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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

# Synthesis and structure elucidation of 1-(2,5/3,5-difluorophenyl)-3-(2,3/2,4/2,5/3,4-dimethoxyphenyl)-2-propen-1-ones as anticancer agents

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**Abstract** The compounds titled 1-(2,5/3,5-difluorophenyl)-3-(2,3/2,4/2,5/3,4-dimethoxyphenyl)-2-propen-1-ones (**1–8**) were synthesized via Claisen-Schmidt condensation under basic condition. The chemical structure of the compounds were identified using several spectroscopic techniques such as $^{1}H$ nuclear magnetic resonance (NMR), $^{13}C$ NMR, $^{19}F$ NMR, DEPT 90, DEPT 135, COSY, HMBC, and HMQC. Cytotoxic activities of the compounds were investigated towards several human tumour cell lines [gingival carcinoma (Ca9-22), oral squamous cell carcinoma derived from tongue (HSC-2)] and human normal oral cells [gingival fibroblasts (HGF), periodontal ligament fibroblasts (HPLF)]. Most of these compounds presented higher cytotoxicity than reference drug 5-fluorouracil while the compounds **7**, [1-(3,5-difluorophenyl)-3-(2,5-dimethoxyphenyl)-2-propen-1-one)], and **2**, [1-(2,5-difluorophenyl)-3-(2,4-dimethoxyphenyl)-2-propen-1-one], were presenting the best activity according to potency selectivity expression values. Type of cell death induced by compound **7** in both HSC-2 and Ca9-22 cells was investigated to understand mechanism of action of the compounds. The compound **7** produced cleaved products of PARP and caspase-3 were produced, suggesting the induction of apoptosis as a possible mechanism of action of the compounds characterized via activation of caspase-3 in both human oral squamous cell carcinomas.

**Keywords** Anticancer · Cytotoxicity · Chalcone · Fluorine · Methoxy · PARP

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## Introduction

Cancer is the second cause of death in the World after cardiovascular diseases from which 22 million people will be affected by 2030 (Mahapatra et al. 2015). Although several cancer chemotherapeutics are available in markets, side effects related to the drugs in clinical use lead the researchers working in this field to investigate new drug candidates which are more safe, potent and selective than the available ones (Mahapatra et al. 2015).

Chalcones and their derivatives are very well known with cytotoxic/anticancer activities (Bilginer et al. 2013; Gul et al. 2009, 2008; Hossain et al. 2016; Karki et al. 2016;

Tugrak et al. 2016; Yerdelen et al. 2015; Yamali et al. 2016a; Yerdelen et al. 2015a). The reported mechanisms for their anticancer activities include inhibition of angiogenesis, inhibition of tubulin polymerization, induction of apoptosis, inhibition of kinases (Karthikeyan et al. 2015; Mahapatra et al. 2015), inhibition of topoisomerase enzyme (Gul et al. 2009) and thiol alkylation (Dimmock et al. 1998).

Methoxylated chalcones have structural similarity to combretastatin and colchicine which are biologically active and have cytotoxic properties. They have similar spatial orientation between two aromatic rings (Kong et al. 2010) (Fig. 1). Similar to combretastatin and colchicine, methoxylated chalcone and their derivatives were reported with the binding ability to the tubulin effectively (Kong et al. 2010). Studies on methoxylated chalcones suggested that the number and the position of methoxy groups on the aromatic rings were critical for their cytotoxicity activities (Boumendjel et al. 2008; Ethiraj et al. 2013; Karthikeyan et al. 2015).

Fluorine bearing compounds or drugs have been reported with several bioactivities including anticancer activities (Fig. 1) (Nakamura et al. 2002; Purser et al. 2008; Zuo et al. 2012). The bioisosteric substitution of fluorine in place of hydrogen in many bioactive molecules has led to potent compounds without extensive stereochemical changes due to its small size (Nakamura et al. 2002; Purser et al. 2008). However, substitution of fluorine is known to modulate overall reactivity and stability of the compounds due to resistance of the carbon-fluorine bond toward metabolic transformations (Kirk 2000; Nakamura et al. 2002). In addition, incorporation of fluorine in a ring system may increase biological half life of molecules by delaying oxidative biotransformation and can increase bioabsorption by lipophilic effects (Nakamura et al. 2002; Purser et al. 2008).

Recently, some fluorinated chalcones were reported as anticancer agents (Burmaoglu et al. 2016). In the study at issue the compound **10** (Fig.1) was found as the most cytotoxic one comparing others towards A549, A498, and A375 cancer cell lines with $IC_{50}$ values as 0.120, 0.030, and 0.060l M, respectively (Burmaoglu et al. 2016).

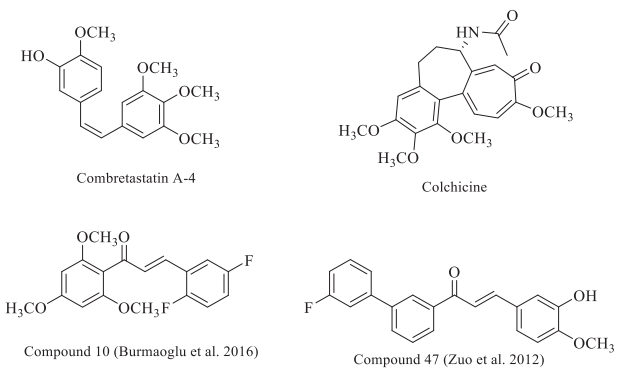



**Fig. 1** Several most potent compounds as anticancer agents

In the light of the literature survey, the aims of this study include the followings: (i) The synthesis of 1-(2,5/3,5-difluorophenyl)-3-(2,3/2,4/2,5/3,4-dimethoxyphenyl)-2-propen-1-ones, **1–8**. (ii) The structure elucidation of these compounds **1–8** with various spectroscopic methods. (iii) To investigate their cytotoxic/anticancer properties and mechanism of action on several cancer cell lines and non-cancer cells.

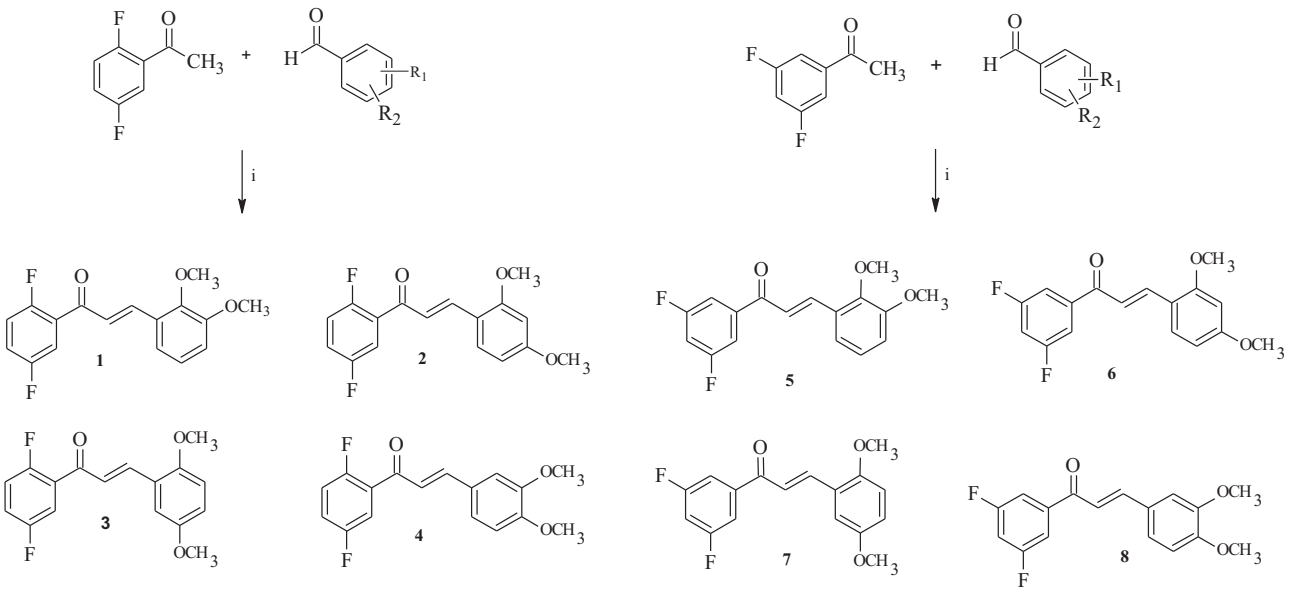
## Materials and methods

The nuclear magnetic resonance (NMR) spectra ($^{1}$H NMR, $^{13}$C NMR, $^{19}$F NMR), distortionless enhancement by polarization transfer (DEPT) (DEPT 90, DEPT 135) correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple-quantum correlation (HMQC) in $CDCl_3$ were recorded on a Bruker AVANCE III 400 MHz (Bruker, Karlsruhe, Germany) spectrometer [400 MHz ($^{1}$H), 400 MHz ($^{19}$F) and 100 MHz ($^{13}$C)]. For 1D, 2D NMR, in Topspin 2.1 NMR program was used. Chemical shifts are given as $\delta$ values in ppm against tetramethylsilane as the internal standard and $J$ values were expressed in Hz. Mass spectra of the compounds were taken using a liquid chromatography ion trap-time of flight tandem mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization mode. Shimadzu's LCMS Solution software was used for data analysis. Melting points were determined using an Electrothermal 9100 instrument (IA9100, Bibby Scientific Limited, Staffordshire, UK) and are uncorrected.

### General procedure for the preperation of chalcones, 1–8, Scheme 1

The title compounds were synthesized by Claisen-Schmidt condensation (Dimmock et al. 1998; Gul et al. 2008; Mete et al. 2016; Yamali et al. 2016a, b; Bilginer et al. 2013; Yerdelen et al. 2015a, b). A mixture of fluorinated acetophenone (6.4 mmol) and methoxylated aldehyde (6.4 mmol) was dissolved in ethanol (5 ml). Aqueous sodium hydroxide solution (30%, 10 ml) was added into the mixture under cold condition (0–5 °C). After overnight stirring at room temperature, the reaction mixture was poured into ice-water mixture and acidified with HCl solution (10%) to pH = 3 (Scheme 1). The solids obtained were crystallized from suitable solvents [It was ethanol-water (**2** and **3**) or ethanol (**5–8**)]. On the other hand, the compounds **1** and **4** were purified by passing through a column of silica gel using chloroform as the eluent.

The chemical structures of the compounds were confirmed by $^{1}$H NMR, $^{13}$C NMR, $^{19}$F NMR and HRMS. The proton and carbon atoms of the compounds were



**Scheme 1** General procedure for the synthesis of the chalcones **1–8**. Reagents and conditions: i: Ethanol, NaOH solution (30%), rt. $R_1$ and $R_2$: Suitable substitutions for the compounds **1–8**. $R_1$ and $R_2$ are methoxy groups at different position of phenyl ring as shown at the compounds **1–8**'s formula

completely assigned by one and two-dimensional (1D and 2D) homonuclear and heteronuclear experiments (DEPT 90–135, $^1$H-$^1$H COSY, $^1$H-$^{13}$C HMQC and HMBC, See Supplementary Material for representative spectra).

*1-(2,5-Difluorophenyl)-3-(2,3-dimethoxyphenyl)-2-propen-1-one (**1**)*

A yellow solid, yield 55%. Mp: 81–83 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 8.09 (dd, $J = 16.0$, 1.9 Hz, 1H, C10-H), 7.53 (ddd, $J = 8.5$, 5.4, 3.2 Hz, 1H, C4-H), 7.45 (dd, $J = 16.0$, 2.8 Hz, 1H, C9-H), 7.26 (d, $J = 1.3$ Hz, 1H, C12-H), 7.25–7.14 (m, 2H, C2-H, C1-H), 7.11 (t, $J = 8.0$ Hz, 1H, C13-H), 7.00 (dd, $J = 7.4$, 1.3 Hz, 1H, C14-H), 3.91 (s, 3H, C18-H, –$OCH_3$), 3.90 (s, 3H, C17-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 188.0 (d, $J_{CO–F} = 3$ Hz, C=O, C7), 158.7 (dd, $J_{CF} = 246.0$, 2.0 Hz, C6 or C3), 157.2 (dd, $J_{CF} = 246.0$, 2.0 Hz, C6 or C3), 153.2 (C15), 149.2 (C16), 140.5 (C10), 128.7 (C11), 128.3 (dd, $J_{CF} = 17.0$, 7.0 Hz, C5), 126.2 (d, $J_{CF} = 7$ Hz, C9), 124.3 (C13), 120.4 (dd, $J_{CF} = 25.0$, 9.0 Hz, C2), 119.7 (C12), 117.9 (dd, $J_{CF} = 27.0$, 8.0 Hz, C1), 117.1 (dd, $J_{CF} = 25.0$, 3.0 Hz, C4), 114.6 (C14), 61.4 (C17), 55.9 (C18). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$) −116.5 (d, $J_{FF} = 20$ Hz), −117.7 (d, $J_{FF} = 20$ Hz) HRMS (ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0983.

*1-(2,5-Difluorophenyl)-3-(2,4-dimethoxyphenyl)-2-propen-1-one (**2**)*

A yellow solid, yield 50 %. Mp: 91–93 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 8.03 (dd, $J = 15.8$, 1.8 Hz, 1H, C10-H), 7.56 (d, $J = 8.6$ Hz, 1H, C12-H), 7.50 (ddd, $J = 8.5$, 5.4, 3.1 Hz, 1H, C4-H), 7.39 (dd, $J = 15.8$, 2.7 Hz, 1H, C9-H), 7.21–7.11 (m, 2H, C2-H, C1-H), 6.54 (dd, $J = 8.6$, 2.3 Hz, 1H, C13-H), 6.47 (d, $J = 2.3$ Hz, 1H, C15-H), 3.89 (s, 3H, C17-H, –$OCH_3$), 3.87 (s, 3H, C18-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 188.2 (d, $J_{CO–F} = 2$ Hz, C=O, C7), 163.5 (C14), 160.6 (C16), 158.6 (dd, $J_{CF} = 245.0$, 2.0 Hz, C6 or C3), 156.9 (dd, $J_{CF} = 245.0$, 2.0 Hz, C6 or C3), 141.5 (C10), 131.1 (C12), 128.8 (dd, $J_{CF} = 16.0$, 6.0 Hz, C5), 123.1 (d, $J_{CF} = 7$ Hz, C9), 119.8 (dd, $J_{CF} = 24.0$, 9.0 Hz, C2), 117.8 (dd, $J_{CF} = 26.0$, 8.0 Hz, C1), 116.9 (dd, $J_{CF} = 25.0$, 3.0 Hz, C4), 116.7 (C11), 105.6 (C15), 98.4 (C13), 55.6 (C17), 55.5 (C18). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$) −117.1 (d, $J_{FF} = 20$ Hz), −118.0 (d, $J_{FF} = 20$ Hz) HRMS (ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0966.

*1-(2,5-Difluorophenyl)-3-(2,5-dimethoxyphenyl)-2-propen-1-one (**3**)*

A yellow solid, yield 52%. Mp: 80–82 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 8.07 (dd, $J = 15.9$, 1.8 Hz, 1H, C10-H), 7.52 (ddd, $J = 8.5$, 5.4, 3.1 Hz, 1H, C4-H), 7.45 (dd, $J = 15.9$, 2.8 Hz, 1H, C9-H), 7.25–7.18 (m, 2H, C2-H, C1-H), 7.16 (d, $J = 2.8$ Hz, 1H, C12-H), 6.98 (dd, $J = 9.0$, 3.0 Hz, 1H, C14-H), 6.89 (d, $J = 9.0$ Hz, 1H, C15-H), 3.88 (s, 3H, C17-H, –$OCH_3$), 3.87 (s, 3H, C18-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 188.1 (d, $J_{CO–F} = 2$ Hz, C=O, C7), 158.6 (d, $J_{CF} = 245.0$ Hz, C6 or C3), 157.1 (d, $J_{CF} = 245.0$ Hz, C6 or C3), 153.5 (d, $J = 4$ Hz, C13, C16), 140.9 (C10), 128.4 (dd, $J_{CF} = 16.0$, 6.0 Hz, C5), 125.7 (d,

$J_{CF}$ = 7 Hz, C9), 124.0 (C11), 120.3 (dd, $J_{CF}$ = 24.0, 9.0 Hz, C2), 118.1 (C12), 117.9 (dd, $J_{CF}$ = 18.0, 12.0 Hz, C1), 117.0 (dd, $J_{CF}$ = 25.0, 4.0 Hz, C4), 113.6 (C14), 112.5 (C15), 56.1 (C17), 55.8 (C18). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$) −116.7 (d, $J_{FF}$ = 20 Hz), −117.8 (d, $J_{FF}$ = 20 Hz) HRMS (ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0970.

*1-(2,5-Difluorophenyl)-3-(3,4-dimethoxyphenyl)-2-propen-1-one (**4**)*

A yellow solid, yield 45%. Mp: 79–81 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 7.72 (dd, $J$ = 15.7, 1.8 Hz, 1H, C10-H), 7.50 (ddd, $J$ = 8.5, 5.4, 3.2 Hz, 1H, C4-H), 7.27–7.15 (m, 4H, C1-H, C2-H, C9-H, C12-H), 7.14 (d, $J$ = 1.6 Hz, 1H, C16-H), 6.90 (d, $J$ = 8.3 Hz, 1H, C13-H), 3.95 (s, 3H, C18-H, –$OCH_3$), 3.94 (s, 3H, C17-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 187.5 (d, $J_{CO–F}$ = 2 Hz, C=O, C7), 158.7 (d, $J_{CF}$ = 246.0 Hz, C6 or C3), 157.0 (d, $J_{CF}$ = 246.0 Hz, C6 or C3), 151.8 (C15), 149.3 (C14), 145.9 (C10), 128.4 (dd, $J_{CF}$ = 17.0, 7.0 Hz, C5), 127.5 (C11), 123.5 (C12), 122.9 (d, $J_{CF}$ = 7 Hz, C9), 120.2 (dd, $J_{CF}$ = 24.0, 9.0 Hz, C2), 117.9 (dd, $J_{CF}$ = 26.0, 8.0 Hz, C1), 116.9 (dd, $J_{CF}$ = 25.0, 4.0 Hz, C4), 111.1 (C13), 110.1 (C16), 56.0 (C18), 55.9 (C17). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$) −116.7 (d, $J_{FF}$ = 20 Hz), −117.8 (d, $J_{FF}$ = 20 Hz) HRMS (ESI-MS) Calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0980.

*1-(3,5-Difluorophenyl)-3-(2,3-dimethoxyphenyl)-2-propen-1-one (**5**)*

A yellow solid, yield 52%. Mp: 96–98 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 8.15 (d, $J$ = 15.8 Hz, 1H, C10-H), 7.54 (dd, $J$ = 8.0, 2.4 Hz, 2H, C4-H, C6-H), 7.50 (d, $J$ = 15.8 Hz, 1H, C9-H), 7.30 (dd, $J$ = 8.0, 1.3 Hz, 1H, C12-H), 7.13 (t, $J$ = 8.0 Hz, 1H, C13-H), 7.07–7.04 (m, 1H, C2-H), 7.01 (dd, $J$ = 8.0, 1.3 Hz, 1H, C14-H), 3.92 (s, 6H, C17-H, C18-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 188.1 (d, $J_{CO–F}$ = 2 Hz, C=O, C7), 163.1 (d, $J_{CF}$ = 246.0 Hz, C1 or C3), 162.9 (d, $J_{CF}$ = 246.0 Hz, C1 or C3), 153.3 (C15), 149.2 (C16), 141.4 (C10), 141.3 (t, $J_{CF}$ = 8.0 Hz, C5), 128.6 (C11), 124.3 (C13), 122.3 (C9), 119.7 (C12), 114.7 (C14), 111.4 (dd, $J_{CF}$ = 19.0, 7.0 Hz, C4, C6), 107.9 (t, $J_{CF}$ = 25.0 Hz, C2), 61.4 (C17), 55.9 (C18). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$)-108.0 (s, 2F). HRMS (ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0955.

*1-(3,5-Difluorophenyl)-3-(2,4-dimethoxyphenyl)-2-propen-1-one (**6**)*

A yellow solid, yield 54%. Mp: 147–148 °C, 144–149 °C (Wu et al. 2012). $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 8.09 (d, $J$ = 15.7 Hz, 1H, C10-H), 7.58 (d, $J$ = 8.6 Hz, 1H, C12-H), 7.52 (dd, $J$ = 8.0, 2.3 Hz, 2H, C4-H, C6-H), 7.45 (d, $J$ = 15.7 Hz, 1H, C9-H), 7.02 (tt, $J$ = 8.7, 2.3 Hz, 1H, C2-H), 6.56 (dd, $J$ = 8.6, 2.4 Hz, 1H, C13-H), 6.50 (d, $J$ = 2.4 Hz, 1H, C15-H), 3.94 (s, 3H, C17-H, –$OCH_3$), 3.88 (s, 3H, C18-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 188.3 (t, $J_{CO–F}$ = 4.1 Hz, C=O, C7), 163.5 (C14), 163.0 (d, $J_{CF}$ = 249.0 Hz, C1 or C3), 162.9 (d, $J_{CF}$ = 249.0 Hz, C1 or C3), 160.7 (C16), 142.2 (C10), 141.9 (t, $J_{CF}$ = 8.0 Hz, C5), 131.5 (C12), 119.1 (C9), 116.7 (C11), 111.3 (dd, $J_{CF}$ = 19.0, 7.0 Hz, C4, C6), 107.5 (t, $J_{CF}$ = 25.0 Hz, C2), 105.6 (C13), 98.5 (C15), 55.6 (C17), 55.5 (C18). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$) −108.6 (s, 2F) HRMS (ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0955.

*1-(3,5-Difluorophenyl)-3-(2,5-dimethoxyphenyl)-2-propen-1-one (**7**)*

A yellow solid, yield 50%. Mp: 110–112 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 8.13 (d, $J$ = 15.8 Hz, 1H, C10-H), 7.56–7.53 (m, 2H, C4-H, C6-H), 7.50 (d, $J$ = 15.8 Hz, 1H, C9-H), 7.17 (d, $J$ = 3 Hz, 1H, C12-H), 7.04 (tt, $J$ = 8.5, 2.4 Hz, 1H, C2-H), 6.99 (dd, $J$ = 9.0, 3.0 Hz, 1H, C14-H), 6.91 (d, $J$ = 9.0 Hz, 1H, C15-H), 3.91 (s, 3H, C17-H, –$OCH_3$), 3.84 (s, 3H, C18-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 188.3 (t, $J_{CO–F}$ = 4.1 Hz, C=O, C7), 163.1 (d, $J_{CF}$ = 249.0 Hz, C1 or C3), 162.9 (d, $J_{CF}$ = 249.0 Hz, C1 or C3), 153.5 (C13, C16), 141.8 (C10), 141.5 (t, $J_{CF}$ = 8.0 Hz, C5), 123.9 (C9), 121.9 (C11), 117.9 (C12), 114.0 (C14), 112.5 (C15), 111.4 (dd, $J_{CF}$ = 18.0, 7.0 Hz, C4, C6), 107.8 (t, $J_{CF}$ = 25.0 Hz, C2), 56.1 (C17), 55.9 (C18). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$) −108.3 (s, 2F) HRMS (ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0956.

*1-(3,5-Difluorophenyl)-3-(3,4-dimethoxyphenyl)-2-propen-1-one (**8**)*

A yellow solid, yield 56%. Mp: 122–123 °C. $^1$H NMR (400 MHz, $\delta$, ppm, $CDCl_3$) 7.81 (d, $J$ = 15.6 Hz, 1H, C10-H), 7.53 (dd, $J$ = 7.9, 2.4 Hz, 2H, C4-H, C6-H), 7.28 (d, $J$ = 15.6 Hz, 1H, C9-H), 7.26 (d, $J$ = 8.3 Hz, 1H, C12-H), 7.17 (d, $J$ = 1.9 Hz, 1H, C16-H), 7.04 (tt, $J$ = 8.4, 2.4 Hz, 1H, C2-H), 6.93 (d, $J$ = 8.3 Hz, 1H, C13-H), 3.99 (s, 3H, C17-H, –$OCH_3$), 3.96 (s, 3H, C18-H, –$OCH_3$). $^{13}$C NMR (100 MHz, $\delta$, ppm, $CDCl_3$) 187.2 (t, $J_{CO-F}$ = 2.2 Hz, C=O, C7), 163.1 (d, $J_{CF}$ = 249.0 Hz, C1 or C3), 162.9 (d, $J_{CF}$ = 249.0 Hz, C1 or C3), 151.9 (C15), 149.4 (C14), 146.6 (C10), 141.5 (d, $J_{CF}$ = 7.0 Hz, C5), 127.4 (C11), 123.7 (C12), 118.7 (C9), 111.3 (dd, $J_{CF}$ = 19.0, 7.0 Hz, C4, C6), 111.1 (C13), 110.1 (C16), 107.8 (t, $J_{CF}$ = 25.0 Hz, C2), 56.1 (C18), 56.0 (C17). $^{19}$F NMR (400 MHz, $\delta$, ppm, $CDCl_3$)-108.2 (s, 2F). HRMS

(ESI-MS) calc. for $C_{17}H_{15}O_3F_2$ $[M+H]^+$ 305.0984; found 305.0975.

### Biological activity

#### *Cytotoxicity assay*

The cytotoxicity of the compounds were assayed towards oral squamous cell carcinoma cell lines (Ca9-22 and HSC-2) and human oral normal cells (HGF and HPLF) as described with some minor modifications (Gul et al. 2016a, b, c, 2017a, b; Yamali et al. 2016a; Tugrak et al. 2016; Yerdelen et al. 2015; Sakagami et al. 2015; Bilginer et al. 2013). In brief, all cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich Inc., St. Louis, MO, USA). The following concentrations of the compounds in dimethylsulfoxide (DMSO) were added to the medium and incubated at 37 °C for 48 h: compounds **1–8** (0.32, 1, 3.2, 10, 31.6, 100, 316, 1000 µM) and 5-fluorouracil (5-FU) (Kyowa, Tokyo, Japan) (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 µM). The media that contained the same concentration of DMSO (0.0078, 0.156, 0.03125, 0.0625, 0.125, 0.25, 0.5 or 1%) were used as controls, since DMSO above 0.25% is cytotoxic. The viable cell numbers were determined by the MTT [3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] method. $CC_{50}$ value was determined from the growth curves plotted at different concentrations of each compounds in triplicate wells.

#### *Western blot analysis*

The cells were washed with phosphate buffer solution and processed for western blot analysis, as described previously (Sakagami et al. 2017). In brief, antibodies against cleaved caspase-3 (Cell Signaling Technology Inc., Beverly, MD, USA), poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology Inc.) and glyceraldehyde 3-phosphate dehydrogenase (Trevigen, Gaithersburg, MD, USA) were used as primary antibodies. As secondary antibodies, we used α-rabbit IgG (DAKO Japan) antibodies which were conjugated with horseradish peroxidase. HSC-2 and Ca9-22 cells were treated with compound **7** for 24 h at the concentrations 8–32 µM. The detached and attached cells were combined and subjected to western blot analysis. Activation of caspase-3 as evidenced by appearance of cleaved caspase-3 and cleavage of PARP, which is one of the caspase-3 substrates, were determined according to literature (Sakagami et al. 2017). Actinomycin D (Act D) was used as a positive control.

## Results and discussion

### Chemistry

The general procedure for the preparation of targeted chalcones **1–8** are outlined in Scheme 1. A suitable acetophenone [2,5-difluoroacetophenone (for compounds **1–4**) or 3,5-difluoroacetophenone (for compounds **5–8**)] in ethanol and aqueous solution of NaOH were reacted with a suitable benzaldehyde derivatives [2,3-dimethoxybenzaldehyde (for compounds **1** and **5**), 2,4-dimethoxybenzaldehyde (for compounds **2** and **6**), 2,5-dimethoxybenzaldehyde (for compounds **3** and **7**), 3,4-dimethoxybenzaldehyde (for compounds **4** and **8**)] at room temperature. Then, the mixture was poured on ice-water and acidified with HCl solution to pH = 3. The compounds were purified by crystallization or column chromatography.

Except compound **6**, all compounds were reported here for the first time with detailed spectroscopic analyzses and cytotoxicity activities. In addition, this study is the first report on the detailed structure elucidation of this type fluorinated chalcones. The chemical structures of the chalcones were identified by $^1$H NMR, $^{13}$C NMR, $^{19}$F NMR and HRMS. The full characterisation of the chalcones **1–8** were presented in the experimental part. Unambiguous chemical shift assignments were made by one and two-dimensional (1D and 2D) homonuclear and heteronuclear experiments (DEPT 90–135, $^1$H-$^1$H COSY, $^1$H-$^{13}$C HMQC and HMBC, See Supplementary Material for representative spectra).

Chalcones, one of the major classes of natural products, are open-chain flavanoids in which two aromatic rings are linked by a three carbon and α,β-unsaturated carbonyl system. They have the tendency to exist both in cis (*Z*) or trans (*E*) forms (Singh et al. 2014). The compounds synthesized in this study were seen as *E* izomer on the basis of coupling constants. The C9-H and C10-H resonances were easily identified by their large trans coupling constants (*J*) in the range of 15–16 Hz. In addition, it was observed that C9-H is more shielded than C10-H because of resonance effects of the carbonyl group.

The chemical shift and splitting patterns of the protons and carbon atoms on the acetophenone moiety and carbon atom of the carbonyl group were affected by the C-F and/or H-F coupling. Interesting splitting patterns were observed in the $^1$H NMR and $^{13}$C NMR spectra for the fluorinated compounds. For compound **1**, which substituted 2,5-difluoro, C4-H was resonated at 7.53 ppm as doublet of doublet of doublet (ddd) splitted by fluorine atoms and C2-H proton, with coupling constants 8.5, 5.4, 3.2 Hz values, actually C4-H should be observed as singlet (s) or doublet (d) with meta coupling. In $^{13}$C NMR, for the compound **1**, the carbonyl carbon, which is away from the fluoro group, was splitted into a doublet (d) at $\delta$ 188.0 ppm with a

**Table 1** Cytotoxic activities of the chalcones **1–8** towards human OSCC cell lines and human oral normal cells

| $CC_{50}$ (μM) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Compounds | Human OSCC cell lines | | | | | | Human oral normal cells | | | | | |
| | Ca9-22 | | HSC-2 | | Mean | Mean | HGF | HPLF | Mean | TS | | PSE |
| | A | SI | | SI | B | SI (E) | C | | D | D/B | C/A | |
| **1** | 5.4 | 6.1 | 8.3 | 4.0 | 6.8 | 5.0 | 46.3 | 19.3 | 32.8 | 4.8 | 8.6 | 73 |
| **2** | 8.7 | 9.7 | 6.5 | 12.9 | 7.6 | 11.3 | 88.9 | 79.9 | 84.4 | 11.1 | 10.2 | 149 |
| **3** | 8.3 | 4.5 | 5.0 | 7.5 | 6.7 | 6.0 | 41.7 | 33.7 | 37.7 | 5.6 | 5.0 | 90 |
| **4** | 42.1 | 4.7 | 32.9 | 6.1 | 37.5 | 5.4 | 200.0 | 200.0 | 200.0 | 5.3 | 4.7 | 14 |
| **5** | 5.1 | 10.9 | 7.6 | 7.3 | 6.4 | 9.1 | 76.5 | 34.8 | 55.6 | 8.8 | 15.0 | 143 |
| **6** | 15.9 | 12.6 | 8.7 | 23.1 | 12.3 | 17.8 | 200.0 | 200.0 | 200.0 | 16.3 | 12.6 | 145 |
| **7** | 3.4 | 14.8 | 3.3 | 15.2 | 3.4 | 15.0 | 69.6 | 31.9 | 50.8 | 15.0 | 20.3 | 442 |
| **8** | 7.6 | 21.2 | 6.8 | 23.7 | 7.2 | 22.5 | 200.0 | 122.6 | 161.3 | 22.4 | 26.3 | 312 |
| 5-Fluorouracil | 32.9 | >30.4 | 37.7 | >26.5 | 35.3 | >28.5 | >1000 | >1000 | >1000 | >28.3 | >30.4 | 80.7 |

$CC_{50}$ values refer to the concentrations of the compounds in μM which reduce the viable cell number by 50%. Oral squamous cell carcinoma (OSCC) cell lines used are Ca9-22 (derived from gingiva) and HSC-2 (derived from tongue). Normal oral cells used are human gingival fibroblasts (HGF) and human periodontal ligament fibroblasts (HPLF). Tumour-specificity (TS) value is calculated by dividing the mean $CC_{50}$ value of each compound against normal cells to mean $CC_{50}$ value against OSCC (D/B or C/A). Selectivity index (SI) figures were generated which are quotients of the average $CC_{50}$ values of non-malignant cells and $CC_{50}$ figure of a compound towards a specific cell line. A potency selectivity expression (PSE) was devised, which is reciprocal of the average $CC_{50}$ value (a measure of potency) and the average SI figure (a determination of tumour selectivity) (Column E/Column B × 100) $CC_{50}$ value was determined from the growth curves plotted at different concentrations of each compounds in triplicate wells

coupling constant of 3 Hz. In addition all carbon atoms on acetophenone moiety were splitted into a doublet of doublet (dd). The splitting of these carbon atoms was because of resonance effects of the fluorine atom. For compound **6**, which substituted 3,5-difluoro, C2-H proton was resonated at 7.02 ppm as tirplet of triplet (tt) splitted by two protons (C4-H and C6-H) and two fluorine atoms, with coupling constants 8.7, 2.3 Hz. Signal of C4-H and C6-H were observed at 7.52 ppm as a doublet of doublet (dd) with coupling constants 8.0 and 2.3 Hz. The carbon of carbonyl group of compound **6** was splitted into a triplet (t) at $\delta$ 188.3 ppm with a coupling constant of 4.1 Hz. (See Supplementary Material for representative spectra).

## Cytotoxic activity

Compounds synthesized **1–8** were screened for their in vitro cytotoxic effects against human tumour cell lines [gingival carcinoma (Ca9-22), oral squamous cell carcinoma (HSC-2)] and human normal oral cells [gingival fibroblasts (HGF), periodontal ligament fibroblasts (HPLF)] via MTT test. The results presented in Table 1 reveal that $CC_{50}$ values of the compounds are in low micro molar range (5.0–42.1 μM) towards cancer cell lines used.

Except **4** towards Ca9-22 cell line, all compounds had 2.1–9.7 times higher cytotoxicity than reference compound 5-FU [The compound and times potency **1** (6.1), **2** (3.8), **3** (4.0), **5** (6.5), **6** (2.1), **7** (9.7), **8** (4.3)] against Ca9-22 cell line. The compounds had 1.1–11.4 times higher cytotoxicity than 5-FU towards HSC-2 cell line [The compound and times potency **1** (4.6), **2** (5.8), **3** (7.5), **4** (1.1), **5** (5.0), **6** (4.3), **7** (11.3), **8** (5.5)]. The conclusion to be drawn is that all compounds in series possess noteworthy antineoplastic potencies.

The second aspect of these compounds to be considered is whether they are tumour specific cytotoxins since tumours are surrounded by different types of normal cells. Hence selectivity index (SI) figure of a compound which reflects whether a compound is tumour specific cytotoxine is very important. SI figures which are quotients of the average $CC_{50}$ values of non-malignant cells and $CC_{50}$ figure of a compound towards a specific cell line were generated (Yamali et al. 2016a; Robles-Escajeda et al. 2016; Tugrak et al. 2016; Bilginer et al. 2013). The results in Table 1 reveal that SI values of greater than 1 were obtained for **1–8**. This suggested that the compounds **1–8** are tumour specific antineoplastic agents based on the literature (Robles-Escajeda et al. 2016). SI values changed in the range of 4.5–21.2 towards Ca9-22 cells while it was in the range of 4.0–23.7 towards HSC-2 cell line. The order of SI values for the compounds towards Ca9-22 cells was **8** (21.2) > **7** (14.8) > **6** (12.6) > **5** (10.9) > **2** (9.7) > **1** (6.1) > **4** (4.7) > **3** (4.5) while it was **8** (23.7) > **6** (23.1) > **7** (15.2) > **2** (12.9) > **3** (7.5) > **5** (7.3) > **4** (6.1) > **1** (4.0) towards HSC-2 cell line.

Tumour selectivity (TS) was also calculated for the compounds by dividing the average $CC_{50}$ value towards normal cells into the average $CC_{50}$ value towards cancer cell lines. (Column D/Column B, Table 1). According to

this calculation the compound having the highest TS value was compound **8** (TS: 22.4). The order of TS values of the compounds was as follows: the compound (TS): **8** (22.4) > **6** (16.3) > **7** (15.0) > **2** (11.1) > **5** (8.8) > **3** (5.6) > **4** (5.3) > **1** (4.8). If any compound having TS value over 10, it can be considered as possible drug candidate for further studies. Hence, it can be said that the compounds **2**, **6**, **7**, and **8** can be considered as lead molecules according to this calculation. By considering the fact that HGF is the corresponding normal cell of cancer cell Ca9-22 having the same origin, TS values were also generated for a compound by dividing the average $CC_{50}$ value towards HGF cells into the $CC_{50}$ value towards Ca9-22 cells. According to this calculation (Column C/Column A, Table 1), the order of TS values was as follows: the compound (TS): **8** (26.3) > **7** (20.3) > **5** (15.0) > **6** (12.6) > **2** (10.2) > **1** (8.6) > **3** (5.0) > **4** (4.7).

Lead compounds should possess both marked cytotoxic potencies and selective toxicity for tumour. In order to identify such molecules, a potency selectivity expression value (PSE) was devised which is reciprocal of the average $CC_{50}$ value (a measure of potency) and the average SI figure (determination of tumour selectivity) (Column E/Column B × 100, Table 1) (Gul et al. 2016a; Yamali et al. 2016a; Bilginer et al. 2013; Tugrak et al. 2016). The order of PSE values of the compounds was as follows: the compound (PSE): **7** (442) > **8** (312) > **2** (149) > **6** (145) > **5** (143) > **3** (90) > **1** (73) > **4** (14). The compound having the highest PSE value (442) was the compound **7**, [1-(3,5-difluorophenyl)-3-(2,5-dimethoxyphenyl)-2-propen-1-one], can be considered as the leader compound of the study (Fig. 2).

Another point considered was whether compound **7** can induce apoptosis in two human oral squamous cell carcinoma cell lines (HSC-2, Ca9-22), derived from both tongue and gingiva, by western blot analysis (Fig. 3). The apoptosis markers which were used in this study were activation of caspase 3 (detected by the active form of caspase 3, that is, cleaved caspase) and the cleavage of PARP, one of the substrates of caspase 3) (Bressenot et al. 2009). When HSC-2 and Ca9-22 cells were treated for 24 h with the increasing concentrations of compound **7**, caspase-3 was dose-dependently cleaved, up to the levels attained by Act D (used as positive apoptosis inducer) in both of these cells, suggesting the activation of caspase-3. This was confirmed by the finding that PARP was cleaved dose-dependently.

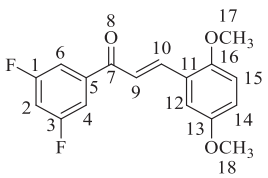



**Fig. 2** Chemical structure and general numbering of leader compound **7** (PSE = 442)

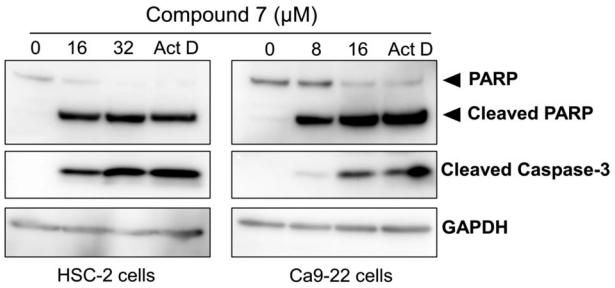



**Fig. 3** Apoptosis induction by compound **7** in both human oral squamous cell carcinoma cell lines. HSC-2 or Ca9-22 cells were treated for 24 h with either vehicle (DMSO 0.08%), the indicated concentrations of compound **7** or 1 μM Actinomycin D (Positive control). Detached and attached cells were combined for western blot analysis

All of these data suggest that compound **7** induced apoptosis in both human oral squamous cell carcinomas.

## Structure–activity relationships (SAR)

In this study, it was designed and tested a series of difluorodimethoxy substituted chalcones (**1–8**) in order to investigate their anticancer effects and also to understand how the positions of fluorine and methoxy groups effect the bioactivity. The compounds **1** and **5**, **2** and **6**, **3** and **7**, **4** and **8** are the couples of compounds which permit comparison the compounds in terms of the effect of fluorine positions to PSE value i.e., the effect of replacement of second fluorine atom on phenyl ring from position 2 to position 3 while the other fluorine which is available at position 5 was kept constant.

When the compound **1** was converted to the compound **5**, the PSE value increased 2.0 times. In the case of conversion of the compound **3** to **7**, PSE value increased 4.9 times. On the other hand, PSE value increased 22.3 times by conversion of **4** to **8**. This suggests that 3,5-positions of fluorine atoms were better positions than 2,5- positions in terms of PSE values in compound couples having the methoxy groups at the same positions which allow comparison.

The effects of methoxy groups to PSE value were considered among compounds **1–4** and compounds **5–8** with each others. The compound **2** had the highest PSE value (149) among **1–4**. This suggests that the best positions of methoxy groups were 2,4 positions comparing to 2,3-; 2,5-; 3,4- positions of methoxy groups while two fluorines were kept constant at 2,5- positions. On the other hand, the compound **7** had the highest PSE value (442) among compounds **5–8**. This suggested that the best positions for methoxy groups were 2,5 positions comparing to 2,3-; 2,4-; 3,4- positions while two fluorines were kept constant at 3,5-positions.

## Conclusion

In summary, all compounds had higher cytotoxicity than reference drug 5-FU. The compounds **2, 5**, **6**, **7** and **8** had higher PSE than 5-FU and their PSE values were over value of 100. Judging from the PSE values, (i) the fluorine positions 3.5- were better than 2,5-positions, (ii) the best positions for methoxy groups were 2,4-positions among 2,5-difluoro derivatives while they were 2,5-dimethoxy position among 3,5-difluoro derivatives. It can be concluded that compound **7** with having the highest PSE value (442) can be used as the leader compound for further studies based on its cytotoxic potency and apoptosis-inducing activity against human oral squamous cell carcinoma cell lines.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

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