

Cytotoxic Activity of Alpinumisoflavone from *Erythrina poeppigiana* (Leguminosae) Against Colon Cancer (WiDr), Cervical Cancer (Hela), and Hepatoma Cancer (HepG₂) Cells

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ABSTRACT

Cancer is the second cause of death after cardiovascular diseases in the world. Anticancer prevention used can cause undesirable things. Flavonoids are secondary metabolites derived from natural products that are useful for anticancer treatment. This study was performed to observe the cytotoxic activity of alpinumisoflavone from *Erythrina poeppigiana*, toward cervical cancer (Hela), colon cancer (WiDr), and hepatoma cancer (HepG₂) cells. The cytotoxic activity of alpinumisoflavone was tested using (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay. The percentage of cell mortality was calculated and the IC₅₀ was calculated using probit analysis. The result shown that alpinumisoflavone has antiproliferative effect to colon cancer (WiDr), cervical cancer (Hela), and hepatoma cancer (HepG₂) cells with the value of IC₅₀ are 5.63, 7.18, and 18.08 µg/ml, respectively. Based on the value of IC₅₀ alpinumisoflavone is very cytotoxic to colon cancer WiDr cell.

1. Introduction

Cancer is a very common problem with many ways of healing and prevention therapy. Chemotherapy is one of the many ways to prevent cancer, but it can cause a patient's health to decrease. Therefore, there is a focus on using alternative treatments and safe therapies against cancer. Medicinal plant species are already being used to treat or prevent development of cancer (Greenwell and Rahman 2015).

Medicinal plants contain secondary metabolites with potential anticancer activities have been reported. The species *Erythrina poeppigiana* which is a family of Leguminosae and famous medicinal plants is used as a traditional medicine for anthelmintic, cancer, malaria, and inflammatory processes. Extracts of the leaves, stem bark, and roots of *E. poeppigiana* are known to contain secondary metabolites such as alkaloids and flavonoids (Herlina *et al.* 2018).

Previously, phytochemical analysis of *E. poeppigiana* has known the presence of erythrina alkaloids and isoflavonoids. Five isoflavonoids, erypogeiins A, B, C, D, and E have been isolated from the roots of *E. poeppigiana* and eleven isoflavones were isolated from the stem bark of *E. poeppigiana* (Tanaka *et al.* 2001, 2002; Herlina *et al.* 2017). Purified flavonoids have also shown anticancer

activities against other human cancers including; hepatoma (Hep-G2), cervical carcinoma (Hela), and breast cancer (MCF-7) (Greenwell and Rahman 2015). The affinity of isoflavonoids for the estrogen receptors ER α and ER β was evaluated using a receptor binding assay (Djiogue *et al.* 2009).

The cytotoxic activity of erypogein D from the stem bark of *E. poeppigiana* showed the highest activity (IC₅₀ 30.12 M) against breast cancer MCF-7 cell compared to cervical cancer HeLa and ovarian cancer SKOV-3 cells in vitro by using MTS assay method, this indicates that erypogein D belongs to the category of moderate cytotoxic activity (Herlina *et al.* 2017). Alpinumisoflavone compound (Figure 1) isolated from the stem bark of *E. poeppigiana* that has never been studied for cytotoxic activity. Thus, in this study, we reported the cytotoxic activity of alpinumisoflavone against cervical cancer (Hela), colon cancer (WiDr), and hepatoma cancer (HepG₂) cells.

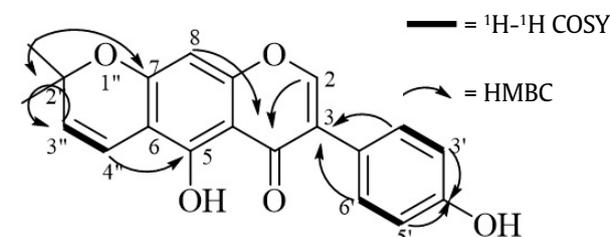


Figure 1. The correlation ¹H-¹H COSY and HMBC

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2. Materials and Methods

2.1. Extraction and Isolation

The alpinumisoflavone was isolated from the stem bark of *E. poeppigiana* in the Organic Chemistry Laboratory, Universitas Padjadjaran, Indonesia. The dried powder of stem bark (2.4 kg) was extracted with methanol at room temperature for @ 3 x 24 h. The evaporated methanol extract (155.9 g) was then partitioned successively with *n*-hexane and ethyl acetate, to yield viscous concentrated of *n*-hexane (24.0 g) and ethyl acetate (30.0 g). The ethyl acetate extract (30.0 g) was fractionated by column chromatography on silica gel using a gradient *n*-hexane-ethyl acetate-methanol to give seven fractions (A–G). Fraction C (8.0 g) eluted with gradient of *n*-hexane/ethyl acetate (from 9:1 to 100:0) was subjected to RF-18 column chromatography eluting with a gradient of methanol/water (from 8:2 to 100:0) to obtain compound 1 (24 mg).

2.2. Cell Culture (WiDr, HeLa, and HepG₂) Cells

The HeLa, WiDr, and HepG₂ cells were used to determine the cytotoxic activity of alpinumisoflavone. The HeLa (ATCC, CCL-2), WiDr (ATCC, HTB-77), and HepG₂ (ATCC, HTB-22) cells were provided by the Faculty of Medicine Research Center, Universitas Gajah Mada, Yogyakarta. The WiDr and HeLa cell lines were maintained in DMEM (Gibco, 1195-065), 10% fetal bovine serum (FBS) (Gibco, 10270), and 1% Abam (Gibco, 15240-062), while HepG₂ cell lines were maintained in McCoy's BA (Gibco, 16600-082), and added the same reagents for HeLa cell lines. HeLa, WiDr, and HepG₂ cells were secured under 37°C, 5% CO₂ atmospheric, and 95% humidity. During an incubation time of 24 hours, calculation of viable number of cells with the

addition of Trypan blue staining and analyzed using haemocytometer (Widowati *et al.* 2013).

2.3. Cytotoxic Assay

Cytotoxic activity of alpinumisoflavone on the treated cells were determined using the (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) MTT colorimetric assay modified method. The treated cells culture (90 µl) of HeLa, WiDr, and HepG₂ cells at density of 104 cells/well were distributed in 96-wells plates. Ten microlitres of the alpinumisoflavone at 100.0, 50.0, 25.0, 12.5, 6.25, and 3.25 (µg/ml) was added to each well. Culture medium alone was served as negative control. The plate was then incubated for 24 h at 37°C in a 5% CO₂. The mixture was further added with 20 µl of using MTT, incubated for 4 hours at 37°C in 5% of CO₂. The absorbance was read at 490 nm wavelength (7). The cell growth inhibition was determined with the equation (2). % of cell growth inhibition=(OD of control cell-OD of treated cell)/(OD of control cell) x 100%. Inhibitory concentration (IC₅₀) were determined using probit analysis. The IC₅₀ values was obtained from average of three times independent experiments (Widowati *et al.* 2013).

3. Results

3.1. Analytical Data of Compound 1

Compound 1 as yellow crystal. Mp: 207-210°C; IR (KBr): 3422, 2977, 1659, 1463, 1060, 800 cm⁻¹; UV/Vis λ_{max} (MeOH) nm (log ε): 282 (4.27), 213 (4.53); HR-TOFMS ES- spectrum showed [M+H]⁺- 335.0877, calcd m/z 335.0861. The ¹H NMR (600 MHz) and ¹³C NMR (150 MHz) spectroscopic data in CDCl₃ (Table 1).

Table 1. ¹H and ¹³C NMR spectroscopic data compound 1 and alpinumisoflavone in CDCl₃

Pos.	Compound 1		Alpinumisoflavone	
	δC ^a (mult)	δH ^b (Int., mult., J=Hz)	δC ^c (mult)	δH ^d (Int., mult., J=Hz)
2	152.7 (d)	7.81 (1H, s)	153.0 (d)	7.78 (1H, s)
3	123.7 (s)	-	-	-
4	181.1 (s)	-	181.4 (s)	-
4a	106.2 (s)	-	106.5 (s)	-
5	156.9 (s)	13.01 (1H, s)	157.2 (s)	13.07 (1H, s)
6	105.7 (s)	-	106.0 (s)	-
7	159.7 (s)	-	160.0 (s)	-
8	95.0 (d)	6.33 (1H, s)	95.3 (d)	6.30 (1H, s)
8a	157.4 (s)	-	156.5 (s)	-
1'	123.0 (s)	-	124.0 (s)	-
2'	130.4 (d)	7.34 (1H, d, 7.8)	130.7 (d)	7.31 (1H, d, 8.1)
3'	115.7 (d)	6.84 (1H, d, 7.8)	116.1 (d)	6.82 (1H, d, 8.1)
4'	156.1 (s)	-	157.7 (s)	-
5'	115.7 (d)	6.84 (1H, d, 7.8)	116.1 (d)	6.82 (1H, d, 8.1)
6'	130.4 (d)	7.34 (1H, d, 7.8)	130.7 (d)	7.31 (1H, d, 8.1)
2''	78.2 (s)	-	78.5 (s)	-
3''	128.3 (d)	5.62 (1H, d, 9.6)	128.6 (d)	5.59 (1H, d, 10)
4''	115.5 (d)	6.71 (1H, d, 9.6)	115.9 (d)	6.69 (1H, d, 10)
2''-CH ₃	28.4 (d)	1.46 (6H, s)	28.7 (d)	1.44 (6H, s)

a= 150 MHz, b= 600 MHz, c = 125 MHz, and d=500 MHz

3.2. Cytotoxic Activity Compound 1 Against Colon Cancer (WiDr), Cervical Cancer (HeLa), and Hepatoma Cancer (HepG₂) Cells

The MTT method was used to determine the cytotoxic activity against WiDr, HeLa, and HepG₂ cells. The results of cytotoxic activities are summarized in Table 2.

3.3. Morphological Appearance of WiDr, HeLa, and HepG₂ Cells

The morphological appearance of WiDr, HeLa, and HepG₂ cells treated with compound 1 was shown in the Figure 2, 3, and 4.

4. Discussion

4.1. Characteristics of Compound 1

The UV absorption maxima of compound 1 in methanol were absorbed at a peak 1 and 2 (287 and 205 nm), which is characteristic of the carbonyl bound

to a benzene group. The compound 1 was added AlCl₃ reactants showed bathochromic and hypochromic shift at a peak I and II (294 and 210 nm), indicating the formation of a compound AlCl₃ complex between the hydroxyl and ketone groups, compound 1 is characteristic of isoflavone. Its IR spectrum showed absorption characteristic of hydroxyl and ketone aromatic (3422, 1659, and 1640 cm⁻¹).

The ¹H NMR spectrum of compound 1 showed the presence of signals typical of an isoflavone nucleus having a pyran ring. The ¹H NMR spectrum revealed a pair of doublets (*J*=9.6 Hz) centered on δ 5.62 and 6.71 and sharp singlets six proton intensities at δ 1.46. The ¹H NMR spectrum also displayed a pair of doublets (*J*=7.8 Hz), each integrating for two protons, at δ 6.84 and 7.34, which were assigned to the H-3' and H-5', and H-2' and H-6' of the para-disubstituted aromatic nucleus. The relatively upfield resonance (δ 6.84) of H-3' and H-5' suggested the presence of an oxygenated substituent at C-4'. The proton signal of hydroxyl group showed the presence of a broad singlet at δ 6.33 (1H), due to a hydroxyl group proton. These were assigned to a 2,2-dimethylchromene ring system. The characteristic C-2 proton of the isoflavone skeleton was evident as a singlet at δ 7.81 (1H).

Table 2. The IC₅₀ value of WiDr, HeLa, and HepG₂ cells

Cells	IC ₅₀ (µg/ml)
WiDr	5.63
HeLa	7.18
HepG ₂	18.08

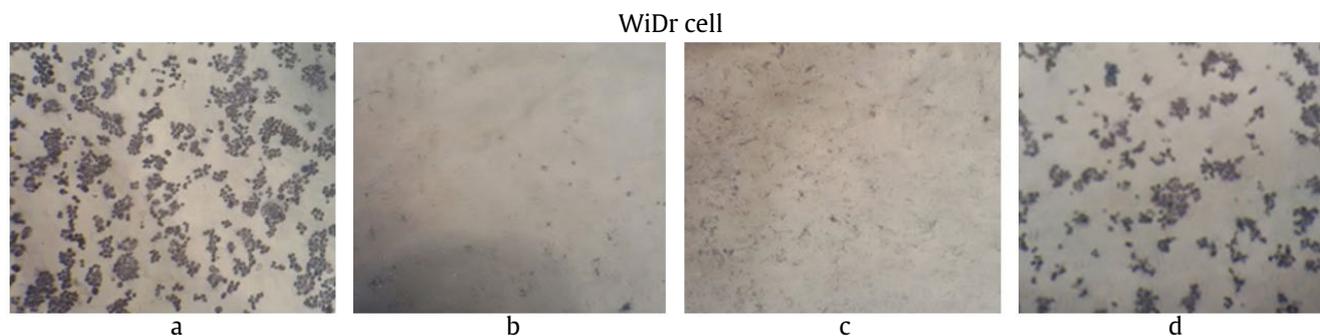


Figure 2. Morphological appearance of WiDr cells with treatment of compound 1: (a) control, (b) 50.0, (c) 12.5, and (d) 3.25 µg/ml. Scale bar: 40x

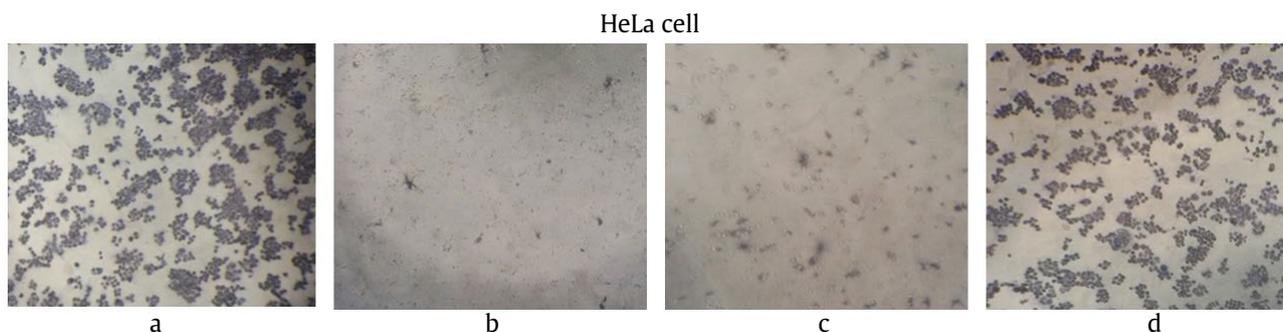


Figure 3. Morphological appearance of HeLa cells with treatment of compound 1: (a) control, (b) 50.0, (c) 12.5, and (d) 1.56 µg/ml. Scale bar: 40x

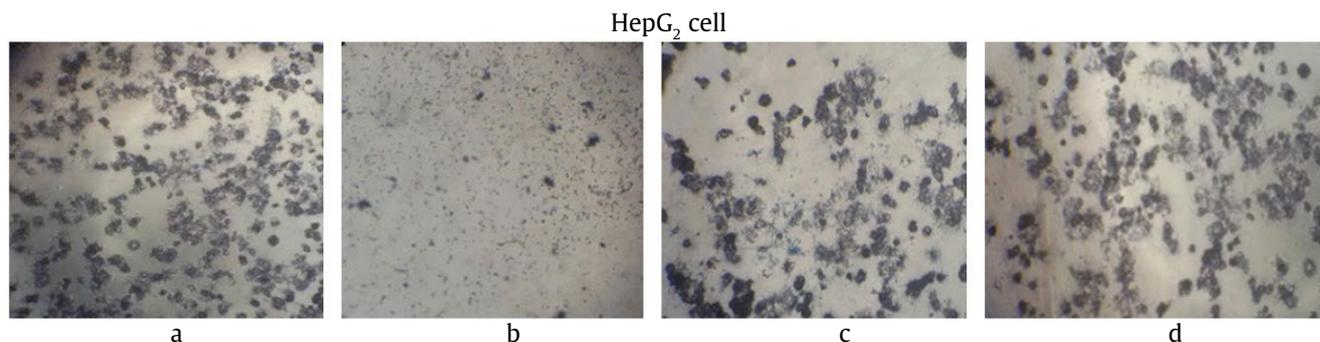


Figure 4. Morphological appearance of HepG₂ cells with treatment of compound 1: (a) control, (b) 50.0, (c) 12.5, and (d) 1.56 µg/ml. Scale bar: 40x

The HMQC spectrum of compound 1 showed signal of H-2 (7.81) was bounded to an oxygenated carbon of C-2 (152.7), this was showed of compound 1 is characteristic of isoflavone. The ¹H-¹H COSY spectrum of compound 1 showed the presence of a cross peak between H-2' (7.34) and H-3' (6.84), H-5' (6.84) and H-6' (7.34) indicated that the protons are *ortho* position, and the crossing peaks between H-3" (5.62) and H-4" (4.71). The HMBC spectrum of compound 1 showed correlation of signal H-3' (6.84) with C-4' (156.1) and H-5' (6.84) with C-4' (156.1), indicated of hydroxyl group position at C-4'. The correlation of signal H-4" (6.71) with C-5 (156.9), indicated of hydroxyl group position at C-5. The correlation of signal proton (1.46) with C-7 (78.2) and C-2" showed of 2,2-dimethylpyran ring position (Figure 4). The ¹H and ¹³C NMR spectroscopic data compound 1 and alpinumisoflavone in CDCl₃ (Table 1). On the basis of the above spectral data and by comparison of these values with those reported for alpinumisoflavone, C₂₀H₁₆O₅. Alpinumisoflavone with another name is 5-hydroxy-7-(p-hydroxyphenyl)-2,2-dimethyl-2H-6Hbenzo-[1,2-b:5,4-b]dipyrans-6-one (Hussaini *et al.* 2011).

4.2. Cytotoxic Activity of Alpinumisoflavone

As shown in Table 2, alpinumisoflavone had the the lowest IC₅₀ in colon cancer WiDr cell (5.63 µg/ml) which indicated highest cytotoxicity compared to cervical cancer cell (Hela) and hepatoma cancer cell (HepG₂). Alpinumisoflavone is the most selective to colon cancer WiDr cell, however the cytotoxic activity of alpinumisoflavone is strong.

Alpinumisoflavone as a major active ingredient commonly used as a traditional Chinese medicine *Derris eriocarpa* in Southwest part of China possesses a variety of pharmacological activities, including atheroprotective, estrogenic, and anti-bacterial (Wang *et al.* 2017). Alpinumisoflavone displayed cytotoxicity against the breast cancer MCF-7, lung cancer NCI-H18, and cervical cancer KB cells line with

IC₅₀ values of >100, >100, and 4.13 µM, respectively (Nkengfack *et al.* 2001; Sudanich *et al.* 2017). Effects of alpinumisoflavone on proliferation after 24, 48, and 72 h incubation of human leukemia U937 cell line showed IC₅₀ values of 35, 32, and 28 µM, respectively (Matsuda *et al.* 2007). Alpinumisoflavone exhibited weak cytotoxic activity against the prostate cancer (PC3) cell line (Iranshahi *et al.* 2012). Wighteone and alpinumisoflavone isolated from *E. indica* (Leguminosae) showed cytotoxic activity against KB nasopharyngeal cancer cells, effective doses of 0.78 and 4.13 µg/ml, respectively (Orang-ojong *et al.* 2013).

Alpinumisoflavone is a flavonoid that has a pyran ring as pyranisoflavonoid. Flavonoid pyran groups isolated from medicinal plants that are responsible as active substances. Alpinumisoflavone isolated from *E. lysistemom* of the African medicinal plant. Alpinumisoflavone reported as a treatment for the prevention of human lung tumor cell death (Namkoong *et al.* 2011).

Flavonoid substituents shown growth inhibitory activity against cervical cancer (Hela) and colon cancer (WiDr) cells. The presence of hydroxyl group in A-ring in positions 5 also increase the cytotoxic activity of a flavonoids. The presence of hydroxyl group in B-ring in positions 4' shown increase the cytotoxicity of a flavonoids. B-ring at heterocyclic C3 position instead of C2 is characteristic of isoflavones. The study of cytotoxic activity of isoflavones showed high activity (López-Lázaro *et al.* 2002).

4.3. The Morphological Appearance

Based on the Figure 1 the morphological appearance of WiDr cell treated with alpinumisoflavone showed the decreasing of cell number, which indicate the effects of treatment toward WiDr cell cells growth. In the Figure 3 and 4, we can see that the morphological appearance of HeLa and HepG₂ cells also showed the decreasing of cell number. Its indicate that treatment with alpinumisoflavone influence the cell growth of WiDr, HeLa, and HepG₂. The result of present study

suggests that the alpinumisoflavone might be a promising agent for anticancer treatment.

5. Conclusion

Alpinumisoflavone compounds isolated from the stem bark of *E. poeppigiana*. Alpinumisoflavone showed the highest cytotoxic activity against colon cancer WiDr cell (5.63 µg/ml) compared with hepatoma cancer HepG₂ and cervical cancer HeLa cells *in vitro* by MTT assay method. Alpinumisoflavone has a category of strong cytotoxic activity.

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