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

Urip Harahap1*, Poppy Anjelisa Zaitun Hasibuan1, Panal Sitorus2, Nur Arfian3, Denny Satria2

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

Asian Pac J Cancer Prev, 19 (2), 565-569

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 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

1Department of Pharmacology, 2Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Sumatera Utara, 3Department of Anatomy, Faculty of Medicine, Gadjah Mada University, Indonesia. *For Correspondence: uripharahap@usu.ac.id
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-streptomycin (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO₂) at 37 °C. The inoculums seeded at 1 x 10⁵ 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 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 (5x10⁵ 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’-gtt gta cca ctc tgt tgg tga a 3’; R: 5’-cag ctg tgt gag agc t-3’), Cox-2 (F: 5’-cga cgt ctc cag cag cga gt-3’; R: 5’-ctg tgt aag cgg tct a-tg-3’), VEGFR-2 (F: 5’-gtg tca gaa tcc tgg tga a-3’; R: 5’-aat gag atg gta cag atg ag-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. 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 (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 was 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 1 x 10⁵ 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 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).

**Antiproliferative Activity**

EAF (10 µg/mL) was submitted for antiproliferative test. In that way, 4T1 cell line (2.5 x 10⁵ 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 (Zihlif et al., 2013).

**Wound Healing Migration Assay**

The migration assay was carried out with 4T1 cells were seeded at 5x10⁴ 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 rapidity after 0, 24, 48 and 72 h. The space from scratch treatment between control and treatment cell culture was quantified using Image J software and defined as cell migration area (Zihlif et al., 2013; Wang et al., 2012).
Antimigration Activity of Ethylacetate Fraction of Zanthoxylum acanthopodium DC. Fruits Against 4T1 Breast Cancer Cells

Antimigration Activity of Ethylacetate Fraction of Zanthoxylum acanthopodium DC. Fruits Against 4T1 Breast Cancer Cells

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 growth 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 processes including inflammation, atherosclerosis, tissue injury, angiogenesis and tumorigenesis (Howe 2007; Sinicropo and Gill, 2004; Singh and Lucci, 2002; Castellone et al., 2005). Flavonoids such as quercetin, genistein 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 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.

Acknowledgements

We gratefully thank to Research Center University of Sumatera Utara through Hibah Talenta “Hibah Penelitian Unggulan Universitas” Research Grant 2017 “No: 5338/UN5.1.R/PPM/2017” for financial support in the study.

References

Abdolmaleki Z, Arab HA, Amanpour S, Muhammadnejad S (2016). Anti-angiogenic effects of ethanolic extract of Artemisia sieberi compared to its active substance Artemisinin. Rev Bras Farmacogn, 26, 326-33.

Aesoy R, Sanchez BC, Norum JH, et al (2008). An autocrien VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells. Mol Cancer Res, 6, 1630-8.

Angrasa R, Hadisahputra S, Silalahi J, Satria D (2014). Combination effects of ethylacetate extract of Zanthoxylum acanthopodium DC. with doxorubicin on T47D breast cancer cells. Int J PharmTech Res, 6, 2032-5.

Carino C, Olawariye AB, Cherrils F, et al (2008). Leptin regulation of proangiogenic molecules in benign and cancerous endometrial cells. Int J Cancer, 123, 2782-90.

Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS (2005). Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science, 310, 1504-9.

Chen JJ, Yang CK, Kuo YH, et al (2015). New coumarin derivatives and other constituents from the stem bark of Zanthoxylum avicennae: Effects of neutrophil pro-inflammatory responses. Int J Mol Sci, 16, 9719-31.

Cui XG, Zhao QJ, Chen QL, et al (2008). Two new benzophenantridine alkaloids from Zanthoxylum nitidum. Helvetica Chimica Acta, 91, 155-8.

Daminiai A, Bakogeorgou E, Kampia M, Notas G, Hatzoglou A (2000). Potent inhibitory action of red wine polyphenols on human. JCB, 78, 429-41.

Fan TP, Yeh JC, Leung KW, Yue PYK, Wong RNS (2006). Angiogenesis: from plants to blood vessels. Trends in Pharmacol Sci, 26, 297-309.

Fernandez CC, Viera PC, daSilva VC, et al (2009). 6-acetylonyl-N-methyl-dihydrodecarine, a New Alkaloid from Zanthoxylum riedelianum. J Braz Chem Soc, 20, 379-82.

Ferrara N, Gerber HP, LeCouter J (2003). The biology of VEGF and its receptors. Nat Med, 9, 669-76.

Hasibuan PAZ, Harahap U, Sitorus P, Satria D (2016). Ethylacetate extract of Zanthoxylum acanthopodium DC. fruit against doxorubicin-resistant T47D cells. Der Pharma Chemica, 8, 172-4.

Hasibuan PAZ, Jessy C, Denny S (2015). Combination effect of ethylaceta extract of Plectranthus ambonicus (Lour.) Spreng. with doxorubicin againsts T47D breast cancer cells. Int J Pharm Pharm Sci, 7, 155-9.

Howe LR (2007). Inflammation and breast cancer. Cyocxooxygenase/prostaglandin signaling and breast cancer. Breast Cancer Res, 9, 210.

Hu J, Shi X, Mao X, Chen J, Li H (2014). Cytotoxic mannopanaryonosides of indole alkaloids from Zanthoxylum nitidum. Chem Biodiversity, 11, 970-4.

Hu J, Zhang WD, Liu RH, et al (2006). Benzophenantridine alkaloids from Zanthoxylum nitidum (Roxb.) DC, and their analgesic and anti-inflammatory activities. Chem Biodiversity, 3, 990-5.

Hynniewta SR, Kumar Y (2008). Herbal remedies among the khasi traditional healers and village folks in meghalaya. Indian J Traditional Knowledge, 7, 581-6.

Lim JH, Park JW, Min DS, et al (2006). NAG-1 up-regulation mediated by EGR-1 and p53 is critica for quercetin induced apoptosis in HCT-116 colon carcinoma cells. Apoptosis, 12, 411-21.

Lirdprapamongkol K, Kramb JP, Daranee C, Chantragan S, Rudef S (2008). Juice of Eclipta prostrata inhibits cell migration in vitro and exhibits anti-angiogenic activity in vivo. In Vivo, 22, 363-8.

Mao P, Ershao Z, Yang C, Likun L, Daqing R (2017). Pinus massoniana bark extract inhibits migration of the lung cancer A549 cell line. Oncol Lett, 13, 1019-23.

Nurrochmad A, Lukitaningsih E, Meiyanto E (2011). Anti cancer activity of rodent tuber (Thyphnomia flagelliforme (lodd.)) Blume on human breast cancer t47d cells. Int J Phytomed, 3, 138-46.

Parhupis A (2006). Kajian Mekanisme Antibakteri Ekstrak Andaliman (Zanthoxylum acanthopodium DC.) terhadap bakteri patogen pangan. tesis. Institut pertanian bogor. Risau W (1997). Mechanism of angiogenesis. Nature, 386, 671-4.

Satria D, Furqan M, Hadisahputra S, Rosidah F (2015). Combinational effects of ethylacetate extract of Picria fel-terrae Lour. and doxorubicin againts T47D breast cancer cells. Int J PharmTech Res, 6, 212-16.

Shalaby F, Rossant J, Yamaguchi TP, et al (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature, 376, 62-66.

Shibuya M, Claesson-Welsh L (2006). Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res, 312, 549-60.

Siegel RL, Miller KD, Jemal A (2015). Cancer statistics. CA Cancer J Clin, 65, 5-29.

Sihotang YM (2015). Uji Aktivitas antikanker payudara dan krisiprotektif dari ekstrak etilasetat daun poguntano (Picria fel-terrae Lour.) dan buah andaliman (Zanthoxylum acanthopodium DC.) secara In-Vivo. Thesis. Fakultas Farmasi USU.

Singh B, Lucci A (2002). Role of cyclooxygenase-2 in breast cancer. J Surg Res, 108, 173-9.

Sinicropo FA, Gill S (2004). Role of cyclooxygenase-2 in colorectal cancer. Cancer Metastasis Rev, 23, 63-75.

Sirait M, Siahaan M, Mangkudidjojo (1991). Pemerkisaan...
Antimigration Activity of Ethylacetate Fraction of Zanthoxylum acanthopodium DC. Fruits Against 4T1 Breast Cancer Cells

DOI:10.22034/APJCP.2018.19.2.565

This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International License.