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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Activation and inhibition effects of some natural products on human cytosolic CAI and CAII

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Abstract

Carbonic anhydrases (CAs) play a significant function in diverse pathological and physiological processes. Their inhibitors and activators are suitable molecules to use as a drug in the treatment of different disease. In the present study, seven natural compounds, namely didymin, retusin isoquercitrin, silymarin, verbascoside, teucroside, and 3′-O-methylhypolaetin 7-O-[6‴-O-acetyl-β-D-allopyranosyl-(1→2)]-6″-O-acetyl-β-D-glucopyranoside were isolated from *Mentha spicata*, *Sideritis libanotica linearis, Platanus orientalis, Teucrium chamaedrys* subsp. *chamaedrys*, and *Silybum marianum*. The influences of compounds on the carbonic anhydrase I(hCAI) and II(hCAII) purified from human erythrocytes were tested. Five phenolic compounds acted as an inhibitor on the activity of hCAI, and IC_{50} values were computed between 18.16 and 172.5 µM. Isozyme hCAII is only inhibited by silymarin with an IC_{50} value of 43.12 µM. This isoenzyme was effectively activated by five natural compounds with AC_{50} values in the range of 2.98–18.53 µM. To understand the binding patterns of molecules that show activation effect against hCAII, molecular docking was done using Leadit 2.3.2 software, and calculated between −19.05 and −14.42 (kJ/mol) binding energies. Both in vitro and in silico results demonstrated that the best activators against hCAII were teucroside and isoquercitrin, with AC_{50} values of 2.98 and 3.17 µM, and binding energies −19.05 and −18.01 (kJ/mol), respectively. According to the ADME results, retusin demonstrated physicochemical and pharmacokinetic properties specific to the drug candidates.

Keywords Carbonic anhydrase · Natural product · Inhibition · Activation · Docking

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) is a zinc metalloenzyme that catalyzes the reversible reactions of water and CO_2: $H_2O + CO_2 \leftrightarrow H^+ + HCO_3^-$. The reaction catalyzed by CAs is vital to in the several tissues. These isoenzymes involve in physiological processes such as metabolism, cell growth, respiration, ionic, fluid balance and acid-base, photosynthesis, and calcification (Gilmour 2010). Owing to the wide distribution and different function of the CAs in multiple cells, tissues, and organs, carbonic anhydrase inhibitors (CAIs) are commonly defined as target enzymes to prevent or treat many illnesses, such as glaucoma, epilepsy, cancer, osteoporosis, and obesity (Supuran 2010). Many researchers have carried out several studies to investigate new and more efficient inhibitors of these enzymes (Alyar and Adem 2014; Senturk et al. 2011). Also, in the last few decades, some reviewers have stressed that carbonic anhydrase deficiency leads to several diseases and in the prevention and treatment of these diseases; their

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activators may be used as a medicinal agent (Supuran 2008; Scozzafava et al. 2006; Bertucci et al. 2010).

Plants contain many bioactive compounds with numerous beneficial health effects. Many of them are used as a drug for the therapy and precaution of many diseases; besides, they provide outstanding contributions to the discovery and development of new medicines (Dykes and Rooney 2007; Ravishankar et al. 2013; Cazarolli et al. 2008). These compounds are also an invaluable source in the searching of enzyme inhibitors or activators. Recent studies have been reported that carbonic anhydrase activities were considerably altered by natural compounds in the low concentration (Innocenti et al. 2010; Davis et al. 2011). So, they are considered to have high potential to influence the activity of these enzymes. We aimed to isolate some natural phenols from five plants and to evaluate influences on the hydratase activity of carbonic anhydrase isoenzyme I (hCAI) and II (hCAII) purified human erythrocytes.

Materials and methods

General

Sepharose-4B, acetic acid, chemicals for electrophoresis, sulfonamide, absolute ethanol, and L-tyrosine and protein assay reagents were bought from Sigma-Aldrich Co. (Taufkirchen, Germany). NMR spectra were recorded on Agilent-600 MHz NMR spectrometer (Santa Clara, USA). The solvent used for NMR study is either $CDCl_3$ or DMSO-d6.

Extraction and isolation of natural products

Isoquercitrin

The powdered with liquid nitrogen leaf parts of *Platanus orientalis* (1480 g) plant were boiled in water for 3 h (120 °C, 10 L distilled water). After cooling to room temperature, the aquatic extract was filtered to remove plant residue. The aquatic extract was partitioned with ethylacetate (1000 mL × 10) to yield ethylacetate extract. The combined EAE was evaporated in vacuum at 30 °C. The total EAE (6.67 g) was fractionated by flash chromatography over a rediself mark flash column (silica column of 40 gr) and partitioned sequentially starting with n-hexane and followed by chloroform, ethylacetate, and methanol with increasing polarity to give 120 fractions. The fraction 27 gave 3-*O*-*rhamnose*-5,7,3′,5′-tetra hydroxy-flavone compound.

Isoquercitrin (1)[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-(((2 S,3 R,4 S,5 S,6 R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2*H*-pyran-2-yl)oxy)-4*H*-chromen-4-one] Yellow amorphous powder (MeOH), m.p. 239-243 °C; 1 H NMR (600 MHz, DMSO-d6, δ ppm): 12.60 (−OH, s, 1 H), 7.64 (1 H, d, J = 8.34 Hz, H-2′), 6.79 (1 H, d, J = 8.34 Hz, H-3′), 7.50 (d, 1 H, J = 1.78, H-6'), 6.18 (s, 1 H, H-6), 6.38 (s, 1 H, H-8), 5.34 (d, 1 H, J = 7,2 Hz, H-1′′), 3.29 (m, 1 H, H-2′′), 3.54 (m, 1 H, H-3′′), 3.62 (m, 1 H, H-4′′), 3.34 (m, 1 H, H-5′′), 3.26–3.43 (m, 2 H, H-6′′); 13 C NMR (150 MHz, DMSO-d6, δ ppm): 156.73 (C-2), 133.93 (C-3), 177.93 (C-4), 161.67 (C-5), 99.10 (C-6), 164.57 (C-7), 93.93 (C-8), 156.67 (C-9), 104.36 (C-10), 121.54 (C-1′), 122.49 (C-2′), 115.74 (C-3′), 148.90 (C-4′), 145.27 (C-5′), 116.42 (C-6′), 102.47 (C-1′′), 76.25 (C-2′′), 71.65 (C-3′′), 68.26 (C-4′′), 73.55 (C-5′′), 60.45 (C-6′′).

Retusin and didymin

The air-dried and powdered aerial parts of *M. spicata* (1000 g) were boiled in water for 2 h. After cooling to room temperature, aquatic extract (AE) was filtered to remove plant residue. The AE was partitioned with ethylacetate (2500 mL × 2) to yield ethylacetate extract (EAE). The combined EAEs were dried in vacuo at 60 °C. The total EAE (25 g) was fractionated by column chromatography on the silica gel using hexane–MeOH solvent gradient with increasing polarity to give 300 fractions each collected 250 mL. The fractions of 161–166 gave retusin and 258–276 gave didymin, respectively.

Retusin (2) [2-(3,4-dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4*H*-chromen-4-one]

Yellow crystals (MeOH/hexane), m.p. 158-160 °C; HPLC/TOF-MS exhibited molecular ion at *m/z* $[M–H]^+$ 359.1159 ($C_{19}H_{18}O_7$), ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm): 12.90 (s, 1 H, −OH), 6.94 (brs, 1 H, H-6), 7.02 (brs, 1 H, H-8), 7.57 (d, 1 H, $J = 2.02$ Hz, H-2′), 7.11 (d, 1 H, $J = 8.51$ Hz, H-5′), 7.70 (dd, 1 H, $J = 8.51$, $J = 2.02$ Hz, H-6′), 3.73 (−OCH_3), 3.92 (−OCH_3), 3.88 (−OCH_3), 3.84 (−OCH_3);^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 164.20 (C-2), 132.40 (C-3), 182.73 (C-4), 163.90 (C-5), 92.19 (C-6), 159.20 (C-7), 104.11 (C-8), 159.30 (C-9), 105.30 (C-10), 123.21 (C-1′), 109.79 (C-2′), 152.49 (C-3′), 149.51 (C-4′), 115.28 (C-5′), 120.59 (C-6′), 60.50 (−OCH_3), 56.92 (−OCH_3), 56.33 (−OCH_3), 56.19 (−OCH_3).

Didymin (3) [5-hydroxy-2-(4-methoxyphenyl)-7-(((2 S,3 R,4 S,5 S,6 R)-3,4,5-trihydroxy-6-((((2 R,3 R,4 R,5 R,6 S)-3,4,5-trihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy) methyl) tetrahydro-2*H*-pyran-2-yl) oxy) chroman-4-one]

Yellow crystals (MeOH/hexane), m.p. 209–210 °C; HPLC-TOF/MS exhibited molecular ion at *m/z* $[M−H]^−$ 593.1966 ($C_{28}H_{34}O_{14}$), ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm): 12.89 (s, 1 H, −OH), 6.92 (s, 1 H, H-3), 6.45 (d, 1 H, $J =$

2.01 Hz, H-6), 6.79 (d, 1 H, $J = 2.01$ Hz, H-8), 8.04 (d, 2 H, $J = 8.52$ Hz, H-2′/H-6′), 7.14 (d, 2 H, $J = 8.52$ Hz, H-3′/H-5′), 5.04 (d, 1 H, $J = 7.08$ Hz, H-1″), 3.32 (m, 1 H, H-2″), 3.28 (m, 1 H, H-3″), 3.15 (m, 1 H, H-4″), 3.66 (m, 1 H, H-5″), 3.46−3.83- (m, 2 H, H-6″), 4.54 (brs, 1 H, H-1‴), 3.46 (m, 1 H, H-2‴), 3.64 (m, 1 H, H-3‴), 3.17 (m, 1 H, H-4‴), 3.40 (m, 1 H, H-5‴), 1.10 (d, 3 H, $J = 6.41$, H-6‴), 3.86 (s, 3 H, $-OCH_3$); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 164.51 (C-2), 104.21 (C-3), 182.80 (C-4), 161.59 (C-5), 100.09 (C-6), 163.31 (C-7), 95.29 (C-8), 157.41 (C-9), 105.72 (C-10), 123.21 (C-1′), 128.98 (C-2′), 115.21 (C-3′), 162.98 (C-4′), 115.21 (C-5′), 128.98 (C-6′), 99.79 (C-1″), 76.68 (C-2″), 73.51 (C-3″), 69.61 (C-4″), 75.54 (C-5″), 66.47 (C-6″), 100.01 (C-1‴), 72.54 (C-2‴), 70.67 (C-3‴), 72.09 (C-4‴), 68.35 (C-5‴), 18.27 (C-6‴), 56.30 ($-OCH_3$).

Silymarin

Defatted seeds of S*ilybum marianum* (100 g) were extracted with acetone (1 L) overnight at room temp. Acetone extract (8 g) chromatographed over silica gel column using hexane: acetone (7:3): as a mobile phase. The whole-acetone extract (8 g) was fractionated by column chromatography on the silica gel n-hexane-methanol solvent gradient elution with increasing polarity to give 20 fractions each collected 250 mL. The fractions 22–24 gave silymarin.

Silymarin (4) [(2 S)-3,5,7-trihydroxy-2-((2 R,3 R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)chroman-4-one]

Yellow powder (MeOH/acetone/hexane), m.p. 230–234 °C ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm): 5.00 (d, 1 H, $J =$ 11.32 Hz, H-2), 4.52 (d, 1 H, $J = 11.32$ Hz, H-3), 5.92 (brs, 1 H, H-6), 5.87 (brs, 1 H, H-8), 6.82 (brs, 1 H, H-2′), 6.88 (brs, 1 H, H-6′), 5.49 (d, 1 H, $J = 6.44$ Hz, H-6′α), 3.48 (m, 1 H, H-6′β), 3.70 (m, 1 H, H-6′γ), 6.97 (brs, 1 H, H-2″), 6.76 (m, 1 H, H-5″), 6.78 (m, 1 H, H-6″), 3.76 (s, 1 H, $-OCH_3$); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 83.70 (C-2), 72.14 (C-3), 198.32 (C-4), 163.76 (C-5), 96.49 (C-6), 167.26 (C-7), 95.45 (C-8), 163.05 (C-9), 100.91 (C-10), 129.52 (C-1′), 115.75 (C-2′), 146.81 (C-3′), 141.19 (C-4′), 130.42 (C-5′), 115.83 (C-6′), 87.49 (C-α), 53.81 (C-β), 63.38 (C-γ), 141.30 (C-1″), 110.85 (C-2″), 148.01 (C-3″), 147.50 (C-4″), 116.09 (C-5″), 119.18 (C-6″), 56.10 ($-OCH_3$).

Extraction and isolation of verbascoside, teucroside, and 3′-*O*-methylhypolaetin 7-O-[6‴-O-acetyl-*β*-D-allopyranosyl-(1 → 2)]-6″-O-acetyl-β-D-glucopyranoside have been carried out according to the methods described previously (Elmastas et al. 2016; Demirtas et al. 2011).

Verbascoside (5) [(2 S,3 S,4 S,5 S,6 S)-6-(3,4-dihydroxyphenethoxy)-5-hydroxy-2-(hydroxymethyl)-4-(((2 S,3 S,4 S,5 S,6 R)-3,4,5-trihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)tetrahydro-2*H*-pyran-3-yl (E)-3-(3,4-dihydroxyphenyl) acrylate]

Yellow crystals (in MeOH), m.p.144-145 °C; HPLC-TOF/MS exhibited molecular ion at *m/z* [M-H]$^-$ 623.1914 ($C_{29}H_{36}O_{15}$); ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm): 6.63 (brs, 1 H, H-2), 6.62 (d, 1 H, $J = 8.0$ Hz, H-5), 6.49 (d, 1 H, $J = 8.0$ Hz, H-6), 3.85–3.69 (t,m, 2 H, $J = 6.7$ Hz, H-6α), 2.68 (t, 2 H, $J = 6.7$ Hz, H-6β), 7.03 (brs, 1 H, H-1′), 6.75 (d, 1 H, $J = 8.3$ Hz, H-5′), 6.98 (d, 1 H, $J = 8.3$ Hz, H-6′), 6.20 (d, 1 H, $J = 15.8$ Hz, H-α′), 7.45 (d, 1 H, $J = 15.8$ Hz, H-β′), 4.37 (d, 1 H, $J = 7.9$ Hz, H-1″), 3.23 (t, 1 H, $J = 8.4$ Hz, H-2″), 3.60 (m, 1 H, H-3″), 4.69 (t, 1 H, $J = 9.7$ Hz, H-4″), 3.52 (m, 1 H, H-5″), 3.31 (m, 1 H, H-6″), 4.78 (d, 1 H, $J = 2.8$ Hz, H-1‴), 3.68 (d, 1 H, $J = 7.81$ Hz, H-2‴), 3.46 (m, 1 H, H-3‴), 3.09 (t, 1 H, $J = 9.32$ Hz, H-4‴), 3.39 (m, 1 H, H-5‴), 0.96 (d, 1 H, $J = 6.23$ Hz, H-6‴); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 129.57 (C-1), 116.22 (C-2), 145.40 (C-3), 143.96 (C-4), 116.74 (C-5), 119.98 (C-6), 72.82 (C-6α), 35.42 (C-6β), 125.93 (C-1′), 115.07 (C-2′), 146.00 (C-3′), 148.92 (C-4′), 116.12 (C-5′), 122.14 (C-6′), 113.99 (C-6′α′), 146.00 (C-6′β′), 166.15 (C = O), 102.70 (C-1″), 74.91 (C-2″), 79.57 (C-3″), 69.56 (C-4″), 74.94 (C-5″), 61.16 (C-6″), 101.65 (C-1‴), 70.95 (C-2‴), 70.69 (C-3‴), 72.10 (C-4‴), 69.17 (C-5‴), 18.57 (C-6‴).

Teucroside (6) [(3 R,6 R)-4-((3,5-dihydroxy-6-methyl-4-((3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)oxy)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(3,4-dihydroxyphenethoxy)-5-hydroxy-2-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yl (E)-3-(3,4-dihydroxyphenyl) acrylate]

Brownish- yellow amorphous powder, HPLC-TOF/MS exhibited molecular ion at *m/z* [M−H]$^-$ 755.2370 ($C_{35}H_{46}O_{19}$); ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm): 6.63 (d, 1 H, $J = 2.58$ Hz, H-2), 6.63 (brs, 1 H, H-5), 6.49 (brs, 1 H, H-6), 3.60 (t, 2 H, $J = 6.76$ Hz, H-α), 2.71 (t, 2 H, $J =$ 6.76 Hz, H-β), 5.18 (brs, 1 H, H-1′), 3.79 (m, 1 H, H-2′), 3.38 (m, 1 H, H-3′), 4.73 (m, 1 H, H-4′), 3.12 (m, 1 H, H-5′), 3.34 (m, 2 H, H-6′), 4.36 (d, 1 H, $J = 7.80$ Hz, H-1″), 3.69 (t, 1 H, $J = 9.28$ Hz, H-2″), 3.48 (m, 1 H, H-3″), 3.20 (m, 1 H, H-4″), 3.36 (m, 1 H, H-5″), 0.94 (d, 3 H, $J = 6.76$ Hz, H-6″), 4.81 (d, 1 H, $J = 2.52$ Hz, H-1‴), 3.60 (m, 1 H, H-2‴), 3.44 (m, 1 H, H-3‴), 3.55 (m, 1 H, H-4‴), 3.46-3.30 (m, 2 H, H-5‴), 7.03 (d, 1 H, $J = 2.80$ Hz, H-2⁗), 6.76 (d, 1 H, $J = 8.04$ Hz, H-5⁗), 6.99 (d, 1 H, $J = 8.04$ Hz, H-6⁗), 6.21 (d, 1 H, $J = 15.80$ Hz, H-α′), 7.46 (d, 1 H, $J = 15.80$ Hz, H-β′); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 129.60 (C-1), 116.75 (C-2), 145.43 (C-3), 143.99 (C-

4), 115.92 (C-5), 120.01 (C-6), 70.75 (C-α), 35.47 (C-β), 100.58 (C-1′), 77.68 (C-2′), 70.75 (C-3′), 69.45 (C-4′), 72.42 (C-5′), 61.13 (C-6′), 102.76 (C-1″), 80.01 (C-2″), 74.91 (C-3″), 74.83 (C-4″), 69.20 (C-5″), 18.64 (C-6″), 102.49 (C-1‴), 70.34 (C-2‴), 71.35 (C-3‴), 67.39 (C-4‴), 64.02 (C-5‴), 125.91 (C-1⁗), 115.14 (C-2⁗), 146.02 (C-3⁗), 148.94 (C-4⁗), 116.24 (C-5⁗), 121.91 (C-6⁗), 113.97 (C-α′), 146.08 (C-β′), 166.15 (C = O).

3′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl-b-D-allopyranosyl-(1 → 2)]-6‴-*O*-acetyl-b-D-glucopyranoside (7) [((3 R,4 S,5 R)-6-(((2 S,4 S,5 S)-6-(acetoxymethyl)-2-((5,8-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl)oxy)-4,5-dihydroxytetrahydro-2*H*-pyran-3-yl)oxy)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl acetate]

Yellow crystals (MeOH/EtOAc), HPLC/TOF-MS exhibited molecular ion at *m/z* $[M-H]^-$ 723.1740 ($C_{32}H_{36}O_{19}$), ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm): 6.78 (s, 1 H, H-3), 6.61 (s, 1 H, H-6), 7.48 (brs, 1 H, H-2′), 7.10 (d, 1 H, J = 8.5 Hz, H-5′), 7.59 (d, 1 H, J = 8.5 Hz, H-6′), 5.15 (d, 1 H, J = 7.6, H-1″), 3.61 (t, 1 H, H-2″), 3.52 (t, 1 H, H-3″), 3.28 (m, H-4″), 3.74 (t, 1 H, H-5″), 4.11-4.31 (d, J = 11.30 Hz, dd, J = 11.30 Hz/7.21 Hz, 2 H, H-6″), 1.85 (s, 3 H, CH_3), 4.90 (d, 1 H, J = 8.00 Hz, H-1‴), 3.30 (m, 1 H, H-2‴), 3.89 (brs, 1 H, H-3‴), 3.38 (t, 1 H, J = 7.9 Hz, H-4‴), 3.82 (brs, 1 H, H-5‴), 3.92–4.14 (m, 2 H, H-6‴), 3.84 (s, 3 H, OCH_3), 2.03 (s, 3 H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 164.40 (C-2), 103.80 (C-3), 182.77 (C-4), 152.63 (C-5), 99.58 (C-6), 150.91 (C-7), 127.86 (C-8), 144.48 (C-9), 106.00 (C-10), 123.51 (C-1′), 113.66 (C-2′), 147.20 (C-3′), 151.76 (C-4′), 112.53 (C-5′), 119.39 (C-6′), 99.88 (C-1″), 82.45 (C-2″), 75.73 (C-3″), 71.86 (C-4″), 74.10 (C-5″), 63.60 (C-6″), 170.72 (C = O), 21.02 ($-CH_3$), 102.65 (C-1‴), 69.90 (C-2‴), 71.27 (C-3‴), 67.34 (C-4‴), 71.87 (C-5‴), 63.92 (C-6‴), 56.24 ($-OCH_3$), 170.62 (C = O), 20.86 ($-CH_3$).

Enzyme studies

Purification of enzymes

Fresh human blood samples were taken in tubes including EDTA, then centrifuged (at 3000 × *g* for 15 min) the plasma and leukocytes removed. Erythrocytes were treated twice with 0.9% NaCl and hemolyzed with five times of ice-cold water. Cell membranes and blast cells were precipitated by centrifugation at 15,000 × *g* for 25 min at 4 °C. After pH of the hemolysate with solid Tris is set 8.7, it was loaded to Sepharose-4B-L-tyrosine sulfanilamide affinity column pre-equilibrated with 0.1 M Na_2SO_4 /25 mM Tris-HCl (pH 8). The column was washed with the 22 mM Na_2SO_4/25 mM Tris-HCl, (pH 8.7) buffer until the absorbance difference between equilibration buffer and recovered solution from the bottom was becoming 0.05 at 280 nm. Isoenzyme I and II were eluted with 25 mM sodium phosphate (pH 6.3)/ 1.0 M NaCl and 0.5 M $NaClO_4$/0.1 M sodium acetate (pH 5.6), respectively. The purification process was fulfilled at 4 °C (Gülçin et al. 2004). The fractions containing the desired isoenzyme were pooled and dialyzed against 10 mM phosphate buffer, pH 7.4.

The protein quantity of samples was detected through the technique of Bradford at 595 nm, using bovine serum albumin as a standard (Bradford 1976).

SDS-PAGE was done to check the purity of enzymes according to the method described by Laemmli (Laemmli 1970) performed in 10% and 3% acrylamide for a lower and an upper gel, respectively, containing 0.1% SDS, using Bio-Rad: The Precision Plus Protein™ Kaleidoscope™ standards as standard proteins. The electrophoretic pattern was photographed.

Activity assay of CA

The activities of enzymes were determined using a slight alteration of the electrometric technic defined by Wilbur and Anderson 1948. The activity measurements were conducted by adding a test sample to 3.5 ml of 15 mM veronal buffer, pH 8.3 at 0–4 °C. The reaction was initiated by addition of 1.5 ml of ice-cold water saturated with CO_2. Activity was stated as the time needed to the pH decline from 8.3 to 7.3. CO_2-hydratase activity as an enzyme unit (EU) was defined by Wilbur-Anderson Units (t_o–t_c/t_c) where t_c and t_o are the times for a pH alteration of with enzyme and without enzyme reactions, respectively.

Effects of natural products

In order to investigate the impact of natural products on hCAI and hCAII, concentrations of isoquercitrin (1–25.6 μM), 3′-O-Methylhypolaetin 7-O-[6‴-O-acetyl-β-D-allopyranosyl-(1 → 2)]-6″-O-acetyl-β-D-glucopyranoside (21-271 μM), retusin (1–23 μM), didymen (1-120 μM), verbascoside (4-50 μM), silymarin (2.52-72 μM), and teucroside (0.7–70 μM) were added to the reaction medium and the enzyme activity was measured. The enzyme activity without isolated compounds was accepted as 100% activity. Three measurements for each experiment were performed, and average values were used for each data point. The IC_{50} and AC_{50} values were derived from activity (%) versus natural product concentration plots.

Docking studies

Possible docking modes between active compounds and carbonic anhydrase II were studied using the FlexX docking

Table 1 The purification results of CAI and CAII isozymes from human erythrocytes

Purification steps		Volume (ml)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Purification fold	Yield (%)
Homogenate		20	13,493	953	14.16	1	100
Sepharose 4B-tyrosine-sulfanilamide affinity gel	hCAI	7.5	5513	2.15	2564.2	181.08	40.65
	hCAII	3	691.11	0.129	5357	378.35	5.12

approach of the LeadIT 2.3.2 suite (BioSolveIT, Germany). The target enzymes CA II (PDB id: 5W8B; Resolution: 1.601 Å) were downloaded in pdb format from protein data bank (http://www.rcsb.org/pdb) (Bhatt et al. 2018). Three-dimensional structures of the compounds were downloaded as the SDF file from the site (https://pubchem.ncbi.nlm.nih.gov). 1-[2-(1H-imidazol-5-yl)ethyl]-4-methyl-2,6-di(propan-2-yl)pyridin-1-ium (A57) used as a reference ligand.

ADME (adsorption, distribution, metabolism, and excretion) characteristics of natural compounds were evaluated using SwissADME online property calculation toolkit. In the present study, we have calculated % ABS (percentage absorption), TPSA (topological polar surface area), n-ROTB (number of rotatable bonds), MV (molecular volume), MW (molecular weight), miLog P (logarithm of partition coeffient of compound between n-octanol and water), n-ON acceptors (number of hydrogen bond acceptors), n-OHNH donors (number of hydrogen bonds). Absorption (% ABS) was calculated by using the equation of $\%\ \mathrm{ABS} = 109 - (0.345 \times \mathrm{TPSA})$.

Results

The enzymes of human isozymes CAI and CAII were purified from human erythrocytes through a Sepharose 4B-L-tyrosine sulfanilamide affinity column chromatography. Using this procedure, CAI, having the specific activity of 5357 EU/mg proteins, was isolated with a yield of 40.65% and 181.08-fold; CAII, having the specific activity of 5357 EU/mg proteins, was purified with a yield of 5.12% and 378.85-fold (Table 1). The analysis of enzyme purity was controlled via the SDS-PAGE and seen single bands in the gel photo (Fig. 1).

The HPLC/TOF-MS, the proton, carbon, DEPT, HMBC, HSQC, and COSY NMR spectra of all compounds have been given in the supplementary data.

Activating and Inhibitory effects of phenolic compounds demonstrated in Fig. 2 on enzyme activities were evaluated under in vitro conditions using hydratase activity. AC_{50} and IC_{50} values were obtained from activity %—natural product [μM] charts (Fig. 3) and results were showed in Table 2. Against the cytosolic isozyme hCAI, natural compounds 1, 4, and 6 behaved as good inhibitors with IC_{50} values in the range of 18.16–49.28 μM. The compound 2 acted as a weak hCAI inhibitor ($IC_{50} = 172.1$ μM). The compounds 2 and 3 did not exhibit inhibitor or activator effects on hCAI in in vitro conditions. Against the cytosolic isozyme hCAII, one of the six compounds acted as inhibitors ($IC_{50} = 43.12$ μM). The compounds 1, 2, 3, 5, and 6 performed as a hCAII activator, AC_{50} values were calculated as 3.17, 7.02, 6.08, 18.53, and 2.98, respectively. The compound 7 did not show any effect on the hCAII enzyme activity.

To evaluate the binding interactions between the compounds and hCAII, molecular docking studies were performed using LeadIT-FlexX 3.2.2. Total binding energies of

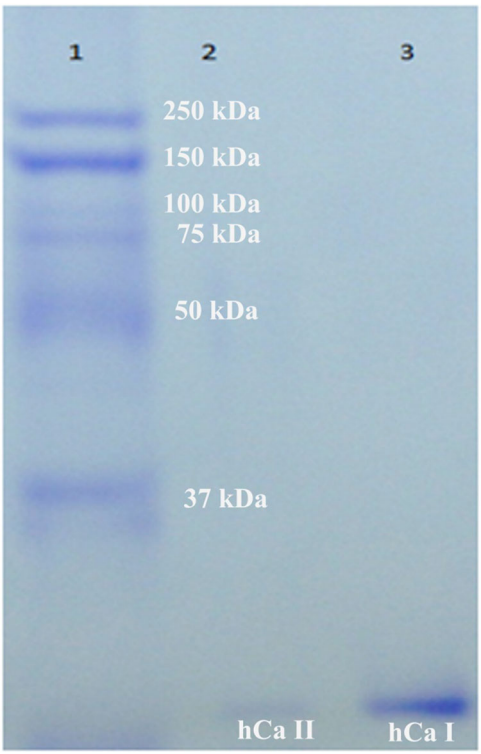

Fig. 1 SDS-polyacrylamide gel electrophoresis of human erythrocytes CA-I and CA-II purified by Sepharose 4B-tyrosine-sulfanilamide affinity gel. Line 1: Bio-Rad, The Precision Plus Protein™ Kaleidoscope™ standards (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa); Line 2: human erythrocytes CA-II, Line 3: human erythrocytes CA-I

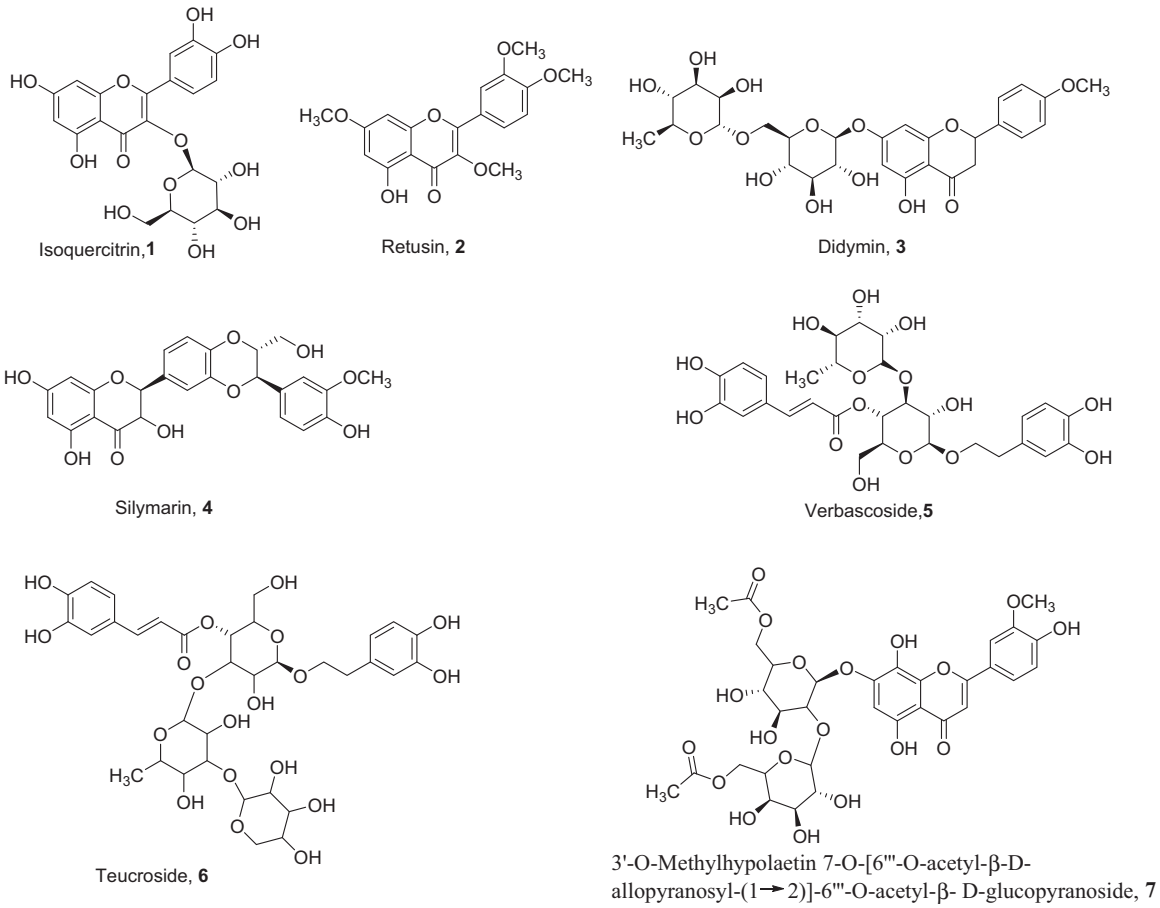

Fig. 2 The structures of molecules

teucroside, isoquercitrin, retusin, verbascoside, and didymin calculated as −19.05, −18.01, −17.78, −14.48, and −14.42, respectively (Table 3). The possible interactions between the molecules and the enzyme are shown in Fig. 4.

In silico ADME study was carried out to define some physicochemical and pharmacokinetics properties. The results are displayed in Table 4.

Discussion

The proton, carbon, DEPT, HMBC, HSQC, and COSY NMR spectra of isoquercitrin confirmed the corresponding flavone (1) as shown in Fig. S1–6.

The compound **2** (**3′-*O*-methylhypolaetin-7-*O*-[6‴-*O*-acetyl-allosyl-(1 → 2)-6‴-*O*-acetyl-glycoside])** was isolated from *Sideritis libanotica* Labill. as yellow crystals. The HPLC/TOF-MS exhibited the molecular ion at *m/z* $[M–H]^-$ 723.1740, which is in accordance with the molecular weight of 724.1732 ($C_{32}H_{36}O_{19}$) (Fig. S7). ^{1}H-NMR spectrum of compound (**2**), 3,4-disubstituted ring B signals as ABX systems at δ_H 7.59 (d, 8.6 Hz), 7.48 (brs), 7.10 (d, 8.6 Hz), H-3 proton at δ_H 6.61 and H-6 proton at δ_H 6.78 observed at singlets. The sharp singlet at δ_H 3.86 corresponds to the presence of three methoxy protons attached to the aromatic ring system and correlate with 151.75 using HMBC experiment to determine the position of methoxy as carbon 3′. Two acetyl groups at δ_H 1.85 and 2.03 ppm were observed in each sugar moieties using proton NMR (Fig. S8). In ^{13}C NMR, all the carbons (32 signals) were in agreement with the chemical structure (Fig. S9). Figures S10-12 confirm the corresponding flavone diglycoside (2) with COSY, HSQC, and HMBC experiments. One-dimensional NMR and mass spectra of retusin observed to confirm the related compound (Figs. S13–15, respectively). The didymin was characterized with 1D NMR and mass spectra as seen in Figs. S16–18.

Verbascoside was extracted and isolated from leaves of *Sideritis germanicopolitana* using methanol and yellow crystals obtained with the chromatographic techniques. The molecular structure of verbascoside was characterized with HPLC-TOF/MS *m/z* $[M–H]^-$ 623.1928 and 1D and 2D-NMR spectra (Figs. S19–23). Two different ABX systems: 3,4-dihydroxy-*β*-phenylethoxy at δ 6.61 (brs), 6.63 (brd,

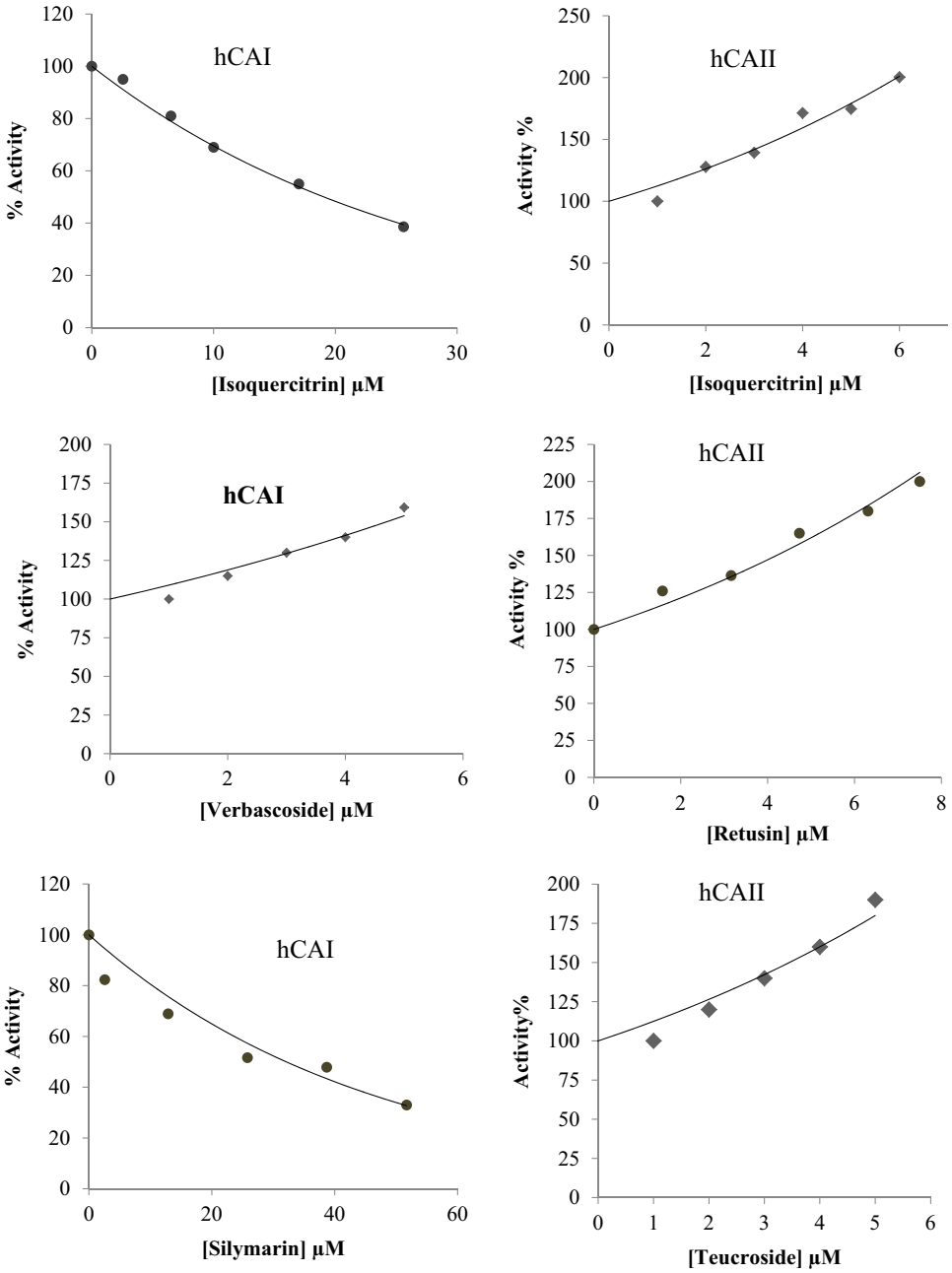

Fig. 3 % Activities—natural product [µM] charts samples

6.61) and 6.48 (d, $J = 8.0$ Hz); for cafeoil moiety 7.01 (brs), 6.74 (d, $J = 7.9$ Hz), and 6.95 (d, $J = 7.9$ Hz), two trans olefinic protons signals at δ 7.44 (d, $J = 15.8$ Hz) and δ 6.18 (d, $J = 15.8$ Hz) and neighbor anomeric protons for *β*-glycoside at δ 4.33 (d, $J = 7.7$ Hz) and for α-rhamnose at δ 5.01 (brs) were in agreement with the chemical structure. The confirmation of the linkages was determined using ^{13}C, COSY, HSQC, and HMBC spectra as 3,4-dihydroxy-*β*-phenylethoxyl moieties to C-1′′, cafeoil moiety to C-4′′, rhamnose moiety to C-3′′. HMBC spectrum exhibited H-1′′ (δ 4.33) and CH_2-α (δ 70.7) interaction with phenylethoxy at C-1′′, H-4′′ (δ 4.69) and carbonyl (δ 166.2) interaction with C-4′′, H-1′′′ (δ 5.01) and C-3′′ (δ 79.6) interaction with α-rhamnose at C-3′′ linked to glycoside moiety. The structure of 3,4-dihydroxy-*β*-phenylethoxy-*O*-(1 → 2)-α-ramnopiranosil-(1 → 3)-4-*O*-cafeoil-*β*-glucopyranoside (verbascoside).

The silymarin was confirmed with 1D and 2D-NMR spectra as given in Figs. S24–29. Teucroside was characterized using 1D and 2D-NMR spectra in Figs. S30–34 and HPLC-TOF/MS in Fig. 35.

Carbonic anhydrases are found in many tissues and involved in various metabolic processes, such as pH regulation, secretion of electrolytes, diuresis, and CO_2 transport

Table 2 Effects on the enzyme activity (hCA-I, II) of natural products (*E* effect, *I* inhibition, *A* activation, *NE* no effect)

Names of the compounds	No.	Source of the compounds	AC_{50}/IC_{50} (μM)			
			hCAI		hCAII	
			E	Values	E	Values
Isoquercitrin	1	*Platanus orientalis*	I	18.16	A	3.17
Retusin	2	*Mentha spicata*	NE		A	7.02
Didymin	3	*Mentha spicata*	NE		A	6.08
Silymarin	4	*Silybum marianum*	I	31.36	I	43.12
Verbascoside	5	*Teucrium chamaedrys* subsp. *chamaedrys*	A	13.00	A	18.53
Teucroside	6	*Teucrium chamaedrys* subsp. *chamaedrys*	I	40.58	A	2.98
3′-O-methylhypolaetin 7-O-[6′′′-O-acetyl-β-D-allopyranosyl-(1 → 2)]-6′′-O-acetyl-β- D-glucopyranoside	7	*Sideritis libanotica linearis*	I	172.5	NE	

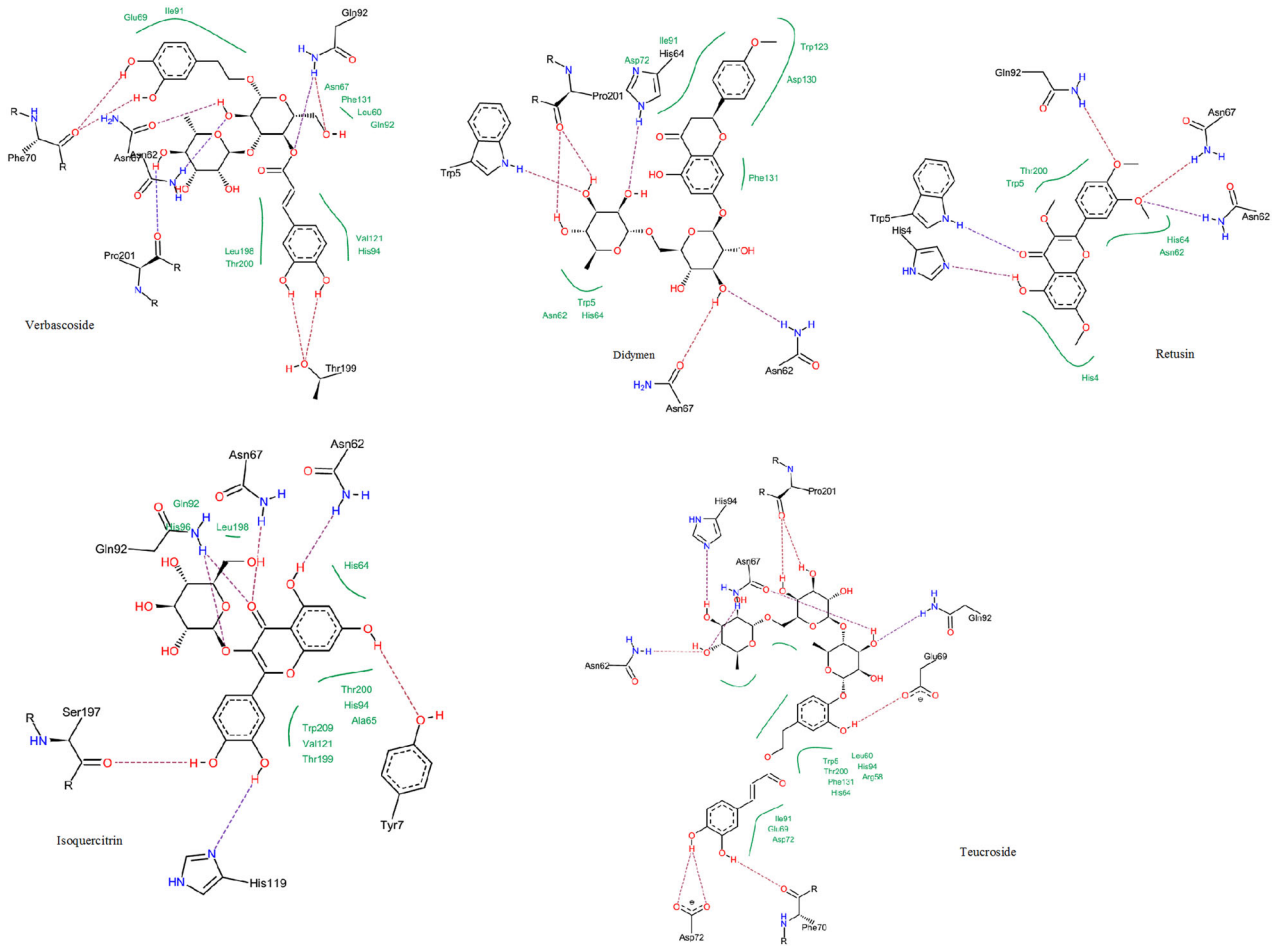

Fig. 4 Potential binding modes of compounds and hCA II

(Supuran and Scozzafava 2007; Gilmour 2010). Carbonic anhydrase enzymes have been chosen as a target enzyme for the treatment of various diseases due to varied tasks and presence in many tissues (Supuran 2010). This enzyme is currently used to treat the different diseases and considered as a target for the amelioration of obesity, cancer, osteoporosis, malaria, and neurodegenerative diseases (Supuran 2008; Alterio et al. 2012). Therefore, the development and

Table 3 Docking Results of the active compounds on CA II using Leadit software (results presented as kJ/mol; software license was granted from BioSolveIT GmbH, Germany)

Compounds	Binding energies	Hydrogen bond energy	The hydrophobic interactions	Van der walls interactions	Steric hindrance energy	The energy of rotatable bonds	The number of total bonds
Teucroside	−19.05	−43.70	−7.35	−11.69	7.5	30.80	16
Isoquercitrin	−18.01	−34.01	−9.39	−10.26	14.85	15.40	23
Didymen	−14.42	−27.98	−7.37	−7.49	3.42	19.60	17
Retusin	−17.78	−22.76	−4.79	−5.91	3.28	7.00	11
Verbascoside	−14.88	−31.40	−10.84	−10.77	7.53	25.20	14

Table 4 In silico ADME analysis

Properties	Rules	1	2	3	4	5	6	7
Molecular weight (g/mol)	<500	464.38	358.34	594.56	482.44	624.59	770.73	724.62
Num. rotatable bonds	<10	4	5	7	4	11	13	12
Num. H-bond acceptors	<10	12	7	14	10	15	19	19
Num. H-bond donors	<5	8	1	7	5	9	11	8
TPSA (Å^2)	20 < TPSA < 130	210.51	87.36	214.06	155.14	245.29	304.21	290.80
ABS (%)		36.37	78.86	35.14	55.47	24,37	4.04	8.67
miLogP	≤4.15	−0.36	3.11	0.17	1.47	−0.45	−1.24	−0.29
nviolations	≤1	2	0	3	0	3	3	3
BBB permeant		No	No	No	No	No	No	No
Log K_p (skin permeation) cm/s	(–9.7 < log kp < –3.5)	−8.88	−6.02	−10.40	−7.89	−10.46	−12.29	−10.81

determination of new and more selective CAIs inhibitors will be an exciting challenge for medicinal chemists, pharmacologists, and physiological studies (Gilmour 2010).

Natural products in search of new drugs have vital importance. They may be used directly as a therapeutic agent or to synthesize more active compounds (Mushtaq and Wani 2013). Many researchers have focused on the detection of the new or known compounds to be obtained from different sources. Phenolic compounds have higher activities because of their structure. They tend to interact effectively with the enzyme (Adem et al. 2016; Aslan and Adem 2015). Recently, some researchers have reported that carbonic anhydrase isoenzymes were affected by phenolic-based compounds (Davis et al. 2013; Öztürk Sarikaya et al. 2010; Senturk et al. 2011).

Mentha spicata has numerous chemically different races throughout the world, such as pulegone-rich and pulegone–piperitone-rich chemotypes of Turkish mints. The further is a new chemotype of *M. spicata* and other compounds were detected in all spearmints with variable proportions (Telci et al. 2010). Retusin and didymin isolated from *Mentha spicata*. It has not been found in the literature that retusin exhibited any effect on enzyme activities or bioactivity as a pure compound. It have been reported that didymin did not exhibited any toxic effect on normal tissues, to have anticancer potential, and to have orally bioavailable and hugely influential (Singhal et al. 2017; Hsu et al. 2016; Cirmi et al. 2016). The first report on the antiproliferative activity of *Sideritis libanotica* ssp. *linearis* against Vero, C6, and HeLa cells was exhibited using methanol extract of endemic herbal tea (Demirtas et al. 2009).

The methanol extract from *S. libanotica* ssp. *linearis* was also studied the antioxidant potential and was obtained the responsible flavone diglycoside from the plant (Demirtas et al. 2011). The findings of the literature were also comparable with this study and the use of *S. libanotica* as a food additive as well as traditional anti-ageing remedy. Based on the above discussion, the flavone diglycoside was tested to determine hCA-I, II in the present study. *Platanus orientalis* L., known as an oriental plane (family Platanaceae), is a deciduous tree native to south–west Asia, south–east Europe, and the Mediterranean regions and the leaves were used in folk medicine in ophthalmia, the bark was boiled in vinegar and given for diarrhea, dysentery, and toothache, while the buds were used as a urinary tract antiseptic and antimicrobial. Flavonoid glycoside, axillarin-3´-O-β-xylopyranoside, in addition to nine flavonoid aglycones and glycosides, phenolic acids were also isolated as anti-hepatotoxic, antioxidant and cytotoxic activities of the total ethanolic extract, total aqueous extract and the flavonoid-rich ethylacetate (Dalia Almahdy et al. 2008). The flavonoid isoquercitrin (quercetin-3-O-β-d-glucopyranoside)

commonly exist in medicinal herbs, fruits, vegetables, plant-derived foods, and beverages. This compound performs many beneficial effects such as antioxidant, anti-inflammatory activities, anticancer, anticarcinogenic, cardioprotective, antidiabetic, and anti-allergic (Valentová et al. 2014). *Teucrium chamaedrys* L. is used as a traditional medicine. The aerial parts of the plant material were extracted to give a new compound named as teuchamaedryn D as well as known compounds of teucrin A, dihydroteugin, syspirensin A, teucroxide. The chromatographic methods were also applied to isolate teucrioside and verbascoside (Elmastas et al. 2016). Verbascoside is one of main active compound of herba cistanche and to have highly bioavailable. It also has potential protective effects at conditions, such as diabetes, liver injuries, Alzheimer's disease and obesity (Cui et al. 2018; Wen et al. 2016). *Silybum marianum* is a herbaceous plant and has been used in medicine for 2000 years to treat gallbladder and liver disorders. *S. marianum* seed contains flavonolignans isomers collectively known as silymarin (Afshar et al. 2015). Silymarin has a wide range of biological and pharmacological influences, including antioxidant activity, antidiabetic activity, cardioprotection, etc. 20–50% of oral silymarin absorbed quickly at 2–4 h in the gastrointestinal tract (Javed et al. 2011).

In this study, the effects of seven natural compounds isolated from the different plants on hCAI and hCAII activities have evaluated under in vitro conditions (Table 2). According to the results, compounds studied were observed to have different effects on the hydratase activity of hCAI and hCAII. Compounds 1, 4, 6, and 7 exhibited inhibitors against hCAI with IC_{50} values in the range 18.16–172.5 µM; whereas, compound 6 with 2.98 µM IC_{50} value acted as an inhibitor of the ubiquitous and dominant rapid cytosolic isozyme hCAII. The previously published study has reported that silymarin inhibited the esterase activities in the range of 1–3 µM of hCAI and hCAII (Senturk et al. 2011). Compared with the results, the hydratase activities of these enzymes less affected by silymarin. Natural compound 2 against hCAI showed inhibitory effect, but against hCAII did not exhibit any effect. Therefore, this compound may be considered as a specific inhibitor for hCAI.

While many researchers widely been studied inhibitors of the enzyme carbonic anhydrase, there is a few research related to their activators. Biochemical and pharmacological data indicate that in some disease causes a decrease in the expression of carbonic anhydrase isoenzymes (Sly and Hu 1995). Reviews on the role of CAAs have been reported that their activation may be used a novel approach to treating disorders, such as Alzheimer's disease and aging (Sun and Alkon 2002; Supuran 2008; Scozzafava et al. 2006). Therefore, enzyme activators are essential as inhibitors from medical aspects. In this study, verbascoside exhibited activation effect on both enzymes at low concentrations in the range of ~13.00–18.53 µM. Isoquercitrin, retusin, didymin, and teucroside have enhanced hCAII activity AC_{50} values under 10 µM. Especially, retusin and didymin only showed activation effect on the hCAII, but they did not exhibit activation or inhibition against hCAI. Therefore, they may be considered as potential hCAII activators.

To figure out the most energetically proper binding position between the activator compounds and hCAII, molecular docking studies were carried out with LeadIT 2.3.2 software. There is generally a correlation between in vitro and in silico results. According to the docking conclusions, teucroside has the best inhibitory potential for hCAII. It was observed that hydrogen bonds have important roles on activation of carbonic anhydrase II. At the interaction of molecules with the active site of the enzyme, amine groups on the side chain of the Asn67, Asn69, and Gln92 are a key player as a hydrogen donor in the formation of hydrogen bonds. Ser197, Pro201, Tyr7 amino acids in the active pocket of the enzyme served as a hydrogen acceptor.

Teucroside interacts with the active site of hCA II five hydrogen bonds between the hydroxyl of the glycoside groups of it's with Gln92, Asn62, Asn62, Pro201, and His94. In another pose, it displayed two hydrogen bonds in the interaction of hydroxyl group bonded to the C4 and 3′ carbon with the carboxyl groups Glu69 and Asp72. Hydrophobic interactions observed between between 7′C and 8′C carbons protons, and alpha and beta protons of position 7 C and 8 C in the teucrol skeleton with amino acids such as Trp5, Thr200, Phe131, His64, His94, Arg58, Leu60, Ile91, Glu69, and Asp72.

The presence of OCH_3 groups in the benzene ring B was essential to form a hydrogen bond with Asn67, Asn62, and Gln92. Oxygen atom at position C4 and unconjugated electron pairs of the oxygen atom in the OH group at the C5 position in the retusin interact indole ring of Trp5 and imidazole side chain of His4 to form two hydrogen bonds.

According to in silico, the hydroxyl of the glycoside groups and the presence of OH groups in the benzene ring play an important role to form a hydrogen bond as H-bond donors. H-bond donors and acceptor groups on the A and B rings of the flavone significantly contribute to the interaction of the enzyme and molecule. When the interaction of verbascoside and teucrosidene with enzyme is examined, it have been seen that branched glycoside structures weaken the enzyme–molecule interaction.

Assessment of in silico ADME is a credible technique to approve the potential of a drug candidate (Van De Waterbeemd and Gifford 2003. Compounds 5, 6, and 7 have greater values than normal parameters in terms of properties, such as molecular weight (MW), Log K_p, topological polar surface are (tPSA), number of hydrogen donors

(nON), and acceptors (nOHNH). According to the ADME data, retusin demonstrated physicochemical and pharmacokinetic properties listed Table 4. specific to the drug candidates.

Conclusion

We have reported the effects of some compounds isolated from the different plants on the human erythrocyte carbonic anhydrase I and II isoform. Both activators and inhibitors of carbonic anhydrases are essential from biochemical, physiological, and pharmacological perspectives. When evaluated in vitro, docking and ADME results, it can be suggested that retusin may be a good lead molecule in activator synthesis for CA II.

Compliance with ethical standards

Conflict of interest This research was financed Cankiri Karatekin University (Project No: BAP 2012-13).

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