ฤทธิ์ความเป็นพิษของสารเคอร์คิวมินอยด์และเคอร์คิวมินอยด์แอนาลอกต่อเซลล์มะเร็งช่องปาก**

Cytotoxic Activity of Curcuminoids and Curcuminoid Analogues Against Human Oral Cancer KB Cells**

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Abstract

The natural curcuminoids, curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**), have been structurally modified to the demethylated analogues **4-6**, methylated analogues **7-12**, acetylated analogues **13-17** and the tetrahydro analogues **18-21**. The curcuminoids **1-3** and the analogues **4-21** were evaluated for their cytotoxic activities against a human oral cancer (KB) cell line. The demethylated analogues **4-6** and methylated analogues **7-8** exhibited high cytotoxic activities against KB cells with IC_{50} values of 7.76, 2.38, 8.17, 7.11 and 3.85 μM, respectively. Interestingly, the analogue **5** exhibited the highest cytotoxicity (IC_{50} value 2.38 μM), which was more potent than ellipticine, the reference anticancer drug (IC_{50} value 5.40 μM). For the acetylated analogues, the results showed that acetylation did not increase cytotoxicity of the parent curcuminoids. Furthermore, it was also found that the tetrahydro analogues were inactive

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in this study. The results indicated that conjugated diketo system is essential for cytotoxicity. Analogues with more of free hydroxyl groups were more potent than the natural curcuminoids, with exception for the mono-*O*-methyl and di-*O*-methyl analogues of curcumin (**1**).

Keywords: Curcuminoid analogues, Structural modification, Cytotoxicity, Oral cancer

บทคัดย่อ

ได้นำเคอร์คิวมินอยด์จากธรรมชาติ 3 ชนิด คือ เคอร์คิวมิน (**1**), ดีเมทอกซีเคอร์คิวมิน (**2**) และบิสดีเมทอกซีเคอร์คิวมิน (**3**) มาปรับเปลี่ยนโครงสร้างเป็น Demethylated analogues **4-6**, Methylated analogues **7-12**, Acetylated analogues **13-17** และ Tetrahydro analogues **18-21** แล้วนำเคอร์คิวมินอยด์ 1-3 และเคอร์คิวมินอยด์แอนาลอก **4-21** มาทดสอบความเป็นพิษต่อเซลล์มะเร็งในช่องปากมนุษย์ (KB) พบว่า Demethylated analogues **4-6** และ Methylated analogues **7-8** แสดงความเป็นพิษต่อเซลล์มะเร็งในช่องปากมนุษย์ โดยมีค่า IC_{50} 7.76, 2.38, 8.17, 7.11 และ 3.85 μM ตามลำดับ โดยเฉพาะ Di-O-demethylcurcumin (**5**) มีฤทธิ์สูงสุด (ค่า IC_{50} คือ 2.38 μM) ซึ่งสูงกว่ายาต้านมะเร็งมาตรฐาน คือ Ellipticine (ค่า IC_{50} 5.40 μM) ส่วน Acetylated analogues นั้นได้แสดงให้เห็นว่า การเติมหมู่อะซีเตตไม่ได้เพิ่มฤทธิ์ความเป็นพิษต่อเซลล์ให้กับสารจากธรรมชาติ นอกจากนี้ยังพบว่า Tetrahydro analogues ไม่มีฤทธิ์ทางชีวภาพที่ทดสอบ ผลจากการศึกษาที่ได้แสดงว่าระบบ Diketo ที่คอนจุเกตเป็นสิ่งจำเป็นต่อการแสดงฤทธิ์ความเป็นพิษต่อเซลล์ แอนาลอกที่มีไฮดรอกซิลอิสระมีฤทธิ์สูงกว่าเคอร์คิวมินอยด์จากธรรมชาติ ยกเว้นกรณีของ Mono-*O*-methyl และ Di-*O*-methyl analogues ของเคอร์คิวมิน (**1**).

คำสำคัญ : เคอร์คิวมินอยด์ การปรับเปลี่ยนโครงสร้าง ฤทธิ์ความเป็นพิษต่อเซลล์ มะเร็งช่องปาก

Introduction

Curcuminoids are the constituents of turmeric (*Curcuma longa* L., family Zingiberaceae) and have been used for over a century as dietary pigments, spices and traditional medicine in India, China and Thailand. Curcumin (**1**) is the major curcuminoid isolated from this plant species, whereas demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) are the minor components (Srivastava et al., 2011). Curcuminoids exhibit many biological activities (Maheshwari et al, 2006), for example, antioxidant (Ruby et al., 1995; Gringburg et al., 1996), anti-inflammatory (Chan et al., 1995; Chan et al., 1998), anticancer (Inano et al., 2000), anti-protozoal (Araujo et al., 1999) and anti-HIV activities (Mazumder et al., 1995). Many researchers have therefore used this class of compounds to study different biological activities. Some investigators have studied the role of the α,β-unsaturated β-diketo functional group and it was concluded that this moiety is essential for its biological activities (Pan et al., 2000). Cytotoxic activities of the natural curcuminoids **1-3** have been evaluated, however, most of them showed relatively low activities when compared with reference anticancer drugs. It is therefore of interest to modify the structures of the natural curcuminoids **1-3** to analogues with higher cytotoxic activity.

Oral cancer is among the non-infective diseases that causes a major health problem in the developing countries. It was estimated that there are more than 300,000 oral cancer cases worldwide and about half of patients died from this type of cancer (Peyrade et al., 2013; Siegel et al., 2014). The five-year relative survival rate of oral cancer patients is less than 35% in advanced stages, of disease at initial diagnosis (Siegel et al., 2014). Late diagnosis, disease recurrence, metastasis and resistance to therapy may attribute to this poor survival rate. Conventional treatments, including surgical treatment combined with radiotherapy and chemotherapy or concomitant chemo-radiotherapy, have limited efficacy and resulted in adverse systemic and cytotoxic effects on normal cells. Chemoprevention is a promising treatment strategy for oral cancer. In general, chemopreventive drugs should be inexpensive, nontoxic or relatively less toxic, and target important pathways involved in the development of this cancer. Combination of chemopreventive agents and conventional therapeutic approaches may reduce toxicities and improving treatment outcomes. The current conventional chemotherapeutic agents,

for example, cisplatin and 5-fluorouracil are rather toxic (Peyrade et al., 2013; Vermorken et al., 2013; Du et al., 2014). Alternative anticancer agents with less undesired side effects are the potential approaches for cancer therapy.

Objectives

1. To modify the structures of natural curcuminoids to analogues.
2. To evaluate cytotoxicity of curcuminoids and the modified analogues against human oral (KB) cells.

Materials and Methods

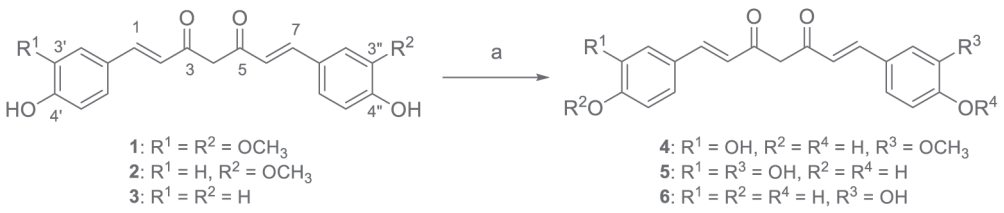
1. Isolation of Curcuminoids 1-3 from Curcuma longa Rhizomes

Crude curcuminoid mixtures from Thai-China Flavours and Fragrances (TCFF) were subjected to column chromatography using silica gel (CH_2Cl_2-MeOH, gradient elution) resulted in the isolation of curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) in 61.0, 14.2 and 2.7 % yields, respectively. The spectroscopic (IR, ^{1}H NMR and mass spectra) data were consistent with the reported values (Venkateswarlu et al., 2005).

2. Chemical Modifications of Curcuminoids

The curcuminoids **1-3** obtained from the foregoing method were used as starting materials for structural modifications to analogues for cytotoxic activity evaluations. Most of the methods were based on the method by Changtam et al. (2010).

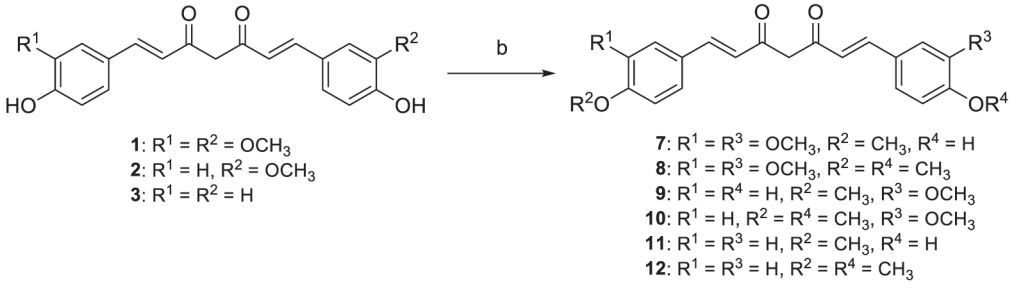
2.1 Demethylated Analogues of Curcuminoid 1. Compound **1** (100 mg, 0.27 mmol) in dry CH_2Cl_2 (15 ml) was stirred at 0° C for 5 min and BBr_3 (0.5 ml) was slowly added. The reaction mixture was allowed to warm up to ambient temperature and stirring was continued for 4 h. Water was added and the mixture was extracted with EtOAc. The combined organic phase was washed with H_2O, dried over anhydrous Na_2SO_4; the solvent was evaporated and the crude mixture was chromatographed using CH_2Cl_2-MeOH-AcOH (50:5:2) to yield mono-*O*-demethylcurcumin (**4**) (38 mg, 41%) as m.p. 167-169° C and di-*O*-demethylcurcumin (**5**) (27 mg, 30%), m.p. 301-303° C (Scheme 1). The spectroscopic (IR, ^{1}H NMR and mass spectra) data were consistent with the reported values (Venkateswarlu et al., 2005).



Scheme 1 Synthesis of demethylated analogues **4-6** from the curcuminoids **1-3**. Reagent and conditions: a, BBr_3 in CH_2Cl_2, from 0° C to ambient temperature.

2.2 Demethylated Analogues of Curcuminoid 2. Compound **2** was subjected to demethylation in a similar manner to that of compound 1 to give *O*-demethyldemethoxycurcumin (**6**) (55%), m.p. 216-218° C (Scheme **1**). The spectroscopic (IR, ^{1}H NMR and mass spectra) data were consistent with the reported values (Venkateswarlu et al., 2005).

2.3 Methylated Analogues of Curcuminoid 1. A mixture of compound **1** (50 mg, 0.14 mmol) in dry acetone (3 ml), anhydrous K_2CO_3 (50 mg) and CH_3I (0.5 ml, excess) was refluxed for 3 h. Water was added and the mixture was extracted with CH_2Cl_2. The combined organic phase was washed with H_2O, dried over anhydrous Na_2SO_4 and the solvent was removed under vacuum. The crude products were purified by column chromatography using CH_2Cl_2 to yield mono-*O*-methylcurcumin (**7**) (12 mg, 23%) and di-*O*-methylcurcumin (**8**) (25 mg, 46%, orange powder; m.p. 128-130° C) (Scheme 2). The spectroscopic (IR, ^{1}H NMR and mass spectra) data were consistent with the reported values (Ohtsu et al., 2002).



Scheme 2 Synthesis of methylated analogues **7-12** from the curcuminoids **1-3**. Reagent and conditions: b, CH_3I in acetone in the presence of anhydrous K_2CO_3 at reflux.

2.4 Methylated Analogues of Curcuminoid 2. Compound **2** was subjected to methylation in a similar manner to that of compound 1 to give 4'-*O*-methyldemethoxycurcumin (**9**) (20%) and di-*O*-methyldemethoxycurcumin **(10)** (54%) (Scheme 2).

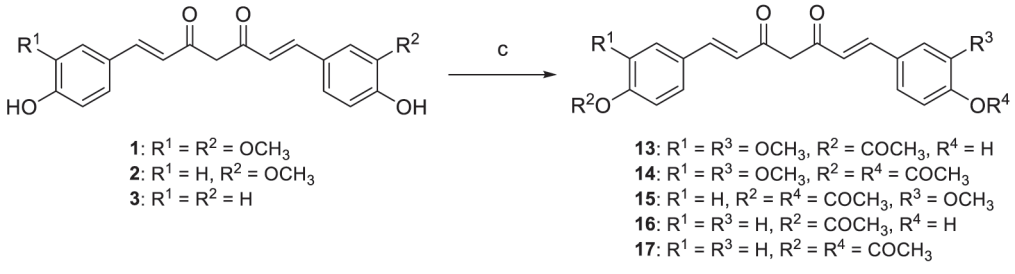
Compound 9: Orange amorphous solid; IR ν_{max}: 3415, 1639, 1584, 1507, 1261, 1135, 1020, 961 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 3.88 and 3.90 (each s, 2×3H, 2×OMe), 5.77 (s, 1H, H-4), 6.45 and 6.46 (each d, *J* = 15.7 Hz, 2×1H, H-2 and H-6), 6.83 (br d, *J* = 8.2 Hz, 2H, H-3' and H-5'), 6.84 (d, *J* = 8.3 Hz, 1H, H-5''), 7.04 (br s, 1H, H-2''), 7.10 (br d, *J* = 8.3 Hz, 1H, H-6''), 7.42 (d, *J* = 8.3 Hz, 2H, H-2' and H-6'), 7.57 (d, *J* = 15.7 Hz, 2H, H-1 and H-7); ESIMS (+ve): *m/z* 727 $[2M+Na]^+$.

Compound 10: Yellow needles, m.p. 160-161° C; IR ν_{max}: 2935, 1623, 1600, 1514, 1457, 1254, 1136, 1026, 967 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 3.82, 3.89 and 3.90 (each s, 3×3H, 3×OMe), 5.77 (s, 1H, H-4), 6.47 (d, *J* = 15.7 Hz, 2H, H-2 and H-6), 6.85 (d, *J* = 8.2 Hz, 1H, H-5''), 6.90 (br d, *J* = 8.5 Hz, 2H, H-3' and H-5'), 7.06 (br s, 1H, H-2''), 7.12 (br d, *J* = 8.2 Hz, 1H, H-6''), 7.49 (d, *J* = 8.5 Hz, 2H, H-2' and H-6'), 7.58 and 7.60 (each d, *J* = 15.7 Hz, 2H, H-1 and H-7); ESIMS (+ve): *m/z* 367 $[M+H]^+$.

2.5 Methylated Analogues of Curcuminoid 3. Compound **3** was subjected to methylation in a similar manner to that of compound **1** to give mono-O-methylbisdemethoxycurcumin (**11**) (17%) and di-*O*-methylbisdemethoxycurcumin (**12**) (30%) (Scheme **2**). The spectroscopic (^1H NMR and mass spectra) data of the later product were consistent with the reported values (Shao et al., 2006).

Compound 11: Orange solid; mp 188-189° C; IR: ν_{max} 3422, 1625, 1602, 1511, 1458, 1262, 1173, 1140, 974, 828 cm^{-1}; ^{1}H-NMR (CDCl3) δ 3.81 (s, 3H, OMe), 5.75 (s, 1H, H-4), 6.44 and 6.46 (each d, *J* = 15.7, 1H, H-2 and H-6), 6.85 (d, *J* = 8.4, 2H, H-3'' and H-5''), 6.88 (d, *J* = 8.5, 2H, H-3' and H-5'), 7.41 (d, *J* = 8.4, 2H, H-2'' and H-6''), 7.47 (d, *J* = 8.5, 2H, H-2' and H-6'), 7.56 and 7.57 (each d, *J* = 15.7, 2x1H, H-1 and H-7); ESIMS *m/z*: 323 $[M+H]^+$.

2.6 Acetylated Analogues of Curcuminoid 1. Acetic anhydride (0.5 ml) was added to a solution of compound **1** (100 mg, 0.27 mmol) in pyridine (2 ml) and the reaction mixture was stirred at ambient temperature for 0.5 h. After the usual work up, the mixture was extracted with EtOAc and the organic phase was washed with H_2O, dried over anhydrous Na_2SO_4 and the solvent evaporated to dryness. The crude products were purified by column chromatography eluting with CH_2Cl_2 to afford mono-*O*-acetylcurcumin (**13**) (12 mg, 11%) and di-*O*-acetylcurcumin (**14**) (60 mg, 49%), m.p. 163-165° C (Scheme 3). The spectroscopic (IR, ^{1}H NMR and mass spectra) data of compounds **13** and **14** were consistent with the reported values (Mishra et al., 2005).



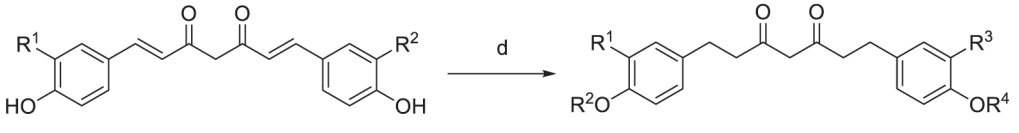
Scheme 3 Synthesis of acetylated analogues 13-17 from the curcuminoids 1-3. Reagent and conditions: c, Ac_2O and pyridine at ambient temperature.

2.7 Acetylated Analogue of Curcuminoid 2. Compound **2** was subjected to acetylation in the same manner to that of compound 1 to give di-*O*-acetyldemethoxycurcumin (**15**) in 85% yield (Scheme **3**). The spectroscopic (IR, ^{1}H NMR and mass spectra) data of compound **15** were consistent with the reported values (Abas et al., 2006).

Compound 15: Yellow aggregated needles, m.p. 140-141° C; IR ν_{max}: 2940, 1760, 1633, 1596, 1560, 1540, 1508, 1367, 1256, 1200, 1013, 969 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 2.28 and 2.30 (each s, 2×3H, 2×OAc), 3.85 (s, 3H, OMe), 5.81 (s, 1H, H-4), 6.53 and 6.54 (each d, *J* = 15.8 Hz, 2×1H, H-2 and H-6), 7.03 (d, *J* = 8.1 Hz, 1H, H-5''), 7.10 (d, *J* = 8.5 Hz, 2H, H-3' and H-5'), 7.11 (obscured signal, 1H, H-6''), 7.54 (d, *J* = 8.4 Hz, 2H, H-2' and H-6'), 7.59 and 7.61 (each d, *J* = 15.8 Hz, 2H, H-1 and H-7); ESIMS (+ve): *m/z* 445 $[M+Na]^+$, 423 $[M+H]^+$.

2.8 Acetylated Analogue of Curcuminoid 3. Compound **3** was acetylated in the same manner to that of compound **1** to give mono-*O*-acetylbisdemethoxycurcumin (16) (15%) and di-*O*-acetylbisdemethoxycurcumin (**17**) (61%, yellow needles, m.p. 173-174° C (Scheme 3). The spectroscopic (IR, ^1H NMR and mass spectra) data of compound **16** and **17** were consistent with the reported values (Mohammadi et al., 2005).

2.9 Catalytic Hydrogenation of Curcuminoid 1. A solution of curcumin (**1**) (50 mg, 0.14 mmol) in EtOH (6 ml) was prepared and 10% Pd-C (20 mg) was then added. After degassing, the mixture was hydrogenated at room temperature and at atmospheric pressure for 3 h. The mixture was filtered through a column of Celite and the solvent was evaporated. The crude product was purified by column chromatography using CH_2Cl_2-MeOH to afford tetrahydrocurcumin (**18**) (35 mg, 69%) as colorless crystals, m.p. 92-94° C (Scheme 4). The spectroscopic (IR, ^1H NMR and mass spectra) data of compound **18** was consistent with the reported values (Ohtsu et al., 2002; Lee et al., 2005).



1: $R^1 = R^2 = OCH_3$
2: $R^1 = H$, $R^2 = OCH_3$
3: $R^1 = R^2 = H$
5: $R^1 = R^2 = OH$

18: $R^1 = R^3 = OCH_3$, $R^2 = R^4 = H$
19: $R^1 = R^2 = R^4 = H$, $R^3 = OCH_3$
20: $R^1 = R^2 = R^3 = R^4 = H$
21: $R^1 = R^3 = OH$, $R^2 = R^4 = H$

Scheme 4 Synthesis of tetrahydro analogues **18-21** from the curcuminoids **1-3**. Reagent and conditions: d, H_2/Pd-C in EtOH.

2.10 Catalytic Hydrogenation of Curcuminoid 2. Demethoxycurcumin (2) was subjected to catalytic hydrogenation in a similar manner to that of compound **1** to afford tetrahydrodemethoxycurcumin (**19**) (62%) as colorless non-crystalline solid (Scheme 4). The spectroscopic (IR, ^{1}H NMR and mass spectra) data of compound **19** were consistent with the reported values (Portes et al., 2007).

2.11 Catalytic Hydrogenation of Curcuminoid 3. Bisdemethoxycurcumin (**3**) was subjected to catalytic hydrogenation in a similar manner to that of compound **1** to give tetrahydrobisdemethoxycurcumin (**20**) (65%) as colorless crystal, m.p. 110-111° C (Scheme 4). The spectroscopic (IR, ^{1}H NMR and mass spectra) data of compound **20** were consistent with the reported values (Portes et al., 2007).

2.12 Catalytic hydrogenation of di-*O*-demethylcurcumin (5). Compound **5** was subjected to catalytic hydrogenation in a similar manner to that of compound 1 to give di-*O*-demethyltetrahydrocurcumin (**21**) (58%) as colorless sticky solid (Scheme **4**). The spectroscopic data of compound **21** were consistent with the reported values (Portes et al., 2007; Ma et al., 2004).

3. Cytotoxicity Assays against Oral Cancer Cell Lines

The cytotoxicity assays against human oral cancer cells were performed employing the established method (Brien et al., 2000). The cytotoxicity assays against human epidermoid carcinoma (KB) cell lines were performed employing resazurin microplate assay (REMA). The standard drug was ellipticine. Briefly, cells at a logarithmic growth phase are gathered and diluted to 7×10^4 cells/ml for KB, in fresh medium. Successively, 5 µl of test sample diluted in 5% DMSO, and 45 µl of cell suspension are added to 384-well plates, incubated at 37° C in 5% CO_2 incubator. After the incubation period (3 days for KB), 12.5 µl of 62.5 µg/ml resazurin solution is added to each well, and the plates are then incubated at 37° C for 4 h. The fluorescence signal is measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Percent inhibition of cell growth is calculated by the following equation: % Inhibition = $[1 - (FU_T/FU_C)] \times 100$ whereas FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively.

The dose response curves were plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibited cell growth by 50% (IC_{50}) were derived using the SOFTMax Pro software (Molecular Devices, USA). The standard drug ellipticine exhibited IC_{50} value against this cell line at 5.40 µM.

Table 1 Cytotoxicity of natural curcuminoids and curcuminoid analogues against human oral cancer (KB) cell lines[a].

Compound	M.W.	IC_{50} (µM)
Natural curcuminoids		
1	368.38	Inactive[b]
2	338.35	Inactive[b]
3	308.33	Inactive[b]
Demethylated analogues		
4	354.35	7.76
5	340.33	2.38
6	324.33	8.17
Methylated analogues		
7	382.41	7.11
8	396.43	3.85
9	352.38	28.40
10	366.41	Inactive[b]
11	322.35	Inactive[b]
12	336.38	Inactive[b]
Acetylated analogues		
13	410.42	15.59
14	452.45	Inactive[b]
15	422.43	Inactive[b]
16	350.36	Inactive[b]
17	392.40	Inactive[b]
Tetrahydro analogues		
18	372.41	Inactive[b]
19	342.39	Inactive[b]
20	312.36	Inactive[b]
21	344.36	Inactive[b]

[a] Ellipticine was used as the reference drug, with IC_{50} value of 5.40 µM.

[b] Inactive at > 20 µg/ml

Results and Discussions

1. Structural Modification of Curcuminoids

Although the natural curcuminoids, curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**), have been reported to exhibit cytotoxic activities (Inano et al., 2000), in most cases the potencies of these three compounds were not high. For example, the curcuminoids **1-3** showed weak cytotoxic activity against oral cancer (KB) cells with IC_{50} values of 21.36, 26.45 and 21.44 μM, respectively (Chuprajob et al., 2014). It is therefore of interest to investigate whether structural modification of these natural compounds would give rise to analogues with high anti-KB activity. General synthetic methodology or reported syntheses of analogues of curcuminoids have been used in this study. The synthetic methodologies are summarized in Schemes **1-4**.

In order to observe the effects of free hydroxyl group and hydrogen bonding of the oxygen function at the aromatic rings, the parent curcuminoids **1-3** were subjected to demethylation and methylation to the appropriate demethylated and methylated analogues (see Materials and Methods). Starting from compound **1**, demethylation to the corresponding mono-*O*-demethyl analogue **4** and di-*O*-demethyl analogue **5** were achieved, by treatment of **1** with boron tribromide. Demethylation of the curcuminoid 2 to the corresponding *O*-demethyl analogue **6** was similarly accomplished. The spectroscopic (IR, ^{1}H NMR and mass spectra) data of compounds **4, 5** and **6** were consistent with the reported values (Venkateswarlu et al., 2005). Methylation of compounds **1, 2** and **3** was achieved by reaction with methyl iodide in acetone in the presence of potassium carbonate to give the corresponding ether analogues **7-12**, respectively. The spectroscopic data of the synthesized compounds were consistent with their structures (see Materials and Methods) and/or were in agreement with the reported values (Ohtsu et al., 2002; Shao et al., 2006).

The acetate esters analogues of the curcuminoids **1-3** were also prepared. Acetylation of the curcuminoid **1** by the conventional method gave the mono-*O*-acetyl analogue **13** and di-*O*-acetyl analogues **14** (Mishra et al., 2005). Acetylation of the curcuminoid **2** by the same process yielded the di-*O*-acetyl analogues **15** (Abas et al., 2006). Acetylation of the curcuminoid **3** in a similar manner afforded the mono-*O*-acetyl analogue **16** and di-*O*-acetyl analogues **17** (Mohammadi et al., 2005). The spectroscopic

data of the synthesized acetates were in agreement with the structures and consistent with the reported values (Mishra et al., 2005; Faridah et al., 2006; Mohammadi et al., 2005).

In order to see if the olefinic bond in the heptyl chain contributed to the cytotoxic activity, a number of non-conjugated analogues of the natural curcuminoids **1, 2** and **3** were prepared for anti-KB activity evaluation. Catalytic hydrogenation of the curcuminoids **1, 2** and **3,** with palladium on charcoal as a catalyst, yielded tetrahydrocurcumin (**18**), tetrahydrodemethoxycurcumin (**19**) and tetrahydrobisdemethoxycurcumin (**20**), respectively. The spectroscopic data of compounds **18-21** were consistent with the reported values (Ishida et al., 2002; Lee et al., 2005; Portes et al., 2007; Ma et al., 2004).

2. Cytotoxic Activity of the Analogues

The natural curcuminoids **1–3** and the chemically modified analogues **4-20** were subjected to cytotoxic activity evaluation against the KB cells using the established assay for cancer cell growth inhibition (Brien et al., 2000) and the results are presented in Table **1**. In the assay according to this work, the cytotoxic activity at the concentration of more than 20 µg/ml is regarded as non-cytotoxic or inactive. Compounds **1–3** were inactive according to this criterion. It has been reported that these compounds exhibited weak cytotoxicity against this cancer cell line, with the IC_{50} values of 21.36, 26.45 and 21.44 µM, respectively, which was much less active than ellipticine, the reference anticancer drug, which exhibited cytotoxicity against KB cells at IC_{50} of 2.25 µM (Chuprajob et al., 2014). The reported low cytotoxicity of the natural curcuminoids was in the same trend as that of our work. Partial demethylation of curcumin (**1**) to mono-*O*-demethylcurcumin (**4**) resulted in a sharp increase in cytotoxic activity (IC_{50} value 7.76 µM). Further demethylation to the di-*O*-demethyl analogue **5** also caused a marked increase in cytotoxic activity (IC_{50} value 2.38 µM). The analogue **5** was more active than ellipticine, which exhibited cytotoxic activity at IC_{50} value of 5.40 µM. The presence of free phenolic hydroxyl groups seemed to contribute to high cytotoxicity of curcuminoids. This was further demonstrated by the increase in activity in going from the inactive curcuminoid **2** to the active analogue **6**, the latter of which exhibited cytotoxic activity at IC_{50} value of 8.17 µM. It is also likely that the presence of 1,2-dihydroxyl groups (as in compounds **4, 5** and **6**) contributed to high cytotoxicity against KB cells.

The effects of free hydroxyl groups on cytotoxicity was further studied by the preparation of the methylated analogues of the curcuminoids **1-3.** However, mono-methylation of compound 1 to the corresponding mono-*O*-methyl analogue **7** resulted in an increase in cytotoxicity against the KB cells; the IC_{50} value of **7** was 7.11 μM. Surprisingly, full methylation of 1 to the corresponding di-*O*-methyl analogue 8 with a high cytotoxicity, an IC_{50} value of 3.85 μM. However, mono- and di-methylations of the parent curcuminoid **2** to the analogues 9 and 10 did not increase (or not much increase) in cytotoxicity; the IC_{50} value of 9 and 10 were 28.40 μM and inactive, respectively. Similar trend was also observed for the mono- and di-*O*-methyl ether analogues 11 and 12.

In order to prove that the presence of extra methyl group(s) in compounds **7** and 8 that gave rise to marked increase in cytotoxicity of the parent compound **1** was just to protect free hydroxyl groups or with some other effect, the curcuminoid **1** was acetylated to the mono-*O*-acetate **13** and the di-*O*-acetate **14**. However, in going from compound 1 to the analogues **13** and **14,** no considerable increase in cytotoxicity of the curcuminoid **1** was observed. The result implied that the marked increase in cytotoxicity of **1** after methylation was not just the effect of protection of free hydroxyl groups. The actual effects should be further studied.

The conjugated system in the heptyl side chain is essential for cytotoxicity, since catalytic hydrogenation of the curcuminoids **1-3** to the corresponding tetrahydro curcuminoid analogues **18, 19** and **20,** respectively, did not improve cytotoxicity of the parent curcuminoids. Catalytic hydrogenation of the most active analogue **5** to the corresponding tetrahydro analogue **21** which, upon cytotoxic activity evaluation, this compound gave the inactive analogue. The results clearly indicated that lack of olefinic bonds in the side chain caused complete loss in cytotoxic activity.

Conclusion

We reported the structural modifications of the natural curcuminoids **1-3** to **18** curcuminoid analogues (**4-21**). These curcuminoids and analogues were evaluated for cytotoxic activity against KB cells. The results have indicated that full conjugated diketo function is essential for cytotoxicity. Analogues with more of free hydroxyl groups are more potent than the curcuminoids, with exception for the mono-*O*-methyl and di-*O*-methyl analogues of curcumin (**1**). Among the potent analogues, di-*O*-demethylcurcumin (**5**) was the most potent compound, with the IC_{50} value of 2.38 μM, which was more cytotoxic than ellipticine, the reference anticancer drug.

Suggestion

Further study of the cytotoxic induction against human oral cancer cell line of potent curcuminoid analogues is warranted in order to evaluate the potential of the highly active compounds for anticancer drug development.

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