Isomers Geometric dan efek sitotoksik pada sel T47D dari analog kurkumin PGV-0 and PGV-1*

Geometric Isomers and Cytotoxic Effect On T47D Cells of Curcumin Analogues PGV-0 and PGV-1*

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* Part of Thesis for doctoral program at Postgraduate School Gadjah Mada University

Abstrak

Analog kurkumin 2,5-bis-(4'-hidroksi-3'-metoksi)-benzilidinsiklopentanon atau PGV-0 dan 2,5-bis-(4'-hidroksi-3',5'-dimetil)-benzilidinsiklopentanon atau PGV-1 memiliki potensi untuk dikembangkan sebagai senyawa yang bersifat sitotoksik. Penelitian ini dilakukan untuk menentukan struktur geometrik kedua senyawa tersebut dan efek sitotoksiknya terhadap sel kanker payudara T47D. Senyawa dielusidasi dengan LC-MS, 1H-NMR dan 13C-NMR, HMBC, HMQC dan NOESY untuk menentukan struktur geometriknya. Senyawa diuji efeksitotoksiknya terhadap sel T47D dengan menggunakan metoda MTT assay. PGV-1 memiliki struktur geometrik engagen-engagen (E-E) dan PGV-0 memiliki struktur geometric Z-Z. Nilai IC_{50} terhadap sel T47D masing-masing 1,74 dan 9,39 μM.
Kata kunci : PGV-0, PGV-1, Sitotoksisitas

Abstract

Curcumin analogues 2,5-bis-(4'-hidroxy-3'-methoxy)-benzilidinecylopentanone (PGV-0) and 2,5-bis-(4'-hidroxy-3',5'-dimethyl)-benzilidinecylopentanone (PGV-1) have a potency to be developed as cytotoxic agent. The aims of this research are to elucidate the geometric isomer and to study the cytotoxic effect on T47D cells of both compounds. To establish the geometric isomer these compounds, they were elucidated by LC-MS, ^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC, NOESY. Their cytotoxic effect were evaluated by MTT assay method on T47D cells. The results concluded that the geometric isomer of PGV-1 is zusammen-zusammen (Z-Z) and PGV-0 is entgegen-entgegen (E-E). The IC_{50} of both compounds are 1.74 and 9.39 μM respectively.
Key words: PGV-0, PGV-1, Cytotoxicity

Introduction

Curcumin has several biological activities, for example anti-inflammatory, antioxidant, and anticancer. Curcumin is unstable compound at pH above 6.5 and on the presence of light (Tonnesen and Karlsen, 1995; van der Goot, 1997). The instability is attributed to the active methylene group. Omission of this group leads to curcumin analogue stabilization with the same biological properties.

Robinson (2003), divided curcumin pharmacopore in three part (Fig. 1). In a continuing research for potent and stable compound, the geometric isomer of these two curcumin analogues, PGV-0 and PGV-1, have been studied together with their cytotoxic properties on T47D cells.

Toxicological studies indicated that both compounds are non toxic even at a high dosage. Biological activities studied for their anti-inflammatory and antioxidant properties showed higher potency than those of the

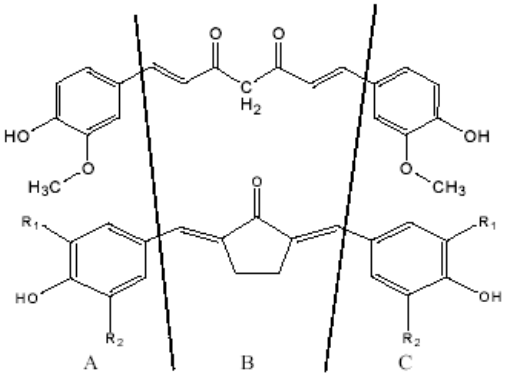


Figure 1. Curcumin and curcumin analogues (PGV) pharmacophore division. Part A and C, both are aromatic ring could be heteroaromatic and also symmetric or not symmetric, these part important for reseptor binding. Part B of curcumin, β-diketone group, has $–CH_2-$ active mathylene group initiate curcumin instabilization. (PGV-0: R_1 is H, R_2 is $-OCH_3$, PGV-1: R_1 and R_2 are $-CH_3$) (Based on Robinson paper, 2003)

curcumin (*Tim Molnas*, 2001). PGV-0 also exhibits cytotoxic effect on Myeloma, HeLa, Raji cell lines better than that of the curcumin (Da'i, 2003). PGV-0 and PGV-1 induce apoptosis on T47D cells that was induced by estrogen hormone (Melannisa, 2005; Nurulita, 2005).

This paper focuses on the elucidation of the geometric isomer of both compounds and their on T47D cells. Structure studies showed that both curcumin analogues have 3 geometric isomers (Fig 2.) These are *entgegen-entgegen* (*E-E*), *entgegen-zusammen* (*E-Z*) and *Z-Z* structures. It seems that, this geometrical structure is responsible for the activity of the compound for cytotoxic effect.

Methodology

Material

PGV-0 and PGV-1 were obtained from the MOLNAS (National Molecule) team UGM, T47D cells were given by Prof. Tatsuo Takeya, NAIST Japan, cells were grown at 37°C in a 5 % CO_2 /95% air atmosphere. Cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) contain

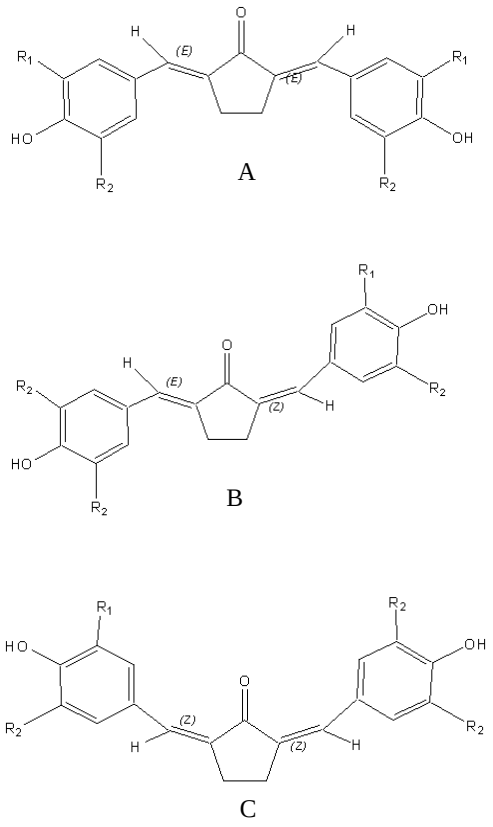


Figure 2. The geometric isomer of PGV (A) *Entgegen-entgegen* (*E-E*) (B) *Entgegen-Zusammen* (*E-Z*) (C) *Z-Z* structures

penicillin-streptomycin antibiotic and fungizon for antifungal, all were purchased from Gibco.

Methods

Structure Elucidation

The purity of these compounds were determined by HPLC method using methanol and water (70:30). Structure elucidation were carried out by using LC-MS, ^{1}H-NMR and ^{13}C-NMR spectra were recorded on 400 MHz Jeol Instrument using d6-DMS0 as solvent. The NMR spectra were recorded using HMBC, HMQC, NOESY and COSY patterns.

In vitro cytotoxic effect test

The cells were seeded at 1,5 X 10^4 cells/ well onto 96 well-plates and than incubated for 24 hours in humidified atmosphere at 37°C and in CO_2 5%. At time 0, medium was replaced with fresh complete

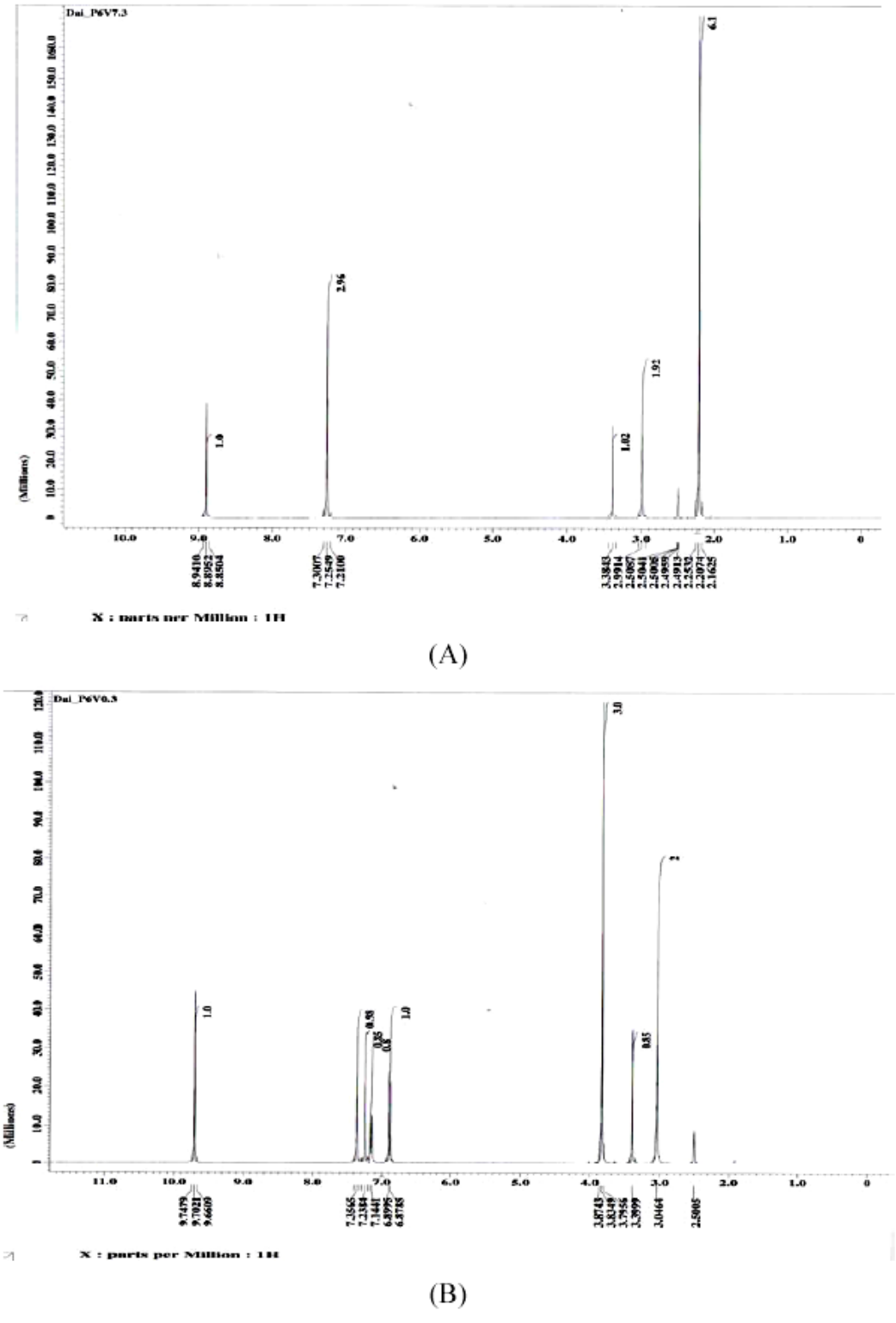
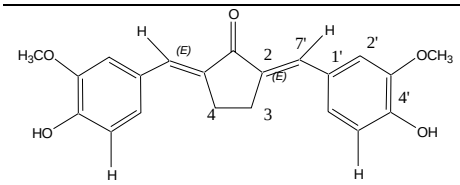
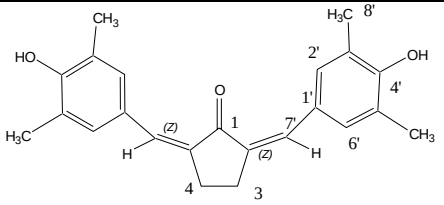


Figure 3. A. The 400 MHz spectras ^{1}H-NMR of PGV-1 (A) and PGV-0 (B), both of samples are diluted using DMSO-*d*6.

Table I. Chemical shift (ppm) of PGV-0 and PGV-1 were measured by ^{1}H-NMR and ^{13}C-NMR





Atom No	δ_H	δ_C	δ_H	δ_C
1		194.80		194.81
2,5		132.82		132.56
3,4	3.06	25.90	2.99	25.94
1'		127.16		126.72
2'	7.24	114.50	7.26	131.41
3'		147.69		124.62
4'		148.50		155.14
5'	6.90 (d/ doublet)	124.76		124.62
6'	7.14 (d)	115.89	7.26	131.41
7'	7.37	148.50	7.26	134.54
8'			2.21	16.74
OH	9.70		8.90	
OCH_3	3.84	55.56		

medium with samples: Curcumin, PGV-0 and PGV-1, each added at concentrations of 100, 50, 25, 10, 5, 2,5 and 1 μMs. Then, plate incubated for 24 hours at humidified atmosphere 37°C in CO_2 5%. At the end of the treatment, medium was replaced and the cells were washed with PBS and added new fresh medium 100 and 15 μL MTT 0,5% in PBS. Plate incubated for 4 hours in humidified atmosphere at 37°C in CO_2 5%. The bio-reduction of the MTT dyes was dissolved in SDS 10 % and assessed by measuring the absorbance of each well at λ 550 nm. Viable cells were expressed as a percentage of the absorbance treatment cells divided by absorbance control cells multiply with 100%. Both absorbance are corrected by using medium absorbance only.

% Viable Cell: ((A – B)/ (C – B))X100%

A : Absorbance of treated cells
B : Absorbance of medium only
C : Absorbance of control cells

The IC_{50} values are from graphic plot of percentage viable cell versus concentration. The IC_{50} values express 50 % cell death induced by the concentration and inform about the potency of such compounds.

Result and Discusion

The purity of these compounds were determined by HPLC and showed that there is no other signal on PGV-1 (data not showed). However, the PGV-0 still contain vanillin about 0.99% (Martono; 2004, Da'i, 2003). LC-MS chemical ionization method confirm the molecular weight of the compounds with MW 352 and 348 which was belonged to PGV-0 and PGV-1 respectively (data not showed).

A series of one and two dimensional NMR spectroscopic experiments using COSY, NOESY, HMBC and HMQC patterns were performed to assign the proton and carbon resonance correlation of the compounds. The experiment resulted that the ^{1}H-NMR and ^{13}C-NMR showed the similarities and differences patterns as indicated each compound (Table 1 and Fig 3). Both compounds were dissolved in DMSO-*d*6, as expected this solvent give additional peak at δ_H 2,50 and 3,39 and δC 39,50 ppms (Williams and Fleming, 1995).

Assignment of both compound showed similar result for cyclopentanone moeities. The signal pattern of the aromatic ring showed the

Table II. HMBC correlations for PGV-1

Proton	Carbon	C1	C2(5)	C3(4)	C1'	C2'(6')	C3'(5')	C4'	C7'	C8'
	ppm	194.81	132.56	25.94	126.72	131.41	124.62	155.14	134.54	16.74
H3(4)	2.99	β	α(β)	Bond					β	
H2'(6')	7.26					Bond				
H7'	7.26	β	α		α	β			Bond	
H8'	2.21					β	α	β		Bond
H(-OH)	8.90						β	α		

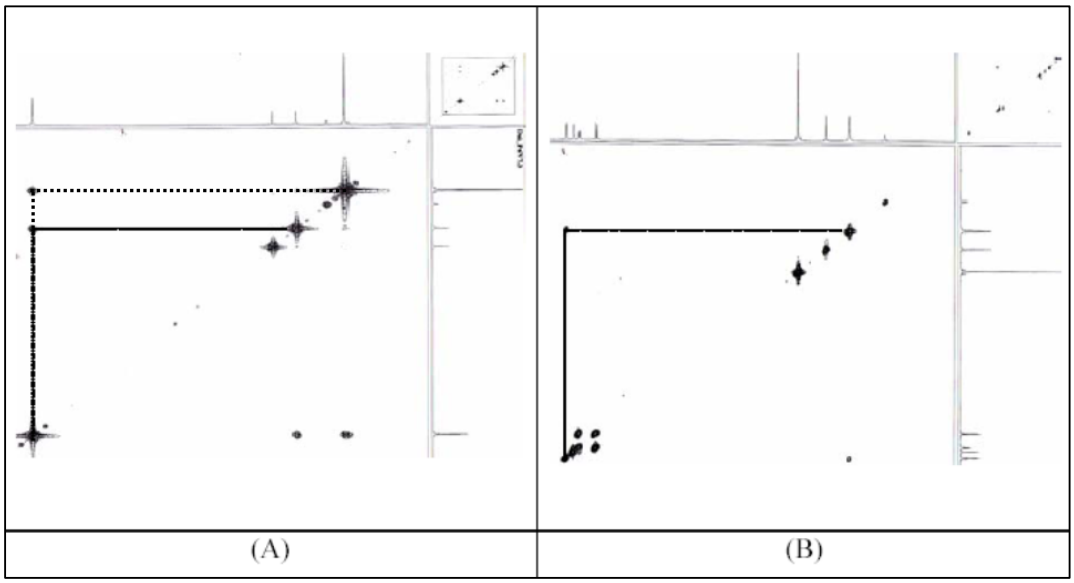


Figure 4. Selected COSY spectra of PGV-1 (A) and PGV-0 (B). Dashes line indicate coupling between $H7^1$ and H-3(4) of pentanone group. The spectra of PGV-1 also indicated cross peak between $H\text{-}2^1(6^1)$ and H of dimethyl group $(H\text{-}8^1)$

influence of methoxy ($-OCH_3$) and methyl ($-CH_3$) as be shown by the δ_C of both compounds for C3. The presence of an electron-withdrawing *meta* ($-OCH_3$) group in *meta* position of PGV-0 resulted in a downfield shift (147.69) of the adjacent C3. The contrast effect was showed by the presence of an electron-donating methyl ($-CH_3$) group in PGV-1, resulted in an up field shift (124.l62). The presence of both methyl groups in PGV-1 also influence the chemical shift for the proton no 7', this electron donating group reduces the positive charge of C7', thus resulted in a up field shift for this proton.

The pattern of chemical shift of the aromatic signal and α,β-unsaturated carbonyl of PGV-1 were established by Heteronuclear Multiple Bond Coherence (HMBC) spectra pattern. This spectrum capitalizes on two and or three bond couplings of our compounds (Silverstein and Webste, 1998). Based on the data, at δ_C 131.41 belongs to C2'(6') due to coupling H7 to this carbon and at δ_C belongs to C2(5) α-carbonyl (Table II). PGV-0 is not too complex to deduce the pattern of the aromatics protons due to no symmetry of the atom in this compound.

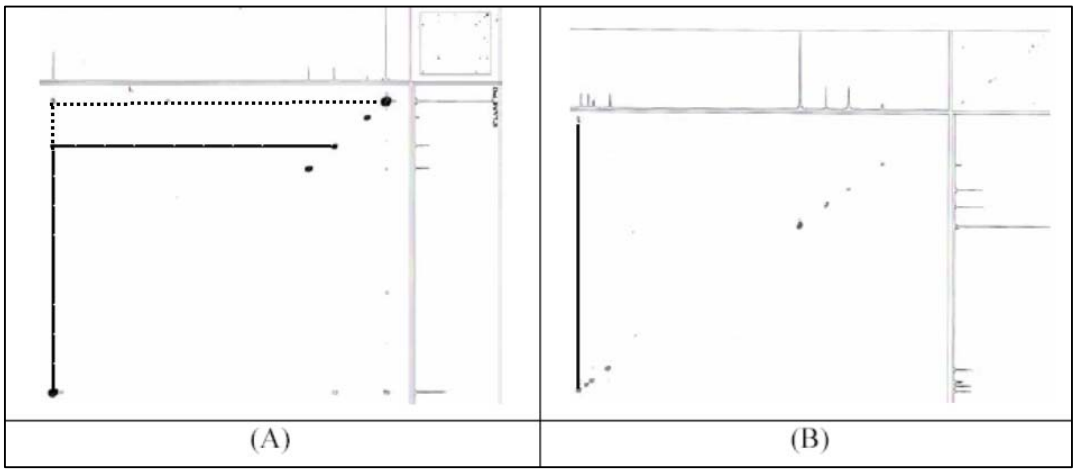


Figure 5. Selected NOESY spectra of PGV-1 (A) and PGV-0 (B) dashed line showed cross peak between H7[1] of PGV-1 and H3(4) but there is no cross peak in PGV-0

For convenience of interpretation it is extremely helpful to use two dimensional spectrum i.e. COSY, NOESY and HMBC. ^{1}H-^{1}H COSY spectrum indicates all the spin-spin coupled proton. COSY spectra can be obtained to emphasize long-range couplings (Williams and Fleming, 1995). NOESY spectrum which indicate protons that are close in space, provides crucial information about the geometry of molecule (Williams and Fleming, 1995). Selected COSY spectrum shown H7' of PGV-0 and PGV-1 couple with H3(4) (Fig 4).

Selected NOESY spectrum shown H-7[1] of PGV-0 has no cross peak with H3(4) of cyclopentanone. This data sugest that H7[1] of PGV-0 is not in the same space with H3(4) proton. The NOESY spectra of PGV-1 showed difference phenomena. The H7[1] proton of PGV-1 showed cross peak with H3(4) of cyclopentanone. Based on this result, the proton H-7[1] may be is in the same space with H-3(4) proton of cyclopentanone group (Fig 5). This result indicated the possibilities that PGV-1 has *Z-Z* isomer. Pentagamavunon-0 can be concluded exactly as *E-E* isomer. Additional cross peak of PGV-1 showed the same space of H-21(61) with H-8[1] of dimethyl group.

Both compounds were evaluated the cytotoxic effect on T47D cells line. The IC_{50} values of PGV-0 and PGV-1 are 11 μM and 5 μM respectively (Fig 6). As comparison the

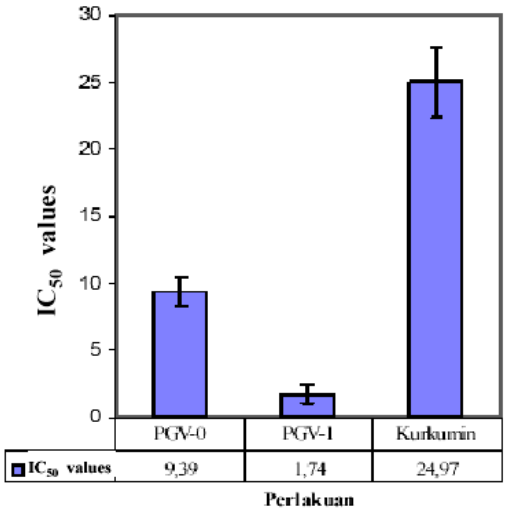


Figure 6. The result of citotoxicity test of PGV-0 and PGV-1 on T47D cell lines showed by IC_{50} values compared with curcumin (n=5). Pentagamavunon-1 (PGV-1) has better potency as cytotoxic agent on T47D cells compare with PGV-0 and curcumin.

IC50 values of curcumin on T47D cells is 22 μM Based on this result, PGV-1 was the most potent compound as cytotoxic agent on T47D.

The activity of PGV-0 and PGV-1 should follow the curcumin activity. Curcumin change the proteins level that involve in the cell cycle and apoptosis process. Incubation of breast cancer cells with 20 and 40 microM

curcumin for 24 h induced G2 block and sub-G0/G1 cell population, respectively (Ramachandran and You, 1999). It is suggest that antiproliferative effect of curcumin modulated cell cycle related gene expression. Curcumin down-regulates cyclin D1 expression through activation of both transcriptional and post-transcriptional mechanisms, and this may contribute to the antiproliferative effects of curcumin against various cell types (Mukhopadhyay et al., 2002). Curcumin also was found to induce G0/G1 and/ or G2/M phase cell cycle arrest, up regulates CDKIs, p21, p27 and p53 and slighly down-regulate cyclin B1 in umblical vein endothelial cells (Park et al., 2002). Curcumin induces apoptosis accompanied by an increase in p53 level as well as its DNA-binding activity followed by Bax expression at the protein level. The research also proved that curcumin increase the precentage of cell with G0/G1 DNA content (Choudhuri et al., 2002). It must be elucidated the mechanism of PGV-1 and PGV-0 to inhibit the cancer cell. The PGV-1 structure must be clarified by using x-ray crystallography to confirm the geometric isomer of PGV-1.

Conclusion

The result concluded that the geometric isomer of PGV-0 is *E-E*. Pentagamavunon-1 has possibility for *Z-Z* isomer. The IC_{50} values of PGV-0 and PGV-1 are 9.39 and 1.74 μM. Pentagmavunon-1 is more potent than the PGV-0 to inhibit the T47D cell growth.

Acknowledgment

This experiment was sponsored by Hibah Bersaing project, DIRJEN DIKTI RI. We also thank to Mrs. Sachiko Iida and Prof. Masashi Kawaichi (Nara Institute of Science and Technology - Japan) for their support to continue this experiment in molecular targets mechanism of PGV-0 and PGV-0.

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