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Full length research paper

Cytotoxicity of fevicordin-A from *Phaleria macrocarpa* (Scheff.) Boerl on P 388, HeLa, CasKi, TE-2, TE-8 and Prepuce's Fibroblast cells

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Cancer is a disease caused by abnormal regulation of cells, particularly the mechanism of cell growth and differentiation. Cancer has high morbidity and mortality. The limitation of medical treatment leads to the findings of an alternative therapy using medicinal plants. *Phaleria macrocarpa* (Boerl.) Scheff family Thymelaeceae, is a plant which grows in tropical area, mainly in Papua island, Indonesia, and has been used empirically to treat various health problems, amongst which are cancer, diabetes mellitus, and hypertension. Fevicordin A, is a bioactive compound of *P. macrocarpa* (Boerl.) Scheff. Previous work of Kurnia and his colleagues concluded that this compound has been successfully isolated from the plant and exhibited toxicity on brine shrimp larvas. The objective of this research was to investigate the cytotoxicity of fevicordin A on P388, HeLa, CasKi, TE-2 and TE-8 cancer cells, while its effect on normal cells was performed on fibroblast cells isolated from human prepuce skin. The method used was MTT assay by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. The results of the work indicated that IC_{50} values of fevicordin-A on the growth of the cancer cells were 0.01 µg/mL (P388), 1.16 µg/mL (HeLa), 12 µg/mL (CasKi), 40 µg/mL (TE-2), and 14.6 µg/mL (TE-8), whereas the IC_{50} on the growth of fibroblast cells was 24.51 µg/mL. These results suggest that fevicordin A may have potential as a cytotoxic agent against leukemia and cervix cancer.

Keywords: Cytotoxicity; Fevicordin A; *Phaleria macrocarpa* (Boerl.) Scheff

INTRODUCTION

Cancer is a disease caused by abnormal regulation of cells, particularly the mechanism of cell growth and differentiation. Cancer arises when a cell, for a variety of reasons, escapes the normal brakes placed on its growth and begins to divide in an uncontrolled mode. This lost of regulation occurs when mutations arise in two broad families of genes that regulate cell growth: (1) oncogenes, which are associated with a dominant gain of function and act as positive signal for growth, and (2) tumour suppressor genes, which are associated with a recessive loss of function. These mutations may be caused by environmental, chemical or biological agents and can result in irreversible alterations in the genome of a cell [Boerner *et al*., 2002].

Since cancer remains a very serious life-threatening disease, efforts have been continually conducted to provide new leads against cancers. Many cancer drugs have been discovered from plant origin (Newman et al., 2003; Balunas et al., 2005; Cragg et al ., 2006) or made synthetically, but currently clinically used drugs have no significant effectiveness and safety. Therefore, this research which aims to search for anticancer drugs is important to the field.

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The fruits and leaves of *Phaleria macrocarpa* (Boerl.) Scheff, known as Mahkota Dewa, are most commonly used in Indonesian traditional medicine for the treatment of cancer, diabetes mellitus, and hypertension. This plant has been proven to contain phenolic glycoside, named mahkoside A, together with other compounds including mangiferin and kaempferol-3-*O*-β-D-glucoside (Zhang *et al*., 2006), icariside C3 and phalerin (Oshimi *et al*, 2008), whilst other researchers have successfully isolated fevicordin A, desacetylfevicordin A, and two other 29-norcucurbitacin compounds from the plant (Kurnia *et a*., 2008). The seeds of *P. macrocarpa* are very toxic and have an unpleasant taste on the tongue when bitten, therefore they are only used for the treatment of skin conditions or as a traditional biopesticide.

The cytotoxic activity of the methanol extracts of pericarp, mesocarp and seeds of this plant on HT-29, MCF-7, HeLa and Chang cell lines has been reported by Hendra and his colleagues (2011). They observed that the methanol extracts of *P. macrocarpa* showed promising *in vitro* cytotoxic activity against MCF-7 and HeLa cell lines with IC50 between 25.5 - 40.8 µg/ml. However, when applied to HT-29 cell line, only seed extract showed promising *in vitro* cytotoxic activity (Hendra *et al*, 2011).This evidence has prompted us to explore cytotoxic activity of this plant against some cancer cell lines and to find out a bioactive compound responsible for its activity. This paper reported the cytotoxicity of fevicordin A isolated from *P. macrocarpa* on cancer cells of leukemia (P388), cervix (HeLa and CasKi), and esophagus (TE-2 and TE-8).

EXPERIMENTAL SECTION

Plant material

The seeds of *P. macrocarpa* were collected from Purworejo, Central of Java, Indonesia, and were determined in the Laboratory of Plant Taxonomy, Faculty of Life Sciences and Technology (http://www.sith.itb.ac.id/en/), Institut Teknologi Bandung, Indonesia.

Extraction and isolation of fevicordin A from P. macrocarpa seeds.

All chemicals used as solvents in this work were purchased from Merck and Sigma Aldrich. The seeds (5 kg) were crushed and extracted with methanol (MeOH). The solvent was evaporated and the viscous extract was partitioned by using ethyl acetate (EtOAc) and water. The EtOAc fraction (5 g) was applied to a Wakogel C-200 column, and eluted with EtOAc-MeOH by a 10% stepwise gradient to obtain seven active fractions from the 80–100% EtOAc and 10–40% MeOH eluates. All the active eluates were collected and chromatographed in a Wakogel C-200 column (n-hexane-acetone in 10% steps) to yield active fractions I and II (60% and 70% acetone eluates). Fraction I was further subjected to ODS column chromatography (H2O-MeOH, 5% stepwise gradient) to afford active fractions I-a and I-b (60% and 70% MeOH eluates). From the fraction I-b, an active compound 1 was isolated and its structure was determined by an analysis of spectroscopic data (^{1}H NMR and ^{13}C NMR with DEPT, HMQC, HMBC, H-H COSY, and NOESY) to yield fevicordin A (18 mg).

Chemicals and Cancer Cell Lines

Murine leukemia cells (P388), cervix cancer cells (HeLa and CasKi), and oesophagus cancer cells (TE-2 and TE-8) were purchased from ATCC, USA. The cell lines were cultured in RPMI-1640 medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and incubated in 5% CO_2 at 37^{o}C.

Isolation of Fibroblast Cells from Human Prepuce Skin

This step was performed in the LAF cabinet (Clyde Apach). Fibroblast primary cells were isolated from human male prepuce skin after circumsicion. The fresh prepuce skin was soaked in povidone iodium solution for 1 hour and washed several times with phosphate buffer saline (PBS) for 30 minutes each. The dermis layer of the prepuce skin was separated (2 mm x 2 mm size), cut into tiny pieces, and placed into culture flask. The cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) which contained D-glucose, L-glutamine, sodium pyruvate (Gibco), supplemented with 10% heat-inactivated FBS (fetal bovine serum), penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone, at 37˚C in 5% CO_2 (Sanyo). The cells were differentiated by incubating them in their culture medium for 24 hours and were collected the next day for further assay.

Cytotoxicity Assay

Cytotoxicity assay was performed onto all cells in the presence of fevicordin A by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. Briefly, cells ($2x10^4$ cells in 100 µl medium/well) were placed in 96-well plates. After the initial cell seeding, different concentrations of fevicordin A were added and incubated for 24 hours. The next day, 10 µl of MTT reagent was added to the wells and incubated for another 3 hours at 37˚C in 5% CO_2 (Sanyo). Cell proliferation was determined by measuring the absorbance of formazan

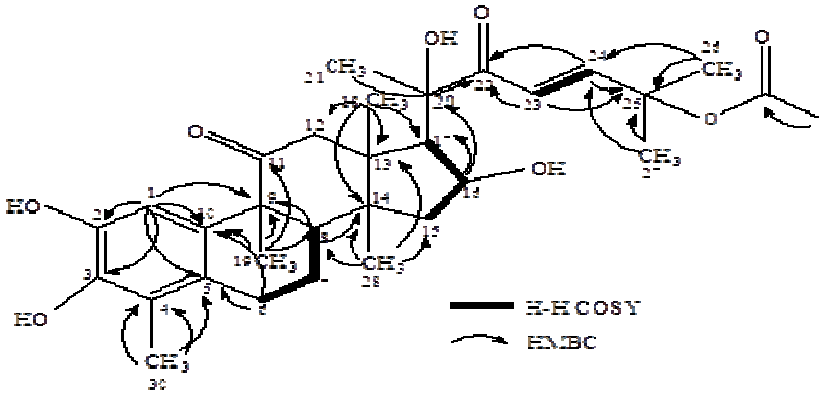

Figure 1. Correlation of H-H COSY and HMBC of fevicordin A

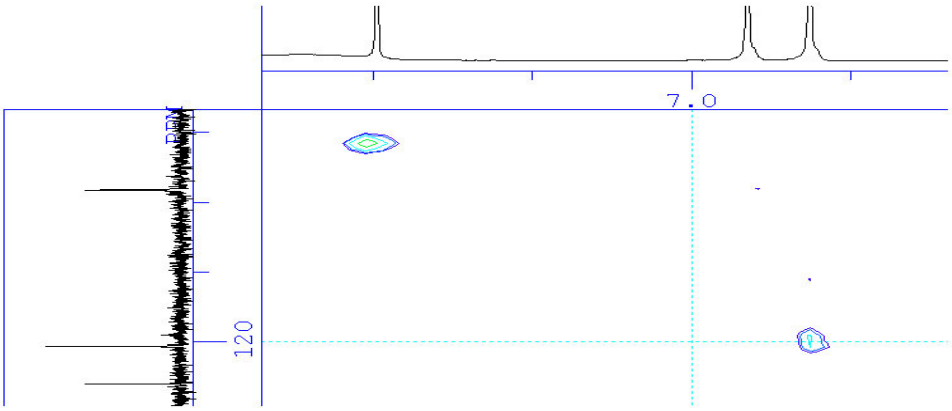

Figure 2. HMQC correlation of ethylenic proton δ_H 6.96 ppm and C-23 carbon δ_C 120.3 ppm

produced from the reaction at 595 nm. Results were derived from triplicate experiments.

RESULT AND DISCUSSION

Structure determination of compound 1

Compound 1 of the fraction I-b was obtained as yellowish amorph with $[\alpha]_D$ +15º (*c* 0.5). It was analysed to have a molecular formula $C_{31}H_{42}O_8$ from its molecular ion peak at m/z 542 in the EI mass spectrum and the number of hydrogens and carbons in the ^1H and ^{13}C NMR spectra. Its H NMR spectrum showed signals due to eight tertiary methyls at δ_H (ppm) 0.98 (s); 1.02(s), 1.31 (s); 1.36 (s); 1.46 (s); 1.48 (s); 1.95 (s), and 2.02 (s) (3H each S), along with signals of methylene hydrogens at 1.44, 1.87, 1.95, 2.17 (1H each m), 2.64, 2.67 (1H each dd), and 2.72, 2.88 (1H each d). In addition, one aromatic hydrogen signal at δ 6.33 (1 H,s) and two olefinic hydrogen signals at δ 6.96 (1 H, d, J= 15.6 Hz) and 7.00 (1H,d, J = 15.6 Hz) were observed. These evidences of ^1H NMR data were supported by the ^{13}C NMR spectrum showing signals due to eight methyl carbons at δ 11.3, 19.6, 19.7, 21.9, 23.8, 25.7, 26.4, and 28.9 and four methyelene carbons at δ 19.2, 23.6, 45.2, and 50.8. The presence of aromatic carbons were shown by the signals appearing at δ 109.1, 123.0, 126.7, 128.4,141.1, and 141.4 and that of olefinic carbons were indicated by the signals at δ 120.3 and 151.9. ^{13}C NMR data suggested that this compound has heterocyclic rings and one aromatic group with a pentasubstituted pattern. The olefinic group might be present at the side chain of the molecule. This skeleton of the compound was assumed to belong to a class of norcucurbitacin.

Correlation of H-H COSY and HMBC (Figure. 1) showed *trans* disubstitution at the double bond C=C (posistion C-23-C-24) as confirmed by ^1H NMR signals at δ_H 6.96 ppm (1H, d, J = 15.6 Hz) and 7.00 ppm (1H, d, J = 15.6 Hz). The ethylenic protons are confirmed bound to C-23 (δ_C 120.3 ppm) and C-24 (δ_C 151.9 ppm), as shown by HMQC correlations at Figure. 2 and Figure. 3, respectively.

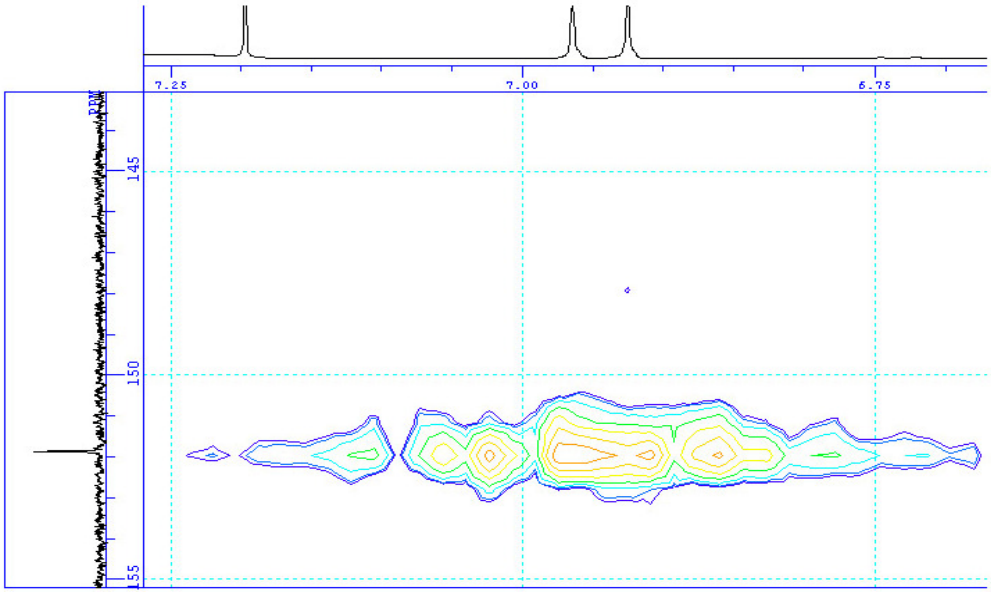

Figure 3. HMQC correlation of ethylenic proton δ_H 7 ppm and C-24 carbon δ_C 151.9 ppm

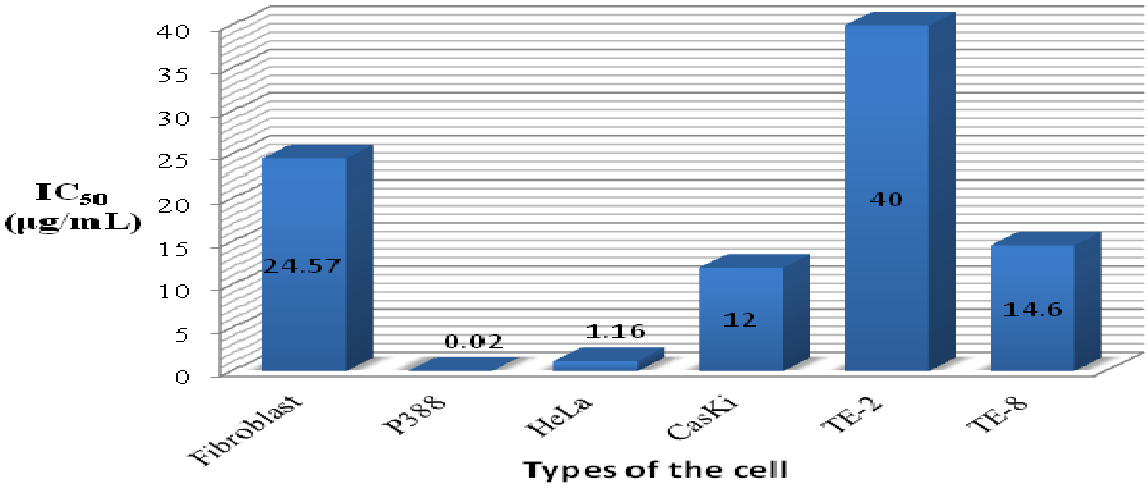

Figure 4. IC_{50} values of fevicordin A against the growth of various cells

The planar structure was determined, without ignoring the double bond equivalency, indicating it to be a C29-tetracyclic triterpenoid including an aromatic A-ring, i.e., the characteristic carbon skeleton of the 29 norcucurbitacin family, and the compound was confirmed as fevicordin A.

Cytotoxicity activity

In the cytotoxic assay, several cancer cells (P388, HeLa, CasKi, TE-2 and TE-8) were used to evaluate cytotoxic activity of fevicordin A of the *P. macrocarpa* seeds. Normal cells, fibroblast cells, cultured and differentiated in DMEM were used to compare the cytotoxicity of fevicordin A on normal cells and cancer cells.

As shown in Figure 4, fevicordin A showed inhibitory activity against the growth of normal and cancer cell lines tested, and selective cytotoxicity was observed on certain types of cancer cells. This compound strongly inhibited proliferation of leukemia cells P 388 (IC_{50} < 0.02 µg/mL) and cervical cancer cells HeLa (IC_{50}: 1.16 µg/mL), but only weakly gave inhibitory activity against the growth of cervical cancer cells CasKi (IC_{50}: 12.0 µg/mL),

esophageal cancer cells TE-8 (IC_{50}: 14.60 µg/mL), and fibroblast cells (IC_{50}: 24.51 µg/mL). Its high cytotoxicity on P 388 cells was predictable because these cells belong to floating cells and very sensitive to tested agents. Moreover, this compound gave different cytotoxicity on the two types of cervical cancer cells (HeLa and CasKi) that might be due to the differences in their differentation. It was also the same that it happened to the two esophageal cancer cells (TE-2 and TE-8). These data indicated that fevicordin A had high selectivity on leukemia cells P 388 and cervical cancer cells HeLa. Further investigation is needed to reveal its mechanism of action.

CONCLUSIONS

This study suggests that fevicordin A isolated from *P. macrocarpa* may have potential as a good candidate for leukemia and cervix cancer drugs.

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