SUPPLEMENTARY MATERIAL

Comparison of antioxidant, anticholinesterase, and antidiabetic activities of three curcuminoids isolated from *Curcuma longa* L.

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Abstract: Bioactive ingredients isolated from medicinal plants are potent drugs. Lately, one of the turmeric ingredients, namely curcumin, has taken great attention due to its several potent bioactivities. However, turmeric contains two curcuminoids beside curcumin, namely demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). In the present study, antioxidant, anticholinesterase, and antidiabetic activities of the three curcuminoids isolated from the *Curcuma longa* were simultaneously tested and compared. The highest antioxidant power was detected for curcumin with the applied methods. The significant anticholinesterase and antidiabetic activities of BDMC compared to its isomers and examination of chemical structures of isomers might be a starting point in designing new drugs for Alzheimer's and Diabetes Mellitus.

Keywords: Turmeric; Curcumin; Demethoxycurcumin; Bisdemethoxycurcumin; Bioactivity; Acetylcholinesterase

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1. Experimental

1.1. Chemicals and spectral measurements

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide (AcI) and butyrylthiocholine chloride (BuCl), galantamine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), *p*-nitrophenyl-*α*-D-glucopyranoside (*p*-NPG), genistein, 2,4,6-tripyridyl-s-triazine (TPTZ) and $FeCl_3.6H_2O$ were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). Acetic acid, methanol, dimethyl sulfoxide (DMSO), sodium acetate trihydrate, hydrochloric acid, ascorbic acid and $FeSO_4.\ 7H_2O$ were purchased from Merck (Darmstadt, Germany).

NMR analyses were performed on an Agilent VNMRS 500 MHz NMR spectrometer (Waldbronn, Germany).

Bioactivity measurements were carried out on a 96-well microplate reader, BioTek Power Wave XS (USA). The measurements and calculations of the activity results were evaluated by using Gen5 Data Analysis software.

1.2. Isolation and identification of curcuminoids

Turmeric rhizomes were purchased from a local market in Turkey. All curcuminoids were isolated as described in our recent work (Kalaycıoğlu et al. 2015).

The chemical structures of curcuminoids are given in Figure S1. ^{1}H and ^{13}C NMR spectral data of the isolated curcuminoids are given in Table S1 and Table S2, respectively.

1.3. DPPH free radical scavenging assay

The free radical-scavenging activity of each curcuminoid was determined by the DPPH assay described by Blois (1958) with slight modification. In its radical form, the absorption wavelength of DPPH is at 517 nm. Under reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 90 μL methanolic 0.1 mM DPPH solution was added to 10 μL sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Inhibition of free radical DPPH in percent (I %) was calculated by using the following equation where A_{sample} is the absorbance of the compounds/references and $A_{control}$ is the absorbance of the control.

Percentage inhibition (I %) = $[(A_{control}-A_{sample})/A_{control}] \times 100$ (1)

The concentration of each curcuminoid causing 50% inhibition (IC_{50}) of DPPH radical was estimated using standard calibration curve. The results were compared with that of the reference compound BHA (butylated hydroxyanisole, a synthetic antioxidant).

1.4. FRAP assay

The ferric-reducing antioxidant power (FRAP) of each curcuminoid was determined, following the method of Benzie & Strain (1996). The FRAP reagent was prepared containing 1:1:10 ratio of 10 mM 2,4,6-tripyridyl-s-tri-azine (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer at pH 3.6, and incubated at 37 °C for 10 min before use. 200 µL of each curcuminoid was allowed to react with 1.8 mL of the FRAP reagent for 10 min at 37 °C in the dark condition. The absorbance of the coloured product (ferrous tripyridyltriazine complex) was measured by a Shimadzu UV-1800 spectrophotometer at 593 nm. Results were expressed as µM Fe(II)/g analyte and compared with that of ascorbic acid. The calibration curve of $FeSO_4.7H_2O$ was constructed in the range of 6.25-40.0 µM with the following equation:

$y=0.0216x-0.0091$, $R^2=0.999$

1.5. Anticholinesterase activity

Acetyl- and butyryl-cholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method developed by Ellman et al. (1961). Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and DTNB were used for the measurement of the anticholinesterase activity. Each curcuminoid was dissolved in methanol to prepare their stock solutions at 4000 µg/mL concentration. One hundred-fifty microliter of 100 mM sodium phosphate buffer (pH 8.0), 10 µg/mL of sample solution and 20 µL AChE (or BChE) enzyme solution were mixed and incubated for 15 min at 25 °C, and then 10 µg/mL of DTNB is added. The reaction was then initiated by the addition of 10 µg/mL acetylthiocholine iodide (or butyrylthiocholine iodide). Final concentrations of the curcuminoids in solutions were 0.04, 7.5, 15 and 30 µg/mL. The hydrolysis of these substrates was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine iodide, at a wavelength of 412 nm. Methanol was used as a solvent to dissolve the samples and controls. Galantamine was used as standard.

1.6. α-Glucosidase inhibitory activity

The isolated curcuminoids were assessed for the inhibition of *α*-glucosidase from *Saccharomyces cerevisiae* according to the slightly modified method of Tsujii et al. (1996). Briefly, a 40 μL solution of *α*-glucosidase (3.0 U/mL, dissolved in phosphate buffer, pH 6.8) was pre-incubated in the presence of 10 μL of each curcuminoid in DMSO at 37 °C for 30 min. The enzymatic reaction was initiated by the addition of 100 μL of *p*-nitrophenyl-*α*-*D*-glucopyranoside (*p*-NPG, final concentration 0.5 mM), and then the mixture was incubated for another 30 min. The *α*-glucosidase activity was determined by monitoring the *p*-nitrophenol released from *p*-NPG at 405 nm. Genistein was used as the positive control.

1.7. Statistical analysis

All data, for antioxidant, anticholinesterase, and antidiabetic activity tests, are the average of triplicate analyses. The data were reported as mean ± standard deviation (SD). The results were analyzed using the IBM SPSS 23.0 (SPSS Inc., Chicago, IL, USA) statistical software program for windows. To compare the significant differences of the mean values at $p < 0.05$, one way analysis (ANOVA) was applied to the result.

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Table S1. ^{1}H NMR spectral data of the isolated curcuminoids.

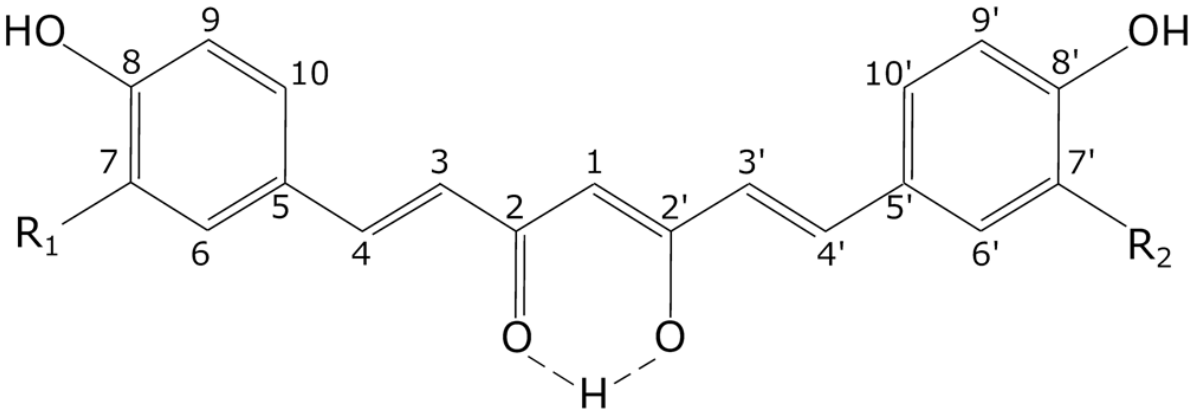
^{1}H	Curcumin	DMC	BDMC
1	6.04 (2H, s)	6.02 (2H, s)	5.99 (2H, s)
2,2'	-	-	-
3,3'	6.75 (1H, d J=15.7 Hz)	6.68 (2H, d J=15.8 Hz)	6.69 (1H, d J=15.7 Hz)
4,4'	7.54 (1H, d J=15.7 Hz)	7.55 (2H, d J=15.8 Hz)	7.55 (1H, m)
5,5'	-	-	-
6,6'	7.30 (1H br. s)	7.32 (1H br. s)	7.55 (1H, m)
7,7'	-	7.53 (1H, d J=8 Hz)	7.55 (1H, m)
8,8'-OH	-	-	-
9,9'	6.82 (1H, d J=7.8 Hz)	6.81 (1H, d J=7.8 Hz)	6.81 (1H, d J=7.8 Hz)
10,10'	7.14 (1H, dd, J=7.8 and 1.8 Hz)	7.14 (1H, dd, J=7.8 and 1.8 Hz)	7.55 (1H, m)
O-CH_3	3.82 (s, 6H)	3.82 (s, 3H)	-

s:singlets; d:doublets; br. s: broad singlet; m: multiple
Conditions of NMR: ^{1}H 500 MHz, DMSO-d_6

Table S2. ^{13}C NMR spectral data of the isolated curcuminoids.

^{13}C	Curcumin	DMC	BDMC
1	101.0	101.0	101.0
2,2'	188.6/188.0	188.6/188.0	188.6/188.0
3,3'	123.4/119.6	123.4/119.6	123.4/119.6
4,4'	152.7/131.2	152.7/131.2	152.7/131.2
5,5'	120.1	127.8/120.1	127.8
6,6'	116.8	115.8/116.8	115.8
7,7'	144.9	157.7/144.9	157.7
8,8'	151.3	115.8/151.3	115.8
9,9'	112.0	127.8/112.0	127.8
10,10'	128.8	127.8/128.8	127.8
O-CH_3	56.2/56.2	56.2	-

Conditions of NMR: ^{13}C 500 MHz, DMSO-d_6



Compound	R_1	R_2
Curcumin	$-OCH_3$	$-OCH_3$
Demethoxycurcumin	H	$-OCH_3$
Bisdemethoxycurcumin	H	H

Figure S1. The chemical structures of three main curcuminoids.