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Cytotoxic Activity Assay of Tinocrisposide from *Tinospora crispa* on Human Cancer Cells

Adek Zamrud Adnan[1], Muhammad Taher[2], Tika Afriani[3], Dewi Imelda Roesma[4] and Andani Eka Putra[5]

[1]Faculty of Pharmacy, Andalas University, Padang 25163, Indonesia
[2]Departmentof Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Malaysia
[3]Department of Pharmacy, Mohammad Natsir University, Bukittinggi 26136, Indonesia
[4]Department of Biology, Andalas University, Indonesia
[5]Medicinal Faculty, Andalas University, Indonesia

ABSTRACT

Tinospora crispa is a climber plant belonging to the family of Menispermaceae. This plant has been used traditionally in Indonesia to treat some diseases and conditions such as fever, malaria, rheumatism, diabetes, cholera and inflammation. The aims of this study was to investigate cytotoxic activity of tinocrisposide extracted from the plant against human cancer cell lines. Cytotoxic activity of tinocrisposide in interval dose of 100; 50; 25; 12.5; 6.25; 3.125 µg/mL has been investigated on H1299 and MCF-7 cell lines and their effects were quantified spectrophotometrically with the 3-(4,5 dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay at wavelength, λ 570 nm. Tinocrisposide in concentrations of 100; 50; 25; 12.5; 6.25; 3.125 µg/mL has been treated against H1299 cell line and showed a percentage viability of 55.5; 67.70; 80.84; 81.09; 82.69; and 87.06% respectively, with IC50 70.9 µg/mL, whereas on MCF-7 cell line showed percentage viability of 80.71; 89.30; 92.76; 96.01; 96.58, and 102.66% respectively, with IC_{50} >100 µg/mL. It can be concluded that tinocrisposide has not shown any cytotoxic activity against H1299 and MCF-7 cell line, but it can be used as a chemopreventive agent toward those cell lines.

Keywords: Cytotoxic activity, MTT assay, tinocrisposide, *Tinospora crispa*

INTRODUCTION

Cancer is one of the leading cause of death all over the world. It is characterized by uncontrolled growth and spreading of normal cells in the body. International Agency for Research on Cancer (IARC), in 2012, has estimated that there would be 14.1 million new cancer cases per year, and it expected the number to rise to 22 million annually within the next two decades. The most common causes of cancer death were lung, liver, and stomach cancer[1]. Medicinal plants have had a long history in cancer treatment [2] and have been used to treat human diseases for centuries [3]. Thus, it is possible for medicinal plants to be used as potential sources for new anticancer drugs development [4].

Tinospora crispa is a climber plant which grows in the wild and covers large area from the Southwestern part of China to Southeast Asia, including Indonesia, Malaysia, Vietnam, Thailand and India. This bitter taste plant that locally known as brotowali [5, 6] has been used as traditional medicine in Indonesia, Malaysia and Thailand to treat fever, diabetes, cholera, rheumatism, reducing thirst, hypertension, low appetite and also used for protection from mosquito bites [5, 7]. Previous studies have reported that, the methanolic and aqueous extract of *T. crispa* displayed a dose-dependent cytotoxic effect on MCF-7, HeLa, Caov-3 cell lines with an IC_{50} value of 33.8, 165 and 100 µg/mL, respectively [8, 9].

T. crispa has been extensively studied chemically and it has been reported to contain flavonoid and quaternary alkaloids including apigenin, berberine, palmatine [10, 11], borapetol A and borapetol B, borapetoside A and borapetoside B, and picroretine [12].

From methanol extract of dried pulvered *T. crispa* stems, a furanoditerpen glycoside with a very bitter taste, tinocrisposide, ($C_{27}H_{36}O_{11}$) has been isolated [13]. It has been observed that tinocrisposide is one of the biological active ingrediens in T. crispa stem extracts. Tinocrisposide have showed some biological activities such as analgetic, antidiabetes, anti-inflammatory[14], antimalarial and antihistamine [15]. However, there is no report available regarding its cytotoxic activity.

The aim of this study was to isolate and investigate the cytotoxic activity of the tinocrisposide from *Tinospora crispa* stems against non-small cell lung cancer (H1299) and breast cancer cell (MCF-7).

MATERIALS AND METHODS

General

IR spectra was measured on Perkin Elmer Spectrum One Fourier Transform Infrared Spectrometer (FT-IR). The UV spectra was obtained on a Shimadzu UV-1800 UV/VIS spectrophotometer. The GCMS spectra was recorded on GC-MS QP2010 SE using a RTX-5 MS column at The University of Islamic Indonesia, Sleman Yogyakarta. Column chromatography was performed on silica gel 60 and Sephadex LH-20 (GE Healthcare). Organic solvents and TLC plates were purchased from Merck, Germany.

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin were purchased from GIBCO. Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) was purchased from Calbiochem, USA. Dimethylsulfoxide (DMSO) was purchased from Fisher Chemical (Thermo Fisher Scientific, USA).

Plant material

Fresh *T. crispa* stem samples were collected from Lubuk Minturun, Padang, Indonesia. This plant was identified and authenticated by Dr. Nurainas at Department of Biology, Faculty of Science, Andalas University. A specimen was deposited in the Herbarium ANDA, University of Andalas.

Cell lines

H1299 and MCF-7 cells were purchased from American Type Culture Collection (ATCC), Virginia, USA.

Extraction and isolation

The dried and powdered stem (3.2 kg) of *T. crispa* was extracted using n-hexane and methanol succesively via maceration process for 3 times in 3 days each at room temperature, and evaporated in vacuo by rotary evaporator. The methanol extracts (273 g) was dissolved in 5 % acetic acid for overnight, decanted and then partitioned, sequentially, with n-hexane and DCM. Each fraction was dried to yield n-hexane, DCM and water fractions. The water fraction was basified with ammonia until pH 9-10 and partitioned with DCM to obtain DCM base extract. The neutral (24.56 g) and base DCM extract (2.64 g) fractions were subjected to column chromatography over silica gel 60 using step gradient elution (1-15%) with mixture of DCM and MeOH. The fractions were combined on the basis of thin layer chromatography (Silica gel TLC- plate) evaluation. TLC was carried out using the mixture of MeOH and DCM (1:9) as mobile phase and the fractions with same R_f values were combined and further purified by column chromatography on Sephadex LH-20 using mixture of methanol and water as mobile phase to obtain tinocrisposide as white amorphous powder (17 mg).

Cytotoxic (MTT) Assay

The cytotoxic effect of the isolated compound on the growth of different human cancer lines was measured by MTT assay [16] with minor modification. A total of 2.0 x 10^5 cells/well were seed into 96-well flat bottom tissue culture plate and then incubated until it reached confluence at 37 °C with 5% CO_2. Then, the cells were treated with different concentration of test compound (3.125–100 µg/mL) in DMSO and incubated for 24 hours at 37 °C with 5% CO_2. Then, supernatant was discarded and washed once with phosphate buffer saline (PBS). Then, 20 µL of MTT stock solution (5 mg/mL PBS) was added to each well and incubate for 4 hours in CO_2 incubator. A total of 100 µL of DMSO was added to each well to solubilize the water-insoluble purple formazan crystal and left at room temperature. The absorbance was measured at wavelength of 570 nm and reference wavelength of 630 nm with a microplate reader (TECAN infinite M200).The untreated cells were used as the control. The results were expressed as a percentage of average absorbance of treated cells when compared with untreated cells. The half maximal

growth inhibitory concentration (IC_{50}) was defined as the concentration of compound that caused a 50% reduction of cell viability. Viability of cells (%) was calculated using the following equation:

$$\text{Viability of cells (\%)} = [\ (A_{Sample} - A_{Blank}) / (A_{Untreated} - A_{Blank})] \times 100$$

Data Analysis

All data were expressed as means ± standard deviation (SD) of result obtained from at least three independent experiments. One-way analysis of variance (ANOVA) followed by Dunnet post hoc test were used to assess differences between the treated and the control. A value of $P < 0.05$ was considered significant. The statistical package IBM SPSS Statistics Version 21 for Windows was used in the analysis.

RESULTS AND DISCUSSION

In this study, we used tinocrisposide as a test compound which was isolated from neutral and base DCM fractions of *Tinospora crispa* stems.The isolation process was conducted according to the method by Pachaly and Adnan [13]. The structures of the compound was identified spectroscopically and confirmed with previous data in literature.

The compound was identified by FTIR, UV spectrophotometry and analysed by GC-MS. Spectral data of compound a white amorphouspowder, FTIR (KBr pellet) $\bar{\nu}\ cm^{-1}$: 3411 is for O-H stretch, 1654 is for C=O stretch, 1708 is for γ-lactone, 2924 is for Ar-H, 1516, 1437 are for C=C, 874, 815, 760 are for C-H-Bending. UV λ_{max}in MeOH nm: 210. GC-MS fragmen (Retention time: 2.500): 83, 98, 238, 295, 310, 327.

MTT assay was conducted to determine the cytotoxic effects of compound on H1299 and MCF-7 cell lines. The cell lines were incubated to various concentrations for 24 hours and cytotoxicity was determined using a microplate reader. Meanwhile, the inhibition concentration (IC_{50}) value was determined from the linear regression of the experimental data.

Based on the results of the study (Table 1), tinocrisposide did not show any cytotoxic activity against H1299 cell with IC_{50} 70.9 µg/mL and MCF-7 cell with IC_{50} values >100 µg/mL (Fig. 1).

Figure 2 showed the H1299 cell lines treated with different concentration (3.125, 6.25, 12.5, 25, 50 and 100) µg/mL of tinocrisposide observed under the phase-contrast inverted microscope. The highest concentration of compound, 100 µg/mL (F) causes cell death. As the concentration of sample decrease, the viable cells will be increased. Thus, at the lowest concentration, 3.125 µg/mL (A), most of the cells were still attached and interacting with the neighboring cells. In contrast, compound concentration used did not affect the cell viability of MCF-7 cell lines even high concentration used (Figure not shown), where the cells were still attached and interacting with neighboring cells.

Table 1. Effect of tinocrisposide on cell viability

Concentration	Average of % viability	
	H1299	MCF-7
3.125	87.06 ± 9.59	102.66 ± 8.26
6.25	82.69 ± 6.84	96.58 ± 3.68
12.5	81.09 ± 8.27	96.01 ± 1.01
25	80.84 ± 4.98	92.76 ± 1.94
50	67.70 ± 4.62	89.30 ± 0.62
100	55.5 ± 1.94	80.71 ± 3.67

Values are expressed as mean ±SD, n=3

Table 2. Cytotoxicity data (IC_{50} value in µg/mL) of tinocrisposide

Cell lines	IC_{50}
H1299	70.9
MCF-7	>100

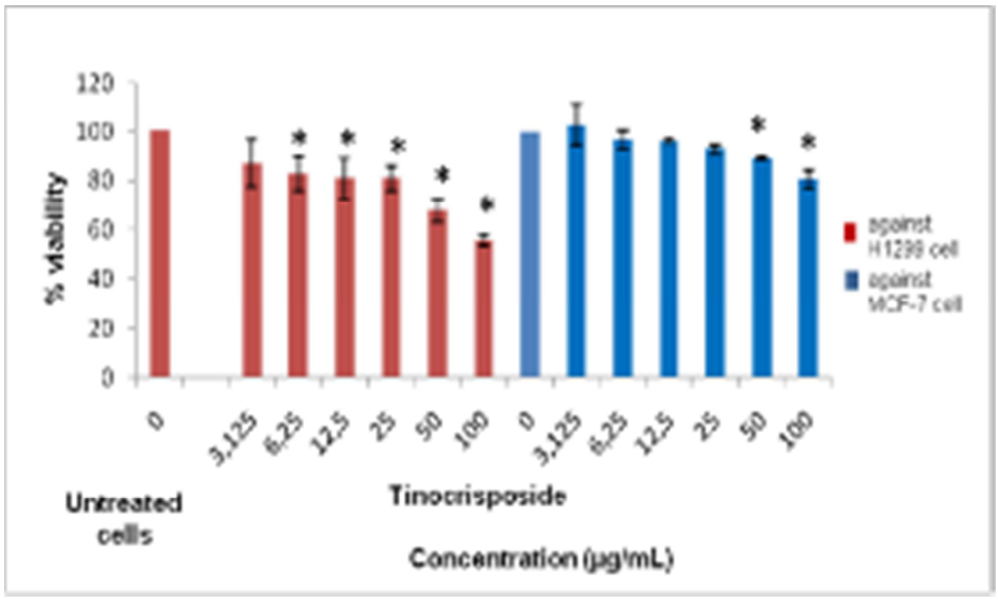

Figure 1. Cytotoxic activity of tinocrisposide.Untreated cells was used as negative control. Error bars represent standard deviation of triplicate values. Asterisks represent statistically significant (*p* value <0.05) data as compared to untreated cells

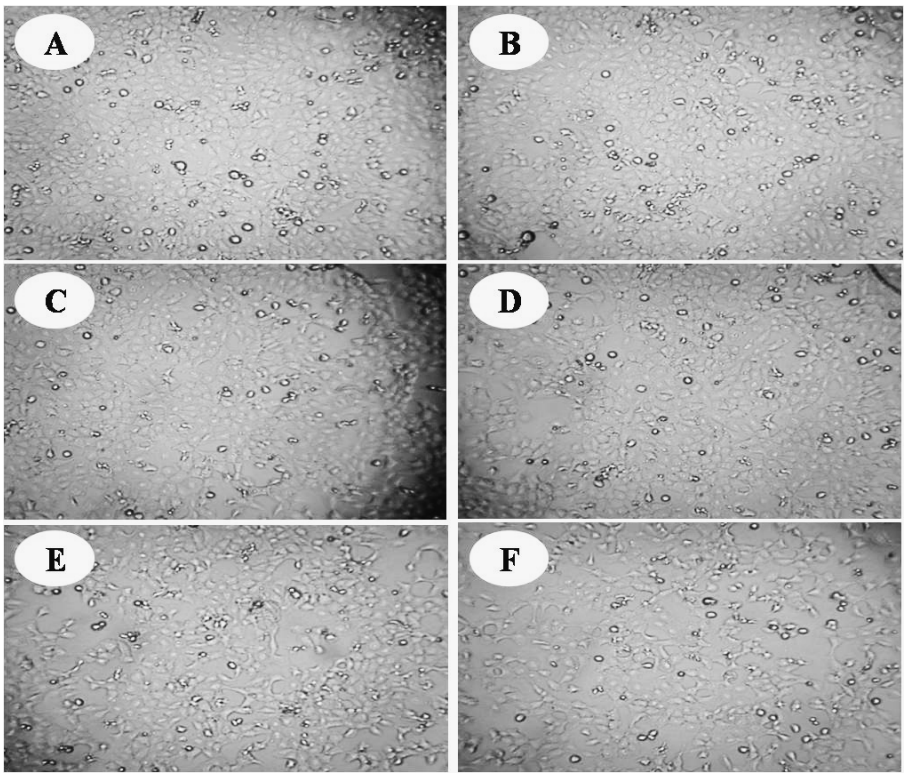

Figure 2. H1299 cells treated with different concentration of tinocrisposide, (A) 3.125 µg/mL (B) 6.25 µg/mL (C) 12.5 µg/mL (D) 25 µg/mL (E) 50 µg/mL and (F) 100 µg/mL observed under the phase-contrast inverted microscope with magnification of 20x

CONCLUSION

It is concluded that tinocrisposide from *Tinospora crispa* stem has not shown any cytotoxic activity against H1299 (IC50 70.9 µg/mL) and MCF-7 cell (IC_{50} values >100 µg/mL), but it can be used as a chemopreventive agent.

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