Nematocidal Activity of Turmeric: Synergistic Action of Curcuminoids[1)]

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A new curcuminoid, cyclocurcumin (IV), was isolated from the nematocidally active fraction of turmeric, the rhizome of *Curcuma longa*, together with three known curcuminoids, curcumin (I), demethoxycurcumin (II) and bisdemethoxycurcumin (III). The structure of IV was elucidated on the basis of spectral data and confirmed by the partial synthesis from curcumin (I). Although the above curcuminoids were ineffective when they were applied independently, the nematocidal activity increased remarkably when they were mixed, suggesting a synergistic action between them.

Keywords turmeric; synergistic effect; cyclocurcumin; nematocidal activity; curcuminoid; *Curcuma longa*

Turmeric, the rhizome of *Curcuma longa* L., is one of the most popular spices and is used as a yellow coloring in foods. In a previous study on the screening of spices for nematocidal activity against the second-stage larvae of dog roundworm, *Toxocara canis*, we observed an appreciable amount of this activity in the water and methanol extracts of turmeric.[2)] In this paper, we report the isolation of a new curcuminoid, cyclocurcumin, and that the nematocidal activity of turmeric is attributable to a synergistic action of curcuminoids.

Results and Discussion

Nematocidal Principles of Turmeric The nematocidal activity of turmeric was concentrated in the chloroform and methanol extracts when the rhizome was successively extracted with hexane, chloroform, methanol, and water (Table I), indicating that the nematocidal constituents are soluble in organic solvents. Turmeric (300 g) was therefore successively extracted as shown in Chart 1. The thin-layer chromatogram (Fig. 1) of the most active fractions ($CHCl_3$ extract supernatant and MeOH extract supernatant) showed three yellow spots, the largest of which was due to curcumin (I). To isolate them, we partitioned the methanol extract between ethyl acetate and water (Chart 1), and chromatographed the organic extract on silica gel to obtain three fractions (fr. 2, fr. 3 and fr. 4), each of which contained one of the above three yellow spots as a major component (Chart 2). Preparative thin-layer chromatography (PTLC) and/or crystallization of these fractions gave three known curcuminoids, curcumin (I), demethoxycurcumin (II), and bisdemethoxycurcumin (III).[3)]

These compounds, however, did not show nematocidal activity, whereas mother liquor II, mother liquor III, and a side fraction (fr. 3-3) were considerably active (Table II), implying the presence of an active principle between compounds II and III, though the amount may be very small. Therefore, a fraction corresponding to fr. 3-3 (165 mg) was newly prepared from the methanol extract and

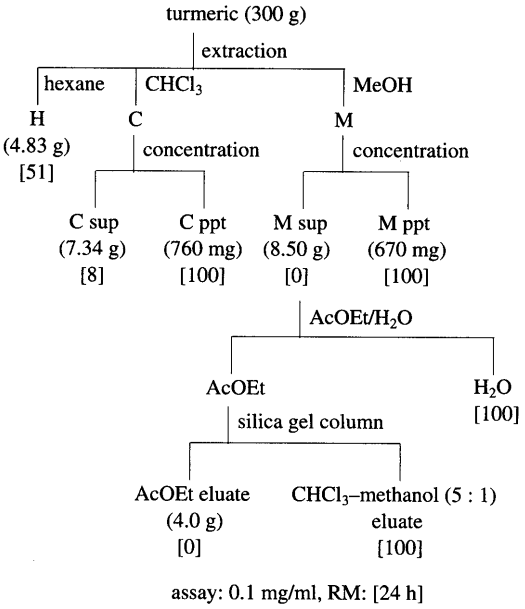

Chart 1. Extraction and Fractionation of Turmeric

TABLE I. Preliminary Test of Nematocidal Activity of Turmeric Extract on the Second-Stage Larvae of *T. canis*

Extract[a)]	Yield (%)	RM value[b)] (0.1 mg/ml)		
		3 h	6 h	24 h
Hexane	3.2	100	100	96
Chloroform	3.0	100	100	34
Methanol	3.1	100	84	0
Water	7.2	100	100	100

a) Extraction was done in the order given (from the top). *b*) Relative mobility value: this value indicates relative mobility of the larvae. When a sample has no effect on the larvae, *i.e.* the larvae move normally, this value is 100. When all larvae die, this value becomes 0 (see ref. 4).

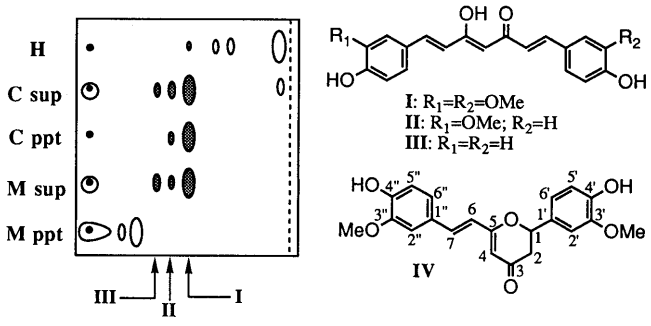

Fig. 1. TLC of the Extracts

Solvent; $CHCl_3$: acetone = 8 : 1. ◉, UV positive; ○, UV negative.

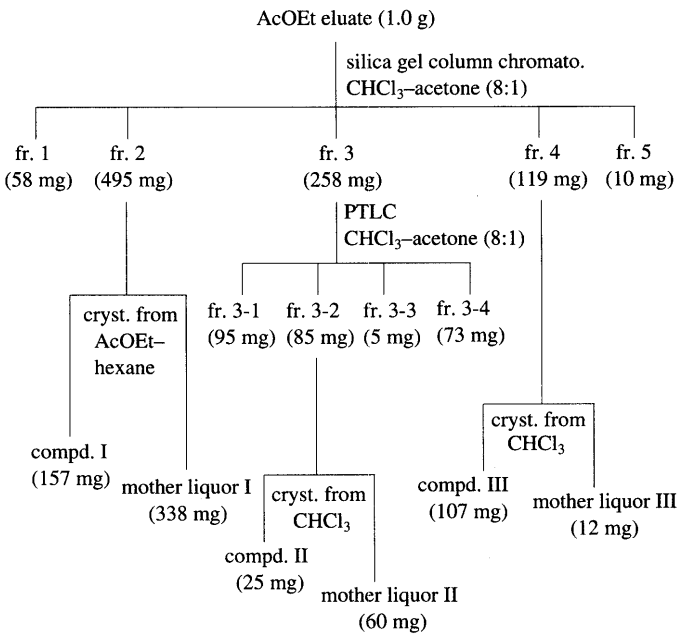

Chart 2. Fractionation of the MeOH Extract

TABLE II. Nematocidal Activity of Curcuminoids, Singly and in Combinations, and Related Fractions

Sample	RM value (0.1 mg/ml)		
	3 h	6 h	24 h
Curcumin (I)	100	100	100
Demethoxycurcumin (II)	100	100	100
Bisdemethoxycurcumin (III)	100	100	100
Cyclocurcumin (IV)	100	100	100
I+II (1:1)	100	93	33
I+III (1:1)	77	67	23
I+IV (1:1)	100	98	98
II+III (1:1)	67	15	0
II+IV (1:1)	99	96	42
III+IV (1:1)	99	96	96
I+II+III (1:1:1)	51	9	0
I+II+IV (1:1:1)	100	98	70
I+III+IV (1:1:1)	87	72	45
II+III+IV (1:1:1)	51	11	0
I+II+III+IV (1:1:1:1)	67	10	0
Mother liquor I	100	100	100
Mother liquor II	83	79	15
Mother liquor III	61	40	0
Fr. 3-1	93	88	58
Fr. 3-3	74	67	0
Fr. 3-4	100	100	88

separated by PTLC ($CHCl_3$: acetone = 10 : 1) with a multiple developing method. A new spot thus obtained was faintly yellow and had strong UV absorption at 373 nm. This spot was purified by recycling HPLC on an octadecyl silica gel (ODS) column to give a new curcuminoid (IV, 4 mg) as a yellow gum, which we called cyclocurcumin. The structure of this compound was determined as shown in Fig. 1 (see below); however, it did not show the expected nematocidal activity at a concentration of 0.1 mg/ml.

Synergistic Action of Curcuminoids in the Nematocidal Activity As indicated above, all curcuminoids isolated from turmeric were ineffective to the larvae of *Toxocara canis* when applied independently. They were inactive even when they were emulsified with the aid of an emulsifier at 1 mg/ml concentration, and were also inactive in alkaline buffer solutions (pH 8—10). When they were mixed, however, a strong nematocidal activity appeared (Table II). The increase in activity was particularly remarkable for combinations which contained demethoxycurcumin (II) and bisdemethoxycurcumin (III). This fact explains the strong activity of the mother liquors II and III. This enhancement in the activity may be attributed to an increase of solubility of the curcuminoids and/or a synergistic action between them. Therefore, the solubility change of the curcuminoids by mixing was examined.

TABLE III. Solubility of Curcuminoids in 2% DMSO–Saline[a)]

Sample	Solubility (μg/ml)			
	I	II	III	Total
I	0.9	—	—	0.9
II	—	1.7	—	1.7
III	—	—	0.4	0.4
I+II	0.4	1.0	—	1.4
I+III	0.5	—	1.8	2.3
II+III	—	1.1	1.8	2.9
I+II+III	0.3	0.8	1.5	2.6

a) Compounds I—III and their equal amount mixtures were dissolved in DMSO and the solution was diluted with saline to make 2% DMSO solution of the curcuminoid(s) at a total concentration of 100 μg/ml. After 30 min, the precipitates formed were removed and the amount of the curcuminoid(s) in the solution was determined by HPLC.

The curcuminoids were practically insoluble in saline and they crystallized out from the assay solution. When they were mixed in an equal amount in different combinations, the solubility of each component was changed. As shown in Table III, the solubility of III increased by *ca.* 4 fold, whereas the concentrations of I and II were decreased. As a result, total concentration of the curcuminoids in the mixture solution increased when it contained bisdemethoxycurcumin (III). However, the total concentration was still too low to explain the above remarkable increase of the activity. In fact, when the precipitates were removed by filtration through an 0.45 μm filter from the assay mixture of I+II+III (which showed RM = 0), the filtrate did not show any activity (RM = 100), suggesting that the insoluble fraction plays an important role in the nematocidal activity.

We therefore conclude that the strong nematocidal activity of turmeric is attributable to a synergistic action of the curcuminoids. The combination of II and III is particularly important, because the addition of I or IV to this combination did not produce further significant increase of the activity, and the other combinations of two of the constituents showed weaker activity. The mechanism of this synergistic action, however, is still to be clarified.

The present example shows that the synergistic action of plural constituents is important for an understanding of the biological effect of crude drugs such as traditional medicines and spices.

Structure of Cyclocurcumin (IV) Cyclocurcumin (IV) has the same molecular formula ($C_{21}H_{20}O_6$) as that of curcumin (I). The ^{1}H-NMR spectrum showed two sets of 1,3,4-substituted benzene ring signals [δ 6.90 (d, J = 8.3 Hz), 7.04 (dd, J = 8.3, 2.0 Hz), 7.22 (d, J = 2.0 Hz), and 6.84 (d, J = 8.3 Hz), 7.11 (dd, J = 8.3, 2.0 Hz), 7.28 (d, J = 2.0 Hz)],

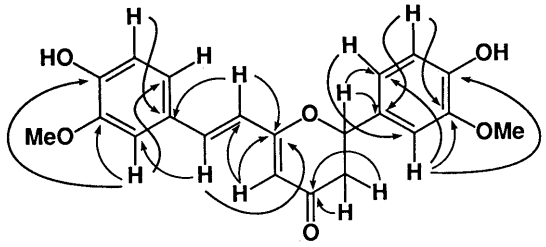

Fig. 2. Observed Long-Range C–H Correlation ($J=10$ Hz) of Cyclocurcumin (IV)

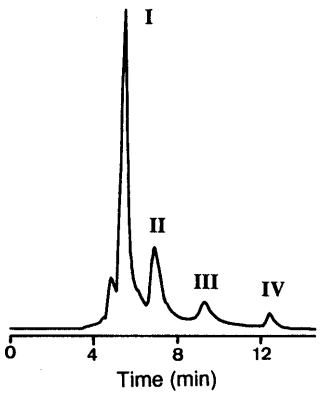

Fig. 3. HPLC Trace of the $CHCl_3$ Extract

HPLC conditions: column, TSKgel Silica-60 (4.6 × 250 mm); solvent, $CHCl_3$: AcOEt: AcOH = 90 : 10 : 2; flow rate, 1 ml/min; detection, UV 370 nm.

showing that they are not equivalent. In addition to a pair of *trans*-double bond protons [δ 6.74 and 7.31 (each 1H, d, $J=16.1$ Hz)], the ^{1}H-NMR spectrum also showed a set of ABX type signals [δ 2.52 (1H, ddd, $J=16.6$, 3.4, 1.0 Hz), 2.93 (1H, dd, $J=16.6$, 13.7 Hz), 5.46 (1H, dd, $J=13.7$, 3.4 Hz)] and an isolated olefin signal [δ 5.50 (br s)]. From these partial structures and its UV, ^{13}C-NMR, C–H COSY, C–H COLOC (Fig. 2) spectra, we concluded that this compound (IV) is the product formed from curcumin (I) by an intramolecular Michael addition of the enol oxygen to the enone group.

The structure (IV) was confirmed by acid catalyzed cyclization of curcumin (I) to cyclocurcumin. For this purpose, trifluoroacetic acid was the most effective. On treatment with trifluoroacetic acid at room temperature, curcumin (I) gave cyclocurcumin (IV) in a moderate yield.

Since cyclocurcumin (IV) isolated from turmeric was racemic, we first supposed that it is an artefact formed from curcumin (I) during an isolation process such as silica gel chromatography. However, curcumin (I) was recovered unchanged after contact with silica gel for several days, and HPLC analysis (Fig. 3) revealed that cyclocurcumin (IV) exists in the chloroform extract from the beginning. Actually, it was isolated from the chloroform extract in 0.8% yield (see Experimental).

Experimental

General Melting points were taken on a Yanagimoto micro hot-stage melting point apparatus and were uncorrected. IR spectra were taken in chloroform solution on a Shimadzu IR-460 spectrometer and data (ν_{max}) are given in cm^{-1}. NMR spectra were measured in acetone-d_6 solution with tetramethylsilane as an internal standard on a JEOL FX-100 (100 MHz for ^{1}H), GX-400 (100 MHz for ^{13}C) or GSX-500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) spectrometer and chemical shifts are given in δ values. Mass spectra (MS) and high resolution mass spectra (HRMS) were taken on a Hitachi M-80 spectrometer and data are given by m/z (%). UV spectra were measured on a Hitachi 323 spectrophotometer in an EtOH solution. Circular dichroism (CD) was measured on a JASCO J-500C spectropolarimeter. Wakogel C-200 (silica gel) was used for column chromatography. For flash column chromatography, Silica gel 60 (Merck 9385, 230—400 mesh) was used. PTLC was run on a precoated Silica gel 60 F_{254} plate (Merck 13792, 200 × 200 × 1 mm) with a concentration zone. Recycling HPLC was performed on an Inertsil Prep-ODS column (20 × 250 mm) using a JAI LC-908 HPLC system (Japan Analytical Industry Co.) equipped with a UV detector (JAI 310). Turmeric was the generous gift of S&B Foods Co.

Assay Method Nematocidal activity was determined and presented in an RM value according to the method previously described.[4] The test sample was dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.75% saline to an appropriate concentration to keep the DMSO concentration at 2%.

Isolation of the Curcuminoids Powdered turmeric (300 g) was successively extracted with hexane, $CHCl_3$, and MeOH (each 600 ml) under reflux. The precipitates formed on cooling the chloroform and methanol extracts were separated and each extract was concentrated to dryness. The yields of these fractions were as follows: hexane extract (H, 4.83 g), $CHCl_3$ extract precipitate (C ppt, 0.76 g), $CHCl_3$ extract supernatant (C sup, 7.34 g), MeOH extract precipitate (M ppt, 0.67 g), MeOH extract supernatant (M sup, 8.50 g). M sup (8.50 g) was partitioned between AcOEt and water and the AcOEt extract was roughly fractionated by silica gel column chromatography to give AcOEt eluate (4.0 g). The AcOEt eluate (1.0 g) was fractionated on silica gel with $CHCl_3$: acetone = 8 : 1 to give five fractions: fr. 1 (58 mg), fr. 2 (495 mg), fr. 3 (258 mg), fr. 4 (119 mg), fr. 5 (10 mg). Crystallization of fr. 2 from AcOEt–hexane gave curcumin (I, 157 mg) and the mother liquor I (338 mg). Fraction 3 was fractionated by PTLC ($CHCl_3$: acetone = 8 : 1) to give four fractions: fr. 3-1 (95 mg), fr. 3-2 (85 mg), fr. 3-3 (5 mg), fr. 3-4 (73 mg). Crystallization of fr. 3-2 from $CHCl_3$ afforded demethoxycurcumin (II, 25 mg) and the mother liquor II (60 mg). Fraction 4 gave bisdemethoxycurcumin (III, 107 mg) and the mother liquor III (12 mg) on crystallization from $CHCl_3$.

Curcumin (I): Yellow fine needles ($CHCl_3$), mp 186—188 °C (lit. 183 °C).[3] ^{1}H-NMR (100 MHz): 8.16 (2H, br s), 7.61 (2H, d, $J=16$ Hz), 7.32 (2H, d, $J=2$ Hz), 7.19 (2H, dd, $J=8$, 2 Hz), 6.88 (2H, d, $J=8$ Hz), 6.69 (2H, d, $J=16$ Hz), 5.97 (1H, s), 3.92 (6H, s). ^{13}C-NMR (100 MHz): 184.5, 150.0, 148.8, 141.4, 128.2, 123.8, 122.3, 116.2, 111.6, 101.6, 56.1.

Demethoxycurcumin (*p*-Hydroxycinnamoylferuloylmethane, II): Orange crystalline powder ($CHCl_3$), mp 175—177 °C (lit. 181—182 °C).[3] ^{1}H-NMR (100 MHz): 8.88 and 8.12 (each 1H, br s), 7.60 (2H, d, $J=16$ Hz), 7.56 (2H, d, $J=8$ Hz), 7.34 (1H, d, $J=1.7$ Hz), 7.27 (1H, dd, $J=8$, 1.7 Hz), 6.90 (2H, d, $J=8$ Hz), 6.88 (1H, d, $J=8$ Hz), 6.69 (1H, d, $J=16$ Hz), 6.64 (1H, d, $J=16$ Hz), 5.97 (1H, s), 3.92 (3H, s). ^{13}C-NMR (100 MHz): 184.5, 184.4, 160.5, 150.0, 148.8, 141.4, 141.0, 130.9, 128.2, 127.7, 123.8, 122.3, 122.1, 116.8, 116.2, 111.5, 101.6, 56.3.

Bisdemethoxycurcumin (*p,p'*-Dihydroxydicinnamoylmethane, III): Dark yellow crystalline powder ($CHCl_3$), mp 230—232 °C (lit. 232—234 °C).[3] ^{1}H-NMR (100 MHz): 8.88 (2H, br s), 7.61 (2H, d, $J=16$ Hz), 7.57 (4H, d, $J=8$ Hz), 6.91 (4H, d, $J=8$ Hz), 6.66 (2H, d, $J=16$ Hz), 5.98 (1H, s). ^{13}C-NMR (125 MHz): 184.5, 160.4, 141.0, 130.9, 127.7, 122.0, 116.7, 101.7.

Isolation of Cyclocurcumin (IV) 1) The AcOEt eluate (100 mg) of the MeOH extract (M sup) was fractionated by PTLC ($CHCl_3$: acetone = 10 : 1, multiple development) to give five fractions. The yields and the RM values after 24 h at 0.1 mg/ml were as follows: fr. 1 (29 mg, 100), fr. 2 (9 mg, 50), fr. 3 (4 mg, 62), fr. 4 (corresponding to fr. 3-3 in Chart 2, 6 mg, 0), fr. 5 (2 mg, 9). The fraction corresponding to fr. 4 (165 mg) was newly prepared from the AcOEt eluate and purified by PTLC ($CHCl_3$: acetone = 10 : 1, multiple development) and recycling HPLC (60% EtOH, 4 cycles) to give cyclocurcumin (IV, 4 mg).

2) The $CHCl_3$ extract (C sup, 2 g) was crystallized from AcOEt–$CHCl_3$ to give crude curcumin (I, 457 mg). The mother liquor was fractionated by flash column chromatography (35 × 140 mm) to give three fractions: fr. 1 ($CHCl_3$: AcOEt = 3 : 1, 600 ml, 649 mg), fr. 2 ($CHCl_3$: AcOEt = 2 : 1, 150 ml, 82 mg), fr. 3 ($CHCl_3$: AcOEt = 2 : 1, 150 ml, 159 mg), fr. 4 (acetone, 200 ml, 355 mg). Fraction 2 was purified by PTLC with AcOEt and then with $CHCl_3$: AcOEt : AcOH = 90 : 10 : 2 to give IV (16 mg).

Cyclocurcumin (IV): Yellow gum. MS: 368 (M^+, 17), 191 (20), 190 (41), 177 (45), 150 (100), 137 (24), 135 (33), 89 (20), 77 (21). HRMS: 368.1222 (M^+, Calcd for $C_{21}H_{20}O_6$: 368.1258). IR: 1644, 1617, 1601, 1559, 1510. ^{1}H-NMR (500 MHz): 2.52 (1H, ddd, $J=16.6$, 3.4, 1.0 Hz, H-2), 2.93 (1H, dd, $J=16.6$, 13.7 Hz, H-2), 3.88 (3H, s, 3''-OMe), 3.91 (3H, s, 3'-OMe),

5.46 (1H, dd, $J=13.7$, 3.4 Hz, H-1), 5.50 (1H, br s, H-4), 6.74 (1H, d, $J=16.1$ Hz, H-6), 6.84 (1H, d, $J=8.3$ Hz, H-5″), 6.90 (1H, d, $J=8.3$ Hz, H-5′), 7.04 (1H, dd, $J=8.3$, 2.0 Hz, H-6′), 7.11 (1H, dd, $J=8.3$, 2.0 Hz, H-6″), 7.22 (1H, d, $J=2.0$ Hz, H-2′), 7.28 (1H, d, $J=2.0$ Hz, H-2″), 7.31 (1H, d, $J=16.1$ Hz, H-7), 7.75 and 8.02 (each 1H, br s, OH). ^{13}C-NMR (125 MHz): 43.8 (t, C-2), 56.35 and 56.39 (each q, OMe), 81.7 (d, C-1), 105.9 (d, C-4), 111.3 × 2 (d, C-2′ and C-2″), 115.8 (d, C-5′), 116.2 (d, C-5″), 119.9 (d, C-6), 120.6 (d, C-6′), 123.0 (d, C-6″), 128.6 (s, C-1″), 131.4 (s, C-1′), 137.7 (d, C-7), 148.0 (s, C-4′), 148.5 (s, C-3′), 148.8 (s, C-3″), 149.4 (s, C-4″), 169.2 (s, C-5), 192.3 (s, C-3). UV λ_{max} (log ε) nm: 233 (4.40), 262 (4.28), 373 (4.89). CD (MeOH): no peak between 220 and 450 nm.

Conversion of Curcumin (I) to Cyclocurcumin (IV) A mixture of curcumin (I, 73 mg) and trifluoroacetic acid (0.6 ml) in dry benzene (20 ml) was stirred at room temperature in the dark for 65 h. The mixture was concentrated and the residue was fractionated by PTLC ($CHCl_3$: AcOEt : AcOH = 90 : 10 : 2, multiple development) to give IV (15 mg, 21%), with recovery of unchanged I (36 mg, 49%).

HPLC Analysis Analytical HPLC was performed on a Toso CCPM pump system and the column temperature was controlled by a Toso CO-8000 column oven. Compounds were detected by a UV detector (Toso UV-8000) at 415 nm for curcuminoids and at 370 nm for cyclocurcumin, and chromatograms were processed by a Shimadzu Chromatopack C-R4A. The analyses of the crude extracts were carried out on a TSK gel Silica-60 column (4.6 × 250 mm, Toso) with $CHCl_3$: AcOEt : AcOH = 90 : 10 : 2 as a solvent at a flow rate of 1 ml/min. Retention time (min): curcumin (I), 5.3; demethoxycurcumin (II), 6.9; bisdemethoxycurcumin (III), 9.2; cyclocurcumin (IV), 12.4.

Quantitative analyses of curcuminoids were performed on a TSK gel NH_2-60 column (7.8 × 300 mm, Toso) at 40 °C with EtOH : H_2O = 94 : 6 as a solvent at a flow rate of 1.2 ml/min.[5] Peaks were detected at 254 nm. Retention time (min): curcumin (I), 15.8; demethoxycurcumin (II), 13.7; bisdemethoxycurcumin (III), 12.1.

Determination of the Solubility of Curcuminoids DMSO solution of each curcuminoid (5 mg/ml) was prepared and equal amounts of the solutions were mixed in a test tube. The mixture was diluted with 0.75% saline to make up 2% DMSO solution of the compounds at a total concentration of 100 μg/ml. After 30 min of the dilution, the solution was filtered through a 0.45 μm filter (Ekicrodisc 13, Gelman Japan), and the filtrate (2 ml) was freeze-dried. The residue was dissolved in EtOH (200 μl), filtered through a 0.45 μm filter, and the filtrate was analyzed by HPLC on a TSK gel NH_2-60 column (7.8 × 300 mm). The weight of the curcuminoid injected showed a linear relationship with the peak height in a range of 0.05—1.0 μg.

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