RESEARCH ARTICLE

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Antimigration Activity of an Ethylacetate Fraction of *Zanthoxylum acanthopodium* DC. Fruits in 4T1 Breast Cancer Cells

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Abstract

Objective: This study was carried out to investigate the antimigration activity of *Zanthoxylum acanthopodium* DC. in the 4T1 breast cancer cell line. **Methods:** *Zanthoxylum acanthopodium* DC. fruit powder was extracted by maceration method with n-hexane and ethylacetate solvents. Cytotoxicity and proliferation were assessed using the MTT method and the cell cycle by flow cytometry. In addition, wound healing assays were conducted by a microscopic method, and expression of COX-2 and VEGFR-2 were determined using qRT-PCR. **Results:** The IC50 of the ethylacetate fraction (EAF) was 48.1 ± 1.06 µg/mL. The EAE at a concentration 10 µg/mL with viable cells was 62.3 ± 0.28% after 72 h incubation, with accumulation in the G2-M phase, inhibition of cell migration in the wound healing assay, and decrease in expression of COX-2 (0.62 ± 0.01) and VEGFR-2 (0.39 ± 0.003). **Conclusion:** The results reveal that an ethylacetate fraction of *Zanthoxylum acanthopodium* DC. fruits provides effective antimigration effects. Further studies are now planned to assess the potential of the ethylacetate fraction to inhibit angiogenesis in breast cancer and determine underlying mechanisms.

Keywords: Antimigration- *Zanthoxylum acanthopodium* DC. fruits- ethylacetate

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Introduction

Cancer cells migration is necessary for tumor development. The spread of cancer in our body is a multistep phenomenon which cancer cells invade surrounding tissues and blood or lymphatic vessel (Lirdprapamongkol et al., 2008; Mao et al., 2016). All solid tumours are dependent on angiogenesis for growth and metastasis. A recent study reported that breast cancer is leading in the estimated new cancer cases, and the second most common death cause of women suffering from cancer in the USA (Siegel et al., 2015).

Traditionally, andaliman fruits (*Zanthoxylum acanthopodium* DC.) has been used as aromaticum substances, tonicum, and treat dysentery. Indian people have used andaliman to treat paralyzed and skin disease such as abscess and leprosy. Andaliman has been used as spices at North Sumatera especially at North Tapanuli (Suryanto et al., 2004; Hynniewta and Kumar, 2008; Sirait et al., 2001). The plants from *Zanthoxylum* genus contain many compounds such as phenol hydroquinones, flavonoids, steroids/ triterpenoids, tannins, glycosides, volatile oils, alkaloids, coumarines, lignans, amides and terpenes (Parhusip, 2006; Fernandez et al., 2009; Yao-Kuassi et al., 2015; Hu et al., 2006; Hu et al., 2014; Yang et al., 2004; Cui et al., 2008; Chen et al., 2015). Ethylacetate extract of andaliman fruits (EAF) was showed to have cytotoxicity effect against MCF-7 and T47D cell lines. EAF was found to have synergistic effect when combined with doxorubicin. EAF was showed to have anticancer activity towards mices induced with benzo (a) pyrene, having cardioprotective effect and active on T47D resistance cells (Sihotang, 2015; Anggraeni et al., 2014; Hasibuan et al., 2016). However, the antimigration activity EAF of *Zanthoxylum acanthopodium* DC. have yet to be elucidated.

The aim of this study was to determine cytotoxic and migration inhibition activity of ethylacetate fraction of *Zanthoxylum acanthopodium* DC. fruits on 4T1 cells.

Materials and Methods

Fractions Preparation

Fresh fruits of *Zanthoxylum acanthopodium* DC. was collected from Onan Rungu village, Samosir regency, Sumatera Utara Province, Indonesia. The air-dried and

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powdered fruits of *Zanthoxylum acanthopodium* DC. (1 kg) were repeatedly extracted by cold maceration with n-hexane (3x3 d, 7.5 L). The powder was dried in the air and extracted with ethylacetate (3x3 d, 7.5 L) at room temperature on a shake. The filtrate was collected, and then evaporated under reduced pressure to give a viscous extract and then freeze dried to give a dried extract (Anggraeni et al., 2014; Hasibuan et al., 2016; Satria et al., 2015; Hasibuan et al., 2015).

Cytotoxicity assay

EAF were submitted for cytotoxicity test. In that way, 4T1 cell line was grown in DMEM medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycine (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO_2) at 37 ^{o}C. The inoculums seeded at 1 x 10^4 cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by fractions. After incubation for 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h at 37 ^{o}C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h in room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using ELISA reader at λ 595 nm. The data which were absorbed from each well were converted to percentage of viable cells (Hasibuan et al., 2015; Satria et al., 2014; Nurrochmad et al., 2014).

Cell cycle inhibition assay

4T1 cells (5x10^5 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with EAF and then incubated for 24 h. Both floating and adherent cells were collected in conical tube using tripsin 0.025%. The cells were washed thrice with cold PBS and centrifuged at 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at 4 ^{o}C for 1 h. The cells were washed thrice with cold PBS and resuspended then centrifuged at 3000 rpm for 3 min and PI kit (containing PI 40 µg/mL and RNAse 100 µg/mL) added to sediment and resuspended and incubated at 37 ^{o}C for 30 min. The samples were analysed using FACScan flow cytometer. Based on DNA content, percentage of cells in each of stage in cell cycle (G1, S and G2/M) were calculated using ModFit Lt. 3.0.s (Anggraeni et al., 2015; Satria et al., 2015; Satria et al., 2017).

Antiproliferative Activity

EAF (10 µg/mL) was submitted for antiproliferative test. In that way, 4T1 cell line (2.5 x 10^3cells/mL) was grown in DMEM complete medium. After 24; 48 and 72 h treatment, MTT assay was performed and cell viability was counted to calculate the antiproliferative activity (Zihlif et al., 2013).

Wound Healing Migration Assay

The migration assay was carried out with 4T1 cells were seeded at 5x10^4 cells/well in 24-well plates and incubated for 24 h at 37°C. Cultured cells were washed with PBS and added culture media which contining 0.5% FBS and incubated for 24 h. Scratch was done in the bottom center of the well within cell layer using yellow tip. Residues cell in the plate were washed with PBS and treated with EAF and incubated for 72 h at 37°C and documented under inverted microscope against cell migration rapidity after 0, 24, 48 and 72 h. The space from scratch treatment between control and treatment cultur cell was quantified using Image J software and defined as cell migration area (Zihlif et al., 2013; Wang et al., 2012).

Expression of COX-2 and VEGFR-2

4T1 cells (5x10^5 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with EAF and then incubated for 24 h. Both floating and adherent cells were collected in conical tube using tripsin 0.025%. The cells were washed thrice with cold PBS and centrifuged at 2500 rpm for 5 min. The supernatant was separated and used for RNA extraction (Genaid, USA) and RNA concentration was determined by spectrophotometric method (Nanodrop) and stored at -80°C until used. Complementary DNA (cDNA) was synthesized from 3.0 µg total RNA using RT-PCR kit (Toyobo, Japan) in a final volume of 20 µL using random primers based on the manufacturer's instructions. qRT-PCR was carried out in AB 7500 Fast (ABI, USA). The reaction mixture consisted of SensiFASTTM SYBR®Lo-ROX kit (10 µL) (Bioline, USA), 1.0 µL of cDNA and 0.8 µL primers in a total volume 19 µL. β-actin was used as internal reference control. The PCR primers were used for β-actin (F: 5'-gtc gta cca ctg gca ttg t-3'; R: 5'-cag ctg tgg tga agc t-3'), Cox-2 (F: 5'-cca gca ctt cac gca tca gt-3'; R: 5'-acg ctg tct agc cag agt ttc ag-3') and VEGFR-2 (F: 5'-gtg tca gaa tcc ctg cga agt a-3'; R: 5'-gaa atg gga ttg gta agg atg-3'). The PCR condition were comprised of first incubation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 sec, annealing/ extension at 60°C 30 sec. Fluoresence was recorded at the end of extension. A negative control (NTC) was run simultaneously with every assay. Quantification of expression was determined by relative method using cycle threshold value (Zihlif et al., 2013; Abdolmaleki et al., 2016; Wang et al., 2012).

Statistical Analysis

The results were presented as means ± SD. The statistical analysis was carried out by using SPSS edition 21.

Results

Inhibitory Concentration 50% (IC50)

MTT method was used to determine cell viability after incubation for 24 h (Figure 1). In every treatment EAF was shown to inhibit cells growth. The IC50 value of EAF was 48.06 ± 1.06 µg/mL.

Effect on Cell Cycle

To evaluate the effect of EAF to increase cell death by modulating cell cycle, we concentrated on it for further studies using flow cytometry method. The effect of EAF

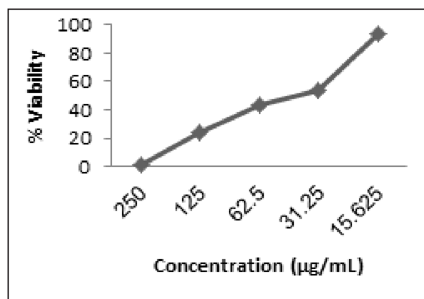

Figure 1. Percentage of Viable Cells VS Concentration of EAF

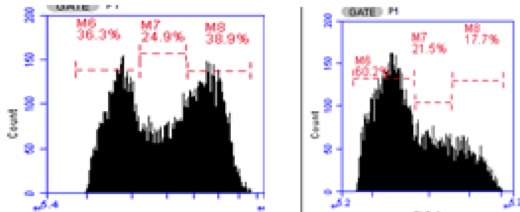

Figure 2. Percentage Phases on Cell Cycle of 4T1 Cells were Treated by (a) Control Cells (b) EAF 10 µg/mL

is given in Figure 2. Whereas treatment of EAF in 10 µg/mL caused cell accumulation at G2/M phase (38.90%) and for control cell (17.70%).

Antiproliferative Activity

To evaluate the effect of EAF to decrease the number of cells by inhibiting cell proliferation. The percentage of viable cells after treatment and incubation for 24, 48 and 72 h (54.08 ± 1.16; 59.88 ± 0.24 and 62.26 ± 0.28) showed the inhibition effect of EAF towards proliferation of 4T1 cells. The effect of EAF is given in Figure 3.

Wound Healing Migration Assay

The scratch wound healing assay was performed to evaluate the effect of EAF on 4T1 migration. The wound healing migration of EAF is given in Figure 4. A little wound repair was observed in wells with EAF at 10 µg/mL after 24; 48 and 72 h incubation with 10.23 ± 1.49%; 47.46 ± 1.46% and 64.15 ± 1.13% respectively closure area.

COX-2 and VEGFR-2 Expression

Two steps qRT-PCR were used to evaluated COX-2 and VEGFR-2 expression in 4T1 cells after the treatment with EAF. EAF were showed a significant

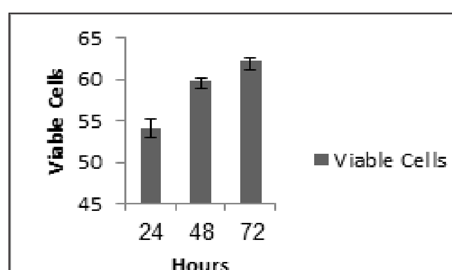

Figure 3. Percentage of Viable Cells of 4T1 Cells were Treated by EAF 10 µg/mL for 24; 48 and 72 h and Measured Viable Cells

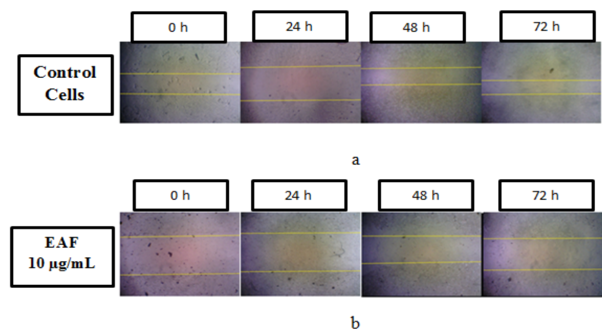

Figure 4. Wound Healing Migration Assay. 4T1 cells were treated by EAF for 24; 48 and 72 h and measured the closure area. (a) control cells; (b) EAF 10 µg/mL. EAF extended migration of 4T1 cells.

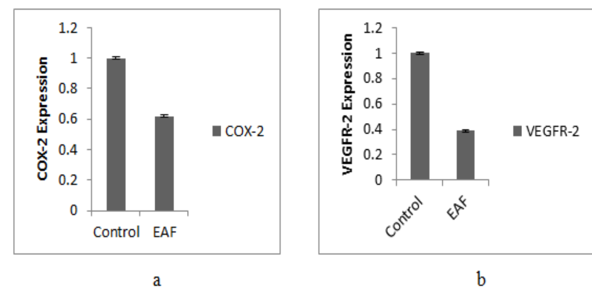

Figure 5. Gene Expression after Treatment with EAF 10 µg/mL. (a) COX-2 expression; (b) VEGFR-2 expression

down-regulatory effect on the expression of COX-2 (0.62 ± 0.01) and VEGFR-2 (0.39 ± 0.003) after treatment at 10 µg/mL. The inhibition of EAF towards COX-2 and VEGFR-2 expression are given in Figure 5.

Discussion

The cytotoxicity estimate of natural product is related to content of active compound in these plants including *Zanthoxylum acanthopodium* DC. Flavonoids, alkaloids and triterpenoids/steroids estimated as active compounds (Yadav et al., 2010). Other phytochemicals such as resveratrol, salvianolic acid B, and ginseng saponins were found to exert inhibitory effect on the vascularization and migration (Fan et al., 2006). This fact was to indicate that EAF can inhibite cell grow that G2/M. EAF were contained polyphenol compound such as flavonoids and tannins (Satria et al., 2015). The isolated polyphenols from plants including kaemferol, quercetin, anthocyanins, coumarin acid, and ellagic acid were shown to inhibit the growth (inhibit cell cycle and induce apoptosis) of human breast (MCF-7), oral (KB, Cal-27), colon (HT-29, HCT-116), and prostate (LNCaP, DU-145) tumor cell lines (Zhang et al., 2008; Daminiaki et al., 2000; Lim et al., 2006; Tang et al., 2007). VEGFR-2 is a transmembrane receptor that plays an important role in endothelial cell development (Risau, 1997; Shalaby et al., 1995) and is thought to mediate the key effect of the endothelial-specific mitogen VEGF on cell proliferation and permeability. Therefore, the majority of VEGFR-2 actions are related to angiogenesis and migration (Ferrara et al., 2003; Shibuya and Claesson, 2006).

VEGFR-2 receptors and VEGFR-2 mRNA are largely expressed in breast cancer (Aesoy et al., 2008; Svensson et al., 2005; Weigand et al., 2005; Carino et al., 2008). Cyclooxygenase-2 (COX-2) is an inducible enzyme which plays a critical role in multiple pathophysiological process including inflammation, atherosclerosis, tissue injury, angiogenesis and tumorigenesis (Howe 2007; Sinicrope and Gill, 2004; Singh and Lucci, 2002; Castellone et al., 2005). Flavonoids such as quercetin, genistein kaemferol inhibited expression of VEGFR-2 and COX-2 which mediated angiogenesis and migration in human breast cancer cells (Xiao et al., 2011). Based on the results and discussion that EAF of *Zanthoxylum acanthopodium* DC. has antimigration activity through inhibition of cell cycle on G2/M phase, wound healing assay, proliferation of cells, and expression of Cox-2 and VEGFR-2.

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