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

Urip Harahap, Poppy Anjelisa Zaitun Hasibuan, Panal Sitorus, Nur Arfian, Denny Satria

Objective: This study was carried out to investigate the antimigration activity of Zanthoxylum acaenthopodium DC. in the 4T1 breast cancer cell line. Methods: Zanthoxylum acaenthopodium 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 ± 0.10 μg/mL. The EAF 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 acaenthopodium 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 acaenthopodium DC. fruits; ethylacetate

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 vessels (Lirdprapanmongkol et al., 2008; Mox et al., 2016). Most solid tumors 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 acaenthopodium DC.) have 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; Srait et al., 2001). The plants from Zanthoxylum genus contain many compounds such as phenol hydroquinones, flavonoids, steroids, triterpenoids, tannins, glycosides, volatile oils, alkaloids, coumaryl, lignans, amides and terpenes (Paraiqvip, 2006; Fernandez et al., 2009; Yoo-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 mice induced with benzo (a) pyrene, having cardioprotective effect and active on T47D resistance cells (Sihotang, 2015; Anggristi et al., 2014; Hasibuan et al., 2016). However, the antimigration activity EAF of Zanthoxylum acaenthopodium DC. have yet to be elucidated.

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

Materials and Methods

Fractious Preparation

Fresh fruits of Zanthoxylum acaenthopodium DC. was collected from Onan Rungu village, Samosir regency, Sumatera Utara Province, Indonesia. The air-dried and
In Ayurvedic medicine, certain fruits of *Zanthoxylum aethiopicum* DC. (1 kg) were repeatedly extracted with cold maceration with n-hexane (3x3 d, 7.5 L). The powder was dried in the air and extracted with ethyl acetate (3x3 d, 7.5 L) at room temperature on a shaker. The filtrate was collected, and then evaporated under reduced pressure to give a viscous extract and then freeze dried to give a dried extract (Anggreni et al., 2014, Hasbuan et al., 2016, Satria et al., 2015, Hasbuan 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-streptomycin (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO2) at 37°C. The inoculums seeded at 1x10^4 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°C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as a stopper (Sigma) in 0.1 N HCI (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 595 nm. The data which were absorbed from each well were converted to percentage of viable cells (Hasbuan et al., 2015, Satria et al., 2014; Nurrochmad et al., 2014).

**Cell cycle inhibition study**

4T1 cells (5x10^4 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 tripin 0.025%. The cells were washed three times 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°C for 1 h. The cells were washed twice with cold PBS and resuspended then centrifuged at 3000 rpm for 5 min and PI kit containing PI 40 μg/L and RNAse 100 μg/mL added to sediment and resuspended and incubated at 37°C for 30 min. The samples were analysed using FACScan flow cytometer. Based on DNA content, percentage of cells in each stage of cell cycle (G1, S and G2/M) were calculated using ModFit LT 3.0 software (Anggreni 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 10^4 cells/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 (Zhilif, 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 containing 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 rapidly after 0, 24, 48 and 72 h. The space from scratch treatment between control and treatment cell was quantified using Image J software and defined as cell migration area (Zhilif et al., 2013; Wang et al., 2012).

**Expression of COX-2 and VEGF-R-2**

4T1 cells (5x10^4 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 tripin 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 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 ABI 7500 Fast (ABI, USA). The reaction mixture consisted of SensiFASTTM SYBR® Lo-ROX kit (10 μL) (Bioline, USA), 10 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 designed for β-actin (F: 5′-gct gca cca tgc gaa ttt tcg ttc a-3′; R: 5′-cag tgg tgg tga agt tgc a-3′), Cox-2 (F: 5′-cag cca ttc cag gca tca tct g-3′; R: 5′-cag tgc tgg agt a-3′) and VEGF-R-2 (F: 5′-cag tca gaa ttc ctc cca cag agt a-3′; R: 3′-atg ttt ctc cag gaa tgc tgc a-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 at 60°C for 1 min and extension at 60°C for 30 sec. Fluorescence 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 (Zhilif 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 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 inhibit cell grow that G2/M. EAF were contained polyphenol compound such as flavonoids and tannins (Satra 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; Minniti et al., 2000; Lim et al., 2006; Tang et al., 2007). VEGFR-2 is a transmembrane receptor that plays an important role in endothelial development (Riusu, 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 processes including inflammation, atherosclerosis, tissue injury, angiogenesis and tumorigenesis (Howe 2007; Sicincro and Gill, 2004; Singh and Luc, 2002; Castellone et al., 2005). Flavonoids such as quercetin, hesperetin kaempferol 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 acanthopodum DC. has antitumor activity through inhibition of cell cycle on G2/M phase, wound healing assay, proliferation of cells, and expression of Cox-2 and VEGFR-2.

Acknowledgements

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