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Synthesis and biological evaluation of novel tris-chalcones as potent carbonic anhydrase, acetylcholinesterase, butyrylcholinesterase and α-glycosidase inhibitors

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ABSTRACT

A novel class of fluoro-substituted tris-chalcones derivatives (**5a-5i**) was synthesized from phloroglucinol and corresponding benzaldehydes. A three step synthesis method was followed for the production of these tris-chalcone compounds. The structures of the newly synthesized compounds (**5a-5i**) were confirmed on the basis of IR, ^{1}H NMR, ^{13}C NMR, and elemental analysis. The compounds' inhibitory activities were tested against human carbonic anhydrase I and II isoenzymes (hCA I and hCA II), acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and α-glycosidase (α-Gly). These chalcone derivatives had K_i values in the range of 19.58–78.73 nM for hCA I, 12.23–41.70 nM for hCA II, 1.09–6.84 nM for AChE, 8.30–32.30 nM for BChE and 0.93 ± 0.20–18.53 ± 5.06 nM against α-glycosidase. These results strongly support the promising nature of the tris-chalcone scaffold as selective carbonic anhydrase, acetylcholinesterase, butyrylcholinesterase, and α-glycosidase inhibitor. Overall, due to these derivatives' inhibitory potential on the tested enzymes, they are promising drug candidates for the treatment of diseases like glaucoma, leukemia, epilepsy; Alzheimer's disease; type-2 diabetes mellitus that are associated with high enzymatic activity of carbonic anhydrase, acetylcholine esterase, butyrylcholinesterase, and α-glycosidase.

1. Introduction

The natural abundance of fluorine atom, such as fluorite, fluorapatite and cryolite, is thought to be at the same level as that of nitrogen, and only 12 organic compounds bearing this special atom have been found so far in nature [1]. Despite this scarcity, compounds containing a large number of synthetic fluorine are widely used in various fields. The presence of fluorine atom or fluorinated groups results in molecules with considerably unique properties that cannot be achieved using any other element [2]. Synthesis of biologically active compounds containing various fluorine groups and effective synthetic procedures that can be applied to fluorine containing organic compounds need to be developed [3].

Since the C–F bond is stronger than the C—H bond, the C—F bond length is short enough to protect the carbon centre from various nucleophilic attacks [4]. Furthermore, the carbon centre of the C—F bond is resistant to oxidation owing to the strong electronegative structure of fluorine. The similarity of the Van der Waals radius of the fluorine atom to the hydrogen atom, its high electronegativity and the stability of the C—F bond make it important to be examined in the class of drug candidate compounds containing fluorine [5]. These properties of the fluorine group contribute to the reduction of adverse effects in addition to the improvement of pharmacokinetic properties, such as absorption, distribution, metabolism and excretion [2,6,7]. Also, biological active structures which are containing these groups have a strong effect on the binding affinity for the receptor and target enzymes [8–11].

Flavonoids are a group of natural compounds that have 1.3-diarylpropane skeleton, which undertake different alicyclic or cyclic

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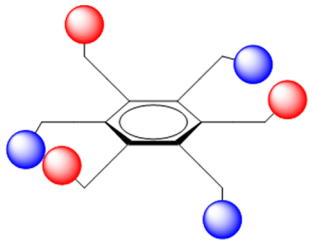
arrangements according to varying levels of oxidation [12]. On the contrary, chalcones are precursor compounds for flavonoids, where the two aromatic rings are fused with α,β-unsaturated ketones, i.e. with 1.3-diphenyl-2-propene-1-one derivatives [13]. It is also known that a great number of enzymes are inhibited by the chalcones in addition to their biological activities, such as antibacterial, antiprotozoal, anti-flammatory, antiulcer, anthelmintic, amoebicidal, insecticidal, immunosuppressive, cytotoxic and anticancer [14]. Most of the naturally occurring chalcones contains hydroxyl (−OH) or methoxy ($-OCH_3$) substituents on the aromatic rings [15]. Synthetic and naturally occurring methoxy chalcones are considerably interesting molecules since they exhibit various pharmacological activity and biological properties [16].

Recently, the presence of multi-structural units, which are multivalent or polyvalent, bound to a central core that may cause simultaneous interactions with another unit or receptor, has become a preferred route for the design of active agents that may be novel drug candidates [17,18].

Thanks to the potential complementarity and diversity of the structures such these units, they enable them to much the same resemble their biochemical interactions with the substrates or inhibitors of the target enzymes [19]. Especially the *hexa*-substituted benzene derivatives allow the target enzymes to be closely similar to existing biochemical interactions with their substrates or inhibitors (Scheme 1) [20–22]. In the case of this conformation provides appropriate synergistic interactions within the binding sites of the target enzyme [23].

Carbonic anhydrase (CA, E.C.4.2.1.1) is a metalloenzyme that contains zinc ions (Zn^{2+}) in its active site. Zn^{2+} ions, which are present in the active site of CA, interact with the substrate molecules directly and cause catalytic effect [24–26]. This enzyme family catalyse the reversible conversion of carbon dioxide (CO_2) and water to bicarbonate (HCO_3^-) and protons (H^+). They are grouped into seven different gene families, namely *α-, β-, γ-, δ-, ζ-, n-* and the last recently discover θ-CAs isoenzymes in prokaryotes and eukaryotes. The gene families do not have similarities with regard to amino acid sequences [27,28]. Human carbonic anhydrase (hCAs) are included in the class of *α*-CAs. Sixteen *α*-CA isoforms have been well defined in humans [29–32]. Among them, CAs I-III, VII and XIII are cytosolic isoenzymes, CAs IV, IX, XII, XIV and XV are membrane-bound isoenzymes, CAs VA and VB are mitochondrial isoenzymes, and CA VI is a secreted isoenzyme [29,30]. The common property of all CA isoenzymes is that they have Zn^{2+} ions bounded to histidine amino acids. Zn^{2+} ions directly interact with the substrate molecules and increase the catalytic activity of the enzyme, thus producing a powerful hydrolysis of water to H^+ and a highly reactive Zn-OH molecule [31,32]. Carbonic anhydrase inhibitors (CAIs) are in clinical use for the treatment of various activities like diuretics, anti-tumour and anti-metastatic agents, anti-glaucoma, and anti-epilepsy [33,34]. For this purpose, development of novel CAIs had a quite importance.

Alzheimer's disease (AD) affects mostly the aged people and above resulting in impaired memory and behaviour. This disorder clinically involves the progressive degeneration of brain tissue that is influenced by the deficit in acetylcholine (ACh) [35,36]. Acetylcholinesterase (AChE), as a major element of the cholinergic system in the peripheral and central nervous system (CNS), is able to convert acetylcholine (ACh) to acetate and choline (Ch) [37,38]. It is caused majorly by environmental and genetic influences. Cerebral amyloid-*β* aggregation, a deficit in ACh and a deficit in cholinergic neurotransmission, was observed in patients with AD [37–40]. Because of serious side effects of the available AChE, there is the need to search for newer effective and safe AChE to treat neurodegenerative damages. AChE inhibitors (AChEIs) or anti-cholinesterases inhibit cholinesterase, increasing the level and length of ACh action [41]. A variety of usages of AChEIs are common in medicine. As a result, a number of AChEIs have been thought for the treatment of AD. They have been used in clinical trials, including natural substances. It was reported that terpenoids, flavonoids and phenolic compounds have been recognized as AChEIs and promising lead compounds for AD [42,43].



Scheme 1. Positions of the substituents on the *hexa*-substituted benzene ring structure.

α-Glycosidase (E.C.3.2.1.20) release from intestine cells and hydrolyses oligosaccharides and polysaccharide to monosaccharide units, such as glucose and fructose in small intestine [44,45]. In human, *α*-glycosidase inhibitors (*α*-GIs) had a great importance for controlling of type-2 diabetes mellitus (T2DM) and hyperglycaemia [46]. Recently, two main chemical classes of *N*-comprising *α*-Glycosidase inhibitors (*α*-GIs) contain sugar-based inhibitors [47]. *α*-GIs can reduce the uptake of dietary carbohydrates and repress postprandial hyperglycaemia and T2DM. Thus, these *α*-GIs are endowed with sugar molecule such as compete and moieties with the oligosaccharides for binding to the active site of the enzyme, hence effectively reducing the postprandial glucose amounts in T2DM [48–50].

In this study, by placing three methoxy groups at 1, 3 and 5 positions, and fluorine substituent, 3-aryl-2-propene-1-one groups, at 2, 4 and 6 positions on the aromatic A ring, the synthesis of the derivatives of this structure, and then human carbonic anhydrase, acetylcholinesterase, butyrylcholinesterase, and *α*-glycosidase inhibition studies of these derivatives containing *hexa*-substitute aromatic system have been tested.

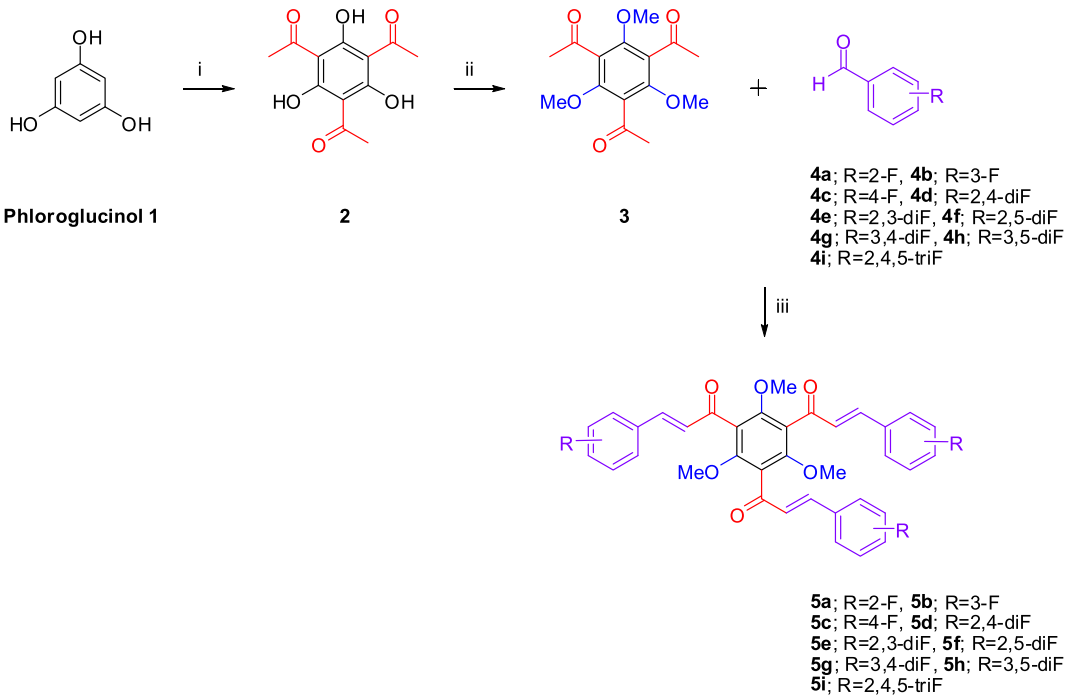
2. Results and discussion

2.1. Chemistry

Phloroglucinol (**1**) was reacted with Ac_2O in the presence of methanesulfonic acid (MSA) at 80 °C gave compound **2** in a yield of 83%. After the successful synthesis of compound **2**, compound **3** was obtained from the reaction of compound **2** with dimethysulfate in 93% yield. The base-catalysed reaction of compound **3** with related benzaldehydes (**4a-4i**) afforded fluoro-substituted trischalcones in good yields (Scheme 2). The reaction progress was checked by TLC monitoring. This is the first report on the preparation of compounds **5a-5i**. All the compounds were isolated as solids and characterized by ^1H NMR, ^{13}C NMR, and elemental analysis (for detailed information see the supplementary data).

2.2. Biochemical studies

Human carbonic anhydrase inhibition has been the subject of several investigations since the discovery of the biological importance of this enzyme in several living organisms [50]. In recent years, many compounds and derivatives have been shaped as main classes of hCA inhibitors, including hCA I, and II [51,52]. Considering the fact that novel fluoro-substituted tris-chalcones (**5a-5i**) are found as effective CA inhibitors. We synthesized novel fluoro-substituted tris-chalcones (**5a-5i**) to explore their possible human carbonic anhydrase isoenzymes I, and II (hCA I, and II), acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and *α*-glycosidase inhibition effects. The inhibition data are summarized in Table 1, along with those referred to acetazolamide (AZA), used as standard inhibitor for both hCA isoenzymes. Acarbose was shown as *α*-glycosidase inhibitor. On the other



Scheme 2. General synthesis method. Reagents: (i) $(CH_3CO)_2O$, CH_3COOH, CH_3SO_2OH, 80 °C; (ii) $(CH_3O)_2SO_2$, K_2CO_3, Acetone, reflux; (iii) 50% KOH, MeOH, r.t.

hand Tacrine (TAC) was used as standard inhibitor for both cholinergic enzymes. In order to evaluate the effect of novel fluoro-substituted tris-chalcones (**5a-5i**) on the indicated metabolic enzymes, the following results has been delineated:

(i) The physiologically relevant hCA I is found at the highest level in erythrocytes and is also expressed in normal colorectal mucosa [53,54]. As for CA I, all novel fluoro-substituted tris-chalcones (**5a-5i**) showed K_i values in the low nanomolar range that gave K_i values ranging from 19.58 ± 2.87 nM to 78.73 ± 25.17 nM. Among the synthesized novel fluoro-substituted tris-chalcones (**5a-5i**), chalcone **5 h**, which posses difluoro in *meta*- position, showed the best inhibition (K_i: 19.58 ± 2.87 nM), followed by chalcone **5i** (K_i: 20.97 ± 8.07 nM) when compared to AZA (Table 1). AZA, which is positive control and used a clinical drug, demonstrated a K_i value of 141.02 ± 50.84 mM. However, there are no dramatically differences observed between inhibition effects of novel fluoro-substituted tris-chalcones (**5a-5i**), except of novel fluoro-substituted tris-chalcones **5a** and **5b,** which possessed a fluoro groups. Recently, it was reported that hCA I isoenzyme was inhibited by a series of new tetrabromo chalcone derivatives in low nanomolar range, with K_i values of 11.30–21.22 nM [55]. Similarly, some chalcone-imide derivatives inhibited this isoenzyme with nanomolar levels (426.47–699.58 nM) [56]. It is well known that grafting of electron-withdrawing groups in an inhibitor enhanced the CA isoenzymes inhibition effects. In contrast to this inhibition effects, incorporation of electron-donating groups resulted in a decreased activity [57]. Also, it was reported that halogen anions such as bromide (Br^-) and coordinate the Zn^{2+} with a distorted tetrahedral geometry, resulting inhibition effects [58].

(ii) Human CA II is physiologically dominant and highly active cytosolic isoform [54]. As shown in Table 1, the inhibition profile of the considered novel fluoro-substituted tris-chalcones (**5a-5i**) against dominant cytosolic hCA II revealed to be quite similar to that shown towards CA II. They demonstrated K_i values between 12.23 ± 2.43–41.70 ± 9.10 nM. The only exception is represented by compound **5a** and **5b** that showed 3.41 and 3.05-folds decreased of the inhibition potency, when compared to compound **5c,** which possessed K_i value of 12.23 ± 2.43 nM. On the other hand AZA, which used to treat glaucoma, altitude sickness, epilepsy, periodic paralysis, heart failure and idiopathic intracranial hypertension [59], had a K_i value of 22.17 ± 0.65 nM against hCA II. In recent studies, it was found that a series of new tetrabromo chalcone derivatives containing 4,7-methanoisoindol-1,3-diones inhibited hCA II isoenzyme in ranging of 8.21–12.86 nM [55]. At the same manner, some chalcone-imide derivatives inhibited dominant hCA II isoenzyme with nanomolar levels (214.92–532.21 nM) [56].

(iii) Another aim of biochemical estimation is the AChE and BChE inhibition effects of novel fluoro-substituted tris-chalcones derivatives (**5a-5i**). Both cholinergic enzymes inhibition properties were recorded according to the procedure of Ellman et al. [60] and described previously [61]. Novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) had K_i values in ranging from 1.09 ± 0.20 to 6.84 ± 1.24 nM for AChE and 85.24 ± 1.71 to 8.35 ± 0.54 nM for BChE. On the other hand, tacrine had K_i value of 5.99 ± 1.79 and 2.43 ± 0.92 nM toward both cholinergic AChE and BChE, respectively. All evaluated novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) showed effective inhibition against both AChE and BChE enzymes, but chalcone **5d**, which posses two fluor groups in benzene ring of chalcone, showed perfect inhibition effect against AChE (K_i: 1.09 ± 0.20 nM) and BChE (K_i: 5.24 ± 1.71 nM) enzymes. In the same context, chalcone-imide derivatives demonstrated cholinergic AChE enzyme inhibition with K_i values in ranging of 70.47–229.42 nM.

(iv) On the other hand, for the α-glycosidase, which present on cells lining the intestine, hydrolysing monosaccharides to be absorbed through the intestine, novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) exhibited IC_{50} and K_i values are between 8.30 ± 3.80–32.30 ± 4.02 nM (Table 1). The results obtained from α-glycosidase assay showed that all novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) had effective α-glycosidase inhibition profiles than that of acarbose (IC_{50}: 22.800 mM) as standard α-glycosidase inhibitor [62]. Also, highly effective K_i values were obtained for chalcone **5c** with K_i value of 8.30 ± 3.80 nM. The inhibition of diggestive enzyme of α-glycosidase had great importance due treating and preventing diabetes, postprandial glucose amounts pand hyperglycemia [63].

Table 1
Enzyme inhibition results of novel fluoro-substituted tris-chalcones derivatives against human carbonic anhydrase I and II isoenzymes (hCA I and hCA II), acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and α-glycosidase (α-Gly).

Compounds	IC_{50} (nM)								K_i (nM)					
	hCA I	r^2	hCA II	r^2	AChE	r^2	BChE	r^2	α-Gly	r^2	hCA I	hCA II	AChE	BChE	α-Gly
5a	33.80	0.9582	33.32	0.9964	9.02	0.9556	12.01	0.9895	37.46	0.9602	78.73 ± 25.17	41.70 ± 9.10	3.23 ± 0.49	6.23 ± 0.74	20.52 ± 1.30
5b	32.84	0.9753	30.66	0.9527	4.63	0.9635	11.07	0.9816	17.95	0.9536	64.37 ± 5.02	37.35 ± 4.31	3.29 ± 0.45	8.35 ± 0.54	22.48 ± 9.02
5c	42.00	0.9628	17.46	0.9415	7.77	0.9873	10.12	0.9945	22.14	0.9534	28.80 ± 7.02	12.23 ± 2.43	3.91 ± 0.92	6.25 ± 1.92	8.30 ± 3.80
5d	26.35	0.9854	22.72	0.9536	7.26	0.9839	10.44	0.9936	24.75	0.9872	42.45 ± 15.25	20.02 ± 4.17	1.09 ± 0.20	5.24 ± 1.71	22.39 ± 7.00
5e	24.75	0.9826	30.53	0.9684	6.53	0.9807	10.81	0.9562	21.39	0.9735	34.73 ± 5.52	19.18 ± 3.45	2.70 ± 0.29	5.50 ± 2.16	32.18 ± 14.22
5f	31.08	0.9755	23.33	0.9566	7.54	0.9515	7.40	0.9895	17.46	0.9692	26.24 ± 9.23	25.94 ± 7.41	2.86 ± 0.27	5.31 ± 0.91	32.30 ± 4.02
5 g	43.04	0.9728	43.04	0.9557	5.33	0.9607	8.67	0.9539	23.57	0.9647	25.18 ± 6.24	25.56 ± 5.74	3.30 ± 0.57	6.51 ± 1.21	24.68 ± 10.37
5 h	35.17	0.9624	33.81	0.9576	7.50	0.9798	7.61	0.9927	25.57	0.9584	19.58 ± 2.87	17.86 ± 6.30	6.84 ± 1.24	6.71 ± 1.51	14.12 ± 0.83
5i	33.00	0.9728	22.35	0.9884	13.80	0.9615	11.23	0.9814	17.54	0.9633	20.97 ± 8.07	17.72 ± 2.28	6.52 ± 1.98	6.97 ± 0.46	10.05 ± 2.05
AZA*	113.79	0.9932	31.79	0.9816	–	–	–	–			141.02 ± 50.84	22.17 ± 0.65			
TAC**	–	–	–	–	5.97	0.9706	8.37	0.9846					5.99 ± 1.79	2.43 ± 0.92	
ACR***									22,800						12600 ± 78

* Acetazolamide (AZA) was used as a standard inhibitor for both hCA I, and II isoenzymes.
** Tacrine (TAC) was used as a standard inhibitor for AChE and BChE enzymes.
*** Acarbose (ACR) was used as a standard inhibitor for α-glycosidase enzyme taken from Ref. [62].

3. Material methods

3.1. Synthesis

3.1.1. General

All reagents used were commercially available unless otherwise specified. Melting points were measured with Gallenkamp melting point devices. IR Spectra: PerkinElmer Spectrum One FT-IR spectrometer. ^{1}H- and ^{13}C NMR Spectra: Varian 400 and Bruker 400 spectrometers. Elemental analysis results were obtained on a Leco CHNS-932 instrument.

3.1.2. 1,1′,1′′-(2,4,6-trihydroxybenzene-1,3,5-triyl)triethanone (2)

The mixture of phloroglucinol (**1**) (1 g, 7.93 mmol), acetic anhydride (2.24 mL, 23.8 mmol), acetic acid (0.45 mL, 7.93 mmol) was heated at 80 °C. After 5 min. MSA (3.1 mL, 15.3 mmol) was added to the mixture and the mixture was heated at 80 °C for 12 h. Completion of reaction was monitored by TLC analysis. Then the reaction was allowed to warm to room temperature and water (5 mL) was added to the mixture. The mixture was then extracted with ethyl acetate (3 × 20 mL). The combined extracts were dried over Na_2SO_4 and the solvent was removed in vacuo. Compound **2** was obtained as a white solid without further purification (1.66 g, 83%). $\mathbf{R_f}$ (EtOAc/Hexanes 40:60) = 0.93, **^{1}H NMR** (400 MHz, $CDCl_3$) δ 17.16 (s, 1H), 2.73 (s, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 205.2, 175.9, 103.3, 33.1. The ^{1}H NMR spectrum is in agreement with reported data [64].

3.1.3. 1,1′,1′′-(2,4,6-Trimethoxybenzene-1,3,5-triyl)triethanone (3)

To a solution of compound **2** (500 mg, 1.98 mmol) in acetone, dimethyl sulfate (0.1 mL, 9.91 mmol) and K_2CO_3 (2.7 g, 19.80 mmol) were added sequentially and stirred under reflux for 2 days. Completion of reaction was monitored by TLC analysis. After 2 days the precipitate was filtered and the solvent was evaporated. The resulted precipitate was washed with water and extracted with ethyl acetate (3 × 30 mL). The combined extracts were dried over Na_2SO_4 and the solvent was removed in vacuo. Compound **3** was obtained as a white solid without further purification (540 mg, yield 93%). $\mathbf{R_f}$ (EtOAc/Hexanes 40:60) = 0.17, **^{1}H NMR** (400 MHz, $CDCl_3$) δ 3.75 (s, 3H), 2.54 (s, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 200.8, 155.5, 127.3, 64.7, 32.7. The ^{1}H NMR and ^{13}C NMR spectra are in agreement with reported data [19].

3.1.4. General procedure for preparation of tris-chalcones (5a-5i)

To a solution of compound **3** (1 eq.) in MeOH (30 mL/1 mmol of substrate) benzaldehyde derivatives (**4a-4i**) (4.5 eq.) and 50% KOH solution (11 mL/1 mmol of substrate) were added sequentially and stirred for 15 h at room temperature. After 15 h solvent was evaporated. Crude material extracted with 2 M HCl solution (2 mL/1 mmol of substrate) and DCM (2 mL/1 mmol of substrate × 3). The combined extracts were dried over Na_2SO_4. The solvent was removed in vacuo and the remaining residue purified via column chromatography over silica gel using gradient elution with EtOAc and hexanes to yield compounds **5a-5i**.

3.1.5. (2E,2′E,2′′E)-1,1′,1′′-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(2-fluorophenyl)prop-2-en-1-one) (5a)

The above general procedure was followed with 2-fluorobenzaldehyde (**4a**) to yield **5a** as a yellow solid (76% yield). $\mathbf{R_f}$ (EtOAc/Hexanes 10:90) = 0.5; **mp** = 175–176 °C; **IR** (KBr, cm-1) Vmax 2945.73, 1654.98, 1607.97, 1574.20; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.63 (dd, 6H, J = 21.8, 11.8 Hz), 7.39 (dd, 3H, J = 12.8, 6.6 Hz), 7.14 (ddd, 9H, J = 24.9, 16.9, 7.6 Hz), 3.77 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.7, 162.8, 160.3, 157.4, 138.5, 132.4, 132.4, 129.9, 129.3, 124.5, 124.0, 122.5, 122.4, 116.4, 116.2, 63.9; **Anal. calcd** for $C_{36}H_{27}F_3O_6$: C, 70.58; H, 4.44; Found: C, 70.20; H, 4.56.

*3.1.6. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(3-fluorophenyl)prop-2-en-1-one) (**5b**)*

The above general procedure was followed with 3-fluorobenzaldehyde (**4b**) to yield **5b** as a yellow solid (54% yield). **R_f** (EtOAc/Hexanes 10:90) = 0.5; **mp** = 157–158 °C; **IR** (KBr, cm-1) Vmax 3066.37, 2946.11, 1653.98, 1606.41, 1580.65; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.49 (d, 3H, J = 16.1 Hz), 7.37 (qd, 6H, J = 7.5, 3.4 Hz), 7.30 (d, 1H, J = 2.1 Hz), 7.28 (d, 2H, J = 2.0 Hz), 7.16–7.10 (m, 3H), 7.07 (d, 3H, J = 16.1 Hz), 3.75 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.3, 164.3, 161.8, 157.5, 144.5, 136.4, 136.4, 130.6, 130.6, 128.7, 124.7, 124.7, 124.0, 117.9, 117.8, 114.9, 114.7, 63.8; **Anal. calcd** for $C_{36}H_{27}F_3O_6$: C, 70.58; H, 4.44; Found: C, 70.36; H, 4.60.

*3.1.7. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(4-fluorophenyl)prop-2-en-1-one) (**5c**)*

The above general procedure was followed with 4-fluorobenzaldehyde (**4c**) to yield **5c** as a white solid (81% yield). **R_f** (EtOAc/Hexanes 10:90) = 0.5; **mp** = 212–213 °C; **IR** (KBr, cm-1) Vmax 2946,63 1697,65 1232,35; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.60–7.54 (m, 6H), 7.48 (d, 3H, J = 16.1 Hz), 7.10 (t, 6H, J = 8.6 Hz), 7.00 (d, 3H, J = 16.1 Hz), 3.74 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.7, 165.8, 163.3, 157.4, 144.9, 130.9, 130.8, 130.6, 130.6, 127.6, 127.6, 124.3, 116.6, 116.34, 63.9; **Anal. calcd** for $C_{36}H_{27}F_3O_6$: C, 70.58; H, 4.44; Found: C, 70.64; H, 4.55.

*3.1.8. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(2,4-difluorophenyl)prop-2-en-1-one) (**5d**)*

The above general procedure was followed with 2,4-difluorobenzaldehyde (**4d**) to yield **5d** as a pale yellow solid (81% yield). **R_f** (EtOAc/Hexanes 30:70) = 0.5; **mp** = 133–134 °C; **IR** (KBr, cm-1) Vmax 3076.77, 2947.33, 1657.55, 1611.05, 1501.23; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.65–7.53 (m, 6H), 7.09 (d, 6H, J = 16.3 Hz), 6.98–6.83 (m, 6H), 3.76 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.4, 163.2, 160.7, 157.4, 137.3, 130.5, 130.4, 129.3, 124.1, 118.9, 112.4, 112.2, 105.0, 104.7, 104.5, 63.9; **Anal. calcd** for $C_{36}H_{24}F_6O_6$: C, 64.87; H, 3.63; Found: C, 65.17; H, 3.55.

*3.1.9. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(2,3-difluorophenyl)prop-2-en-1-one) (**5e**)*

The above general procedure was followed with 2,3-difluorobenzaldehyde (**4e**) to yield **5e** as a pale yellow solid (76% yield). **R_f** (EtOAc/Hexanes 30:70) = 0.5; **mp** = 121–122 °C; **IR** (KBr, cm-1) Vmax 2947.41, 1658.39, 1607.22, 1485.02; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.62 (d, 3H, J = 16.3 Hz), 7.37 (t, 3H, J = 6.6 Hz), 7.26–7.08 (m, 9H), 3.77 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.1, 157.70, 152.2, 152.1, 150.9, 150.8, 149.8, 149.6, 136.9, 130.8, 130.7, 124.6, 124.6, 124.4, 124.0, 123.9, 123.9, 119.3, 119.1, 64.0; **Anal. calcd** for $C_{36}H_{24}F_6O_6$: C, 64.87; H, 3.63; Found: C, 65.05; H, 3.48.

*3.1.10. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(2,5-difluorophenyl)prop-2-en-1-one) (**5f**)*

The above general procedure was followed with 2,5-difluorobenzaldehyde (**4f**) to yield **5f** as a yellow solid (75% yield). **R_f** (EtOAc/Hexanes 30:70) = 0.63; **mp** = 131–132 °C; **IR** (KBr, cm-1) Vmax 3076.07, 1660.65, 1606.98, 1574.49; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.58 (d, 3H, J = 16.2 Hz), 7.29 (dd, 3H, J = 12.0, 5.6 Hz), 7.12 (s, 3H, J = 7.8 Hz), 7.10–7.03 (m, 6H), 3.77 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.0, 159.9, 159.9, 158.8, 158.8, 157.7, 157.5, 157.5, 156.3, 156.3, 136.9, 130.5, 124.2, 124.1, 123.1, 123.3, 123.6, 123.0, 119.5, 118.2, 117.3, 117.4, 117.4, 117.3, 114.9, 114.9, 114.6, 64.1; **Anal. calcd** for $C_{36}H_{24}F_6O_6$: C, 64.87; H, 3.63; Found: C, 64.65; H, 3.72.

*3.1.11. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(3,4-difluorophenyl)prop-2-en-1-one) (**5 g**)*

The above general procedure was followed with 3,4-difluorobenzaldehyde (**4 g**) to yield **5 g** as a yellow solid (62% yield). **R_f** (EtOAc/Hexanes 30:70) = 0.5; **mp** = 186–187 °C; **IR** (KBr, cm-1) Vmax 2947.41, 1651.88, 1607.24, 1573.82, 1515.96; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.48–7.36 (m, 6H), 7.36–7.28 (m, 6H), 7.21 (dt, 6H, J = 16.6, 8.3 Hz), 6.99 (d, 3H, J = 16.0 Hz), 3.74 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 191.9, 157.5, 153.1, 151.9, 150.7, 150.6, 149.5, 143.3, 131.4, 128.3, 125.6, 124.1, 118.1, 117.9, 116.9, 116.7, 63.8; **Anal. calcd** for $C_{36}H_{24}F_6O_6$: C, 64.87; H, 3.63; Found: C, 65.17; H, 3.52.

*3.1.12. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(3,5-difluorophenyl)prop-2-en-1-one) (**5 h**)*

The above general procedure was followed with 3,5-difluorobenzaldehyde (**4 h**) to yield **5 h** as a yellow solid (55% yield). **R_f** (EtOAc/Hexanes 30:70) = 0.5; **mp** = 79–80 °C; **IR** (KBr, cm-1) Vmax 2948.54, 1655.08, 1570.62, 1519.62, 1344.67; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.43 (d, 3H, J = 16.0 Hz), 7.15–7.00 (m, 9H), 6.87 (tt, 3H, J = 8.6, 2.2 Hz), 3.75 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 191.6, 164.5, 164.4, 162.1, 161.9, 157.76, 142.8, 137.5, 137.4, 137.3, 129.5, 124.0, 111.3, 111.3, 111.2, 111.1, 106.3, 106.1, 105.8, 63.9; **Anal. calcd** for $C_{36}H_{24}F_6O_6$: C, 64.87; H, 3.63; Found: C, 64.98; H, 3.77.

*3.1.13. (2E,2′E,2″E)-1,1′,1″-(2,4,6-trimethoxybenzene-1,3,5-triyl)tris(3-(2,4,5-trifluorophenyl)prop-2-en-1-one) (**5i**)*

The above general procedure was followed with 2,4,5-trifluorobenzaldehyde (**4i**) to yield **5i** as a yellow solid (45% yield). **R_f** (EtOAc/Hexanes 30:70) = 0.53; **mp** = 74–75 °C; **IR** (KBr, cm-1) Vmax 3063.84, 1657,49, 1509.15; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.53 (d, 3H, J = 15.9 Hz), 7.43 (dd, 3H, J = 15.1, 8.1 Hz), 7.09–6.94 (m, 6H), 3.76 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 191.8, 157.7, 135.8, 130.1, 124.1, 119.0, 116.3, 116.1, 106.7, 106.5, 106.4, 106.2, 64.1; **Anal. calcd** for $C_{36}H_{21}F_9O_6$: C, 60.01; H, 2.94; Found: C, 59.78; H, 3.17.

3.2. Biochemical studies

3.2.1. hCA inhibition studies

In this work, both hCA I, and II isoenzymes were purified by Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography [65,66]. In this affinity technic, Sepharose-4B-L-tyrosine-sulfanilamide was used as an affinity matrix for selective retention of CA isoenzymes [67,68]. CA activity determination was spectrophotometrically measured according to Verpoorte et al. [69] as described previously [70]. *p*-Nitrophenylacetate (PNA) was consumed as substrate for both isoenzymes and enzymatically transformed to p-nitrophenolate ions [69]. One CA activity unit is the amount of enzyme, which had absorbance change at 348 nm of PNA to 4-nitrophenylate over a period of 3 min at 25 °C [71].

Bradford technique was used for the investigation of protein amount during the purification stages [71]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDSPAGE) was used for fixation of both isoenzymes [72] described in previous studies [73]. Bovine serum albumin was used as the standard protein [74]. For determining the inhibition parameters of each novel fluoro-substituted tris-chalcones derivative and an activity (%) [Chalcones derivatives] graph was drawn. To calculate K_i values, three different novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) concentrations were tested [75].

3.2.2. AChE/BChE inhibition studies

The inhibitory effect of novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) on AChE/BChE activities was performed according to Ellman's method [60] as described previously [76]. Acetylthiocholine iodide/butrylcholine iodide (AChI/BChI) were used as substrate for both cholinergic reactions. In brief, an aliquot (100 µL) of Tris/HCl buffer (pH 8.0, 1.0 M) and different concentration of sample solutions (10–30 µg/mL) were added to 50 µL of AChE/BChE enzymes solution (5.32×10^{-3} EU). The solutions were incubated at 20 °C for 10 min. An aliquot (50 µL, 0.5 mM) of DTNB (5,5′-dithio-bis(2-nitro-benzoic)acid) and AChI/BChI were added to incubated mixture and enzymatic

reactions were initiated. AChE/BChE activities were spectrophotometrically determined at 412 nm [77].

3.2.3. α-Glycosidase inhibition studies

α-Glycosidase inhibition effect of novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) was evaluated according to the method of Tao et al. [40]. Firstly, phosphate buffer (pH 7.4, 75 μL) was mixed with of 5 μL of the sample and α-glycosidase enzyme solution (20 μL), which prepared in phosphate buffer (0.15 U/mL, pH 7.4). After preincubation 50 μL of *p*-Nitrophenyl-D-glycopyranoside (*p*-NPG) in phosphate buffer (5 mM, pH 7.4) was added and solution was re-incubated at 37 °C. The absorbance o mixtures were recorded at 405 nm [78]. For the determination of K_i values, three different novel fluoro-substituted tris-chalcones derivatives (**5a-5i**) concentrations were used. Then, the Lineweaver-Burk graphs were drawn [78].

4. Conclusions

A new series of tris-chalcone derivatives were successfully produced with high yields and they were evaluated for their inhibitory potentials on the hCA I, hCA II, AChE, BChE and α-glycosidase enzymes whose high and uncontrolled activities have been associated with the certain diseases. Our compounds were potent inhibitors against the tested enzymes. They exerted better inhibitory potential than the conventional inhibitors that were purchased commercially except that of BChE enzyme. These molecules represent a promising structural scaffold that can be further explored in order to generate other synthetics with enhanced inhibitory potential as well as selectivity against these enzymes. Reported molecules constitute strong drug candidates against the diseases associated with the aberrant activity of the tested enzymes. These diseases can be listed as glaucoma, epilepsy, altitude sickness, periodic paralysis, idiopathic intracranial hypertension, and heart failure (as CA inhibitors), to treat myasthenia gravis, postural tachycardia syndrome, Alzheimer's disease (as cholinergic enzymes inhibitors) and treatment of type 2 diabetes mellitus (as α-glycosidase inhibitors). In our future studies we will be designing and synthesizing similar molecules with potent biological activities and further dissecting these molecules efficiencies and biological activities by using *in vitro* and *in vivo* disease models.

Declaration of interest

The authors report no financial and non-financial conflict of interest.

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Appendix A. Supplementary material

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