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Synthesis and anti-inflammatory activity of fluorinated isocoumarins and 3,4-dihydroisocoumarins

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Abstract

The synthesis of several fluorinated isocoumarins and 3,4-dihydroisocoumarins are described. The structures of the synthesized compounds were confirmed by spectral and elemental analysis. All synthesized compounds were evaluated for their anti-inflammatory and antioxidant activity. These compounds were found to present antioxidant and anti-inflammatory activities. A few of them were found to be significantly active in vivo against ear edema in mice produced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and also good radical scavengers.

Keywords: Synthesis; Fluorinated isocoumarins; 3,4-Dihydroisocoumarins; Anti-inflammatory activity; Antioxidant activity

1. Introduction

Inflammation is a complex phenomenon involving interrelationships between humoral or cellular reactions and a number of inflammatory mediators. It is a usual symptom covering different pathologies, and there are still many questions to be answered in order to understand the inflammatory process as well as a need for better-tolerated and more efficient non-steroidal anti-inflammatory drugs. In the pathways of the inflammatory process, the implication of free radicals is particularly important. It has also been reported that anti-inflammatory drugs may be effective in the prevention of free radical-mediated damage [1].

Isocoumarins and 3,4-dihydroisocoumarins have been reported to have multiple biological activities. The isocoumarin nucleus is an abundant structural motif in natural products [2]. Many constituents of the steadily growing class of known isocoumarins exhibit valuable biological properties such as antifungal [3,4], antitumor or cytotoxic [5–7], anti-inflammatory, anti-allergic and enzyme inhibitory activity [8]. Naturally occurring isocoumarins containing halogens have been seldom reported, examples of naturally occurring isocoumarins containing fluorine are not known yet. However a few examples of naturally occurring isocoumarins containing chlorine and bromine have been reported. 4-Chloro-3-[(4-fluorophenyl) methoxy] isocoumarin has been found [9] to be quite effective inhibitor for human Q31 granzyme A, murine and human granzyme A isolated from cytotoxic T lymphocytes. This isocoumarin derivative has also been found [10] to be useful in the treatment of emphysema as serine protease inhibitor. 6-(2-Chloro-4-trifluromethylphenoxy)-3,4-dihydroisocoumarin has been used [11] as an herbicide, which almost totally controlled the growth of *Schinochloa crusgall, Sinapis alba* and other weeds. In continuation of our previous studies [12–14] and important biological activities associated with fluoro substituted isocoumarins and 3,4-dihydroisocoumarins prompted us to synthesize some new 3-difluorophenylisocoumarins and their conversion to the corresponding 3,4-dihydroisocoumarin in order to check their anti-inflammatory and antioxidant activities. General synthetic scheme is shown as follows (Scheme 1).

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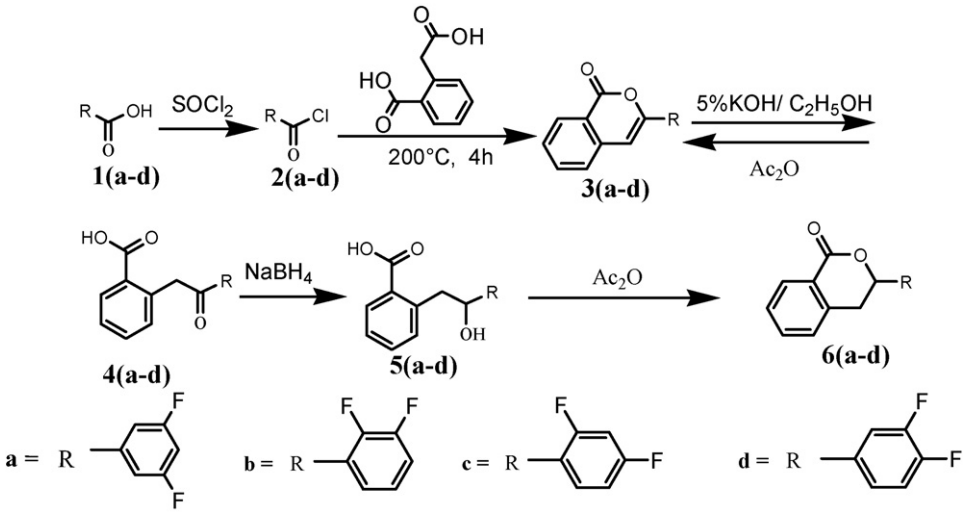

Scheme 1. Synthesis of fluoro-substituted isocoumarins.

2. Results and discussion

2.1. Synthesis

Condensation of the acid chloride with homophthalic acid is useful for the preparation of 3-substituated isocoumarins skelton [14]. A short and efficient synthesis of 3-(difluorophenyl)isocoumarins **3**(**a**–**d**) using this method and conversion of **3**(**a**–**d**) into corresponding racemic 3-(difluorophenyl)-3,4-dihydroisocoumarins **6**(**a**–**d**) were achieved and anti-inflammatory and antioxidant activities of the synthesized compounds were examined. Difluorobenzoic acids **1**(**a**–**d**) were converted into their respective acid chlorides **2**(**a**–**d**) by reaction with thionyl chloride. Direct condensation of acid chlorides **2**(**a**–**d**) with homophthalic acid at 200 °C afforded 3-difluorophenylisocoumarins **3**(**a**–**d**) in 77–80% yield, which were purified by column chromatography. These isocoumarins **3**(**a**–**d**) exhibited characteristic 1H-singlet at δ 6.86–6.93 ppm for C_4–H in ^{1}H NMR. In IR spectra of isocoumarins **3**(**a**–**d**) lactonic carbonyl absorptions were observed at 1703–1709 cm^{-1}. The molecular ion peak in the mass spectrum of isocoumarin **3a** was obtained at *m*/*z* 258. Alkaline hydrolysis of isocoumarins **3**(**a**–**d**) afforded 2-(difluoro benzoylmethyl)benzoic acids **4**(**a**–**d**). Isocoumarins **3**(**a**–**d**) were obtained on refluxing keto-acids **4**(**a**–**d**) with acetic anhydride (reversible reaction). Sodium borohydride reduction of keto-acids **4**(**a**–**d**) afforded the corresponding racemic hydroxy acids **5**(**a**–**d**), which were cyclodehydrated with acetic anhydride to produce 3-difluorophenyl-3,4-dihydroisocoumarin **6**(**a**–**c**) which exhibited the carbonyl absorptions at 1704, 1707, 1708 and 1703 cm^{-1}, respectively. The typical AB pattern for C_3–H and ABX pattern for C_4–H protons were observed in ^{1}H NMR spectrum of the compound **6a**. Thus, each of the C_4–H showed a doublet of doublet at δ 3.46 and 3.81 ppm and another doublet of doublet observed at δ 5.92 ppm for C_3–H.

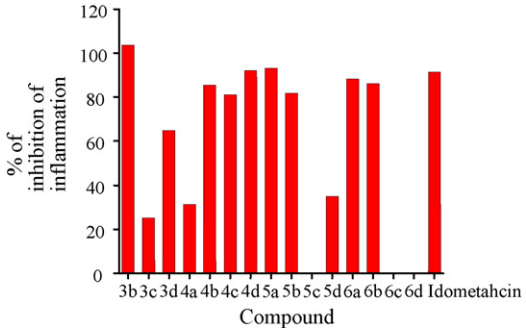

Fig. 1. Anti-inflammatory activity of the synthesized compounds.

2.2. Biological activities

2.2.1. Anti-inflammatory activity

The synthesized compounds **3**(**a**–**d**), **4**(**a**–**d**), **5**(**a**–**d**) and **6**(**a**–**d**) were evaluated by measuring their anti-inflammatory activity against TPA-induced inflammation in mice, and the inhibitory activities were compared with that of indomethacin, a commercially available anti-inflammatory drug. At a dose of 1.0 mg/ear, the compounds **3b**, **4d** and **5a** showed good activity while the remaining compounds showed lower or nearly equal activity compared to that of indomethacin (91.35% at a dose of 1.0 mg/ear). For anti-inflammatory evaluation, these compounds did not achieve a structural similar analysis as for the antioxidant activity, because, in case of anti-inflammatory activity we have other factors that intervene in the biological response (solubility, absorption, distribution, etc.). Due to the lack of exact data related to the solubility, absorption and distribution of each compound tested, it is difficult to clearly show the structure–activity relationship concerning the anti-inflammatory activity. However, we can qualitatively evaluate the activity by taking account of these factors.

The in vivo anti-inflammatory activity of these compounds indicated these compounds as a possible candidate for the development of new drugs to treat symptoms associated with inflammatory diseases, such as osteoarthritis and arteriosclerosis. Further studies on the assessment of the COX-1/COX-2 selectivity index and inhibitory potency are in progress (Fig. 1).

2.2.2. Antioxidant activity

The radical scavenging effects of synthetic compounds against stable free radical DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) were measured spectrophotometrically. Values for their IC_{50} are shown in Table 1. Compounds **4a**, **4d**, **5b** and **5d** were found to be good radical scavengers with IC_{50} below 100, 97.0, 93.3, 99.5 and 94.5 μM, respectively. Compound **5b** was the most effective DPPH scavenger with an IC_{50} value of 93.3. Compounds **3**(**a**–**d**) and **6**(**a**–**d**) were considerably inactive with IC_{50} values over 1000 μM.

3. Experimental

The difluorobenzoic acids were purchased from Aldrich. Melting points were determined in open capillaries using Gallenkemp melting point apparatus and were uncorrected. The infrared spectra were recorded on a Hitachi model 270-50 spectrophotometer as KBr disks or as neat liquids. ^{1}H NMR (400 MHz) spectra were recorded on a Bruker AM-400 as $CDCl_3$ solution using TMS as an internal standard while EIMS were recorded on a MAT-112-S machine. The petroleum ether used corresponds to the fraction with a boiling range of 40–80 °C.

*3.1. General synthetic procedure for the isocoumarin derivatives **3(a–d)***

A mixture of difluorobenzoic acids **1**(**a**–**d**) (63.3 mmol) and thionyl chloride (76.0 mmol) was heated for 30 min in the presence of a drop of DMF under reflux. Completion of the reaction was determined by the stoppage of SO_2 evolution. Removal of excess of thionyl chloride under reduced pressure afforded difluorobenzoyl chlorides **2**(**a**–**d**).

Table 1
Anti-inflammatory and antioxidant activities of the synthesized compounds **3**(**a**–**d**), **4**(**a**–**d**), **5**(**a**–**d**) and **6**(**a**–**d**)

Entry	Compound	% of inhibition of inflammation (1 mg/ear)	Antioxidant IC_{50} (μM)
01	**3a**	−12.5[a]	>1000
02	**3b**	103.7	>1000
03	**3c**	25.3	>1000
04	**3d**	64.8	>1000
05	**4a**	31.3	97.0
06	**4b**	85.4	322.4
07	**4c**	81.0	463.3
08	**4d**	92.2	93.3
09	**5a**	93.1	337.2
10	**5b**	81.8	99.5
11	**5c**	−64.9[a]	862
12	**5d**	35.1	94.5
13	**6a**	88.2	>1000
14	**6b**	86.1	>1000
15	**6c**	−14.8[a]	>1000
16	**6d**	−54.7[a]	>1000
17	Indomethacin	91.4	Not determine
18	Quercetin	Not determine	29.4

[a] Values with negative sign represent pro-inflammatory activity.

A mixture of homophthalic acid (11.3 mmol) and difluorobenzoyl chlorides (62.0 mmol) **2**(**a**–**d**) was heated at 200 °C under reflux for 4 h. The reaction mixture was dissolved in ethyl acetate and aqueous solution of sodium carbonate was added in order to remove the unreacted homophthalic acid. The organic layer was separated, concentrated and chromatographed on silica gel using petroleum ether (40–80 °C) as eluent to afford isocoumarins **3**(**a**–**d**) as solids, which were further purified by recrystallization from methanol.

*3.1.1. Preparation of 3-(3′,5′-difluorophenyl) isocoumarin (**3a**)*

Yield, 80% white solid; mp, 149–151 °C. IR (KBr, ν, cm^{-1}): 2920, 1704, 1569, 1241, 1142. ^{1}H NMR ($CDCl_3$) δ 6.81 (1H, m, H-4′), 6.94 (1H, s, H-4), 7.40 (2H, dd, J = 1.98, 8.30 Hz, H-2′, 6′), 7.50 (1H, dd, J = 7.88 Hz, H-7), 7.54 (1H, d, J = 7.40 Hz, H-5), 7.74 (1H, m, H-6), 8.31 (1H, d, J = 7.92 Hz, H-8); EIMS (70 eV): *m/z* (%) 258.10 (100%) (M^+). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.77; H, 3.12; O, 12.39; F, 14.71. Found: C, 69.73; H, 3.10; O, 12.44; F, 14.73%.

*3.1.2. Preparation of 3-(2′,3′-difluorophenyl) isocoumarin (**3b**)*

Yield, 77% white solid; mp, 156–158 °C. IR (KBr, ν, cm^{-1}): 2935, 1707, 1566, 1242, 1141. ^{1}H NMR ($CDCl_3$) δ 7.19 (1H, s, H-4), 7.36 (1H, m, H-4′), 7.44 (1H, m, H-5′), 7.54 (1H, dd, J = 8.2 Hz, H-7), 7.68 (1H, d, J = 7.36 Hz, H-5), 7.74 (1H, m, H-6′), 7.92 (1H, m, H-6), 8.31 (1H, d, J = 7.90 Hz, H-8); EIMS (70 eV): *m/z* (%) 258.10 (100%) (M^+). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.77; H, 3.12; O, 12.39; F, 14.71. Found: C, 69.79; H, 3.13; O, 12.31; F, 14.77%.

*3.1.3. Preparation of 3-(2′,4′-difluorophenyl) isocoumarin (**3c**)*

Yield, 78% white solid; mp, 131–133 °C. IR (KBr, ν, cm^{-1}): 2927, 1709, 1562, 1248, 1141. ^{1}H NMR ($CDCl_3$) δ 6.93 (1H, m, H-3′), 6.99 (1H, m, H-5′), 7.12 (1H, s, H-4), 7.50 (1H, dd, J = 3.0, 7.8 Hz, H-7), 7.53 (1H, d, J = 7.56 Hz, H-5), 7.73 (1H, m, H-6), 7.99 (1H, m, H-6′), 8.30 (1H, d, J = 7.89 Hz, H-8); EIMS (70 eV): *m/z* (%) 258.00 (83.22%) (M^+). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.77; H, 3.12; O, 12.39; F, 14.71. Found: C, 69.72; H, 3.11; O, 12.48; F, 14.69%.

*3.1.4. Preparation of 3-(3′,4′-difluorophenyl) isocoumarin (**3d**)*

Yield, 79% white solid; mp, 156–159 °C. IR (KBr, ν, cm^{-1}): 2923, 1703, 1565, 1247, 1189. ^{1}H NMR ($CDCl_3$) δ 6.88 (1H, s, H-4), 7.23 (1H, m), 7.51 (1H, m, H-2′), 7.53 (1H, dd, J = 1.0, 7.8 Hz, H-5), 7.72 (1H, m, H-5′), 7.74 (1H, m, H-6), 7.75 (1H, d, J = 1.24, 7.8, 11.2 Hz, H-6′), 8.30 (1H, dd, J = 8.0 Hz, H-8); EIMS (70 eV): *m/z* (%) 258.10 (100%) (M^+). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.77; H, 3.12; O, 12.39; F, 14.71. Found: C, 69.71; H, 3.13; O, 12.41; F, 14.75%.

3.2. General synthetic procedure for the keto-acid derivatives 4(a–d)

A solution of isocoumarins **3**(**a**–**d**) in ethanol (50 mL) and 5% potassium hydroxide (100 mL) were refluxed for 4 h. Ethanol was removed from the reaction mixture by distillation. Ice-cold water (20 mL) was added and the reaction mixtures were acidified with hydrochloric acid. The reaction mixture was then extracted with dichloromethane (3× 20 mL). The extracts were dried (Na_2SO_4) and solvent was rotary evaporated to yield crude solid benzoic acids **4**(**a**–**d**), which were recrystallized from methanol.

3.2.1. Preparation of 2′-(3″,5″-difluorobenzoylmethyl) benzoic acid (4a)

Yield, 80% colorless crystals; mp, 124–126 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2920, 2733, 1708, 1470, 1142. ^{1}H NMR (D_2O) δ 4.61 (1H, s, H-1′), 6.98 (1H, dd, *J* = 8.25, 16.6 Hz, H-4″), 7.23 (1H, d, *J* = 7.4 Hz, H-3), 7.37 (1H, dd, *J* = 7.6 Hz, H-4), 7.51 (2H, m, H-2″,6″), 7.77 (1H, m, H-4), 8.08 (1H, d, *J* = 7.23 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 276.20 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 65.22; H, 3.65; O, 17.38; F, 13.76. Found: C, 65.24; H, 3.66; O, 17.34; F, 13.76%.

3.2.2. Preparation of 2′-(2″,3″-difluorobenzoylmethyl) benzoic acid (4b)

Yield, 70% colorless crystals; mp, 151–154 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2920, 2735, 1704, 1471, 1145. ^{1}H NMR (D_2O) δ 4.65 (1H, s, H-1′), 6.98 (1H, dd, *J* = 8.02, 16.6 Hz, H-4″,5″), 7.27 (1H, d, *J* = 7.14 Hz, H-3), 7.31 (1H, dd, *J* = 7.6 Hz, H-5), 7.51 (2H, m, H-6″), 7.73 (1H, m, H-4), 8.05 (1H, d, *J* = 7.21 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 276.20 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 65.22; H, 3.65; O, 17.38; F, 13.76. Found: C, 65.29; H, 3.62; O, 17.38; F, 13.71%.

3.2.3. Preparation of 2′-(2″,4″-difluorobenzoylmethyl) benzoic acid (4c)

Yield, 77% colorless crystals; mp, 174–177 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2924, 2731, 1708, 1477, 1140. ^{1}H NMR (D_2O) δ 4.62 (1H, s, H-1′), 6.79 (1H, m, H-3″), 6.97 (1H, dd, *J* = 8.23, 16.6 Hz, H-5″), 7.23 (1H, d, *J* = 7.4 Hz, H-3), 7.35 (1H, dd, *J* = 7.6 Hz, H-5), 7.51 (2H, m, H-6″), 7.71 (1H, m, H-4), 8.10 (1H, d, *J* = 7.25 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 276.20 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 65.22; H, 3.65; O, 17.38; F, 13.76. Found: C, 65.19; H, 3.67; O, 17.39; F, 13.75%.

3.2.4. Preparation of 2′-(3″,4″-difluorobenzoylmethyl) benzoic acid (4d)

Yield, 69% colorless crystals; mp, 132–135 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2927, 2737, 1710, 1473, 1141. ^{1}H NMR (D_2O) δ 4.62 (1H, s, H-1′), 6.89 (1H, dd, *J* = 8.22, 16.16 Hz, H-5″), 7.24 (1H, d, *J* = 7.14 Hz, H-3), 7.33 (1H, dd, *J* = 7.16 Hz, H-5), 7.55 (2H, m, H-2″,6″), 7.73 (1H, m, H-4), 8.03 (1H, d, *J* = 7.21 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 276.20 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 65.22; H, 3.65; O, 17.38; F, 13.76. Found: C, 65.22; H, 3.69; O, 17.30; F, 13.79%.

3.3. General synthetic procedure for the dihydroisocoumarin derivatives 6(a–d)

To a solution of the keto-acids **4**(**a**–**d**) (2.07 mmol) dissolved in 1% potassium hydroxide solution (25 mL), sodium borohydride (0.25 g) was added and the reaction mixture was stirred for 1 h at room temperature. After acidification with hydrochloric acid, the reaction mixture was extracted with ethyl acetate (2× 50 mL). The usual workup gave the crude hydroxy-acids **5**(**a**–**d**), which were dissolved in acetic anhydride (1 mL) and heated under reflux for 2 h. The reaction mixture was cooled, water (25 mL) was added and the reaction mixture was stirred overnight. The crystals that deposited were collected by filtration and the filtrate was extracted with dichloromethane (2× 20 mL). The solvent was removed under reduced pressure. The crude dihydroisocoumarin **6**(**a**–**d**) was purified by column chromatography on silica gel using petroleum ether as an eluent.

3.3.1. Preparation of 2-[2′-hydroxy-2′-(3″,5″-difluorophenyl)ethyl] benzoic acid (5a)

Yield, 69% colorless crystals; mp, 132–133 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2920, 2733, 1708, 1470, 1142. ^{1}H NMR (D_2O) δ 2.33 (1H, dd, *J* = 8.22, 15.6 Hz, H-1′b), 2.67 (1H, dd, *J* = 6.24, 15.6 Hz, H-1′a), 4.49 (1H, dd, *J* = 7.12, 14.20 Hz, H-2′), 6.91 (3H, m, H-2″,4″,6″), 7.23 (1H, d, *J* = 7.4 Hz, H-3), 7.37 (1H, dd, *J* = 7.6 Hz, H-5), 7.77 (1H, m, H-4), 8.08 (1H, d, *J* = 7.23 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 278.00 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 64.75; H, 4.35; O, 17.25; F, 13.66. Found: C, 64.24; H, 4.66; O, 17.34; F, 13.76%.

3.3.2. Preparation of 2-[2′-hydroxy-2′-(2″,3″-difluorophenyl)ethyl] benzoic acid (5b)

Yield, 75% colorless crystals; mp, 144–146 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2922, 2737, 1701, 1475, 1141. ^{1}H NMR (D_2O) δ 2.53 (1H, dd, *J* = 8.20, 15.16 Hz, H-1′b), 3.01 (1H, dd, *J* = 6.14, 15.06 Hz, H-1′a), 4.62 (1H, dd, *J* = 7.10, 14.29 Hz, H-2′), 6.69 (1H, m, H–H-4″), 6.99 (2H, m, H-5″,6″), 7.43 (1H, d, *J* = 7.4 Hz, H-3), 7.39 (1H, dd, *J* = 7.6 Hz, H-5), 7.67 (m, H-4), 8.03 (1H, d, *J* = 7.21 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 278.00 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 64.75; H, 4.35; O, 17.25; F, 13.66. Found: C, 64.77; H, 4.46; O, 17.07; F, 13.70%.

3.3.3. Preparation of 2-[2′-hydroxy-2′-(2″,4″-difluorophenyl)ethyl] benzoic acid (5c)

Yield, 79% colorless crystals; mp, 129–130 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2923, 2730, 1705, 1477, 1145. ^{1}H NMR (D_2O) δ 2.57 (1H, dd, *J* = 8.20, 15.56 Hz, H-1′b), 2.99 (1H, dd, *J* = 6.21, 15.56 Hz, H-1′a), 4.75 (1H, dd, *J* = 7.10, 14.25 Hz, H-2′), 6.55 (2H, m, H-3″,5″), 7.01 (1H, dd, *J* = 8.25, 16.6 Hz, H-6″), 7.23 (1H, d, *J* = 7.4 Hz, H-3), 7.45 (1H, dd, *J* = 7.6 Hz, H-5), 7.69 (1H, m, H-4), 8.10 (1H, d, *J* = 7.23 Hz, H-6); EIMS (70 eV): *m*/*z* (%) 278.00 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 64.75; H, 4.35; O, 17.25; F, 13.66. Found: C, 64.67; H, 4.26; O, 17.40; F, 13.67%.

*3.3.4. Preparation of 2-[2′-hydroxy-2′-(3″,4″-difluorophenyl) ethyl] benzoic acid (**5d**)*

Yield, 75% colorless crystals; mp, 121–123 °C. IR (KBr, ν, cm^{-1}): 3300–3250, 2933, 2733, 1703, 1467, 1143. ^1H NMR (D_2O) δ 2.55 (1H, dd, J = 8.02, 15.36 Hz, H-1′b), 2.89 (1H, dd, J = 6.12, 15.6 Hz, H-1′a), 4.85 (1H, dd, J = 7.01, 14.22 Hz, H-2′), 6.71 (2H, m, H-2″,5″), 6.87 (1H, dd, J = 8.20, 16.6 Hz, H-6″), 7.43 (1H, d, J = 7.4 Hz, H-3), 7.55 (1H, dd, J = 7.6 Hz, H-5), 7.61 (1H, m, J = 1.6, 7.90 Hz, H-4), 8.09 (1H, d, J = 7.23 Hz, H-6); EIMS (70 eV): *m/z* (%) 278.00 (M^+). Anal. Calcd. for $C_{15}H_{10}F_2O_3$: C, 64.75; H, 4.35; O, 17.25; F, 13.66. Found: C, 64.60; H, 4.28; O, 17.49; F, 13.63%.

*3.3.5. Preparation of (DL)-3-(3′,5′-difluorophenyl)-3,4-dihydroisocoumarin (**6a**)*

Yield, 69% white solid; mp, 121–122 °C. IR (KBr, ν, cm^{-1}): 2920, 1704, 1244, 1142. ^1H NMR ($CDCl_3$) δ 3.46 (1H, dd, J = 2.92, 16.37 Hz, H-4b), 3.81 (1H, dd, J = 4.09, 13.04 Hz, H-4a), 5.92 (1H, dd, J = 2.95, 13.10 Hz, H-3), 6.90 (1H, m, H-4′), 7.12 (2H, m, H-2′,6′), 7.28 (1H, m, H-7), 7.51 (1H, dd, J = 2.15, 7.68 Hz, H-5), 7.67 (1H, dd, J = 3.37, 5.67 Hz, H-6), 8.14 (1H, d, J = 7.58 Hz, H-8); EIMS (70 eV): *m/z* (%) 261.20 (4%) (M + 1). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.23; H, 3.87; O, 12.30; F, 14.60. Found: C, 69.23; H, 3.03; O 13.07; F, 14.62%.

*3.3.6. Preparation of (DL)-3-(2′,3′-difluorophenyl)-3,4-dihydroisocoumarin (**6b**)*

Yield, 80% white solid; mp, 118–121 °C. IR (KBr, ν, cm^{-1}): 2935, 1707, 1244, 1141. ^1H NMR ($CDCl_3$) δ 3.09 (1H, dd, J = 2.92, 16.37 Hz, H-4b), 3.31 (1H, dd, J = 4.09, 16.31 Hz, H-4a), 5.76 (1H, dd, J = 2.94, 12.16 Hz, H-3), 6.61 (1H, m, H-4′), 6.71 (m, H-6′), 7.46 (1H, m, H-5′), 7.54 (1H, dd, J = 2.24, 7.90 Hz, H-7), 7.62 (1H, dd, J = 2.5, 7.57 Hz, H-5), 7.68 (1H, dd, J = 3.20, 5.72 Hz, H-6), 8.13 (1H, d, J = 7.27 Hz, H-8); EIMS (70 eV): *m/z* (%) 261.20 (51%) (M + 1). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.23; H, 3.87; O, 12.30; F, 14.60. Found: C, 69.23; H, 3.03; O 13.07; F, 14.62%.

*3.3.7. Preparation of (DL)-3-(2′,4′-difluorophenyl)-3,4-dihydroisocoumarin (**6c**)*

Yield, 80% white solid; mp, 121–122 °C. IR (KBr, ν, cm^{-1}): 2935, 1707, 1244, 1141. ^1H NMR ($CDCl_3$) δ 3.09 (1H, dd, J = 2.92, 16.37 Hz, H-4b), 3.31 (1H, dd, J = 4.09, 16.31 Hz, H-4a), 5.76 (1H, dd, J = 2.94, 12.16 Hz, H-3), 6.61 (1H, m, H-4′), 6.71 (m, H-6′), 7.46 (1H, m, H-5′), 7.54 (1H, dd, J = 2.24, 7.90 Hz, H-7), 7.62 (1H, dd, J = 2.5, 7.57 Hz, H-5), 7.68 (1H, dd, J = 3.20, 5.72 Hz, H-6), 8.13 (1H, d, J = 7.27 Hz, H-8); EIMS (70 eV): *m/z* (%) 261.20 (4%) (M + 1). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.23; H, 3.87; O, 12.30; F, 14.60. Found: C, 69.23; H, 3.03; O 13.07; F, 14.62%.

*3.3.8. Preparation of (DL)-3-(3′,4′-difluorophenyl)-3,4-dihydroisocoumarin (**6d**)*

Yield, 69% white solid; mp, 121–122 °C. IR (KBr, ν, cm^{-1}): 2923, 1703, 1243, 1189. ^1H NMR ($CDCl_3$) δ 3.08 (1H, dd, J = 3.10, 16.39 Hz, H-4b), 3.29 (1H, dd, J = 4.4, 16.38 Hz, H-4a), 5.46 (1H, dd, J = 3.09, 11.91 Hz, H-3), 7.15 (2H, m, H-2′,5′), 7.21 (1H, m, H-6′), 7.41 (1H, m, H-7), 7.55 (1H, dd, J = 1.22, 7.48 Hz, H-5), 7.88 (1H, m, J = 1.8, 6.90 Hz, H-6), 8.13 (1H, d, J = 7.79 Hz, H-8); EIMS (70 eV): *m/z* (%) 260.00 (4.5%) (M + 1). Anal. Calcd. for $C_{15}H_8F_2O_2$: C, 69.23; H, 3.87; O, 12.30; F, 14.60. Found: C, 69.23; H, 3.03; O 13.07; F, 14.62%.

4. Biological assay

4.1. Anti-inflammatory activity

4.1.1. Experimental animals

Adult male Wistar CD-1 mice with a body weight ranging from 20 to 25 g were used. All animals had free access to food and water and were kept on a 12/12 h light–dark cycle.

4.1.2. TPA-induced mouse ear edema

Mouse ear edema was evaluated following the protocol previously described [15], using groups of three male CD-1 mice. Edema was induced by topical application of 2.5 μg per ear of TPA dissolved in EtOH. Solutions of the compounds (1 mg/ear) and the standard drug indomethacin (1 mg/ear) as reference, dissolved in different solvents according to their solubility, respectively, were applied to both sides of the right ear (10 μL each side) simultaneously with TPA. The ear swelling was measured before TPA application and 4 h after, and the edema was expressed as the increase in thickness. The results are shown in Table 1.

4.2. Antioxidant activity

4.2.1. DPPH radical scavenging assay

Radical scavenging activity of compounds against stable DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate, Sigma–Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were measured at 515 nm on a UV–vis light spectrophotometer (Spectronic Genesys 8, Rochester, USA).

Radical scavenging activity of compounds was measured by slightly modified method of Brand-Williams [16], as described below. Compound solutions were prepared by dissolving an appropriate amount of each compound in ethanol. The solution of DPPH in ethanol was prepared just before UV measurements. 1.5 mL of this solution was mixed with 0.5 mL of compound solutions in 1 dm path length disposable microcuvettes (final mass ratio of compound with DPPH was approximately 3:1). The samples (at 1, 10, 100, 500 and 1000 μM) were kept in the dark for 30 min at room temperature and then the decrease in absorption was measured. For the control absorption of blank sample containing the same amount of ethanol and DPPH solution was measured. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\%\text{inhibition} = \left[\frac{A_B - A_A}{A_B}\right] \times 100$$

where A_B is the absorption of blank sample ($t = 0$ min); A_A is the absorption of tested extract solution ($t = 15$ min).

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