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Aloe vera stimulate cell proliferation, cell migration, expression of vascular endothelial growth factor-A (VEGF-A), and c-Jun N-terminal kinase-1 (JNK-1) on fibroblast of diabetic rat models

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ABSTRACT

The disturbance of cell migration and cell proliferation,diminished production of vascular endothelial growth factor-A (VEGF-A) and c-Jun N-terminal kinase-1 (JNK-1) are important factors in wound healing process. *Aloe vera* contains active compounds which can help in the wound healing process. Thestudy aimed to investigate the effect of ethanol extract of*A. vera*on cell proliferation, cell migration, VEGF-A and JNK-1 expression of skin fibroblast cells of diabetic rats. The primary skin fibroblast cells were isolated from diabetic Wistar rat and incubated with the *A. vera* extract in various concentrations i.e. 500 (AV500), 250 (AV250), and 125 µg/Ml (AV125) for 24, 48 and 72 h.The cell proliferation was examined visually by counting the cells number, the cell migration was observed using *in vitro* scratch assay, whereas VEGF-A and JNK-1 expression were examined using RT-PCR. In 24 and 48h incubation,the cell proliferation of AV500 and AV250 groups had higher number of cells than negative control group,but there was no significant difference ($p>0.05$). However in72 h incubation,the cell proliferation of AV500 group ($29.33\pm1.28x10^4$ cells/mL)was significantly different compared to negative control group ($22.91\pm3.21x10^4$ cells/mL) ($p<0.05$). In 24 h incubation, the cell migration of AV500(78.13±7.18%), AV250 (73.88±4.75%) and AV125 (68.80±17.11%) groupswere significantly higher thanthat of negative control group (53.91±2.74%) ($p<0.05$). In contrastin 48 and 72 hincubation,there were no significantly different in cell migration ($p>0.05$).The expression of VEGF-A and JNK-1 after incubation with the AV500 for 48 h, weresignificantly higher than those of negative control group ($p<0.05$). In conclusion, *A. vera* increases cell proliferation, cell migration, VEGF-A and JNK-1 expression offibroblast cellof diabetic rat skin.

ABSTRAK

Gangguan migrasi dan proliferasi sel, juga pengurangan produksi VEGF dan JNK-1 yang merupakan faktor penting dalam proses penyembuhan luka.Lidah buaya (*Aloe verra*) mengandung berbagai senyawa aktif yang dapat membantu dalam proses penyembuhan luka. Penelitian ini bertujuan untuk mengkaji efek ekstrak etanol *A.vera*terhadap proliferasi sel, migrasi sel, ekspresi VEGF-A dan JNK-1dalam kultur fibroblast kulit tikus diabetes. Kultur sel primerfibroblast diisolasi dari kulit tikus Wistar diabetic dan diinkubasikan dengan ekstrak *A. vera* dengan konsentrasi 500 (AV500), 250 (AV250), dan 125 µg/Ml (AV125). Proliferasi diperiksa secara visual dengan menghitung jumlah sel, migrasi sel diamati dengan metode uji gores *in vitro*, dan ekspresi VEGF-A dan JNK-1 diperiksa dengan RT-PCR. Pada inkubasi 24 dan 48 jam, proliferasi sel kelompok V500 dan AV250 lebih tinggi dibandingan kelompok controlnegatif, tetapi tidak bermakna secara nyata ($p>0.05$). Namun pada inkubasi 72 jam, proliferasi sel kelompok AV500 ($29,33\pm1,28x10^4$ cells/mL) berbeda nyata dengan

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kelompok controlnegatif (22,91±3,21x10^4 cells/mL) (p<0.05). Pada inkubasi 24 jam, migrasi sel kelompok AV500 (78,13±7,18%), AV250 (73,88±4,75%) and AV125 (68,80±17,11%) lebih tinggi secara nyata dibandingkan kelompok kontrolnegatif (53,91±2,74%) (p<0.05). Sebaliknya pada inkubasi 48 dan 72 jam, tidak terdapat perbedaan migrasi sel secara nyata(p>0,05). Ekspresi VEGF-A dan JNK-1 setelah inkubasi dengan AV500 selama 48 jam lebih tinggi secara nyata dibandingkan dengan kelompok kontrol negatif (p<0,05). Dapat disimpulkan, lidah buaya dapat meningkatkan ekspresi VEGF-A dan JNK-1 sel fibroblastkulit tikus diabetes.

INTRODUCTION

Patients with diabetes mellitus (DM) can develop neuropathy which can cause diabetic foot ulcer. The neuropathy caused by disruption of vascular system and sensory nerve damage in extremities area leads to loss of sensation and skin integrity damage.[1,2] This is a port ofthe entry for microbe to cause a chronic wound, which is difficult to cure. Moreover patients with severe diabetic foot ulcer can be amputated.[3]

Hyperglycemia condition changes metabolism of tissue connection because of disturbance in synthesis process and increased the speed of collagen degradation, which results to delayed wound healing process.[4,5] Delayed diabetic wound healing is related to low number of vascular endothelial growth factor (VEGF) expression and other growth factors which are the main factors of angiogenesis, cell proliferation, and cell migration on wound healing process.[3,6] Cell migration activity decreased in hyperglycemia due to the excessive production of reactive oxygen species (ROS) that causes oxidative stress and DNA damage, also failure in cell function.[7-10] Hyperglycemia obstructs c-Jun N-terminal kinase(JNK) phosphorylation which is the downstream of VEGF, as a result there are disturbance on migration activity and cell proliferation.[9,11-13]

*Aloevera*isa medicinal plant traditionally used to treat acute or chronic wound.[14,15] Topical form of *A.vera* can accelerate healing process of diabetic wound and plays a role in all stages of wound healing process.[4,16] *Aloevera* is nontoxic and can maintain or increase epithelial cell and fibroblast cellviability.[17,18] *Aloevera* contains variety of active compounds such as aloe emodin, β-sitosterol, and acemannanwhich can stimulate VEGF production in ischemia reperfusion damaged brain of Mongolian gerbil and fibroblast cellculture of gingival rats.[19,20] *Aloe vera*oral administration significantly stimulates production of TGF-β1 and bFGF on wound radiation and increases wound contraction, and increase fibroblast migration, also endothelial cells.[21] This studyaimed to invetigate the effect of *A. vera* ethanol extracton cell proliferation, cell migration, VEGF-A and JNK-1 expression of fibroblast cell culture of diabetic rat skin.

MATERIALS AND METHODS

Preparation of *A. vera* ethanolic extract

Aloe verabarbadensis Millerwas collected from *Unit Pelaksana Teknis Daerah*(UPTD) *Agribisnis* Pontianak, West Borneo, Indonesia. The extract was prepared according to Moniruzzaman *et al.*[22] after modification. The leaves of *A. vera* were washed in several times then chopped and homogenized by using blender. About one kg of*A. vera*wasmacerated using two L of 80% ethanol for24 h. The macerates were then evaporatedunder reduced pressure using a rotary evaporator. The semi-dried ethanol extract was then freeze-dried at-55^{o}C and transferred to a reagent bottle to be stored in a freezerat-8^{o}C.

Phytochemical screening

The ethanolic extract of *A.vera*was subjected to various qualitative tests for identification ofitsconstituentsusing thin-layer chromatography (TLC). Separation was performed using silica gel GF_{254}as the stationary phaseand mixture of chloroform : methanol : ethyl acetate (5:1:1,v/v/v) as mobile phases. The chromatogram was observed under UV light atwave length 254 and 366 nm and sprayed with citroborateto detect flavonoids in the extract. Mannose examination was performed using high performance liquid chromatography (HPLC). Sample was added one g to volumetric flask and added 10 mL H_2O, furthermore vortexedandcentrifugedat 10.000 rpm for 5 min. The product was filtered by Millex 0.45 µm and injected to HPLC as much 50 µL. The chromatographic separation was conducted with MetaCarb 87P columnkept in column oven at 85 ^oC, H_2O as mobile phase at a flow rate of 0.6 mL/min and RID detector for detection.

Animal preparation

Protocol of the study has been approvedby the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (ref. KE/FK/422/EC/2016). Male albino rats of the *Wistar* strain weighing ±200 g obtained from the Department of Pharmacology and Therapy, Public Health and Nursing, Universitas Gadjah Mada were maintained on standard rat feed and water *ad libitum.* The animals were fasted for 12 h but had been allowed free access to water. After fasting, DM was induced by intraperitoneal injection of streptozotocin 60 mg/kg body weight (BW) and nicotinamide 120 mg/kgBW in 0.05 M dissolved in citrate buffer (pH 4.5).[23] After three days, blood glucose level were measured and only animal with glycaemia higher than 250 mg/dL were considered diabetic.

Fibroblast cell isolation and culture

Rats were sacrificed with an overdose of ketamine and xylazine. Fibroblast cells were isolated according to Seluanov *et al.*[24] after modification. Underarm skin area of animal were shaved and dissected to collect skin samples. Skin wascutapproximately 6 cm^2 using sterile technique and cultured using explant method. The cells were cultured under standard conditions, with Dubelco's modified Eagle's medium (DMEM low glucose) containing 10% fetal bovine serum (FBS), 1% penstrep, and 0.5% fungizone (GIBCO®, Grandlisland, NY, USA), then incubated at 37°C and 5% CO_2. The medium was changed thrice a week and cells were trypsinized at 80% confluence. Passages 4-7 of cell were used for all experiments. The sample then divided into six groups i.e. KN asnormal fibroblast cell + low glucose medium (5 mM); K(+) asfibroblast of diabetic rat + high glucose medium (25 mM) + Algisite-M® dressing; K(-) asfibroblast of diabetic rat + high glucose medium (25 mM); AV500 asfibroblast of diabetic rat + high glucose medium (25 mM) + *A. vera* 500 µg/mL; AV250 asfibroblast of diabetic rat + high glucose medium (25 mM) + *A. vera* 250 µg/mL; AV125 asfibroblast of diabetic rat + high glucose medium (25 mM) + *A. vera* 125 µg/mL.

Preparation of solution

About 5 mg of the ethanolic extract of *A. vera* was diluted in 100 µL dimethyl sulphoxide (DMSO; SIGMA®; St Louis, MO, USA) to obtain a stock solution (50.000 µg/mL) and then dissolved inhigh glucose medium (DMEM 25 mM glucose, FBS 10%, penstrep 1%, fungizone 0.5%) and created in various concentration of *A. vera* (500, 250, 125µg/mL). Algsite-M®dressing extractsolution was created

by using 2 $cm^2$of Algsite-M®dressing (Smith & Nephew®) and incubated in 8 mL of high glucose medium at 37°C for 24 h.Positive control (Algsite-M®) was used for each assay.

Cell proliferation examination

Cell proliferation examinationwas performed by counting the number of cells in hemocytometer slide Neubauer. Cells were seeded in microplate-96 wells with 10^4cells/mL per well in volume 100 µL, and incubated in high glucose medium (DMEM 25 mM glucose) for 24 hours at 37°C and 5% CO_2. Furthermore, cellswere incubated with *A. vera* extract concentration 500, 250, 125µg/mL and Algisite-M®extract in high glucose medium. The number of cells was then counted in 24, 48 and 72 h after incubation.

Cell migration examination

Cell migration test carried out using in vitro scratch assay method. The number of cells to examine cell migration was$5x10^4$cellsper sampleinmicroplate-24 well. Cells were incubated in high glucose medium (DMEM 25mM glucose) at 37°Cand 5% $CO_2$for 24 h.Furthermore, cells were incubated with *A. vera*extract concentration 500, 250, 125µg/mLand Algisite-M®extract in high glucose medium after creating a wound across the cell monolayer with a yellow plastic tip. Cell migration into the wound surface was then measured after 24, 48, 72 h after incubation and analyzed byImageJ program.

Expression of VEGF-A and JNK-1 examination

The number of cells to examine VEGF-A and JNK-1 expression was $2x10^5$ cells/mL per well in volume one mL. Cells were incubated in high glucose medium (DMEM 25mM glucose) at 37°C and 5% CO_2 for 24 h. Furthermore, cells were incubated with *A. vera* extract concentration 500, 250, 125µg/mL and Algisite-M® dressing in high glucose medium and incubatedat 37°C and 5% CO_2 for 48 h. Total RNA isolated by using RNA ISO Plus or GENEzol™ (GZR100) with recommended procedures. RNA were quantified by using spectrophotometer. PCR cDNA mixwere carried out bymixed 5x buffer (Toyobo, Cat. No. TRT-101), *random primer* (TaKaRa, Cat. No. 3801), *deoxyribonucleotide triphosphate* (dNTP) (TaKaRa, Cat. No. 4030), and Rever TraAce (Toyobo, Cat. No. TRT-101). cDNA product, PCR*water*, and PCR mixture were mixed and carried out PCR running with conditions: denaturationat 30°C (10 min), annealingat 42°C (60 min), and extension at 99°C (5 min).Gene amplification was performed by using polymerase chain reaction (PCR) method with specific primer. GoTaq® Green Master Mix (Promega, USA) in 12.5 µL were used to amplifications andusing total reaction volume total 25µL. Furthermore, 10µL amplification productswere visualized by using electrophoresis.

TABLE 1. Primer used to examineVEGF-A, JNK-1 dan GAPDH gene expressions

Primer	Urutan
VEGF-A	F: 5'-ACCTCCACCATGCCAAGT-3'
	R: 5'-TTGGTCTGCATTCACATCTG-3'
JNK-1	F: 5'-GCCAGTCAGGCGAGAGATTT-3'
	R: 5'-GGACGCATCTATCACCAGCA-3'
GAPDH	F: 5'-ATGACATCAAGAAGGTGGTG-3'
	R: 5'-CATACCAGGAAATGAGCTTG-3'

TABLE 2. PCR conditionsused to examine VEGF-A, JNK-1andGAPDH gene expressions

PCR condition	Pre Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycle	Sample
VEGF-A	94°2 min	94°10 sec	65°30 sec	72°1 min	72°10 min	35x	4µL
JNK-1	94°2 min	94°10 sec	60°30 sec	72°1 min	72°10 min	35x	4µL
GAPDH	94°2 min	94°10 sec	60°30 sec	72°1 min	72°10 min	35x	4µL

Statistical analysis

Statistical analysis was carried out with the SPSS programfor windows. Datawere expressed as the mean ± standard deviation (SD). The differences among the mean values from four independent experiments were analyzedwith one-way ANOVA followed by post hoc LSD test analysis. A p value <0.05 was considered significant.

RESULTS

Phytochemical screeningof *A. vera*

Phytochemical analysis of the *A. vera*extract by TLC showedthe presence of compounds on length of wave 366 nm withrate of flow (Rf) valueof 0.32; 0.45; 0.64; 0.84; and 0.90, respectively. HPLCexamination of *A. vera* extract resultshowedthe level of mannose compound<32 µg/g (FIGURE 1).

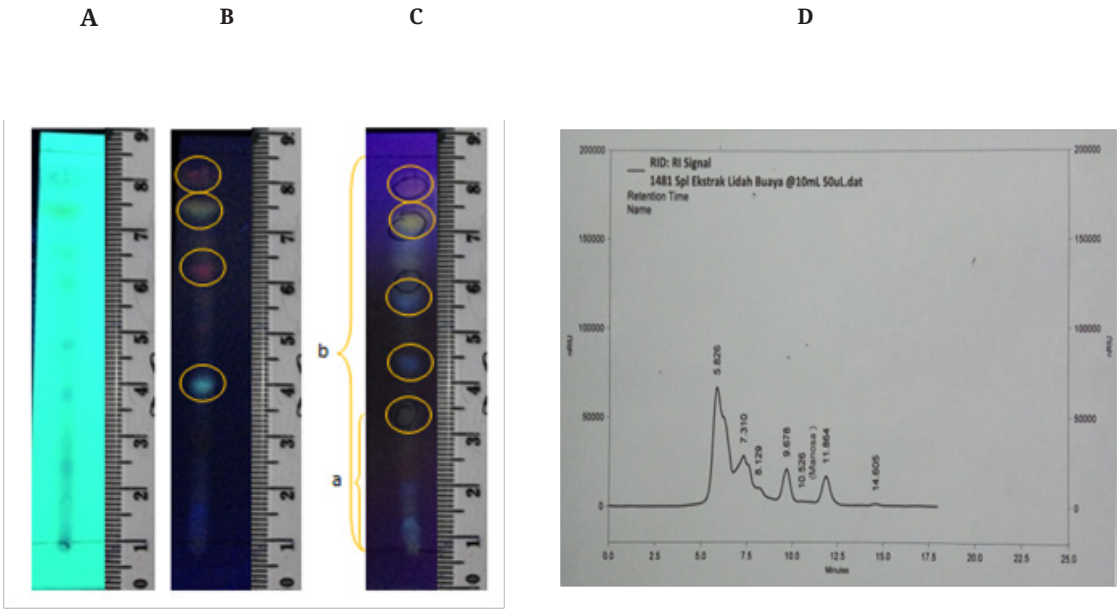

FIGURE 1. The TLC and HPLC chromatogram of *A. vera* extract. (A) Examinationby UV 254 nm; (B) Examination by UV 366. nm. (C) Examinationby UV 366 nm after Sitroborat spraying. (D) Graphic of HPLC examination (the arrow shows mannose level).

Cell proliferation

Cell proliferation is presented in FIGURE 2. The cell proliferation of the negative control groupafter 24, 48, 72 h incubations were significantly lower than that normal fibroblast cell group ($p<0.05$). The cell proliferation of the AV500 and AV250 groups after24 and 48 h incubations were higher than that the negative control group, but they were not significantly different ($p>0.05$). However, after72 h incubation, significantly different between AV500, AV250, and AV125 groupscompared to that the negative control groupwas observed ($p< 0.05$).

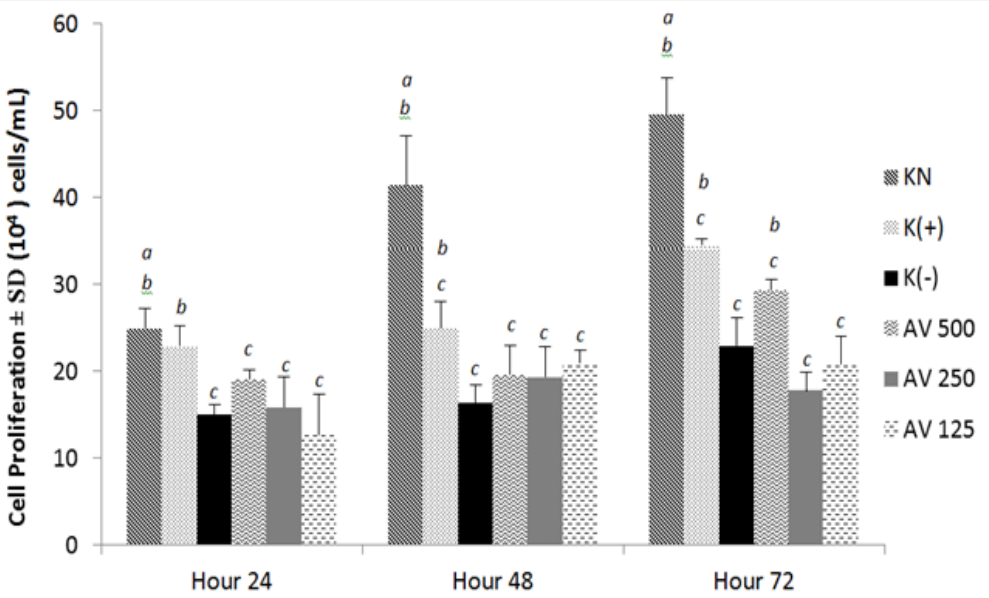

FIGURE 2. Cell proliferation (mean ± SD 10^4 cells/mL) after 24, 48 and 72 h incubations.One way ANOVA examinationin 24h ($p = 0.001$),48 h ($p = 0.000$)and 72 h ($p = 0.000$).Post hoc LSD: *a* ($p < 0.05$) compared to positive control; *b* ($p < 0.05$) compared to negative control; *c* ($p < 0.05$) compared to normal fibroblast cell control.

Cell migrtion

The cell migration of the normal fibroblast cell groupafter 24 h incubation was highestcompared toall of the groups ($p<0.05$). The cell migration of the AV500, AV250 and AV125 groupafter 24 h incubation was significantlyhigher thanthat the negativecontrol group ($p<0.05$). However, after 48 and 72 h incubation, no significantly different in all groups were observed ($p>0.05$).It was indicated that scratch wound has closed at the hour 48 after treatments. The cell migration of all groups arepresented inFIGURE 4.

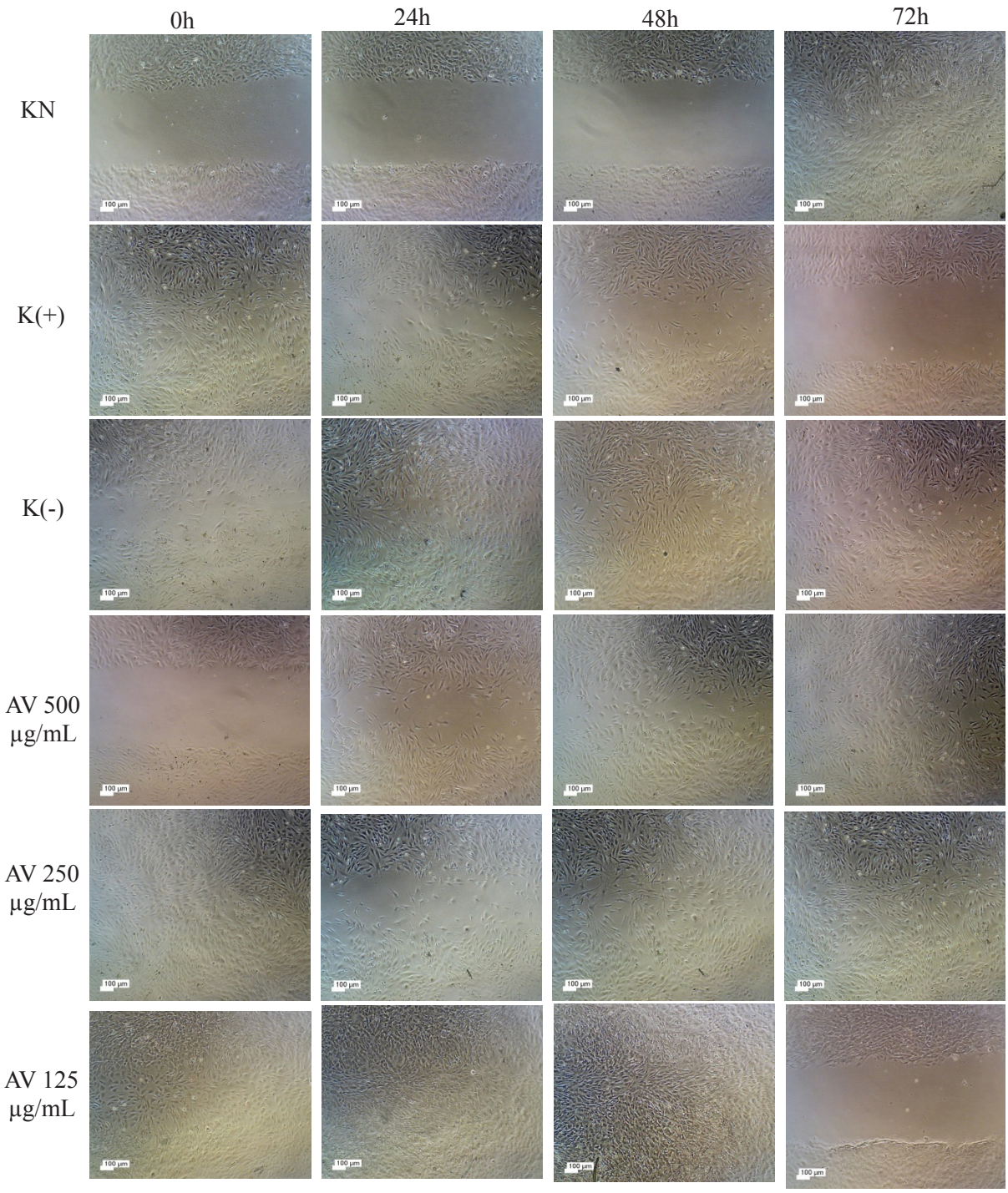

FIGURE 3. Image of cell migration in hour 0, 24, 48,and 72 after treatments. The yellow line was the margin of the scratch. Magnification 40x. The scale of measurement was 100 µm. The migration rate was quantified by the Image-J software and data were expressed as mean ± SD.

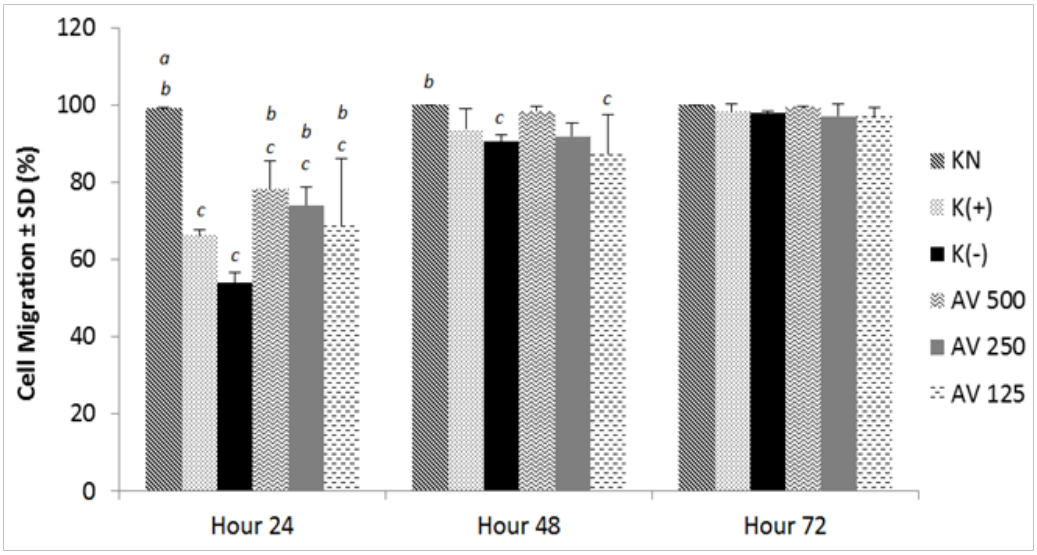

FIGURE 4. Cell migration (mean ± SD%) in 24, 48, 72 h after incubations. One way ANOVAexamination in 24 h (p = 0.000), 48 and 72 h (p > 0.05). Posthoc LSD: *a* (p < 0.05) compared to positive control; *b* (p < 0.05) compared to negative control; *c* (p < 0.05) compared to normal fibroblast cell control.

VEGF-Aexpression

The normal fibroblast cell control group showed highest VEGF-A/GAPDH expression compared to all of the groups (p<0.05).The AV500 group had higher VEGF-A/GAPDH expression compared to that positive control, negative control, AV250, andAV125 groups(p<0.05).

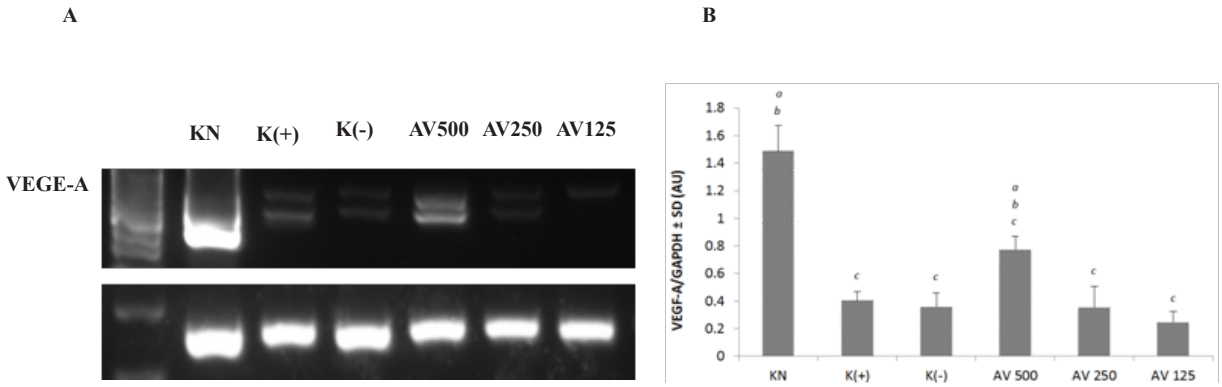

FIGURE 5. (A) Resultsof electrophoresis VEGF-A and GAPDH geneafter 48 hours incubation.(B) Chart bar of mean± SD VEGF-A/GAPDH in hour 48. One way ANOVAtest (p = 0.000). Posthoc LSD: a(p < 0.05)compared to positive control; b(p < 0.05)compared to negative control; c (p < 0.05)compared to normal fibroblast cell control

JNK-1 expression

The normal fibroblast cell control groups howed highest JNK-1/GAPDH expression compared to all of the groups (p<0.05). The AV500 group had higher JNK-1/GAPDH expression compared to that negative control, AV250, and AV125group(p<0.05).

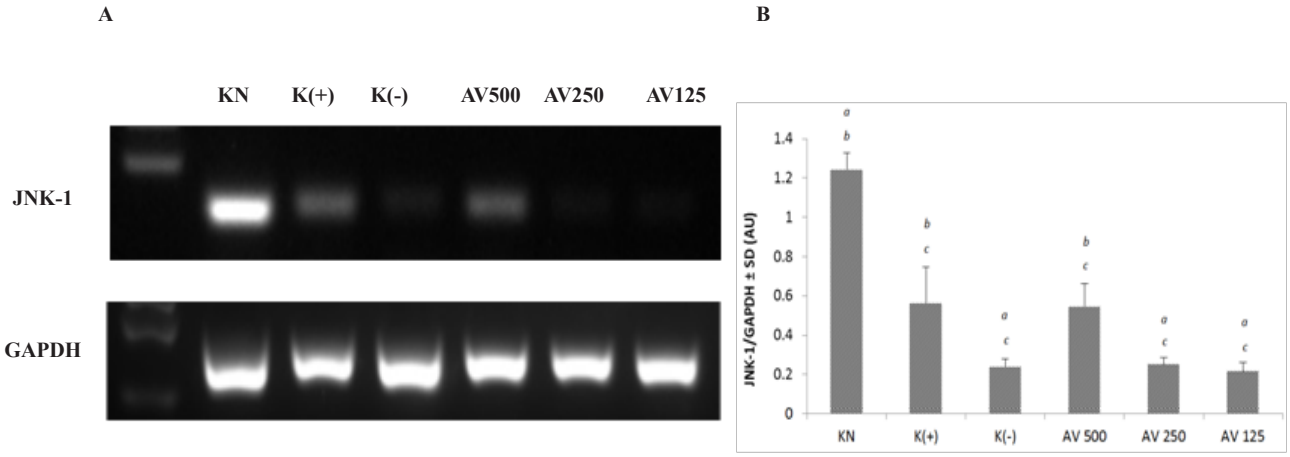

FIGURE 6. (A) Resultsof electrophoresisJNK-1and GAPDH geneafter 48 hours incubation(B) Chart bar of mean± SD JNK-1/GAPDH in hour 48. One way ANOVAtest (p = 0.000). Posthoc LSD: *a*(p< 0.05)compared to positive control; *b*(p< 0.05)compared to negative control; *c* (p< 0.05)compared to normal fibroblast cell control.

DISCUSSION

In this study, hyperglycemia condition caused cell proliferation and cell migration disturbances, also decreased VEGF-A and JNK-1 gene expression on fibroblast of diabetic rat. It was indicated by significantly fewer cell proliferation of the negative control group compared to that control normal group after 24, 48 and 72 h incubations (p<0.05). In addition, cell migration of negative control group was significantly lower compared to that control normal group (p=0.000) and the scracth wounds of control normal group was almost completely closed compared to the other groups. The VEGF-A and JNK-1 gene expression of negative control group after 24 h incubation was significantly lower compared to that normal control group (p=0.000).

The result of this study was supported by previous studies which confirmed that hyperglycemia condition causedthe cell proliferation and cell migration disturbance.[7,8,11,12] Moreover, hyperglycemia condition can diminish VEGF production and JNK phosphorylation lead to disturbance of cell proliferation and cell migrationon wound healing process.[8,10,13]

The impairment of cell proliferation on hyperglycemia can caused by several factors such as increase of L-lactate production,[11] inflammatory mediators and advanced glycation end-product (AGE) which is stimulate apoptosis through activation of ROS, pro-apoptotic transcription factor FOXOI and caspase 3.[12] The increase of ROS production affectsthe protein structure and function, as well asinhibition ofcell migration directly by over-activation of the small Rho GTPase Racl and altered polarity and morphology of cells.[7,13] Diminished VEGF production on hyperglycemia occurs by inhibition of JAK-2 and

STAT3 (Signal transducer and activator of transcription-3).[25] Hyperglycemia obstructs JNK phosphorylation which is the downstream of VEGF, as a result there are disturbance on cell proliferation and cell migration.[11-13,26]

Ethanolic *A. vera*extract can stimulate proliferation and migration of fibroblast cell of diabetic rat in this study. The cell proliferation after 24 and 48 h incubations with *A. vera* extract at concentration of 500 and 250 µg/mL were higher than that the negative control group, but they were not significantly different ($p>0.05$). However, after 72 h incubation, significantly different between *A. vera*extract at concentration of 500, 250 and 125 µg/mL compared to that the negative control group was observed ($p < 0.05$). The cell migration after 24 h incubation with *A. vera* extract at concentration of 500, 250 and 125 µg/mL were significantly higher than that the negative control group ($p <0.05$).The result of this study was also support by the previous studies whichconfirmed that *A. vera* can increase cell proliferation and cell migration.[17,27] The increase of cell proliferation and cell migration occurred in concentration dependent manner. The scratches wounds almost completely closed in each group after 48h incubation. The increase of the cell migration activity is likely occurred before 24 h incubation, so after 24 h the migration has been achieved>50% in each group.

The VEGF-A and JNK-1 expressions after incubation with *A. vera* extract at concentration of 500 µg/mL were significantly higher compared to that the negative control group ($p<0.05$). It was indicated that *A. vera* can stimulate VEGF production and the other growth factors which has a role in wound healing process as previously reported.[20,28] The incubationof *A.vera* extract at concentrationof 250 and 125 µg/mL on the VEGF-A and JNK-1 expressionswere not significantly different compared to the negative control. This may be related to the small concentrations that do not affect the increased expression of VEGF-A and JNK-1.

The glycoproteins and polysaccharides components in *A.vera* can trigger cell growth and significantly increase cell proliferation and cell migration by stimulating TGF-β1 and bFGF production, also affecting Gap junctional intercellular communication (GJIC) which is the important factors in growth of tissue and organs.[28,29] Aloesincompound of *A. vera* stimulates cell growth through induce cell cycleprotein regulatorsuch ascyclin E, cyclin dependen kinase-2 (CDK2), and cell division cycle-25A (CDC25A) which synergistically up-regulate the cyclin E/CDK2 kinase activity.[30] β-Sitosterol compound of *A. vera* increase VEGF expression onbrain ischemic reperfusion animals.[27]

The phytochemical analysis of the *A.vera*extract by TLC showedthe presence of compounds on length of wave 366 nm withRf value of 0.32; 0.45; 0.64; 0.84; and 0.90.It was indicated that *A.vera* extract has various compounds that taken along by mobile phase with various distance based on polarity of compounds. Citroborate spraying was used to examine of flavonoid existence in the *A.vera* extract. The major polysaccharide compound of *A. vera* is acemannan (mannose-6-phosphat) which is composed of one or more polymers of various chain length with molecular weights 30-40 kDa and consists of repeating units of glucose and mannose.[31] In this research, mannose compound was analyzed byusing HPLC and had mannose content <32 µg/gr.

Previous studies reported that *A. vera* accelerates diabetic wound healing[4,16] by increase the glycosaminoglycans (GAGs), and plasma insulin level, also diminish blood glucose level.[32] Acemannan accelerates oral and diabetic wound healingof animal

modelsby stimulates KGF-1, VEGF production, collagen synthesis, and also increase collagen cross link activity for wound contraction.[20,28] The active accemanan should bind to mannose receptor on the cell surface by recognize polysaccharide chains terminating with mannose, fucose or N-acetylglosamine and stimulate growth factor production and cell proliferationby induce the intracellular signaling pathway.[20,33] Acemanan promotes skin wound healing and accelerates cell proliferation through stimulate synthesis of protein translationcyclin D1 which is mediated by AKT/mTOR signaling pathway.[34]

Antioxidant activity of *A. vera* like flavonoid significantly decreases ROS, lipid peroxidase and glycosylated hemoglobin level which are the cause of cell proliferation and cell migration impairment, also diminished of VEGF on diabetic wound.[7,10,35] This antioxidant activity also has a role in radical scavenging by increase in activity ofsuperoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes.[7,10,35]

CONCLUSION

Ethanolic extract of *A. vera* increases cell proliferation, cell migration, VEGF-Aand JNK-1expression onfibroblast cell cultured of diabetic rat skin.

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