

นิพนธ์ต้นฉบับ**Abstract : The cytotoxic effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin purified from Turmeric powder on leukemic cell lines****Songyot Anuchapreeda^a, Wittawat Sadjapong^a, Chadarat Duangrat^b, and Pornngarm Limtrakul^c**

Curcuminoids, major active components of the food flavor turmeric (*Curcuma longa* Linn.); consist of curcumin, demethoxycurcumin and bisdemethoxycurcumin that exhibit anticarcinogenic properties *in vivo*. This study was aimed to investigate the effect of curcuminoids on the cytotoxicity of the leukemic cells, HL60 (human promyeloid leukemia), U937 (human monocytic leukemia) and K562 (human erythroid leukemia). In this experiment, three major natural curcuminoids, pure curcumin, demethoxycurcumin and bisdemethoxycurcumin, isolated from turmeric powder, were compared for cytotoxicity on leukemic cell lines by MTT assay and trypan blue exclusion method. The result showed that all three curcuminoids exhibited an excellent cytotoxic activity on leukemic cell lines with the inhibitory concentration at 50% (IC_{50}) approximately 7 μ g/mL in HL60 and U937 and 20 μ g/mL in K562 cell line. Demethoxycurcumin seem to be the most effective in all leukemic cell lines. The inhibitory effect of three forms of curcuminoids was not statistically different in each leukemic cell line. These results indicate that three major forms of curcuminoids affect the cell viability in all leukemic cell lines by MTT assay and Trypan blue exclusion method. Thus curcuminoid treatment may provide a guideline for molecular study and clinical treatment in leukemic patients in future. Bull Chiang Mai Assoc Med Sci 2006; 39: 60-71.

Key words: Curcumin; Demethoxycurcumin; Bisdemethoxycurcumin; Leukemic cell lines

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1. Introduction

Cancer is one of the major health concerns in the world today. An estimated 6 million new cases are diagnosed each year worldwide.¹ Cancer is a main cause of death in Thailand.² Almost half of the new cases of cancer are diagnosed early enough to be treated with surgery or radiotherapy. In the remaining cases, however, the cancer has metastasized and must be treated with chemotherapy. Chemotherapy can effectively cure several cancers, such as leukemia, lymphomas, choriocarcinoma, and testicular cancers. Chemotherapy can improve the long-term survival for patients with breast cancer, the most common malignancy in women.

Herbs were used as drugs, spices, and a few types were used for food element in daily life: for example, turmeric, ginger and pepper. These herbs show various biological activities, such as anti-inflammatory, anti-mutagen, strong antioxidant, anti-thrombotic action, antimicrobial action, antiparasitic action and oncogene expression inhibitor. One of the pharmacologically safe compounds that inhibit the proliferation of tumor cells and have a potential to be used as anticancer agents, is a group of

turmeric curcuminoids.

Curcumin (diferuloyl methane), a phenolic compound believed to be the main pharmacological agent in turmeric, possesses antioxidant activity *in vitro*,³⁻⁶ and is used in lipid peroxidation tests.⁷⁻⁹ Curcumin is effective in preventing and ameliorating gastric lesion. It also possesses anti-inflammatory,^{10,11} antibacterial,¹²⁻¹⁴ anti-fungal and anti-yeast,¹⁵ antihypocholesterolemic,^{6, 17} anticancer [18-23], antimutagen,^{24, 25} antiparasitic [26], antitumor-promoting²⁷ antiproliferative,²⁸ MDR modulator effect,²⁹⁻³¹ etc.

Curcuminoids are composed of three major compounds: curcumin, demethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III). These 3 major pigments can absorb the visible light at the wave length between 420-425 nm.

The structures of the three main curcuminoids isolated from turmeric powder are shown in Figure 1. Commercial grade curcuminoids (such as Sigma-Aldrich) when isolated from the powdered dry rhizome of *Curcuma longa* Linn contains approximately 77% curcumin, 17% demethoxycurcumin and 3% bisdemethoxycurcumin.

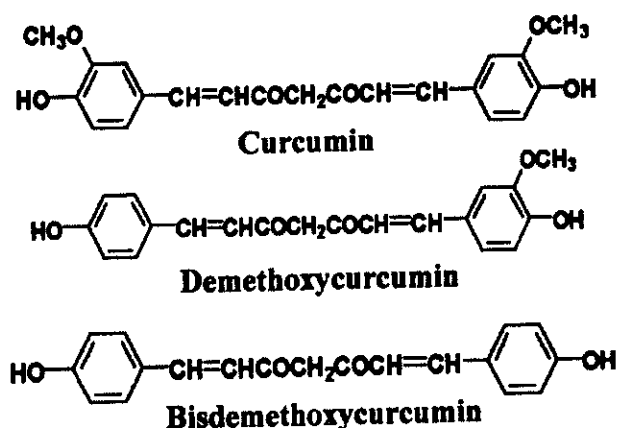


Fig. 1 Chemical structures of curcumin, demethoxycurcumin and bisdemethoxycurcumin

The safety of *Curcuma longa* and its derivatives has been studied in various animal models.³² It is clear that turmeric is not toxic to animals even at high doses. A single feeding of a 30 percent turmeric diet to rats did not produce any toxic effects. In a 24 h acute toxicity study, mice were fed turmeric extract at daily dosages of 0.5, 1.0 and 3.0 g/kg. There was no increase in mortality rate when compared to the respective controls. A 90-day feeding of turmeric extract resulted in no significant weight gain.³³

Studies of the effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin and tetrahydrocurcumin on TPA induced tumor promotion have found that pure curcumin and demethoxycurcumin had an equally potent inhibitory effect on TPA induced increases in tumor promotion in DMBA-initiated mouse skin. Bisdemethoxycurcumin and tetrahydrocurcumin were less active.³⁴

The cytotoxicity of curcumin was examined in cancer cell lines; Hep-2 (human larynx), PC-9 and PC-14 (human lung cancers), Hep-1 (mouse hepatoma) and F-25 (mutate H-ras transfected NIH mouse fibroblast) by MTT assay. It was found that curcumin is a potent antiproliferative agent for several cancer cell lines.³⁵

Cytotoxicity evaluation *in vitro* is usually made by using cell viability assays, such as the uptake of a dye by dead cells after breakdown of the cellular permeability barrier (ex. Trypan blue, eosin Y, etc.) or mitochondrial function (ex. MTT or XTT assay), but other parameters, such as changes in cell morphology under microscopic examination, have also been used as indicators of compound toxicity. In this experiment, we determine the cytotoxicity of curcuminoids by MTT assay and trypan blue exclusion method. This study showed which curcuminoids exhibited the most effect in leukemic cell lines. The reduction of MTT ([3-(4,5-dimethylthiazol-2-yl)2,

5-diphenyl tetrazolium bromide]) in the cell assesses the functional intactness of mitochondria based on the enzymatic reduction of the tetrazolium salt by the mitochondrial dehydrogenase in viable cells. MTT is probably the most commonly used colorimetric indicator of cell viability and it has been used to evaluate cytotoxicity in a quantitative way in contrast with cell morphology evaluation by inverted light microscopy which is qualitative and more subjective.

2. Materials and methods

2.1 Chemicals

Silica gel 60 and petroleum ether was purchased from Merck. An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) and trypan blue dye were purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640, Penicillin-Streptomycin, Fetal bovine serum and L-glutamine were purchased from InvitrogenTM Life Technology (Carlsbad, CA, USA).

2.2 Extraction and isolation of curcuminoids

Chiang Mai turmeric powder (1 Kg) was successively extracted with hexane (2.5 L) and 95% ethanol (7.5 L) at room temperature. Turmeric curcuminoids were then precipitated with petroleum ether yielding 50 g crude curcuminoid mixtures (78% curcumin, 16% demethoxycurcumin and 5% bisdemethoxycurcumin). The crude curcuminoids (3 g) were further fractionated by Silica gel 60 column chromatography (44 X 1.6 cm) using first CHCl_3 and followed by $\text{CHCl}_3/\text{MeOH}$ with increasing polarity. Fractions containing curcumin (1.11 g) were eluted with 100% CHCl_3 (0.6 L). Fractions containing demethoxycurcumin (200 mg) and

bisdemethoxycurcumin (40 mg) were further eluted with $\text{CHCl}_3/\text{MeOH}$ (98:2, 0.8 L) and $\text{CHCl}_3/\text{MeOH}$ (95:5, 1 L), respectively.^{30, 31} The purity of curcumin, demethoxycurcumin and bisdemethoxycurcumin by HPLC analysis was 100%. These curcuminoids were used in the experiments described here.

2.3. Cell culture conditions

The erythroid leukemic cell line (K562) was a generous gift from Dr. Chaisuree Supawilai (Research Institute for Health Sciences, Chiang Mai, Thailand). Human promyeloid leukemia (HL60) was a generous gift from Dr. Rattana Bunjertpongchai (Department of Biochemistry, Faculty of Medicine, Chiang Mai University) and human monocytic leukemia (U937) was a generous gift from Dr. Watchara Kasinrek (Department of Immunology, Faculty of Associated Medical Science). These leukemic cell lines were cultured in RPMI 1640 medium supplemented with 1 mM L-glutamine, 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin, 10% inactivated FCS, and adjusted to pH 7.2 by the addition of 15 mM HEPES. All cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C.

2.4 Determination of cell viability

Cell viability was determined by MTT test method and confirmed by trypan blue exclusion test [36]. MTT (5 mg/mL) was dissolved in PBS. The solution is filtered through a 0.2 μm filter and stored at 2–8°C for frequent use. Cells were cultured in 96-well plates ($3.0 \times 10^4/\text{well}$) containing 100 μL medium, before curcuminoid treatment at 37°C for 24 hrs. After 24 hrs, the cultured medium was added with 100 μL fresh medium containing various con-

centrations of curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin), and incubated for another 24 hrs. Diluted curcuminoid solutions were freshly prepared in DMSO prior to each experiment. The metabolic activity of each well was determined by the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay and compared with those in untreated cells. After removal of 100 μL medium, MTT dye solution was added (15 μL / 100 μL medium) and the plates were incubated at 37°C for 4 hours in humidified 5% CO_2 atmosphere. After 4 hours, the solvent (DMSO), 100 μL , was added to each well, mixed thoroughly to dissolve the dye crystals and the absorbance was measured using an ELISA plate reader (Biotek EL 311) at 570 nm with a reference wavelength of 630 nm. High optical density readings correspond to a high intensity of dye color, i.e., to a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated by the following formula:

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

Trypan blue exclusion method [36] is a typical method to measure the cell viability. The intact membrane of viable cells excludes the trypan blue dye. Trypan blue enters dead cells whose membrane becomes permeable. Trypan blue assay was used to evaluate cell viability as described previously.³⁷ This microscopic assay for cell death was carried out by assessing the ability of live cells to exclude trypan blue dye. A 50 μL aliquot of the cells, after curcuminoid treatment as described by MTT assay, was stained with 50 μL of 0.2% trypan blue and cells were monitored under a light microscope (40X magnification). Stained cells were considered to be no longer viable.

2.5. Statistical analysis

Data were the mean \pm standard deviation of mean from triplicate samples of three independent experiments. Differences between the means were analyzed by one-way ANOVA analysis of variance. Statistical significance was considered when $P < 0.05$.

3. Results

The crude curcuminoids were separated into three components by silica gel 60 column chroma-

tography. In order to further confirm the purity of curcuminoids, HPLC was employed. The standard curcuminoids (Sigma-Aldrich) showed three peaks, with the retention time of 7.087, 8.115 and 9.275 min corresponding to curcumin, demethoxycurcumin and bisdemethoxycurcumin, respectively. This was verified by subjecting purified curcuminoids to HPLC analysis and curcumin, demethoxycurcumin and bisdemethoxycurcumin eluted at 7.072, 8.128 and 9.258, respectively. The purity of each curcuminoid was 100% (Fig. 2).

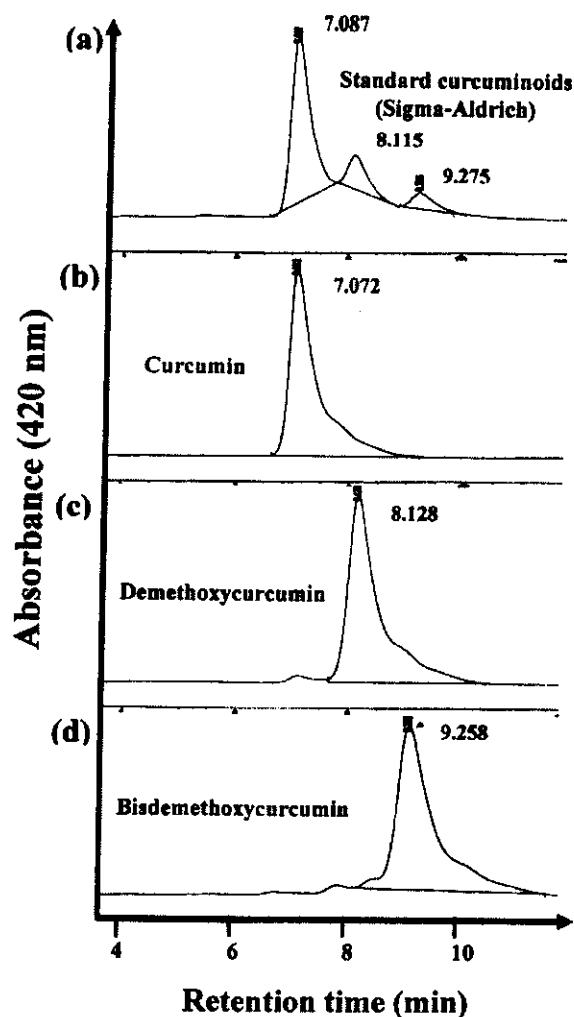


Fig. 2 The HPLC elution profile of pure curcumin, demethoxycurcumin and bisdemethoxycurcumin. The HPLC histogram of standard curcuminoids (Sigma-Aldrich), pure curcumin, demethoxycurcumin and bisdemethoxycurcumin as depicted in panels (a), (b), (c) and (d), respectively.

Curcuminoids have been reported to contain anti-carcinogen against human cancer cells. The activity has usually been reported on the basis of curcuminoid mixture (curcumin, demethoxycurcumin and bisdemethoxycurcumin). Thus, previous results did not show which forms of curcuminoids caused the maximum activity in cancer cells, especially in leukemic cell lines. The cytotoxic effects of curcumin, demethoxycurcumin and bisdemethoxycurcumin at various concentrations (0-50%) on HL60 cell line for 24 h by MTT assay was shown in Fig. 1. All

curcuminoids are capable of inhibiting cell growth of HL60. The IC_{50} of demethoxycurcumin, bisdemethoxycurcumin and curcumin were $9.8 \pm 1.30 \mu\text{g/mL}$, $10.3 \pm 1.73 \mu\text{g/mL}$ and $11.2 \pm 1.23 \mu\text{g/mL}$, respectively (Fig. 3a and 3c). Three curcuminoids did not show different inhibitory effect. These results were supported by using trypan blue exclusion method which has shown that the IC_{50} of demethoxycurcumin, bisdemethoxycurcumin and curcumin were $3.2 \pm 0.12 \mu\text{g/mL}$, $3.5 \pm 0.52 \mu\text{g/mL}$ and $4.2 \pm 0.5 \mu\text{g/mL}$, respectively (Fig. 3b and 3c).

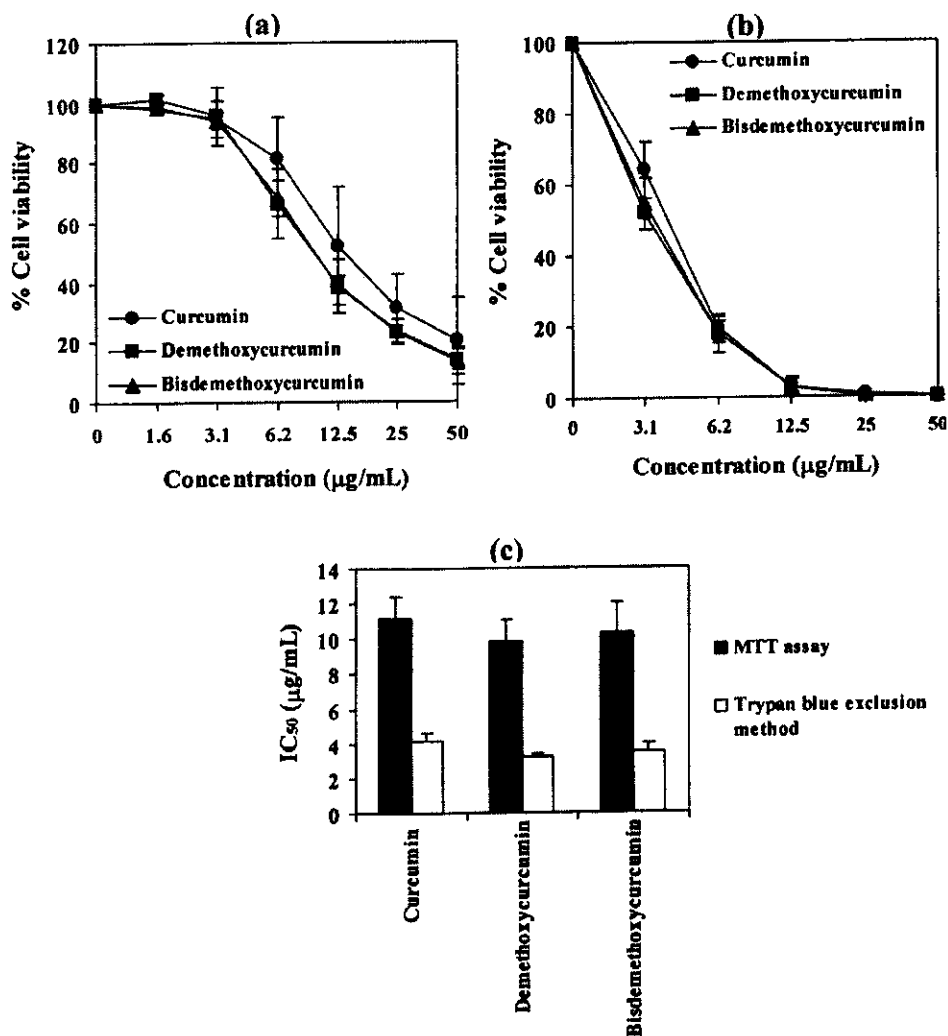


Fig. 3 Effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin on HL60 cell line. Cells were grown in the presence of various concentrations of curcumin, demethoxycurcumin and bisdemethoxycurcumin. The number of viable cells was determined by MTT assay (a) in triplicate, trypan blue exclusion method (b). IC_{50} values in three independent experiment of MTT assay and trypan blue exclusion method were plot for the comparison (c).

The cytotoxic effect of curcuminoids on U937 cell line by MTT assay demonstrated that the IC_{50} of demethoxycurcumin, bisdemethoxycurcumin and curcumin were $7.0 \pm 1.34 \mu\text{g/mL}$, $8.6 \pm 0.70 \mu\text{g/mL}$ and $8.7 \pm 0.97 \mu\text{g/mL}$, respectively (Fig. 4a and 4c). In this case, three curcuminoids did not show

different significant cytotoxic effect. These results were supported by using trypan blue exclusion method which the IC_{50} of demethoxycurcumin, bisdemethoxycurcumin and curcumin were $5.5 \pm 1.41 \mu\text{g/mL}$, $6.7 \pm 0.24 \mu\text{g/mL}$ and $8.2 \pm 0.48 \mu\text{g/mL}$, respectively (Fig. 4b and 4c).

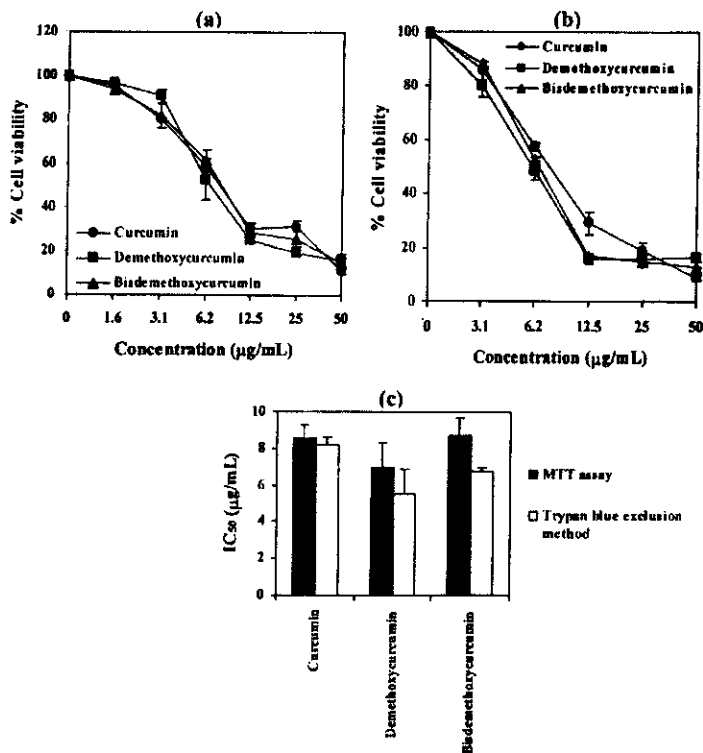


Fig. 4 Effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin on U937 cell line. Cells were grown in the presence of various concentrations of curcumin, demethoxycurcumin and bisdemethoxycurcumin. The number of viable cells was determined by MTT assay (a) in triplicate, trypan blue exclusion method (b). IC_{50} values in three independent experiment of MTT assay and trypan blue exclusion method were plot for the comparison (c).

The concentration of curcuminoids that caused the cytotoxic effect in K562 cell line was higher than that in HL60 and U937 (0-100 $\mu\text{g/mL}$). After curcuminoid treatment for 24 hrs and determined by MTT assay, it was found that the IC_{50} of demethoxycurcumin was $21.7 \pm 2.14 \mu\text{g/mL}$, followed by curcumin and bisdemethoxycurcumin at $25.9 \pm 8.67 \mu\text{g/mL}$ and $27.4 \pm 5.8 \mu\text{g/mL}$, respectively (Fig. 5a and 5c). These three curcuminoids did not show any

different cytotoxic effect. However, the IC_{50} for cell cytotoxicity by trypan blue exclusion method were $14.1 \pm 0.63 \mu\text{g/mL}$, $23.4 \pm 0.81 \mu\text{g/mL}$ and $27.3 \pm 1.13 \mu\text{g/mL}$ for demethoxycurcumin, curcumin and bisdemethoxycurcumin, respectively (Fig 5b and 5c). By trypan blue exclusion method, demethoxycurcumin significantly inhibited in growth of K562 cell line as compared to curcumin and bisdemethoxycurcumin by 40% and 48%, respectively ($P < 0.05$) (Fig. 5c).

However, the overview of the cytotoxic effect result showed that all three ingredients of curcuminoids exhibited an excellent cytotoxic

activity on leukemic cell lines with the inhibitory concentration at 50% (IC_{50}) approximately 7 $\mu\text{g/mL}$ in HL60 and U937 and 20 $\mu\text{g/mL}$ in K562 cell line.

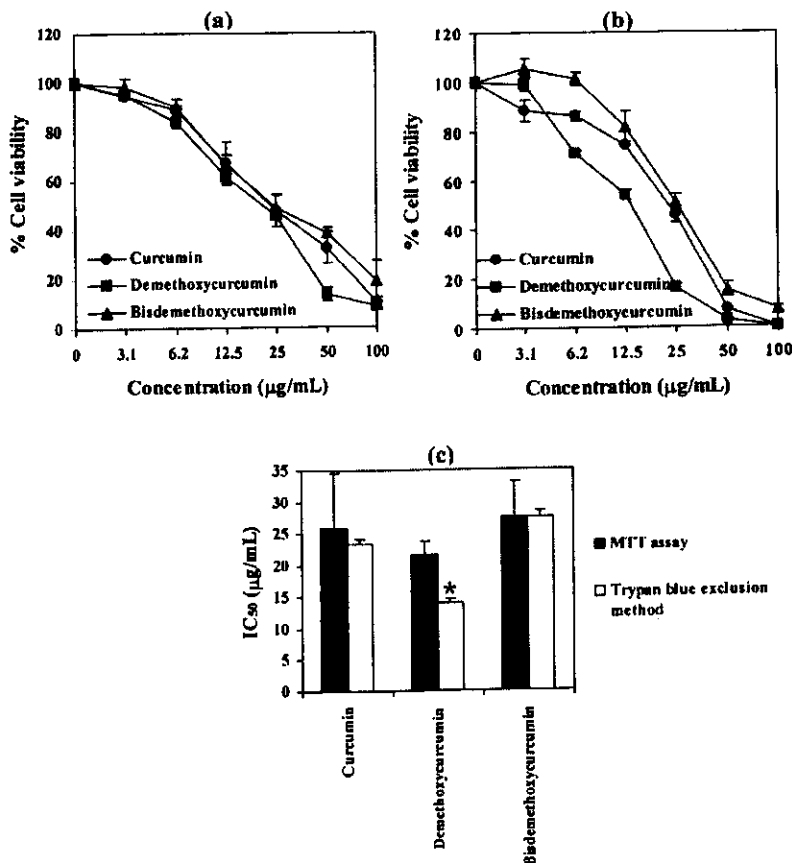


Fig. 5 Effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin on K562 cell line. Cells were grown in the presence of various concentrations of curcumin, demethoxycurcumin and bisdemethoxycurcumin. The number of viable cells was determined by MTT assay (a) in triplicate, trypan blue exclusion method (b). IC_{50} values in three independent experiment of MTT assay and trypan blue exclusion method were plot for the comparison (c). Key: (*) significantly different from curcumin and bisdemethoxycurcumin ($P < 0.05$).

4. Discussion

Our previous study indicated that curcumin (Sigma-Aldrich), a natural plant phenolic compound, exhibited anti-carcinogenic activities *in vitro* [35] and *in vivo* [18]. Since curcuminoids are non-toxic, non-mutagenic and non-carcinogenic, fraction of these compounds that can inhibit the proliferation of tumor cells have potential as anticancer agents. In the

present studies we demonstrated the cytotoxic effect of curcuminoids against leukemic cell lines, HL60, U937 and K562. The cytotoxic effect was monitored by MTT assay and trypan blue exclusion method. The principle of the MTT assay is to measure mitochondria enzyme activities of viable cells. The viable cells convert the tetrazolium component of the dye solution into a formazan crystal product. The

formazan product was solubilized by an organic solvent, and the violet color solution was determined by the absorbance at 570 nm. The 570 nm absorbance reading is directly proportional to number of viable cells.

The trypan blue exclusion method is the classical and the most widely accepted method and used for differentiating living and dead cells in culture. The intact membrane of viable cell excludes the trypan blue dye. These methods can be used for both proliferation and complement-mediated cytotoxicity assays. Cell viable test by trypan blue dye exclusion method is more suitable to cells grown in suspension than the monolayers because dead cells can detach from monolayers and therefore be lost from the assay.

Although the cytotoxicity of curcuminoid mixture (the commercial curcuminoid mixture which is usually sold as "curcumin" is a mixture of the three curcuminoids) has been previously reported, the cytotoxic effect of the individual compound had not been investigated. The results obtained from this study have show that curcuminoids exhibited the most effect in leukemic cell lines. All curcuminoids showed cytotoxic effect on all leukemic cell lines, with demethoxycurcumin appeared to be the most cytotoxic fraction. However, the effect of demethoxycurcumin on K562, which was determined by trypan blue assay, showed the significant difference from curcumin and bisdemethoxycurcumin.

The results obtained from MTT and trypan blue assay showed similar degree of cell cytotoxicity after curcuminoids treatment. The trypan blue test is reliable and correlates well with the MTT assay. Nevertheless, the IC_{50} values of both methods were not absolutely the same, which may due to the fact that different parts of the cell were examined. MTT

assay is used to investigate tumor cell viability. This assay involves mitochondria succinate dehydrogenase enzyme found in metabolically active cell. On the other hand, trypan blue involves the intact membrane of viable cell. This study, MTT assay exhibited higher IC_{50} values than trypan blue exclusion method. It was possible that some dead cells still contained the mitochondria enzyme inside but the cell permeability was lost.

The results of the present study are in agreement with other reports on the inhibitory action of commercial curcumin (Sigma-Aldrich) on cell growth and proliferation. The growth inhibitory effect of curcumin against several cancer cell lines; breast tumor cell line [38], Hep-2, PC-9, PC-14, Hep-1 and F-25 [35] were reported. It has been reported that curcumin reduced the proliferation rate of HT-29 and HCT-15 human colon cancer cell lines *in vitro* mainly by accumulating cells in the G2/M phase [39], the proliferation of cell cycle progression of human umbilical vein endothelial cell [40] and the proliferation of human promyelocytic leukemia HL60 cells [41]. But curcumin showed no significant effect on inhibiting proliferation or inducing apoptosis on human PBMC [42]. Thus curcuminoid treatment may provide a guideline for molecular study and clinical treatment in leukemic patients in future.

Acknowledgements

This work was financially supported by the Thailand Research Fund (TRF), the Commission of Higher Education, and Chiang Mai University Endowment Fund. We gratefully acknowledge Dr. Floyd W Dunn, Dr. Roongsiri Chotpadiwetkul, and Mr. Singkome Tima for their excellent help.

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