Biological Evaluation of Curcumin and Related Diarylheptanoids

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Nine derivatives of three natural diarylheptanoids, curcumin, demethoxycurcumin and bisdemethoxycurcumin, were prepared. Their antioxidant, free radical scavenging, nitric oxide (NO) inhibitory and cytotoxic activities were evaluated and compared with those of the respective natural compounds. Curcumin (**1**), demethoxycurcumin (**2**), demethyldemethoxycurcumin (**C3**), diacetyldemethoxycurcumin (**AC2**) and triacetyldemethylcurcumin (**AC5**) exhibited higher antioxidant activity than quercetin while products from demethylation of **1** and **2** exhibited higher free radical scavenging activity. Compounds **AC2** and **AC5** were found to be most active in inhibiting breast cancer cells (MCF-7) proliferation with IC_{50} values of 6.7 and 3.6 μM, respectively. The activity of **AC2** is almost doubled and of **AC5** almost tripled as compared to curcumin. Their selectivity towards different cell lines is also more noticeable. Compounds **AC2** and **AC5** also showed increased activity against a human prostate cancer cell line (DU-145) and non-small lung cancer cell line (NCI-H460) with IC_{50} values of 20.4, 16.3 and 18.3, 10.7 μM, respectively.

Key words: Curcumin Derivatives, Antioxidant, Nitric Oxide Inhibitory and Cytotoxic Activity

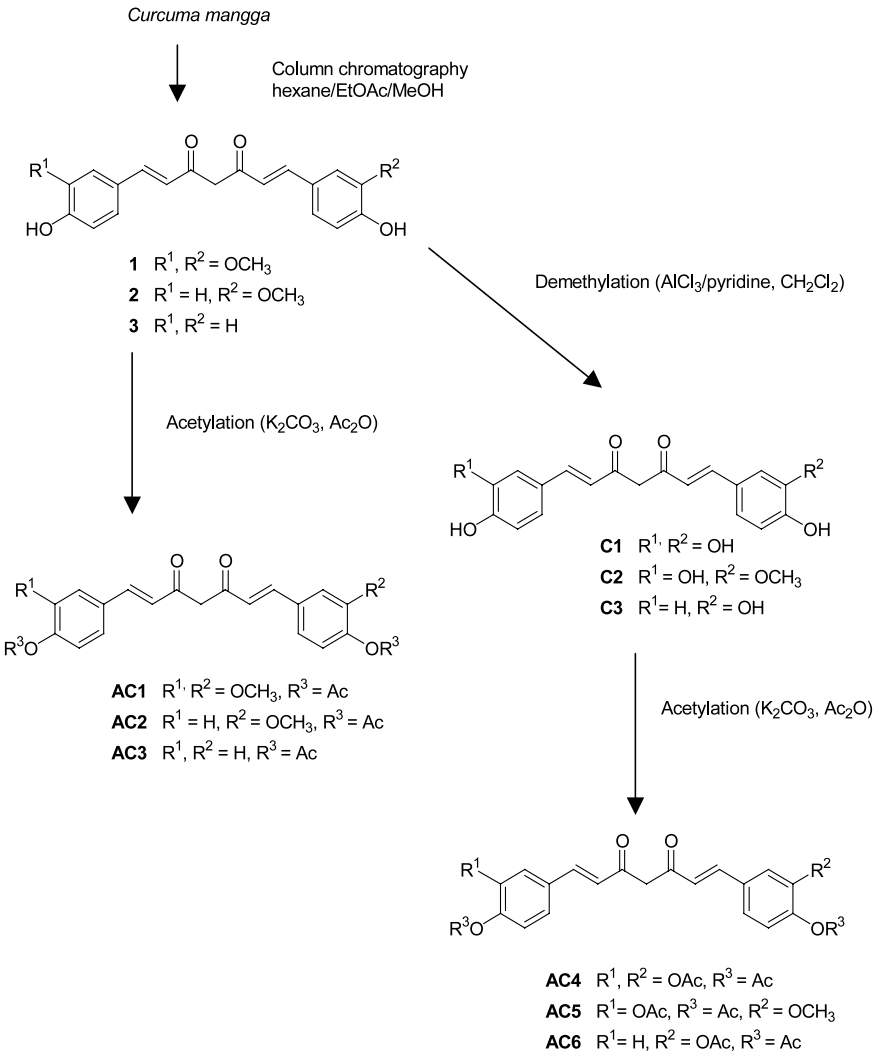
Introduction

Curcumin (diferuloylmethane) is a phenolic compound, occurring as the major pigment in tumeric (*Curcuma domestica*). It is widely used as colouring and flavouring agent in food (Ishida *et al*., 2002). Curcumin is known to have diverse biological functions, such as choleretic, antioxidative, antihepatotoxic, cytotoxic, anti-inflammatory, antifungal, antibacterial and antirheumatic activities (Nurfina *et al*., 1997). However, studies have demonstrated the limited use of curcumin due to its relatively low potency and its poor absorption characteristic (Ruby *et al*., 1995). In view of the potential importance of curcumin as pharmacophore, we prepared several of its derivatives based on its demethylated, demethoxylated, as well as acetylated analogues. Our hypothesis is that since the bioactivity of curcumin is limited due to its hydrophilic character resulting from the presence of two phenolic groups at both ends of the molecule (Ruby *et al*., 1995), by changing the phenolic functional group to acetate we anticipate that the molecule ends will become more lipophilic, and thus its transport through the cell membrane is facilitated. Acetate functionality is however rather labile towards hydrolysis and therefore its activity shall be reestablished when the hydrolysis into a free OH group occurs in the cell. Therefore, the main objective of the present study is to investigate whether the substitution pattern of the aromatic moiety in curcumin pharmacophore as well as making it more lipophilic will alter its activities.

Results and Discussion

Preparation of curcumin and related diarylheptanoids

Curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) were obtained by column chromatographic separation (silica gel, $CHCl_3$/MeOH) of the ethyl acetate fraction from the crude acetone extract of the rhizomes of *Curcuma mangga* (Scheme 1). Bisdemethylcurcumin [1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione] (**C1**), demethylcurcumin [1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-



Scheme 1. Demethylation and acetylation reactions of curcumin and derivatives.

dione] (**C2**), and demethyldemethoxycurcumin [1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione] (**C3**) were prepared by demethylation of **1** and **2**, respectively, with $AlCl_3$ and pyridine in CH_2Cl_2 (Mazumder *et al.*, 1997). Diacetylcurcumin [1,7-bis(4-acetoxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (**AC1**), diacetyldemethoxycurcumin [1-(4-acetoxyphenyl)-7-(4-acetoxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (**AC2**), and diacetylbisdemethoxycurcumin [1,7-bis(4-acetoxyphenyl)-1,6-heptadiene-3,5-dione] (**AC3**) were obtained after acetylation of **1**, **2** and **3**, respectively, with Ac_2O (neat) in the presence of K_2CO_3. In the same manner, acetylation of compounds **C1**, **C2** and **C3** gave the respective tetraacetylbisdemethylcurcumin [1,7-bis(3,4-diacetoxyphenyl)-1,6-heptadiene-3,5-dione] (**AC4**), triacetyldemethylcurcumin [1-(4-acetoxy-3-methoxyphenyl)-7-(3,4-diacetoxyphenyl)-1,6-hepta-diene-3,5-dione] (**AC5**), and triacetyldemethyldemethoxycurcumin [1-(4-acetoxyphenyl)-7-(3,4-diacetoxyphenyl)-1,6-heptadiene-3,5-dione] (**AC6**). Compounds **1**, **2**, **3**, **C1**, **C2** and **AC1** (Scheme 1), were identified by spectral data (UV, MS, IR, ^1H NMR and ^{13}C NMR) and by comparison with literature values of curcumin (**1**), demethoxycurcumin (**2**), bisdemethoxycurcumin (**3**) (Syu *et al.*, 1998), bisdemethylcurcumin (**C1**) and demethyl-

curcumin (**C2**) (Mazumder *et al.*, 1997). All other new compounds were determined based on the analysis of spectroscopic data and comparison of these data with those of related compounds. Detailed spectroscopic data for all these compounds may be obtained from us upon request.

Antioxidant activity

The antioxidant activity of **1** and its derivatives was determined using the ferric thiocyanate (FTC) method (Kikuzaki and Nakatani, 1993). Lower absorbance values indicated a high level of antioxidant activity (Fig. 1). Compounds **1**, **2**, **3**, **C1**, **C2**, **C3**, **AC2** and **AC5** exhibited higher activity than α-tocopherol, while **AC1**, **AC3** and **AC4** apparently were inactive. However, none of the derivatives were more active than **1** and butylated hydroxytoluene (BHT), a commercial antioxidant. It has been reported that the demethylated derivatives of curcumin are the most potent inhibitors of lipid peroxidation, while methylation of these compounds leads to a decrease in the potency of the antioxidant activity (Ruby *et al.*, 1995). The presence of olefinic double bonds in curcumin is important for the antioxidant activity. Furthermore, it has been reported that the delocalization

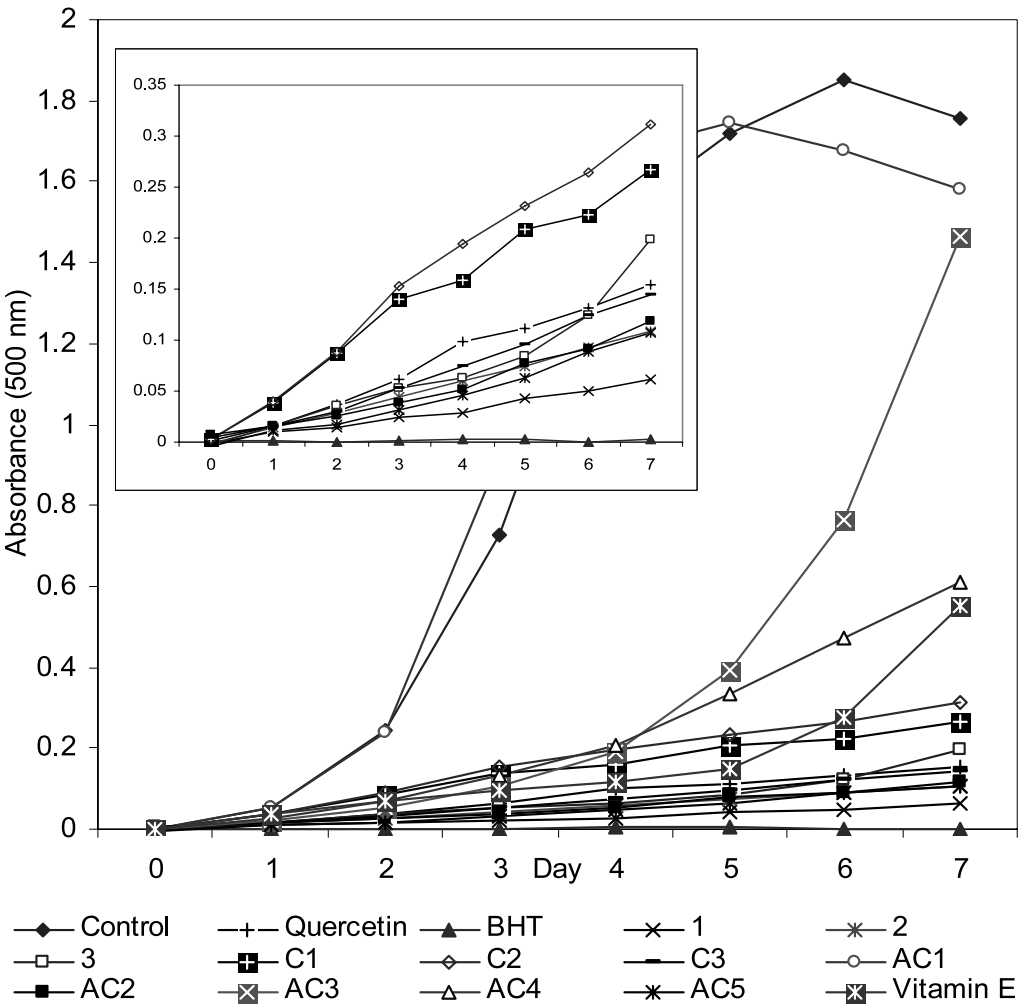


Fig. 1. Lipid oxidation monitored daily using the FTC method. Low absorbance values indicate strong antioxidant activity. Standard deviation of each point is $<10\%$ of the mean value. Inset shows the expanded plots of the nine most active compounds and BHT. The final content of each sample was 0.01% w/v.

of the phenolic radical to the conjugated alkyl chain is important for the antioxidant activity of curcuminoids (Osawa *et al.*, 1985). However, the substitution pattern of the aromatic moiety is also an important factor. It is interesting to note that, introducing the acetyl group at the *meta* and *para* positions of both aromatic rings producing the symmetrical analogues tetraacetylbisdemethylcurcumin has actually reduced the activity. On the other hand, an increase in activity is observed when the two aromatic ends of the heptadienedione system are unsymmetrical as exemplified by **AC2** and **AC5**. From this experiment it appears that the unsymmetrical nature of these two aromatic ends has an important role in affecting its antioxidant activity.

Radical scavenging activity

Compounds **1**, **2** and **3** displayed free radical scavenging activity with IC_{50} values ranging from 32–104 μM (Table I). The activities exhibited by **C1**, **C2** and **C3** were significantly higher than those of **1** and **2**. It is obvious that demethylation of **1** and **2** has drastically increased the activity. All of the acetylated products were found to be inactive (**AC1**–**AC6**). From this observation it may be concluded that the presence of a free OH group in the aromatic rings in curcumin derivatives is important for free radical scavenging activities. The activity is enhanced when *ortho*-dihydroxyphenyl moieties are present as shown by compounds **C1**, **C2** and **C3**.

Table I. Free radical scavenging and NO inhibitory activity of curcumin and derivatives.

Compound	IC_{50} [μM]	
	DPPH [a]	NO [b]
1	31.8 ± 3.2	18.5
2	92.5 ± 4.1	10
3	104.4 ± 6.8	12.5
C1	5.8 ± 3.8	na
C2	16.5 ± 4.1	na
C3	24.1 ± 5.9	na
AC1	na	50
AC2	na	30
AC3	na	na
AC4	214.5 ± 9.8	na
AC5	260.4 ± 12.3	14.5
AC6	nd	na
Quercetin	16.2 ± 2.1	
Vitamin C	33.3 ± 2.8	
L-Name		59.5 ± 2.2

na, no activity.
nd, not determined.
[a] Data represent the mean ± SD of 2 experiments performed in triplicate.
[b] Data represent the mean of one experiment performed in triplicate.

Nitric oxide (NO) inhibitory activity

Demethoxylation of curcumin did not significantly affect the activity, as shown by **2** and **3** (Table I). Demethylation of curcumin yielded **C1** and **C2** which were inactive in inhibiting nitric oxide production from macrophages (RAW 264.7 cells) induced by recombinant murine interferon-γ (IFN-γ) and lipopolysaccharide (LPS). On the other hand, the acetylated compounds such as **AC1**, **AC2**, **AC3**, **AC4** and **AC6** were two to three times less active than compound **1** as well as **2**. However, acetylation of **C2**, which yielded the unsymmetrical compound **AC5**, was found to regenerate the activity. Inhibition of nitrite production by **AC5** is not due to its cytotoxicity, as indicated by its cell viability values (data not shown). Previous study on the structure-activity relationships of a series of curcumin analogues showed that the presence of olefinic double bonds and 4-hydroxy groups in the diaryl moiety are important for anti-inflammatory activity (Nurfina *et al.*, 1997). From this investigation, it appeared that demethoxycurcumin (**2**) was the most potent among the curcumin analogues. From this observation, we therefore suggest that, besides the olefinic double bonds and the 4-OH groups, the presence of one $-OCH_3$ group is also important for the anti-inflammatory activity.

Cytotoxic activity

The cytotoxic activity of the isolated curcuminoids from *C. mangga* and their derivatives is shown in Table II. Demethylation of **1** and **2** significantly increased the cytotoxic activity against all cell lines tested, as shown by compounds **C1**, **C2** and **C3**. Thus, the presence of a catechol moiety has in general enhanced the cytotoxic properties. Acetylation of the free hydroxy group of **1**, **2** and **3**, as well as the free hydroxy group of their demethylated products reduced or significantly affected the activity except for **AC2** and **AC5**. Compounds **AC2** and **AC5** representing unsymmetrical acetylated curcuminoids were found to be most ac-

Table II. Cytotoxicity of curcuminoids against human cancer cell lines.

Compound	IC_{50} [μM][a]		
	MCF-7	NCI-H460	DU-145
1	10.44 ± 1.3	20.6 ± 4.9	27.6 ± 0.1
2	18.51 ± 8.4	21.7 ± 4.2	25.7 ± 6.1
3	33.78 ± 1.3	30.1 ± 2.4	88.1 ± 6.8
C1	15.60 ± 10.6	16.0 ± 4.1	22.5 ± 2.0
C2	11.56 ± 6.3	23.3 ± 7.6	23.1 ± 7.8
C3	11.52 ± 7.11	17.4 ± 1.0	20.1 ± 2.4
AC1	33.29 ± 24.7	38.4 ± 12.3	52.4 ± 20.1
AC2	6.72 ± 2.1	18.3 ± 8.2	20.4 ± 9.2
AC3	20.75 ± 8.9	28.1 ± 2.5	42.2 ± 11.2
AC4	20.92 ± 16.4	27.1 ± 3.2	30.2 ± 4.1
AC5	3.63 ± 1.9	10.7 ± 6.2	16.3 ± 5.7

[a] 50% inhibitory concentration (molar concentration of compounds, which causes a 50% inhibition as compared to control). Data represent the mean ± SD of 3 experiments performed in triplicate.

tive in inhibiting MCF-7 cells proliferation with IC_{50} values of 6.7 and 3.6 μM, respectively. The activity of these two compounds on a human prostate cancer cell line (DU-145) and the non-small lung cancer cell line (NCI-H460) was also enhanced although less active than on the MCF-7 cell line.

Experimental

General experimental procedures

Analytical TLC was carried out on silica gel F_{254} precoated (0.2 mm thickness; Merck) plastic TLC sheets. The TLC plates were spotted with samples using a fine glass capillary tube and developed in a chromatographic tank saturated with solvent vapour at room temperature. Column chromatography (CC) was performed on silica gel Merck 7734 (70–230 mesh ASTM) or Merck 9385 (230–400 mesh ASTM). Melting points were determined using a hot stage melting point apparatus equipped with a microscope, XSP-12 model 500X, and are uncorrected. Ultraviolet (UV) spectra were recorded on a Varian UV-VIS 500 instrument in absolute methanol, and infrared (IR) spectra were recorded on a Perkin Elmer 1650 FTIR spectrometer (ν expressed in cm^{-1}). ^1H and ^{13}C NMR spectra were recorded on a Varian Unity 500 spectrometer (Varian Inc., Palo Alto, CA) and measured at 500 and 125 MHz, respectively. Deuterated chloroform ($CDCl_3$), acetone-d_6 and deuterated methanol (CD_3OD) were used as solvents. Chemical shifts (δ) were recorded in ppm. Mass spectra (MS) have been recorded on a Polaris Q ThermoFinnigan instrument (San Jose, CA) with ionization induced by electron impacts (EIMS) at 70 eV.

Plant material

25 kg of the fresh *C. mangga* rhizomes were collected in Johor, Malaysia in October 2000. The rhizomes were cleaned, chopped into smaller pieces (3–5 mm thickness) and dried under the shade. A voucher specimen (No. SK 149/02) was deposited at the Herbarium of the Laboratory of Natural Products, Institute of Bioscience, University Putra Malaysia.

*Isolation of curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**)*

8 kg of the dried powdered rhizomes (32% w/w of fresh rhizomes) of *Curcuma mangga* were extracted three times with acetone to give 900 g extract (11.25% of the dried material). 250 g of the acetone extract were partitioned into a hexane/H_2O mixture to furnish the 150 g hexane-soluble portion and aqueous phase. The aqueous phase was further extracted with ethyl acetate (EtOAc) to give 45 g EtOAc and 25 g H_2O-soluble portions. 40 g of the ethyl acetate fraction were absorbed onto 40 g silica gel (7734), dried and then loaded onto the top of a glass column (9.5 cm × 90 cm) bed packed with 1 kg of silica gel (7734). The column was eluted in a gradient manner with a hexane/EtOAc mixture, followed by EtOAc/MeOH to afford 13 fractions (A–M). Fractions D, E, F and G were combined (5 g) and subjected to silica gel column chromatography using a CH_2Cl_2/MeOH gradient and further purified by gel permeation chromatography on a Sephadex LH-20 column to afford compounds **1** (150 mg), **2** (250 mg) and **3** (20 mg). Compounds **1**, **2**, and **3** were identified by spectral data and by comparison with literature data.

*General procedure for demethylation of **1** and **2***

Compounds **C1**, **C2** and **C3** were prepared as described by Mazumder *et al.* (1997). Compounds **C1** and **C2** were obtained after heating of **1** with $AlCl_3$ and pyridine (1:7:0.03) in anhydrous CH_2Cl_2 in a 250-ml single neck round bottom flask, and refluxing for 24 h while compound **C3** was obtained after heating of **2** using the same reagent and procedure.

*Demethyldemethoxycurcumin (**C3**)*

Yellow powder (acetone); m.p. 106–108 °C. – UV (MeOH): λ_{max} (log ε) = 423 nm (5.84). – IR (KBr): ν_{max} = 3425 (–OH), 1600 cm^{-1} (C=O). – MS (EI, 70 eV): *m/z* (rel. int.) = 324 (M^+, 27), 306 (85), 176 (50), 147 (100).

General procedure for acetylation

Into a 50-ml round bottomed flask, curcumin (1 mmol), K_2CO_3 (2 mmol) and anhydrous Ac_2O (2 mmol) were introduced. The reaction mixture was stirred at room temperature and the reaction progress was monitored by TLC for about 2 h. The reaction mixture was then filtered and washed with water (30 ml). The aqueous layer was extracted with EtOAc (3 × 30 ml). The combined organic extract was dried over anhydrous sodium sulfate. The product, obtained after removal of the solvent under reduced pressure, was crystallized from an appropriate solvent.

*Diacetyldemethoxycurcumin (**AC2**)*

Yellow powder (acetone); m.p. 130–131 °C. – UV (MeOH): λ_{max} (log ε) = 396 nm (5.91). – IR (KBr): ν_{max} = 3435, 1763, 1621, 1192 cm^{-1}. – MS (EI, 70 eV): *m/z* (rel. int.) = 422 (M^+, 5), 362 (40), 320 (100), 190 (55), 147 (44).

*Diacetylbisdemethoxycurcumin (**AC3**)*

Yellow powder (acetone). – UV (MeOH): λ_{max} (log ε) = 309 (5.78), 392 nm (5.88). – IR (KBr): ν_{max} = 3370, 1624, 1602 cm^{-1}. – MS (EI, 70 eV): *m/z* (rel. int.) = 392 (M^+, 10), 350 (24), 308 (30).

*Tetraacetylbisdemethylcurcumin (**AC4**)*

Yellow powder (acetone); m.p. 165–167 °C. – UV (MeOH): λ_{max} (log ε) = 309 (5.89), 395 nm (5.99). – IR (KBr): ν_{max} = 3436, 1763, 1620, 1217 cm^{-1}. – MS (EI, 70 eV): *m/z* (rel. int.) = 508 (M^+, 5), 466, (12), 424 (42), 364 (64), 322 (74), 176 (80), 163 (100).

*Triacetyldemethylcurcumin (**AC5**)*

Yellow powder (acetone); m.p. 122–125 °C. – UV(MeOH): λ_{max} (log ε) = 309 (5.88), 392 nm (5.99). – IR (KBr): ν_{max} = 3441, 1771, 1629 cm^{-1}. – MS (EI, 70 eV): *m/z* (rel. int.) = 480 (M^+, 5), 438 (16), 396 (24), 336 (100), 190 (60), 177 (50), 145 (44).

*Triacetyldemethyldemethoxycurcumin (**AC6**)*

Yellow powder (acetone); m.p. 126–129 °C. – UV (MeOH): λ_{max} (log ε) = 307 (5.88), 391 nm (5.99). – IR (KBr): ν_{max} = 3448, 1763, 1629, 1219 cm^{-1}. – MS (EI, 70 eV): *m/z* (rel. int.) = 450 (M^+, 5), 408 (16), 366 (60), 348 (56), 306 (100), 176 (68), 147 (88).

Antioxidative activity

The antioxidant activity of curcumin and its analogues was established using the ferric thiocyanate (FTC) method. This assay was carried out according to the method of Kikuzaki and Nakatani (1993).

DPPH free radical scavenging activity assay

The potential antioxidant activity of curcumin and its analogues was assessed on the basis of its scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to Cotelle *et al.* (1996).

NO inhibitory activity

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Minimal (DMEM) essential medium. For nitrite measurement, RAW 264.7 cells were seeded at 1×10^6 cells per well in flat bottom 96-well microtiter plates and then stimulated with 200 U/ml recombinant murine interferon-γ (IFN-γ) and 10 μg/ml lipopolysaccharide (LPS) in the presence or absence of various concentrations of compounds for 17–20 h. Levels of NO were estimated by the accumulation of the stable NO metabolite nitrite by the Griess assay (Dirsch *et al.*, 1998). An equal volume of culture supernatants (50 μl) and Griess reagents [50 μl 1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4] were incubated at room temperature for 10 min, and the absorbance was

measured at 550 nm. L-Name was used as standard. The amounts of nitrite were calculated against a $NaNO_2$ standard curve.

Cytotoxicity assay

In vitro cytotoxicity assay was carried out according to the established procedures by Stanslas *et al.* (2000). The absorbance of the formazan solution was determined at 550 nm using a microplate reader (Spectramax Plus, USA). The IC_{50} values (concentration of a drug that produces 50% reduction in the absorbance compared with untreated controls) were determined from the dose-response curves.

Conclusion

In summary, modification of curcumin produced compounds which are more active as inhibitors of lipid peroxidation and radical scavenger. However, these derivatives do not significantly alter the NO production activity. The studies also demonstrate that the cytotoxic activity of compounds **AC2** and **AC5** were more efficacious than curcumin. The substitution pattern of the aromatic moiety resulted in symmetrical and unsymmetrical analogues indicating that they have a significant role in determining their activities. From the results of this study, the unsymmetrical structures seemed to be a key structural element for the enhancement of their activities. Further studies are required to determine the precise molecular requirements and the mechanisms involved in the biological activities of these compounds.

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