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Curcuminoids—Cellular Uptake by Human Primary Colon Cancer Cells As Quantitated by a Sensitive Hplc Assay and Its Relation with the Inhibition of Proliferation and Apoptosis

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Curcumin, which is a bright orange-yellow pigment of turmeric with antioxidant properties, has been shown to produce a potent preventative action against several types of cancers in recent studies. It has also been reported to protect the development of colon tumor in animals being fed with carcinogen. In the colon cancer cells, curcumin was illustrated to inhibit cell proliferation and induce apoptosis. As an antioxidant, it acts as an anti-inflammatory as well as an antitumor agent. Curcumin has been detected to exist in nature in the form of curcuminoids, a mixture of curcumin, the major component, with two of its related demethoxy compounds (demethoxycurcumin and bisdemethoxycurcumin). In the present study, we have investigated the antiproliferation and induced apoptosis effects of curcuminoids on colon cancer, using the primary cancer cells isolated from Taiwanese colon cancer patients as the model for colorectal cancer. Results showed that curcuminoids inhibited cell proliferation and induced apoptosis of these human primary colon cancer cells. The effects were observed in a dose-dependent manner as dose increased from 12.5 to 100 μM. With the aim of furthering the fundamental understanding of the mechanisms underlying the antiproliferation and induced apoptosis effects of curcuminoids on these human colon cancer cells, we developed a sensitive, rapid, and reproducible assay method based on high-performance liquid chromatography (HPLC). This HPLC technique developed was found to successfully determine, in a quantitative manner, the cellular uptake of curcuminoids. The uptake of these curcuminoids by the colon cancer cells was shown to increase as the dose of curcuminoids was increased. The observations of inhibited proliferation and increased apoptosis in the colon cancer cells appeared to be associated with the cellular uptake of curcuminoids.

KEYWORDS: Curcuminoids; curcumin; HPLC (high-performance liquid chromatography); human primary colon cancer cells

INTRODUCTION

Turmeric (curcumin) is an herb, from the rhizome of the plant called *Curcuma Longa*, to which many cancer-preventive properties have been attributed (*1*–*3*). It has been a widely known and popularly used spice and coloring agent. It is present in curry and mustard; both have been used extensively in the Asian countries, and in traditional medicine.

Interest in this herb has grown in recent years because of its putative beneficial pharmacological effects, which include antioxidant (*4*), anti inflammatory (*5*), and cancer chemopreventive actions (*6*).

Curcumin is also a potent scavenger of various reactive oxygen species (ROS), including superoxide anions (*7*) and hydroxyl radicals (*8*). In addition, there have been indications that curcumin may help prevent and treat patients with Alzheimer's disease by reducing oxidative damage, plaque burden, and suppressing specific inflammatory factors (*9*, *10*). The low incidence of colon cancer in Asian countries could be attributed to the regular uses of curcumin in the diet besides the low intake of meat (*11*, *12*). In rats and mice, curcumin has been shown to produce a profound effect on colon carcinogenesis, including the rats treated with colon carcinogen azoxymethane (AOM). Also, in a mouse model with a mutation in the APC gene, curcumin was reported to reduce the number of colon tumors (*13*) or decrease the multiplicity of colon adenomas (*14*). Moreover, curcumin was found to protect against the develop-

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ment of colon cancer during both the initiation and the promotion stages in both rats and mice that were fed with AOM (*15, 16*). In addition to the effect on colon cancer, curcumin showed anticancer effects on intestinal cancer, stomach cancer, and hepatocellular carcinoma (*17*).

Curcumin has been demonstrated by in vitro studies to cause a dose-dependent reduction in the proliferation of hepatoma cells and the cell accumulation in the G2/M phase (*18*). In exposure to curcumin, the apoptosis of hepatoma cells was noted to increase (*19*). Also, in other cell lines, e.g., breast cancer cells, prostate cancer cells, and leukemia cells, curcumin has been reported to inhibit cell proliferation and induce apoptosis (*20*). In addition to the inhibition of cell proliferation and the increase in apoptosis, many mechanisms have been proposed to justify the anticarcinogenic effect of curcumin: anti-inflammatory action, antioxidant activity, the induction of phase II detoxification enzymes, the inhibition of cyclooxygenase 2 (COX-2), effect on AP-1 and NFκB transcription factors, the inhibition of matrix metalloproteinase (MMP), effect on protein kinases and others (*21, 22*).

In rats, the oral intake of curcumin has been reported to yield an intestinal absorption of ~60% (*23*). Curcumin and the metabolites formed in the intestine and in the liver were found to be mostly excreted with the feces (*24*). As the colon is known to be exposed to both curcumin and its metabolites, it is a likely target for the anticarcinogenic activity of curcumin. Moreover, the fact that humans have been reported to be capable of consuming curcumin of up to 8 g a day without a toxic effect (*25*) makes curcumin a very interesting chemopreventive agent. Studying the inhibition of cell proliferation and the induction of apoptosis by curcumin could achieve a better insight into the mechanisms underlying the cancer chemoprevention by curcumin.

A variety of methods have been reported for the detection and analysis of curcuminoids (*26–28*). Most are spectrophotometric methods, which express the total yellow color content of the samples (*29*). Although commercial curcumin products contain mixtures of curcumin and its related compounds (demethoxycurcumin and bisdemethoxycurcumin) (*33*), it has been found not possible to quantify these individual ingredients of curcuminoids by the spectrophotometric method. A literature search indicated that liquid chromatography–mass spectrophotometry and radiolabeled determinations have been reported for the detection of curcumin (*30*). Moreover, high-performance liquid chromatography has also been developed for the quantitation of curcumin in biological samples (*31*).

In this report, studies have been initiated to investigate whether the curcuminoids could contribute to the antiproliferation and apoptosis of the primary cancer cells isolated from the colon cancer patients. The aims are to investigate whether the antiproliferation and apoptosis activities of curcuminoids on primary colon cancer cells that were isolated from the colon cancer patients could be explained, qualitatively as well as quantitatively, by the bioavailability of curcuminoids. To help the accomplishment of these research aims, we thus developed a sensitive and reproducible high-performance liquid chromatographic method to quantitate the cellular uptake of curcumin as well as its related compounds by the primary colon cancer cells.

MATERIALS AND METHODS

Human Colon Cancer Specimens. The specimens of human colon cancer were obtained from a total of five colon patients suffering from adenocarcinoma (CP-1, -2, -,3 and -5) or tubulovillous adenoma (CP-4) on the colon (2 cases), sigmoid colon (2 cases), or descending colon (one case). These patients consist of three men (54–82 years old) and two women (62–72 years old) who were receiving treatment in the Kaohsiung Medical University Hospital (KMUH). The specimens were taken during operation for colon cancer removal between the period of Jan 13, 2004, and May 10, 2005. Specimens were removed from only the typical and clinically clear-cut (Grade II) cases (*32*). Prior written informed consent was obtained from the patients and all procedures used had been reviewed and approved by the ethics board at KMUH in adherence to the Helsinki Principles.

Cell Culture. The five primary cell lines of colon cancer cells were derived, as a gift, from the cell bank maintained in the MedicoGenomics Research Center at KMU (*32*). The cells were grown at 37 °C in the Dulbecco's Modified Eagle Medium (GibcoBRL) supplemented with 10% (v/v) fetal bovine serum (HyClone) and a combination of antibiotics (penicillin, 200 unit/ml, and streptomycin, 200 g/mL) (HyClone) under a CO_2/air (5%) atmosphere for this series of studies.

LDH Cell Survival/Cytotoxicity Assay. The survival/cytotoxicity of the primary colon cancer cells following exposure to curcuminoids was assessed by a lactate dehydrogenase (LDH) assay (CytoTox96 nonradioactive cytotoxicity assay kit) using instructions provided by the manufacturer (Promega). Briefly, the cells were first incubated with curcuminoids for 24 h. The cells were then harvested and treated with Substrate Mix for 30 min; the reaction was then stopped by the stop solution (50 μL/well). The extent of the reaction was determined by Powerwave XS reader (Bio-Tek) at 490nm. Each assay was carried out in triplicate and the results were expressed as the mean (−/−SEM). Cell survival was then expressed as the percentage of the assay data for the control group.

Cell Proliferation Assay. The proliferation of the primary colon cancer cells after exposure to curcuminoids was assessed by the CellTiter96 Aqueous one solution cell proliferation assay kit (Promega). Briefly, cells were first incubated with curcuminoids for 24 h and then treated with MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymeth-oxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) for 4 h. The absorbance was determined by the Powerwave XS reader (Bio-Tek) at 490nm. Each assay was carried out in triplicate and the results were expressed as the mean (−/−SEM). Cell proliferation was expressed as the percentage of the assay data determined for the control group.

Evaluation of Apoptosis. The apoptosis was assessed by the APOPercentage Apoptosis Assay kit (Biocolor Ltd. United Kingdom). Briefly, the primary colon cancer cells (5×10^3) were seeded in the culture medium containing 100 μL of serum and dispensed into the 96-well microplates. After being treated with curcuminoids for 24 h, the culture medium was replaced with a fresh medium containing the APOPercentage Dye Label. The change in cell morphology was determined microscopically by Olympus CKX41. The APOP% Dye Release Reagent was added to each well to aid cell lysis and the release of bound dye from the apoptotic cells. The Powerwave XS reader (Bio-Tek) was used to measure the amount of the cell-bound dye recovered in the solution. A reference wavelength of 655 nm was used to estimate the apoptotic index at a measurement wavelength of 550 nm. Each assay was carried out in triplicate and the results were expressed as the mean (−/−SEM) of the absorption at 550 nm.

Mitochondrial Membrane Potential (MMP). The primary colon cancer cells were first seeded in 24-well plates (Orange Scientific. E.U.) for 16–24 h. Following treatment with curcuminoids, Rhodamine 123 (10 μg/mL, Sigma) was added to the culture medium (50 μL/well) and then incubated (37 °C, 20 min) for mitochondria staining. After being washed twice with a warm PBS, the cells were fixed with 2% paraformaldehyde andinspected by fluorescence microscopy (Olympus CKX41 and U-RFLT 50), and the RFU (relative fluorescence unit) was detected for the BioTek FLx800 TBI. For Rhodamine 123, the wavelength settings were 504 and 534 nm. Each assay was carried out in triplicate and the results were expressed as the mean (−/−SEM) of RFU and reported as the percentage of the RFU for the control group.

Preparation of Extracting Buffer. To achieve an efficient recovery of curcuminoids from the colon cancer cells and the culture medium, we developed an extracting solution. It was prepared from the

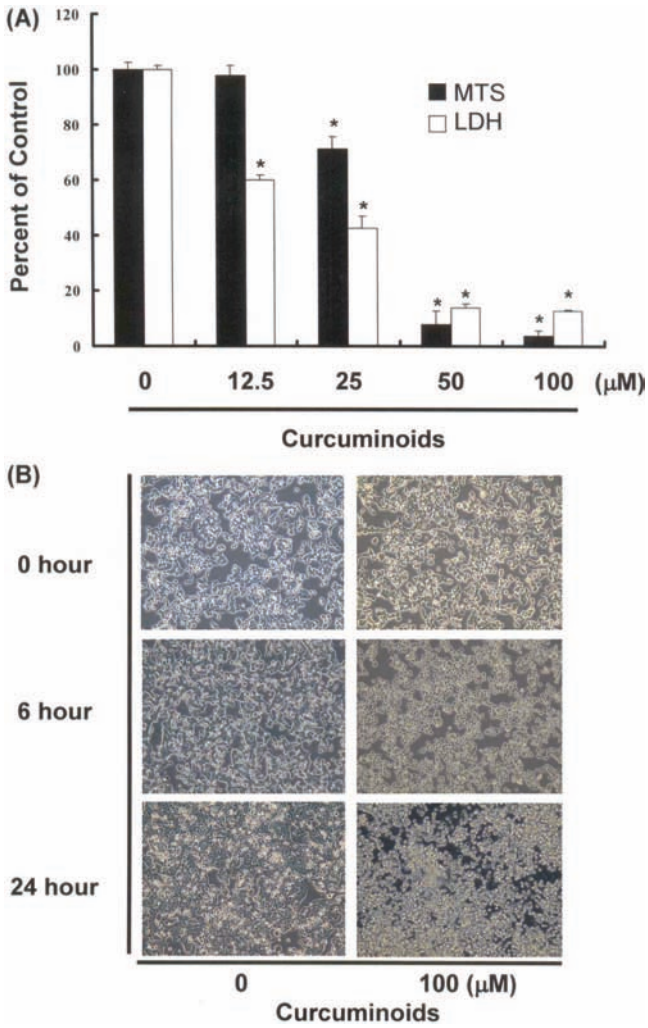

Figure 1. Effect of curcuminoids on the survival/proliferation of human primary colon cancer cells. (**A**) Primary colon cancer cells (five groups, $n = 6$ per group) were treated with curcuminoids (0, 12.5, 25, 50, and 100 μM) and their survival and proliferation were assessed, respectively, by lactate dehydrogenase (LDH) and by MTS assays. The solid bars (for MTS) and open bars (for LDH) represent, respectively, the levels of cell survival and proliferation, shown as the percentage of the controls (treated with 0 μM curcuminoids). All the data shown are the mean (−/−SEM) of at least three independent experiments. The symbol (*) on each bar denotes the difference is statistically significant at the $P < 0.05$ level as compared to the control value. (**B**) The morphology of the human primary colon cancer cells before and after the treatment with curcuminoids (100 μM) for 6 and 24 h. The cells were suspended in the medium.

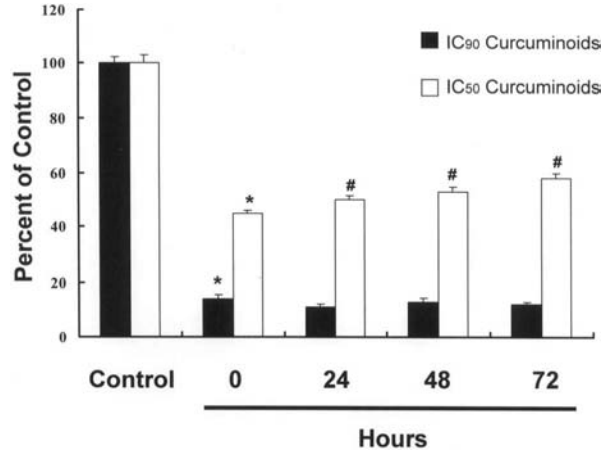

Figure 2. Reversibility of the growth inhibitory effect of curcuminoids. The primary colon cancer cells (five groups; $n = 6$ per group) were each first treated with curcuminoids at IC_{50} (in open bars) or IC_{90} (in solid bars) for 24 h. After the treatment was terminated by washing off curcuminoids, the cultures were reincubated for 24–72 h to check the extend of recovery of cancer cells. All the data shown are the mean (−/−SEM) of at least three independent experiments. The symbol (*) on the bar at the time zero of recovery study denotes the difference is statistically significant at the $P < 0.05$ level as compared to the control value, whereas the symbol (#) on the bar denotes the difference is statistically significant at the $P < 0.05$ level as compared to time 0 of the recovery study (for IC_{50}).

combination of ethyl acetate and isopropanol (9:1 (v/v)) and stored at room temperature until use (the solution was found to be stable for at least one month).

Recovery of Curcuminoids from Culture Medium for Assay. Culture medium (500 μL) was first acidified with 6N HCl (1:1 (v/v)) and vortexed for 30 s. Following addition of the extracting buffer (500 μL) prepared above, samples were each vortexed again and then shaken in the Orbited shaker (at 100 rpm) for 15 min. After centrifugation at 18000 rpm for 20 min, the upper organic layer was filtered through a membrane filter (0.22 μm) and then transferred to a clean injection sample vial (about 100 μL) for the quantitative analysis by the HPLC methods described below.

Recovery of Curcuminoids from Colon Cancer Cells for Assay. The cell pellets were each resuspended in the RIPA buffer (20 mM Tris/HCl at pH 8.0, 137 mM NaCl, 10 % of glycerol, 5 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride, 1.5 mg of leupeptin, and protease inhibitor cocktail) and the cell-liquid extraction was carried out. The cell extracts were cleared by centrifugation at 18000 rpm for 15 min. The amount of total cellular proteins in the extracts was determined using the Bradford method (Bio-Rad, USA). The cell extract (100 μg) obtained was each acidified by 6N HCl (1:1 v/v) and vortexed for 30 s. The extracting buffer (500 μL) was then added to each of the acidified cell extracts, and samples were vortexed again and shaken in the Orbited shaker (at 100 rpm) for 15 min. After centrifugation at 18000 rpm for 20 min, the upper organic layer was filtered by a membrane filter (0.22 μm) and transferred to a clean injection sample vial (about 100 μL) for quantitative analysis by the HPLC methods described below.

HPLC Analysis of Curcuminoids. the HPLC system used to carry out the HPLC analysis of curcuminoids was a Hitachi L-2000 series high-performance liquid chromatograph system (Hitachi, Japan), which consisted of a solvent delivery system (L-2130 model) equipped with a fluorescence detector (L-2480 model), an autosampler (L-2130 model), and a reversed-phase column (Purospher STAR RP-18e, 5 μm, Merck) maintained at 37 °C by a column oven (L-2350 model). A mobile phase, which was composed of acetic acid (2 %), pH 2.5, and acetonitrile (at a ratio of 47:53) and delivered at a flow rate of 0.8 mL/min, was used, and the samples were each injected at an injection volume of 20 μL. With the use of an excitation wavelength at 420 nm and emission wavelength at 540 nm, curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (bDMC) were detected.

Statistical Evaluation. All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis was conducted using a one-way ANOVA test, with the significant differences determined at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Curcuminoids Inhibit the Cell Survival/Proliferation of Primary Colon Cancer Cells. We hypothesized that curcuminoids could mediate the survival of primary colon cancer cells and thus inhibit their proliferation. To explore this antitumor activity of curcuminoids against the primary colon cancer cells, we initiated an in vitro study and exposed the primary colon cancer cells to varying doses of curcuminoids for 24 h. The survival and proliferation of cancer cells were then assessed, respectively, by MTS and LDH assays. The results summarized in **Figure 1A** indicate that the survival and proliferation of the

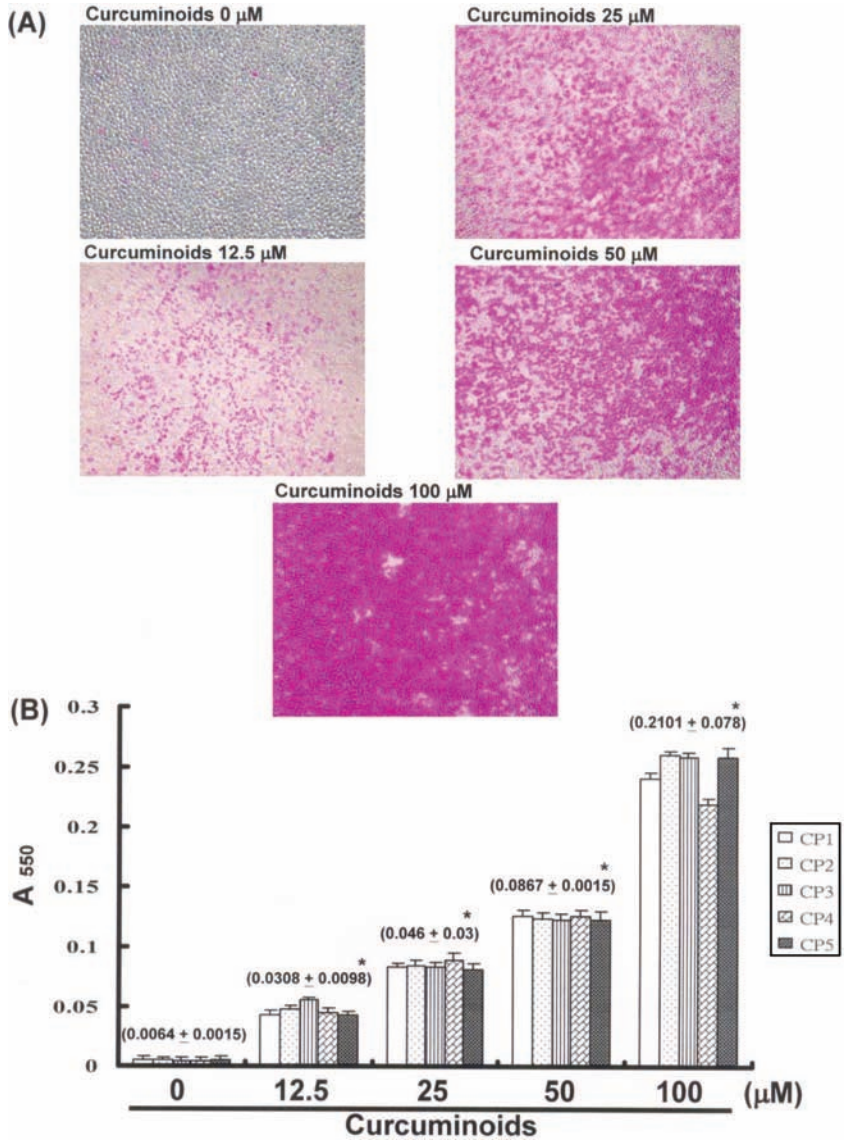

Figure 3. Apoptosis of primary colon cancer cells induced by curcuminoids treatment. The apoptosis was determined by APOPercentage Apoptosis Assay kit after cells had been exposed to curcuminoids (0, 12.5, 25, 50, and 100 μM) for 24 h. The assay was performed to identify apoptotic cells in the curcuminoid-treated cultures: (**A**) the pink-colored deposits shown are indicative of the positive presence of apoptotic cells. The cell morphology was determined microscopically by Olympus CKX41. (**B**) The bars represent the levels of apoptotic cell in the primary colon cancer cells (isolated from five Taiwanese colon cancer patients (CPs)). All the data shown are the mean (−/−SEM) of at least three independent experiments. The symbol (*) on each set of data denotes that the difference from the treatment with 0 μM curcuminoids is statistically significant at the $P < 0.05$ level.

primary colon cancer cells both decrease as the dose of curcuminoids added into the cell culture increases, which shows a dose- dependent reduction (The IC_{50} and IC_{90} of curcuminoids in the five primary colon cancer cells were determined, respectively, to be 23–28 μM and 47–54 μM). Moreover, curcuminoids were noted to induce a morphological change in the primary colon cancer cells. A microscopic examination showed that following the exposure to curcuminoids (100 μM) for 6–24 h, the primary colon cancer cells displayed a remarkable change in their morphology (**Figure 1B**). The curcuminoids induced the death of cancer cells, which formed a suspension in the medium.

Growth-Inhibitory Effect of Curcuminoids Is Partially Irreversible. To study whether the growth-inhibitory effect of curcuminoids is reversible, the primary colon cancer cells were recultivated in a fresh culture medium after their exposure to curcuminoids (at either IC_{50} or IC_{90}) for 24 h; the recovery of cell proliferation was then assessed for additional 24–72 h and analyzed by the MTS assay. The results in **Figure 2** suggest that the cancer cells have substantially lost their ability to proliferate following the curcuminoids treatment for 24 h. The loss of recoverability appears to depend on the dose of curcuminoids used in the treatment: The cancer cells treated with curcuminoids at the dose of IC_{90} have a remarkably lower recoverability than those treated with IC_{50}. No significant difference was noted in the recovery of proliferation for the IC_{90}-treated cancer cells even after incubation for up to 72 h (**Figure 2**). The observations could imply that the primary colon cancer cells have undergone an irreversible change, such as apoptosis, at least to a partial extend.

Curcuminoids Induce Apoptosis of Primary Colon Cancer Cells. To explore the potential role that curcuminoids could play in the apoptosis of primary colon cancer cells, we used the APOPercentage Apoptosis Assay kit to identify the existence of apoptotic cells in the five primary colon cancer cells after the 24 h exposure to curcuminoids. A typical set of results for APOPercentage Apoptosis assay is illustrated in **Figure 3A**, in which the pink-colored deposits are indicative of the positive

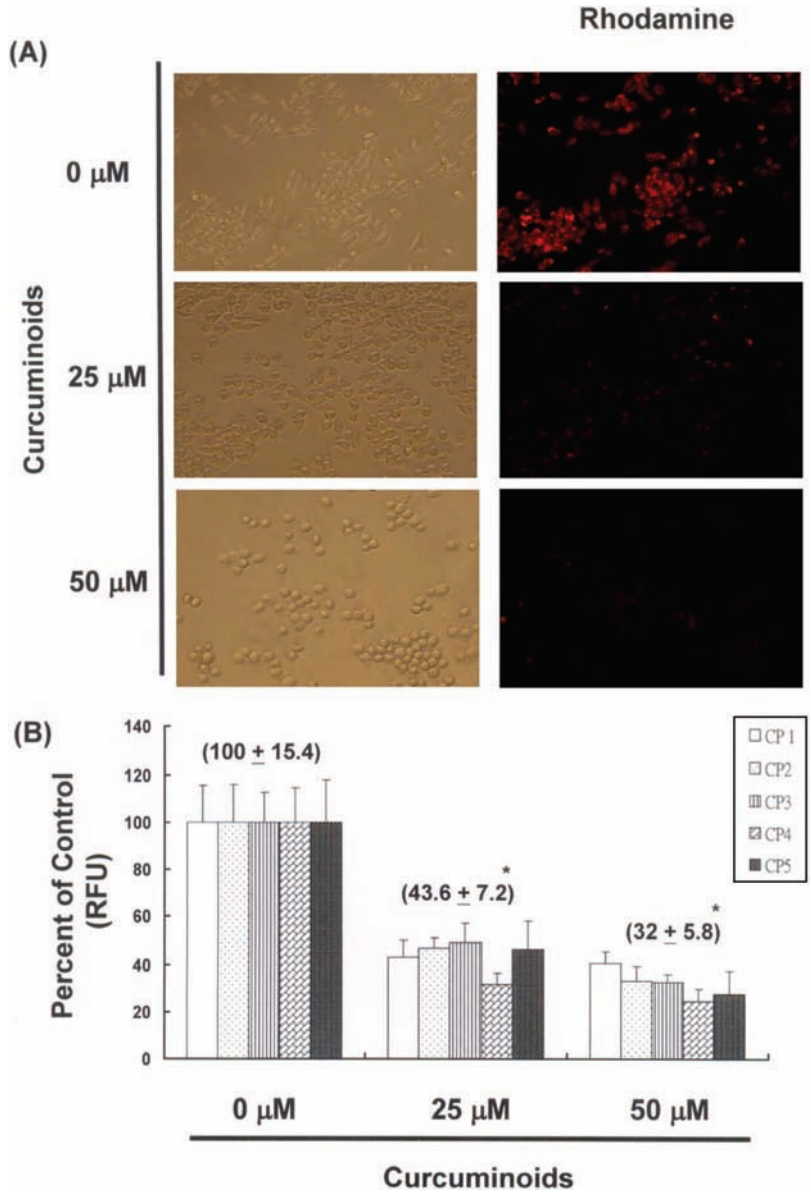

Figure 4. Reduction of the mitochondrial membrane potential (MMP) in the primary colon cancer cells by curcuminoids, which was determined by Rhodamine staining and detected by fluorescence microscopy: (**A**) By the measurement of red fluorescence, MMP is shown to be significantly reduced in the primary colon cancer cells treated with curcuminoids (25 and 50 μM) compared to those in the controls (treated with 0 μM curcuminoids). (**B**) Using BioTek FLx800 TBI fluorescence reader, we determined the intensity of fluorescence and found it to decline (presented as the percentage of the controls) as the concentration of curcuminoids used to treat the primary colon cancer cells (isolated from five Taiwanese colon cancer patients (CPs)) was increased. All the data shown are the mean (−/−SEM) of at least three independent experiments. The symbol (*) on each group of bars denotes that difference from the treatment with 0 μM curcuminoids is statistically significant at the $P < 0.05$ level.

existence of apoptotic cells. A dose-dependent increase in apoptosis was observed, that is, the higher the dose of curcuminoids (0, 12.5, 25, 50, 100 μM), the greater the extent of apoptosis (**Figure 3A**). The quantitative measurements of the apoptosis are outlined in **Figure 3B** and indicate that the curcuminoid-induced apoptosis of primary colon cancer cells has increased in proportion to the dose of curcuminoids used. The data indicate that the extent of apoptosis shows a 4.8- to 32.8-fold increase over the control as the dose of curcuminoids varies in the range of 12.5–100 μM (all are significantly different from the control group at $P < 0.05$). Taken together, the observations imply that the curcuminoids have significantly elevated the apoptosis of all five primary colon cancer cells.

Curcuminoids Reduces the Mitochondrial Membrane Potential in Primary Colon Cancer Cells. To explore the possible effect of curcuminoids on the mitochondrial membrane potential (MMP) in the primary colon cancer cells, we used Rhodamine 123 to determine the MMP in the curcuminoid-treated cancer cells. The results compared in **Figure 4A** indicate that the MMP of the primary colon cancer cells has been significantly reduced after treatment with 25 or 50 μM curcuminoids. The results summarized in **Figure 4B** indicate that the intensity of fluorescence, as determined by the BioTek FLx800 TBI fluorescence reader, decreases as the curcuminoid dose increases. The observations imply that the reduction of MMP in the primary colon cancer cells depends on the dosage of curcuminoids used.

Development of a Sensitive and Reproducible Hplc Assay Method for Curcuminoids Assay. To characterize the kinetics of uptake of curcuminoids by the primary colon cancer cells, we have developed a sensitive HPLC method for achieving the

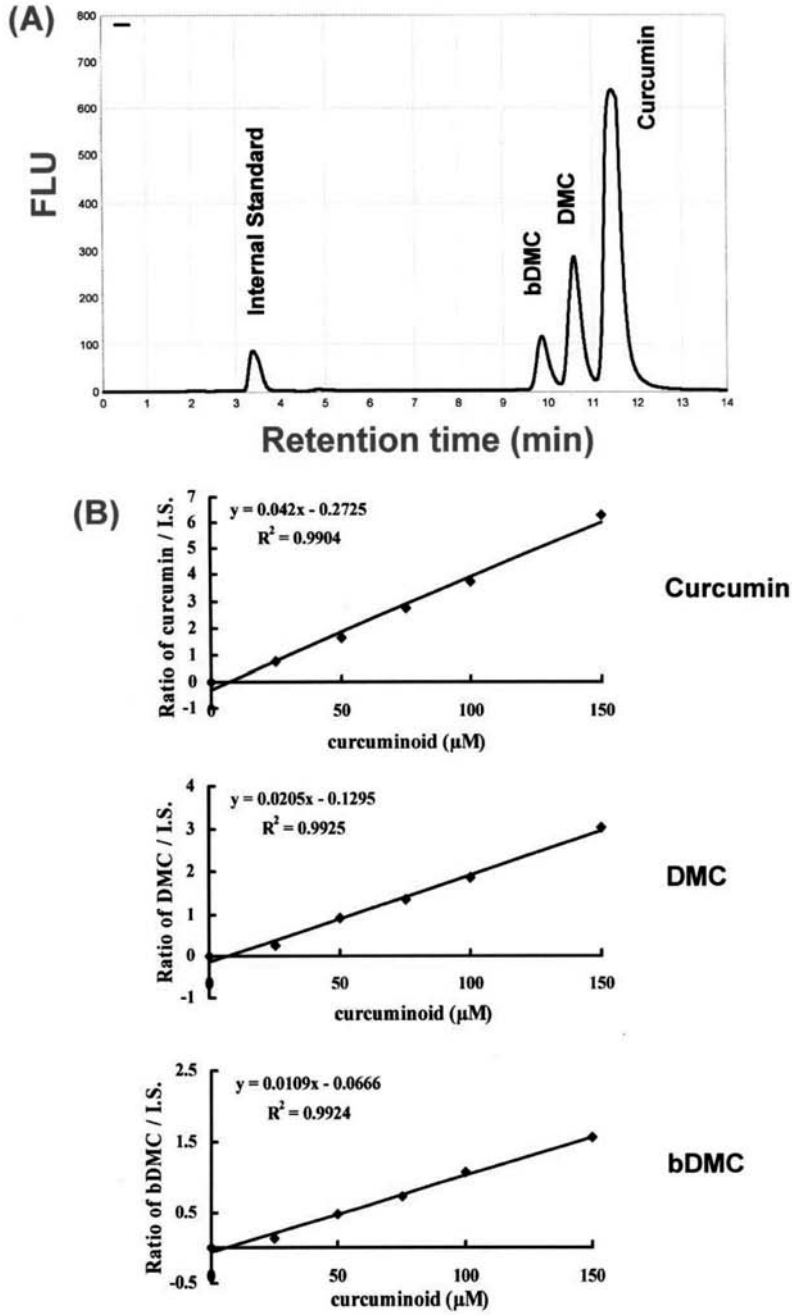

Figure 5. HPLC chromatogram of the curcuminoids recovered from the medium used for the culture of primary colon cancer cells. (**A**) Using the HPLC conditions developed, the characteristic peaks for the components in the curcuminoids, curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (bDMC), have been well separated. (**B**) Linear relationship between the peak area of HPLC peaks and the concentrations has been well-established for curcumin, DMC, and bDMC.

reproducible and quantitative analysis of curcuminoids. Using the HPLC system and conditions developed, as outlined above in the Methods, we found the major components of the curcuminoids, which consist of curcumin, demethoxycurcumin (DMC), and bisdemethoxy-curcumin (bDMC) (*33*), were found to yield three well-defined peaks (**Figure 5A**). Furthermore, the results in **Figure 5B** indicate that the area under the peaks for curcumin (**Figure 5A**), produced from a series of 6 standard curcuminoids solutions with concentrations in the range of 0–150 μM has yielded a perfect linearity with the concentrations of curcuminoids (with a correlation coefficient of 0.99). The same linearity has also been observed to exist for the peak areas determined for DMC and bDMC (with a correlation coefficient above 0.99).

Determination of Accuracy and Precision of Hplc Analysis. The accuracy and precision of the HPLC analytical method developed for the assay of curcuminoids was confirmed by attainment of a correlation coefficient of >0.99 for all the standard curves constructed from the assay of six standard solutions of curcuminoids (**Figure 5B**). Furthermore, the preparation and assay of these standard solutions were repeated on three different days to check the day-to-day reproducibility. Results indicated that analyses of the standard solutions prepared on three different dates have all yielded the same linear peak area–concentration relationships, similar to **Figure 5B**, for crcumin, DMC, and bDMC. Statistical analysis of these slope values showed that the HPLC assay achieved a day-to-day reproducibility of 2.98% for curcumin, 1.39% for DMC, and 3.12% for bDMC.

Table 1. Quantitative Assay of Curcumin, Demethoxycurcumin (DMC), and Bisdemethoxycurcumin (bDMC) Uptake by the Primary Colon Cancer Cells, Presented as μG/mg of Total Cellular Proteins, and that Remaining in the Culture Medium, Presented as μG/Culture, Following the Curcuminoids Treatment (0, 50, 100, or 150 μM) for 24 h [a]

	culture medium (area)			primary colon cancer cells (area/mg of total protein)		
curcuminoids (μM)	curcumin	DMC	bDMC	curcumin	DMC	bDMC
0	ND[b]	ND	ND	ND	ND	ND
50	83847 ± 2634[c]	82354 ± 1747[c]	57204 ± 3107[c]	15639 ± 2531[c]	11903 ± 1489[c]	8138 ± 564[c]
100	788879 ± 7362[c]	411499 ± 5912[c]	304717 ± 4055[c]	227096 ± 6827[c]	75709 ± 3379[c]	42821 ± 2113[c]
150	17818069 ± 23580[c]	7646524 ± 16837[c]	4100983 ± 37882[c]	642224 ± 11240[c]	132462 ± 4921[c]	105863 ± 5568[c]

[a] Data shown are the mean (+/−SEM) of at least three independent experiments (as those shown in Figure 6). The symbol (*) on each set of mean (+/−SEM) data denotes the difference from the treatment with 0 μM curcuminoid is statistically significant at $P < 0.05$. [b] ND = non-detectable. [c] $P < 0.05$ versus curcuminoid 0 μM.

Determination of Efficiency and Precision of Extraction. the efficiency and precision of the extraction method developed for the recovery of curcuminoids was assessed by analyzing the results obtained by replicating the extraction procedure on the sample pools of curcuminoids at three concentrations, high (150 μM), medium (100 μM), and low (50 μM), prepared with the culture medium as well as with the pellets of primary colon cancer cells. Assay of the samples after the extraction was found to yield a recovery rate of 61.57(−/−1.17)% and 77.79(−/−1.07)% for curcumin, 56.27-(−/−7.65)% and 66.95(−/−4.1)% for DMC, and 50.78-(−/−5.32)% and 68.92(−/−3.64)% for BDMC (respectively, from the cell pellets and the culture medium). These rates of recovery have been utilized in the accurate determination of the amounts of curcumin, DMC, and bDMC recovered from the cancer cells and culture medium (**Table 1**). With this information, we could determine the uptake of curcumin, DMC, and bDMC by the cancer cells as a function of the dose of curcuminoids (**Figure 7**). Similarly, the drug distribution between the cancer cells and the culture medium could also be assessed.

Uptake of Curcuminoids in Primary Colon Cancer Cells. To study the kinetics of cellular uptake of curcuminoids by human primary colon cancer cells during the course of 24 h treatment with curcuminoids, we extracted the levels of curcuminoids in the cancer cells, using the cell-liquid method described above, while the content of curcuminoids in the culture medium was also extracted, using the liquid–liquid method described earlier. The results in **Figure 6** indicate that using the extraction methods developed, we have satisfactorily recovered the major components of curcuminoids, curcumin, DMC, and bDMC, from the cancer cells and their culture medium. Compared to the controls, the HPLC chromatogram for the cell culture treated with curcuminoids (150 μM) for 24 h have displayed the well-defined peaks for the curcumin, DMC, and bDMC (**Figure 6B** for the cancer cells, and **Figure 6A** for the culture medium). From the peak area under the specific peaks, the amounts of curcumin, DMC, and bDMC taken up by the colon cancer cells and those remaining in the culture medium could be separately determined.

The results summarized in **Table 1** indicate that the cellular uptake of Curcumin, DMC, and bDMC by the primary colon cancer cells, presented as the ratio of μg/mg of total proteins, has increased in a dose-dependent manner with the increase in the dose of curcuminoids added into the cell culture. It is interesting to note that the amount of curcuminoids, which is the sum of curcumin, DMC, and bDMC, taken up by the primary colon cancer cells has increased in proportion to the dose of curcuminoids up to 100 μM (**Figure 7A**), whereas the distribution of curcumin, DMC, and bDMC, presented as the percentage of the amount of curcuminoids taken up into the cancel cells, was observed to increase at first and then level off after

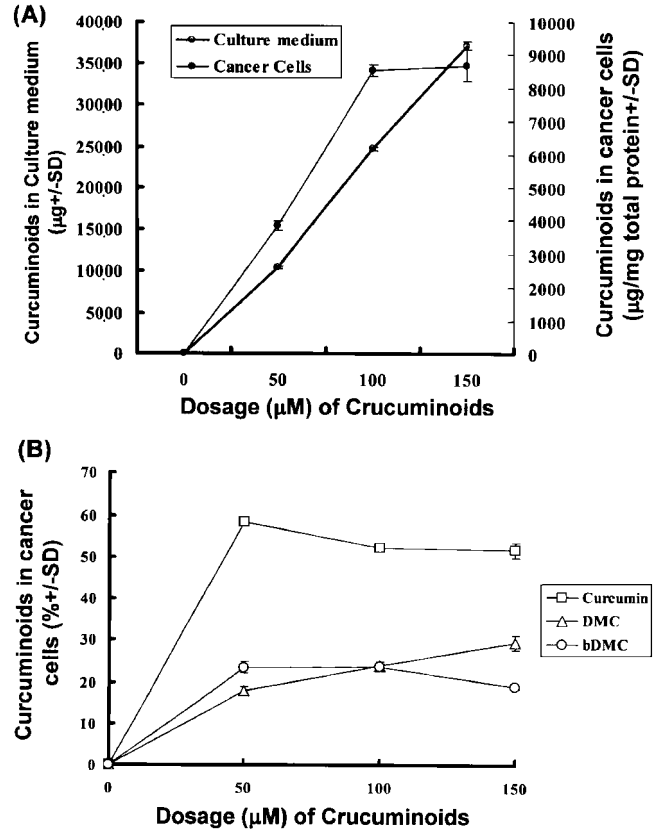

Figure 6. HPLC assay of curcuminoids in the culture medium and in the primary colon cancer cells: (**A**) The HPLC chromatograms of curcuminoids in culture medium having been treated with curcuminoids (at 150 μM), versus the control (at 0 μM), for 24 h. (**B**) The HPLC chromatograms of curcuminoids recovered from the primary colon cancer cells having been exposed to curcuminoids (at 150 μM), versus the control (at 0 μM), for 24 h.

curcuminoids had reached the dose of 50 μM. Comparing the dose-dependent effect of curcuminoids on the survival of primary colon cancer cells (LDH assay in **Figure 1A**) with the cellular uptake profile of curcumin (**Figure 7A**) suggests that the reduction in the survival of colon cancer cells could be attributed to the increased uptake of curcuminoids into the cancer cells. The same correlation may also be established for the inhibited proliferation of cancer cells (MTS assay in **Figure 1A**) with the increased cellular uptake of curcuminoids (**Figure 7A**). Similarly, the increased apoptosis observed in the primary colon cancer ells (**Figure 3**) may also be attributed to the increase in the uptake of curcuminoids by the cancer cells (**Figure 7A**).

Collectively, the data illustrated by **Figures 1**, **3**, and **7** could imply that the increased uptake of curcumin, DMC, and bDMC

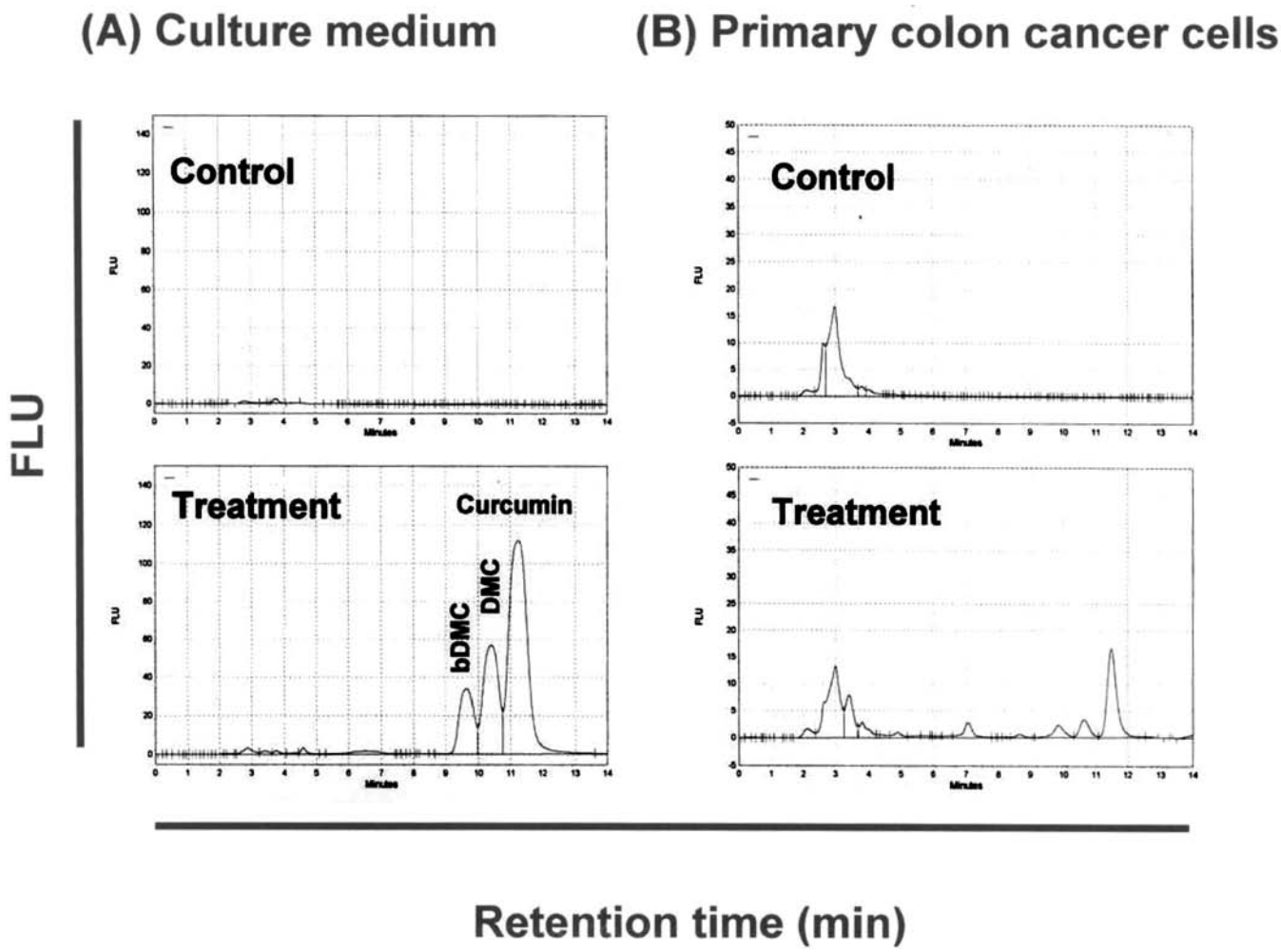

Figure 7. The cellular uptake of curcuminoids by the primary colon cancer cells and the differential distribution of curcumin, DMC, and bDMC in the cancer cells: (**A**) The uptake of curcuminoids by the primary colon cancer cells as a function of the dose of curcuminoids added into the cell culture. The amounts of curcuminoids remained in the culture medium are also presented for comparison. Plotted from the data in Table 1. (**B**) The distribution of curcumin and its related compound (DMC and bDMC), presented as the percentage of the total amount of curcuminoids taken up by the primary colon cancer cells (determined from the data in Table 1), as a function of the dose of curcuminoids added into the culture of primary colon cancer cells.

and their accumulation in the primary colon cancer cells could play an important role in their growth arrest and cell death.

The elimination of cell proliferation and apoptosis has been regarded as one of the key strategies for cancer prevention (*33*). Development of rapid, sensitive, and reliable assays for measurement of cell proliferation and apoptosis should benefit the studies of cancer chemoprevention.

Curcumin is the major yellow pigment extracted from turmeric, a commonly used spice derived from the rhizome of the herb *Curcuma longa* Linn. In the Indian subcontinent and Southeast Asia, curcumin has been used as a traditional treatment of inflammation, skin wounds, and tumors (*34–36*). In several animal models, curcumin has been shown to have cancer chemopreventive, antineoplastic, and anti-inflammatory properties (*37*). Curcumin is also a potent scavenger of various reactive oxygen species (ROS), including superoxide. There have been indications that curcumin may help in the prevention and treatment of patients with Alzheimer's disease by reducing the oxidative damage, plaque burden, and suppression of specific inflammatory factors (*38*). In rodents, curcumin has been reported to yield a poor systemic bioavailability following an oral dosing (*39*), which may be related to its inadequate intestinal absorption and extensive metabolism in the GI tract. Curcumin bioavailability may also be poor in humans, as disclosed by a recent pilot study of the standardized *Curcuma* extract in the colorectal cancer patients (*40*).

In a number of cell types, the curcumin was found to produce an anticancer activity and induce apoptosis. However, no direct evidence has been shown on the effect of curcumin-stimulated anticancer in the primary colorectal cancer cells. Our studies have provided a strong evidence to indicate that curcumin may irreversibly induce the apoptosis of primary cancer cells isolated from the colon cancer patients.

The results in **Figures** 5 and **6** demonstrated that the analytical methods developed for the analysis of curcuminoids has shown several advantages, which include: (i) the short running time ($<$15 min) for the quantitative assay of curcumin and its related compounds (DMC abd bDMC) coexisting in the curcuminoids, (ii) a simple extraction procedure for achieving a highly efficient recovery of curcumin and its related compounds from the cell culture and culture medium, and (iii) high degree of assay sensitivity with good correlation. Moreover, the method developed in this series of studies has achieved a sensitivity of detection better than those reported in the literature (*41–43*).

Compared with the previously described methodologies, the HPLC method developed in the present study for the assay of curcumin and its related compounds is rapid, sensitive, accurate, reproducible, and easily standardized. We believe the method

reported here will be a useful analytical tool in the future for the qualitative and quantitative determination of curcuminoids in cells, tissues, or organs for a wide range of research purposes and clinical applications.

ABBREVIATIONS USED

DMC, dimethoxycurcumin; bDMC, bis-dimethoxycurcumin; HPLC, high performance liquid chromatography; AOM, azoxymethane; MTS,(3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymeth-oxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium); LDH; lactate dehydrogenase.

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