetic organic chemistry due to their biological capabilities as antioxidant. The antioxidants are known to possess extensive biological effects that include antiviral, antibacterial, anti-inflammatory, antithrombotic, and vasodilatory activities. The simple flavone (**F1**) and substituted flavone derivatives (**F2**–**F5**) have been synthesized from *o*-hydroxyacetophenone and benzaldehyde derivatives in good yield. The structures have been established by different spectroscopic techniques like ^{1}H NMR, ^{13}C NMR, IR, and elemental analysis. Antioxidant profile of these compounds was established using DPPH and H_2O_2 free radical scavenging assay. The findings showed that halogenated flavones showed more enzyme inhibitions and antioxidant activities than simple flavones and are potential candidates for the treatment of wide range of diseases.

1. Introduction

Reactive oxygen species (ROS) and the free radicals like superoxide, hydroxyl, alkoxyl, hydroperoxyl, and peroxyl are produced during normal metabolism in humans [1]. Free radicals are produced by an important chemical process known as oxidation that in turn initiates chain reactions to damage the cells and originate oxidative stress. This process leads to the development of different disorders like Alzheimer's disease [2, 3], Parkinson's disease [4], the pathologies caused by diabetes [5, 6], rheumatoid arthritis [7], and neurodegeneration in motor neuron [8]. The antioxidants have been used specifically as to stop the chain reactions by the removal of free radical intermediates and slow down other oxidation processes by oxidizing themselves and act as reducing agents like polyphenols or ascorbic acid [9]. Naturally, a complex system of enzymes and antioxidant metabolites work in coordination to stop oxidative damage to the cellular components like proteins, DNA, and lipids by preventing the formation or removal of these reactive species before damaging the important components of the cells [10, 11].

Lipoxygenases (LOX) are the members of a class of nonheme iron containing dioxygenases that catalyze the first step in the arachidonic acid cascade that lead to formation of lipoxins and leukotrienes involved in the variety of inflammatory responses [12]. Alzheimer's disease is the common reason of mental illness (dementia) that shows a progressive loss of cholinergic synapses in the brain regions. Mostly, a decreased level of neurotransmitter acetylcholine

(ACh) at neuromuscular junction plays a critical role in this disease. Hence, these types of disorders can be overcome by restoring the adequate level of neurotransmitter to inhibit cholinesterase using agents known as anticholinesterase [13].

In general, the flavonoids are reported to possess potent antioxidant activity [14] by scavenging hydroxyl radicals, superoxide anions, and lipid peroxyradicals [15, 16]. Based upon the significance of flavonoids, an attempt was made to synthesize the flavones derivatives for their possible enzyme inhibition and antioxidant potentials. Here, the first objective was to report synthesis of flavones derivatives and its evaluation for enzyme inhibition and antioxidant activities. The second objective was to establish the structure activity relationship (SAR) of the flavone derivatives.

2. Experimental

2.1. Materials. Ketone and benzaldehyde derivatives, DPPH, ascorbic acid, quercetin, rutin, silica, DTNB 5,5-dithiobisnitrobenzoic acid, enzymes including 15-lipoxigenase (soybean), AChE *Electric eel*, substrates acetylthiocholine iodide, and galantamine hydrobromide were of Sigma Aldrich Chemical Company. TLC plates were of Merck 60 F254, Darmstadt, Germany. Solvents and chemicals like ethanol, n-hexane, ethyl acetate, dipotassium hydrogen phosphate, and potassium dihydrogen phosphate used were of extra pure analytical grade and were purchased from E. Merck.

^{1}H-NMR and ^{13}C NMR spectra were recorded in deuterated chloroform (CDCl3) on Bruker SF spectrometers operating at 300 and 75 megahertz (MHz) frequencies, respectively. Chemical shifts values are expressed in δ (ppm) downfield relative to TMS which was used as an internal standard. Infrared spectra were recorded on Thermo Scientific USA (Nicolet 6700) Infrared spectrometer on KBr disk method. All melting points are uncorrected and were taken in open capillary tubes using Electrothermal 9100 apparatus (Barnstead, UK). Reaction extents and final products purities were checked on TLC plates (Merck 60 F254, Darmstadt, Germany) and spots were visualized under UV Lamp (180–365 nm) and with subsequent staining with iodine vapours.

*2.2. General Procedure for the Synthesis of Flavone Derivatives (**F1**, **F2**, **F3**, **F4**, and **F5**).* To an ethanolic solution of 2-hydroxyacetophenone (15 mili mol), sodium hydroxide (10 mL, 40% ethanolic) was added dropwise at room temperature. Then corresponding benzaldehyde derivatives (15 mili mol) were added dropwise to this mixture and stirred for 24 hours at room temperature (25 ± 2°C). The reaction was monitored by TLC and upon completion of the reaction, it was poured into crushed ice and neutralized with 1N HCl solution resulting in yellow precipitates of corresponding chalcones. The chalcones were filtered and washed with water to remove the impurities.

In the next step, the respective chalcones were cyclized to flavone derivatives in 15 mL DMSO in the presence of iodine (375 mg) at 140°C for 1 hour separately. Upon completion of reactions, the mixtures were cooled to room temperature and poured into water followed by extraction with ethyl acetate (25 mL × 3), treated with sodium thiosulfate solution (20%) and brine solution, and dried over sodium sulfate. The final products (mixture of flavone and chalcone) were subjected to column chromatography using n-hexane: ethyl acetate (9 : 1) to purify flavones derivatives (Figure 1) [17].

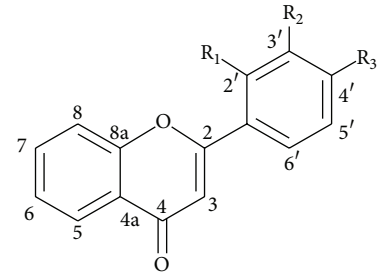

Figure 1: Structure of flavone derivatives.

*2-Phenyl-4H-chromen-4-one (**F1**).* ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.22 (dd, J = 8.0, 1.7 Hz, 1H), 7.75–7.53 (m, 5H), 7.50–7.37 (m, 4H), 6.85 (s, 1H).^{13}C NMR (75 MHz, $CDCl_3$) δ 178.48, 163.42, 156.26, 133.79, 131.77, 131.61, 129.04, 126.66, 125.71, 125.24, 123.95, 118.09, 107.58 IR (KBr), υ, cm^{-1}, 1635.4, 1463.3, 1372.4, 766.0. Found, %: C 81.07; H 4.54. $C_{15}H_{10}O_2$. Calculated, %: C 81.19; H 4.60 [18, 19].

*2-(4-(Dimethylamino)phenyl)-4H-chromen-4-one (**F2**).* ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.24 (dd, J = 7.9, 1.7 Hz, 1H), 7.89–7.81 (m, 2H), 7.68 (ddd, J = 8.7, 7.1, 1.7 Hz, 1H), 7.55 (dd, J = 8.4, 1.3 Hz, 1H), 7.40 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H), 6.80–6.76 (m, 2H), 6.73 (s, 1H), 3.10 (s, 6H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ ppm = 178.20, 163.7, 156.50, 152.60, 133.22, 127.75, 125.58, 124.81, 124.03, 117.84, 111.66, 104.39, 40.10. IR (KBr) υ, cm^{-1}, 2919.4 (CH) 1730.3 (C=O), 1197.8 and 1363.2 (C–N), 1558.1 (C=C), 3311.5 (=C–H) 1127.2 (C–O). Found, %: C 76.96; H 5.70; N 5.28. $C_{17}H_{15}N_{O2}$. Calculated, %: C 76.59; H 5.60; N 5.60.

*2-(2,4-Dichlorophenyl)-4H-chromen-4-one (**F3**).* ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.27 (dd, J = 8.0, 1.7 Hz, 1H, H-3′), 7.74 (ddd, J = 8.7, 7.1, 1.7 Hz, 1H, 5′-H), 7.64–7.56 (m, 2H, 5-H, 6′-H), 7.55–7.40 (m, 3H, 6-H, 7-H, 8-H), 6.68 (s, 1H, H-3).^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 177.98, 161.51, 156.54, 137.43, 134.06, 133.81, 131.42, 130.77, 130.40, 127.58, 125.82, 125.50, 123.81, 118.18, 113.16. IR (KBr) V_{max} cm^{-1}: 3066.5, 2920.6, 1734.1, 1645.4, 1221.1, 748.2. Found, %: C 60.99; H 2.28. $C_{15}H_8Cl_2O_2$. Calculated, %: C 61.88; H 2.77.

*2-(2,3-Dichlorophenyl)-4H-chromen-4-one (**F4**).* ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.32–8.17 (m, 1H, H4′), 7.86–7.61 (m, 2H, H5′,6′), 7.56–7.21 (m, 4H, H-5, 6, 7, 8), 6.67 (s, 1H, H3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.39, 162.62, 156.58, 134.60, 134.26, 134.08, 132.62, 129.80, 128.93, 127.65, 125.85, 125.61, 123.69, 118.23, 112.98. IR (KBr) V_{max} cm^{-1}: 3048.9, 2918.5, 1714.7, 1659.3, 1191.3, 747.8. Found, %: C 62.02; H 2.46. $C_{15}H_8Cl_2O_2$. Calculated, %: C 61.88; H 2.77.

*2-(3,4-Dichlorophenyl)-4H-chromen-4-one (**F5**).* ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.24 (dd, J = 8.0, 1.7 Hz, 1H),

8.04 (d, J = 2.1 Hz, 1H), 7.74 (tq, J = 7.0, 2.2 Hz, 2H), 7.65–7.56 (m, 2H), 7.46 (ddd, J = 8.2, 7.1, 1.1 Hz, 1H), 6.80 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.03, 160.82, 156.08, 135.96, 134.09, 133.70, 131.69, 131.11, 128.06, 125.78, 125.55, 125.25, 123.87, 118.06, 108.19. IR (KBr) υ, cm^{-1}, 1659.3, 1413.7, 1378.8, 750.04, 747.8. Found, %: C 61.92; H 2.53. $C_{15}H_8Cl_2O_2$. Calculated, %: C 61.88; H 2.77.

2.3. In Vitro Antioxidant and Enzyme Inhibition Activity

2.3.1. DPPH Radical Scavenging Activity. The antioxidant activity of the synthesized compounds, ascorbic acid, tocopherol, and rutin was measured with the slight modifications using DPPH. 2% methanolic solution of DPPH was freshly prepared and 1 mL from this solution was added to each 1 mL of different concentrations of the tested flavone derivatives ranging from 25 to 150 μg/mL.

After 30 minutes, the absorbance was measured at 517 nm. Ascorbic acid, tocopherol, and rutin were used as a positive control. The scavenging activity of tested samples was calculated by the following formula in triplicate [20]:

$$\%\text{scavenging} = \left\{\frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})}\right\} \times 100, \quad (1)$$

where A_{control} = absorbance of DPPH + methanol and A_{sample} = absorbance of DPPH + sample/standard.

2.3.2. Hydrogen Peroxide Scavenging Activity. The antioxidant activity of the synthesized compounds, ascorbic acid, tocopherol, and rutin was measured with the slight modifications using hydrogen peroxide. A 2 mM solution of hydrogen peroxide was prepared in phosphate buffer (50 mM, pH 7.4). 0.1 mL of flavone derivatives (25–150 μg/mL) was transferred into test tubes and their volumes were made up to 0.4 mL with phosphate buffer or solvent. After careful addition of hydrogen peroxide solution (0.6 mL), tubes were then incubated for 10 minutes and were determined against a blank (50 mM phosphate buffer). Ascorbic acid, tocopherol, and rutin were used as a positive control [20].

The scavenging ability was calculated (in triplicate) by the following formula:

$$\%\text{scavenging activity} = \left\{\frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})}\right\} \times 100, \quad (2)$$

where A_{control} = absorbance of H_2O_2 + methanol and A_{sample} = absorbance of H_2O_2 + sample/standard.

2.3.3. In Vitro Lipoxygenase Activity. The lipoxygenase activity of synthesized flavones was determined by spectrophotometric method with slight modification. Inhibition was determined by measuring the loss of soybean 15-LOX activity (5 μg) with 0.2 μM linoleic acid as the substrate prepared in borate buffer (0.2 M, pH 9.0). The inhibition in triplicate at various concentrations of synthetic flavones (12.5, 25, 50, 100, 150, 200, and 250 μg/mL) was recorded at 234 nm using UV-Vis spectrophotometer. Indomethacin and quercetin were used as positive control, while methanol was used as negative control. IC_{50} indicating the concentration of 50% inhibition was also calculated [21].

2.3.4. In Vitro Anticholinesterase Activity. The *in vitro* anticholinesterase activity of synthesized flavones was determined by spectrophotometric method with slight modification as per our previous reported method. Various concentrations of synthetic flavones further diluted in phosphate buffer (0.1 M) in different concentrations (12.5, 25, 50, 100, 150, 200, and 250 μg/mL) were achieved. AChE (518 U/mg) was diluted in 0.1 M phosphate buffer (pH 8.0) until final concentration of 0.03 U/mL (AChE) was obtained. Solutions of DTNB (0.2273 mM) and ATchI (0.5 mM) were prepared in distilled water. For each sample, enzyme solution of 5 μL followed by flavones derivatives (205 μL) and DTNB reagent (5 μL) were mixed. The solution mixture was maintained at 30°C for 15 min using water bath with subsequent addition of substrate solution (5 μL). A double beam spectrophotometer (Thermo Electron Corporation, USA) was used to measure the absorbance at 412 nm. IC_{50} indicating the concentration of 50% inhibition was also calculated [22].

2.4. Statistical Analysis.

Data are presented as mean ± SEM. Analysis of variance and Dunnett's test are statistically manipulated with GraphPad Prism 5 version 5.01 software.

3. Results and Discussion

The general structure and physical parameters of flavone derivatives are given in Figure 1 and Table 1. The *in vitro* enzyme inhibition potentials capacity of the flavones derivatives was determined and IC_{50} values are given in Table 2. It is evident that halogenated derived flavones (**F3**, **F4**, and **F5**) showed good activity in comparison with other flavones derivatives (**F1** and **F2**). These results suggest that change in the position or additional moiety may increase or decrease the potency of individual flavones. The antioxidant capacity of the flavones derivatives was estimated with DPPH and H_2O_2 scavenging systems and results are shown in Tables 3 and 4. The concentration dependent DPPH scavenging effects of flavones derivatives are given. Among the synthesized flavones, the maximum concentration dependent DPPH scavenging effects of 83.16 ($P < 0.001$) at 120 μg/mL were observed by **F5** while mild effects of 51.06 ($P < 0.05$) were produced by **F2** at ahigh concentration of 120 μg/mL and are comparable with standard ascorbic acid, tocopherol, and rutin. Similar type of findings was observed using H_2O_2 scavenging system and is given in Table 4. It is evident from the results (Table 1) that nitrogen containing flavone derivative (**F2**) showed less activity in comparison with halogenated one (**F5**). Other halogens containing flavones (**F3** and **F4**) also showed antioxidant activity but it was less in comparison to **F5**, thus suggesting that positioning of halogens may increase or decrease the antioxidant effects as

Table 1: Physical parameters of flavone derivatives.

Flavone	R_1	R_2	R_3	Yield	Appearance	R_f	M.P. (°C)
F1	–H	–H	–H	68.7%	Creamy white solid	0.58	96–98
F2	–H	–H	$-N(CH_3)_2$	73.6%	Brick red solid	0.67	107–109
F3	–Cl	–H	–Cl	87.0%	White solid	0.57	90.5
F4	–Cl	–Cl	–H	81.0%	White solid	0.64	88.3
F5	–H	–Cl	–Cl	79.3%	White solid	0.61	195–197

Table 2: *In vitro* enzyme inhibition potentials of flavone derivatives.

Sample test	LOX IC_{50} (μg/mL)	AChE IC_{50} (μg/mL)
F1	77.29 ± 1.32	187.23 ± 1.65
F2	98.63 ± 1.91	≥250
F3	65.75 ± 1.45	131.33 ± 1.05
F4	61.62 ± 1.15	126.29 ± 1.39
F5	58.33 ± 1.69	112.33 ± 1.68
Indomethacin	53.66 ± 1.38	—
Quercetin	38.50 ± 1.72	—
Galantamine	—	25.66 ± 1.09

All the values were expressed as mean ± SEM ($n = 3$).

evident from the findings. Simple flavone (**F1**) also produced the effects that were almost similar to halogenated ones. These findings may help the future research and open a new window for the synthesis of potent antioxidants for the treatment of wide range of diseases associated with ROS.

In normal situations, the free radicals as by-products are constantly formed by the body's cells from the cellular redox process using oxygen, an essential element of life [23]. These are generally called reactive oxygen species (ROS) and have a special attraction for proteins, carbohydrates, lipids, and nucleic acids [24]. It has been reported that ROS can be both beneficial and harmful based on the concentration and environment in the biological systems [25, 26]. The beneficial effects involve a defense against infections, function of cellular signaling, and gene expression. On the other hand, the ROS can mediate injury to cell structures and often referred to as an "oxidative stress" [27]. The harmful effects of ROS are counteracted by antioxidants, some of which are enzymes present in the body [28].

Natural antioxidants like α-tocopherol, carotenoids, ascorbic acid, flavonoids, and other phenolic compounds might also play a significant role as physiological and dietary antioxidants [29, 30]. The natural antioxidants are known to possess extensive biological effects that include anticancer, antiviral, antibacterial, anti-inflammatory, antithrombotic, and vasodilatory activities [31].

One method of estimating the antioxidant activity is based on the use of a stable free radical known as DPPH [32–35] and the electron donation ability of antioxidants can be determined by DPPH purple-colored solution bleaching [36]. This method is based on scavenging of DPPH through the addition of an antioxidant that decolorizes the DPPH solution and degree of decolorization is proportional to the free radical scavenging activity indicating its potency [37]. Hydrogen peroxide occurs naturally at low concentration in the air, water, food, plants, microorganisms, and human body [38]. H_2O_2 rapidly converts into water and oxygen and may produce hydroxyl radicals ($^{\bullet}OH$) that can initiate the lipid peroxidation and cause damage to DNA [39].

LOXs are sensitive to antioxidants as they are involved in inhibition of lipid hydroperoxide formation due to scavenging of lipid oxy- or peroxyradicals and can minimize LOX catalysis by less availability of lipid hydroperoxide substrate [40]. Studies have implicated oxygen free radicals in the process of inflammation and phenolic compounds like flavonoids may block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity and may serve as a scavenger of reactive free radicals which are produced during arachidonic acid metabolism [41].

Flavonoids are phenolic compounds that are mostly present in fruits, vegetables, and tea; they are an integral part of the human diet [42]. Synthetic as well as naturally occurring flavonoid derivatives have many interesting pharmacological activities. These include antitumor [43, 44], anticonvulsant [45], vasorelaxant [46], analgesic [47], antioxidant [48], and anti-inflammatory [49] activities. Various synthetic halogenated flavonoids derivatives have been found to be very potent natural flavonoids [50, 51].

Several studies have reported that natural antioxidants are associated with low rate of heart, cancer, diabetes, and other diseases associated with ageing [52, 53]. These findings will help the researcher to explore the development of synthetic flavones derivatives for the treatment of wide range of diseases associated with ROS like inflammation and Alzheimer's disease.

4. Conclusion

In conclusion, the present study confirms the enzyme inhibition and antioxidant activities of flavone derivatives. These findings will open a new channel to synthesize halogenated flavones and explore the development of synthetic flavones derivatives for the treatment of wide range of diseases associated with ROS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 3: DPPH radical scavenging activity of flavone derivatives.

	% Scavenging activity (DPPH)					
	25 μg/mL	50 μg/mL	75 μg/mL	100 μg/mL	125 μg/mL	150 μg/mL
Ascorbic acid	65.23 ± 0.41	71.56 ± 0.61	75.26 ± 0.68	81.09 ± 0.42	86.11 ± 0.87	90.68 ± 0.58
Tocopherol	72.32 ± 0.36	79.16 ± 0.57	84.41 ± 0.61	90.18 ± 0.53	92.24 ± 0.75	94.33 ± 0.49
Rutin	79.03 ± 0.76	83.11 ± 0.43	87.63 ± 0.81	91.25 ± 0.61	95.35 ± 0.66	97.67 ± 0.51
F1	32.12 ± 0.61	38.46 ± 1.17	46.48 ± 0.57*	55.81 ± 0.81**	61.65 ± 1.06**	63.44 ± 0.75**
F2	16.65 ± 1.04	21.62 ± 0.67	30.94 ± 0.63	37.63 ± 0.39	44.74 ± 0.86*	47.76 ± 0.82*
F3	42.47 ± 0.84*	51.62 ± 1.21**	59.74 ± 0.75**	63.18 ± 0.54***	68.21 ± 0.61***	70.16 ± 0.69***
F4	35.31 ± 0.92	38.32 ± 0.62*	55.27 ± 0.43**	61.65 ± 0.49***	65.36 ± 0.72***	68.14 ± 0.55***
F5	41.96 ± 0.54*	53.78 ± 1.31**	61.21 ± 0.69***	65.23 ± 0.71***	71.96 ± 0.47***	73.12 ± 0.48***

All the values were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ when compared to control group (one-way ANOVA followed by Dunnett's: compare all versus control test).

Table 4: Hydrogen peroxide radical scavenging activity of flavone derivatives.

	% Scavenging activity (H_2O_2)					
	25 μg/mL	50 μg/mL	75 μg/mL	100 μg/mL	125 μg/mL	150 μg/mL
Ascorbic acid	71.23 ± 0.65	76.57 ± 0.49	84.06 ± 0.81	88.19 ± 0.56	92.21 ± 0.71	96.63 ± 0.63
Tocopherol	73.02 ± 0.43	76.26 ± 0.66	82.43 ± 0.73	90.38 ± 0.87	93.65 ± 0.63	95.47 ± 0.48
Rutin	77.13 ± 0.69	82.15 ± 0.87	88.43 ± 0.34	92.35 ± 0.61	97.16 ± 0.46	98.84 ± 0.74
F1	28.21 ± 0.84	32.52 ± 0.77	38.33 ± 0.87*	47.41 ± 0.36**	55.34 ± 0.67**	57.84 ± 0.69**
F2	13.75 ± 0.60	17.12 ± 0.97	25.77 ± 0.68	32.35 ± 0.79	36.47 ± 0.68	42.19 ± 0.48*
F3	37.43 ± 0.48	48.35 ± 0.91**	53.19 ± 0.57**	56.78 ± 0.45***	60.46 ± 0.46***	63.16 ± 0.84***
F4	31.62 ± 0.54	35.13 ± 0.77	46.91 ± 0.74*	51.85 ± 0.78**	57.74 ± 0.56**	61.39 ± 0.43**
F5	43.07 ± 0.48*	51.72 ± 0.76**	57.68 ± 0.49**	62.86 ± 0.65***	70.21 ± 0.71***	72.26 ± 0.78***

All the values were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ when compared to control group (one-way ANOVA followed by Dunnett's: compare all versus control test).

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