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The Activity of Combination of Ethanol Extract of *Artocarpus lacucha* Buch.-Ham and *Anredera cordifolia* Steenis Leaves to Increase Wound Healing Process on NIH-3T3 Cell Line

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Abstract

AIM: This study aims to determine the effect of the combination of ethanol extract of *Artocarpus lacucha* Buch.-Ham and *Anredera cordifolia* Steenis leaves to increase cell proliferation and increase VEGFR-2 expression of NIH-3T3.

METHODS: The samples used were *Artocarpus lacucha* Buch.-Ham and *Anredera cordifilia* Steenis leaves. The powder of simples was extracted using ethanol 80% with maceration method. The effect of extract combination on proliferation was evaluated using the MTT method. Wound healing assay was established by a cell migration method, and VEGFR-2 expression was determined using RT-PCR.

RESULTS: The effect of combination of ethanol extract of Artocarpus lacucha leaves (EEALL) and ethanol extract of Anredera cordifolia leaves (EEACL) on cell proliferation after 24h, 48h and 72h incubation found as viable cells were showed (124.33 ± 0.32%; 128.52 ± 0.41%; 118.35 ± 0.22%). Percent of wound closed after 24 h and 48 h incubation are 64.88 ± 0.90% and 100.00 ± 0.00%, and expression of VEGFR-2 increased from 1 (control) to 1.58 ± 0.02.

CONCLUSION: The results suggest that a combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) is effective in increasing cells proliferation and hence wound healing process.

Introduction

Wound healing is a complex process involving many cells consisting of four phases, namely hemostasis, inflammation, proliferation, and remodeling. In the hemostasis, phase is the beginning of the wound healing process by involving platelets. During the inflammatory phase, fibroblasts function as cytokine secretions, and growth factors to activate the body's defense system. During the proliferation and remodeling phases, fibroblasts are important for granulating and reorganizing tissues of the extracellular matrix [1], [2], [3], [4].

Artocarpus lacucha Buch.-Ham belongs to the family of Moraceae, popularly regarded as a medicinal plant, commonly called as monkey jack and in Indonesia, it is called mobe. This plant is widely distributed in the tropical regions of south and southeast Asia, mainly Nepal, Srilanka, India, Myanmar, Indonesia, Vietnam, and Thailand. It has many pharmacological activities such as anti-inflammatory, antiviral, anticancer, antibacterial, and anti-HIV. In Thailand, the dried aqueous extract of its heartwood has been used as a traditional anthelmintic agent [5], [6], [7].

Anredera cordifolia (Ten.) Steenis is also known as Binahong is a family of Basellaceae, which is a medicinal plant that has been growing very well for long. In Indonesia, the Binahong plant is still uncommon but in Vietnam, this plant has a high demand and is often used as a vegetable in Taiwan. In China and Taiwan, this plant is known to have tremendous benefits and has been consumed more than a thousand years ago. Almost all parts of the Binahong plant such as tubers, stems, and leaves can be used in herbal therapy. Binahong leaf extract can stimulate fibroblasts and collagen formation, which accelerates the process of wound healing [8], [9], [10]. The aim of this study was

to analyze the proliferation activity of the combination of ethanol extract of *Artocarpus lacucha* leaves (ALLE) and *Anredera cordifolia* leaves (ACLE).

Materials and Methods

Materials

NIH-3T3 fibroblasts were obtained from the Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%. Fetal bovine serum (FBS) and kept at 37°C with a CO_2 supply of 5%. Moreover, reagents used were agarose (Promega), FluoroVue (Smobio), DNA Ladder 100 bp (Smobio), ethanol (Merck), HEPES (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2,5 difeniltetrazolium bromida (MTT) (Sigma), nuclease-free water, distilled water, DMSO (Merck), phosphate-buffered saline (PBS) (Gibco), ReverTra Ace (Toyobo), total RNA isolation kit (Geneaid), tripsin-EDTA 0.25% (Gibco), and fetal bovine serum (Gibco).

Preparation of extract

The air-dried and powdered leaves of *Artocarpus lacucha* Buch.-Ham. and *Anredera cordifolia* Steenis (500 g) was extracted by maceration method with ethanol 80% (Merck). The filtrate was collected and then evaporated under reduced pressure to give a viscous fraction and then dried to dry [11].

Analysis of proliferative activity

Combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) in *cosolvent* DMSO (Sigma) was submitted for proliferative test. In that way, the NIH-3T3 cell line (1 × 10^4cells/mL) was grown in DMEM complete medium. After 24 h, 48 h, and 72 h treatment, MTT assay was performed, and cell viability was counted to determine the proliferative activity [12], [13].

Wound healing migration assay

The migration assay was carried out with NIH-3T3 cells seeded at 5 × 10^4 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. Cell residues in the plate were washed with PBS and treated with the combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) and incubated for 48 h at 37°C and documented under inverted microscope against cell migration rapidity after 0, 24, and 48 h. The space from scratch treatment between control and treatment culture cells was quantified using ImageJ software and defined as cell migration area [14], [15], [16].

Expression of VEGFR-2

NIH-3T3 cells (5 × 10^4 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with the combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) and then incubated for 24 h. Both floating and adherent cells were collected in a conical tube using trypsin 0.25%. 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 (Geneaid, 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. RT-PCR was carried out in Applied Biosystems ProFlex. The reaction mixture consisted of GoTaq Green (12.5 μL) (Promega), 1.0 μL of cDNA, 1 μL of forward primers, 1 μL of reverse primers, and 9.5 μL of nuclease-free water to make a total volume of 25 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'), 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 was comprised first incubation at 95°C for 2 min, 95°C for 30 s, annealing at 55.5°C 30 s for VEGFR-2, and 58°C 30 s for β-actin, extension at 72° for 1 min, and 35 cycles. The PCR products were detected by electrophoresis in 2% agarose gels and added gel red 10 μL. Then, they were visualized with gel doc [13], [17], [18].

Statistic analysis

The results were presented as means ± SD. The statistical analysis was carried out by using the SPSS edition 22.

Results

Wound healing migration assay

A little wound repair was observed in wells with the combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) after 24 and 48 h incubation with 64.88 ± 0.90% and 100.00 ± 0.00%, respectively, closure area. The wound healing migration of a combination of

EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) is given in Figure 1.

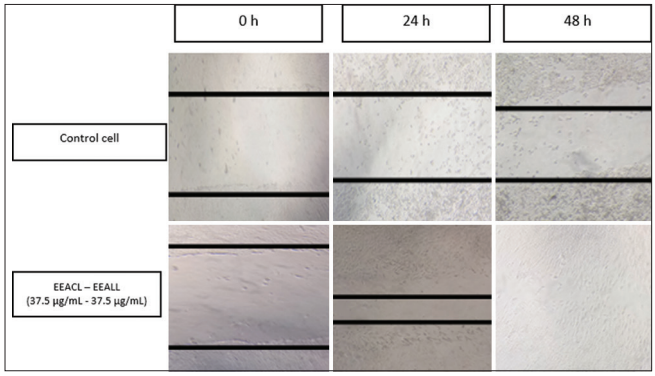

Figure 1: Wound healing migration assay. NIH-3T3 cells were treated by combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) for 0; 24 and 48 h and measured the closure area. (a) Control cells; (b) combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL)

Proliferative activity

The percentage of viable cells after treatment and incubation for 24 h, 48 h, and 72 h were showed (124.33 ± 0.32; 128.52 ± 0.41; and 118.35 ± 0.22) showed the stimulation effect of the combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) toward the proliferation of NIH-3T3 cells. The effect of the combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) is given in Figure 2.

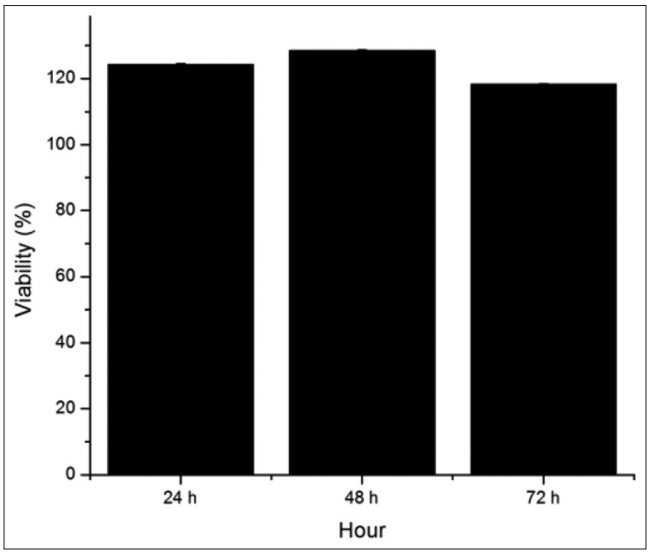

Figure 2: Percentage of viable cells of NIH-3T3 cells were treated by a combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) for 24 h, 48, and 72 h and measured viable cells

COX-2 expression

Combination of EEACL and EEALL (37.5 μg/mL−37.5 μg/mL) was showed no significant change on the expression of β-actin expression (housekeeping gene) but have a significant up-regulatory effect on the expression of VEGFR-2 (1.58 ± 0.02). VEGFR-2 expression is given in Figure 3.

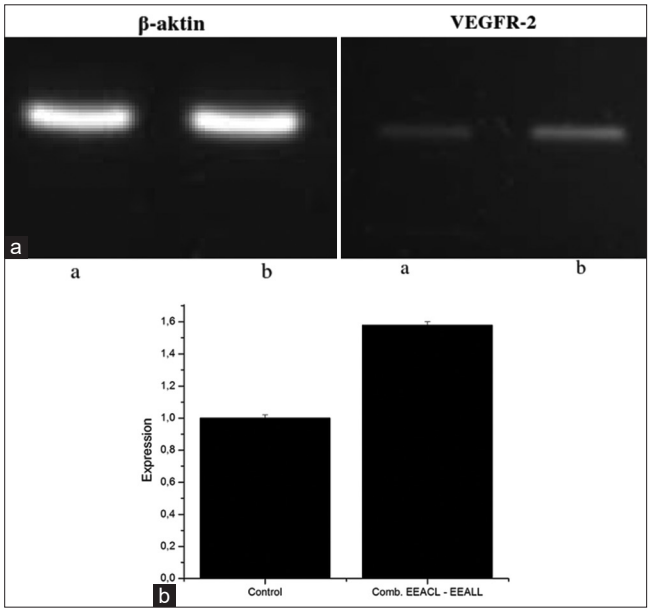

Figure 3: Representative figures showing VEGFR-2 expression after treatment with combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL) (a) bands of VEGFR-2 expressions (A: Control Cell; B: combination of EEALL and EEACL (37.5 μg/mL−37.5 μg/mL), and (b) chart of VEGFR-2 expressions

Discussion

The binahong plant contains various secondary metabolites that function in the proliferation of fibroblasts, osteoblasts, and osteocytes, optimizing wound healing and in turn lowering the value of RSV. In the binahong leaf extract, there is saponin, which can increase the expression of transforming growth factor-alpha (TGF-α) and transforming growth factor-beta (TGF-ß) [1], [19], [20]. TGF-ß can activate fibroblasts and TGF-a can activate osterix, which will function in osteoblast differentiation [21]. Saponin is also antiseptic and it can affect cell membrane integrity and cause the lysis of pathogens, especially fungi [8]. Apigenin, a flavonoid found in binahong, can increase the expression of TGF-ß and platelet-derived growth factor (PDGF) that also function in fibroblast activation so that it can migrate toward the clot. PDGF is also a protein that affects fibroblast proliferation [2], [3], [22], [23], [24], [25], [26]. Apigenin also has anti-inflammatory properties because it can inhibit the activation of nuclear factor-kappa B (NF-κB). Inhibited NF-κB can prevent the production of inflammatory mediators that can increase inflammation [24], [25], [26]. The other flavonoids in the binahong plant that function in socket wound healing are quercetin and vitexin. Quercetin has the ability to reduce osteoclast formation through inhibiting interleukin-17

(IL-17), which is induced by receptor activator of nuclear factor kappa-B ligand (RANKL), and quercetin can also increase osteogenesis, angiogenesis, and function as an antioxidant [27], [28], [29], [30], [31]. Vitexin can affect bone formation by increasing osteoblast differentiation through phosphorylation-small mothers against (p-Smad) and runt-related transcription factor 2 (Runx2) [30], [31]. The tannin in the binahong plant functions in fibroblast migration because it can increase vascular endothelial growth factor (VEGF) in the early phase of wound healing [32], [33], [34]. Tannin is also an antioxidant. Antioxidant is needed to neutralize free radicals that are produced during wound healing. Free radicals can damage cell protein structure, which will prevent cell proliferation [35]. VEGF is a protein that plays a role in fibroblast migration [21]. *Artocarpus lacucha* Buch.-Ham is one of the plants of the *Artocarpus* genus which contains high concentration of flavonoids such as artocarpin that are anti-inflammatory, antioxidant, anticancer, and antimicrobial and accelerate the wound healing process by enhancing collagen deposition, re-epithelialization, and angiogenesis [6], [7].

Conclusion

The combination of extract ethanol of *Artocarpus lacucha* Buch.-Ham leaves (EEALL) and extract ethanol of *Anredera cordifolia* Steenis leaves (EEACL) in concentration (37.5 μg/mL−37.5 μg/mL) is increased wound healing process on NIH-3T3 cell lines through increase proliferation, migration of cells, and expression of VEGFR-2 genes.

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