

Cytotoxicity, Proliferation and Migration Rate Assessments of Human Dermal Fibroblast Adult Cells using *Zingiber zerumbet* Extract

(Penilaian Kesitotoksikan, Proliferasi dan Kadar Migrasi Sel Dewasa Fibroblas Derma Manusia menggunakan Ekstrak *Zingiber zerumbet*)

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ABSTRACT

Zingiber zerumbet is a plant that is traditionally consumed in many countries, including Malaysia due to its therapeutic properties. Previously it has been proven to inhibit migration and proliferation of cancer cells. However, its effects on normal cells are still unknown and would be the main focus of this study. The cytotoxicity along with proliferation and migration activities were evaluated against human dermal fibroblast adult cell line (HDF-a) using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium], proliferation and scratch assays. The cytotoxicity effect was determined following 24 h of treatment by ethyl acetate *Z. zerumbet* extracts while proliferation assay was conducted at different concentrations (500, 750, 1000, 1250 and 1500 µg/mL) at 24, 48 and 72 h. The cell migration rate was determined by scratch assay in 6-well culture plate using 10 µL pipette tip following 80% confluency at optimum concentration (750 µg/mL) of *Z. zerumbet* at different time points. Migration rate was scored at every 24 h using digital camera connected to the inverted microscope. The results from cytotoxicity test showed no IC_{50} values were observed for *Z. zerumbet* extract as compared to the positive control (menadione). Proliferation assay exhibited highest cell viability (137%) at 750 µg/mL. The rate of cell migration increased following treatment with *Z. zerumbet* extract after 48 h. However, the result was not significantly different when compared to the control. In conclusion, *Z. zerumbet* ethyl acetate extract is able to induce cell proliferation and potentially promoting migration rate of wound healing in in-vitro model.

Keywords: Dermal; fibroblast; migration; proliferation; wound healing; *Zingiber zerumbet*

ABSTRAK

Zingiber zerumbet merupakan tumbuhan ubatan tradisi yang digunakan di kebanyakan negara termasuk Malaysia disebabkan kesan teraputiknya. Kajian terdahulu telah membuktikan ia dapat menghalang migrasi dan proliferasi sel kanser. Namun, kesan pada sel normal masih belum diketahui dan menjadi fokus kajian ini. Kesan kesitotoksikan serta aktiviti proliferasi dan migrasi terhadap sel dewasa derma fibroblas manusia (HDF-a) dikaji menggunakan asai MTT [3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium], proliferasi dan calaran. Kesan kesitotoksikan ditentukan selepas rawatan selama 24 jam ekstrak etil asetat *Z. zerumbet* dan asai proliferasi ditentukan pada kepekatan berbeza (500, 750, 1000, 1250 dan 1500 µg/mL) pada 24, 48 dan 72 jam. Kadar migrasi sel ditentukan dengan asai calaran pada jangka waktu berbeza dalam piring kultur 6 telaga menggunakan hujung tip pipet 10 µL selepas sel mencapai 80% konfluensi pada kepekatan optimum (750 µg/mL) *Z. zerumbet*. Kadar migrasi sel ditentukan setiap 24 jam menggunakan kamera digital yang disambung pada mikroskop. Keputusan daripada ujian kesitotoksikan menunjukkan tiada nilai IC_{50} diperhatikan pada ekstrak *Z. zerumbet* apabila dibandingkan dengan kawalan positif (menadion). Asai proliferasi menunjukkan keviabelan sel yang tinggi (137%) pada kepekatan 750 µg/mL. Kadar migrasi sel meningkat selepas 48 jam rawatan dengan ekstrak *Z. zerumbet*. Namun, keputusan menunjukkan tiada perbezaan signifikan apabila dibandingkan dengan kawalan. Kesimpulannya, ekstrak etil asetat *Z. zerumbet* merangsang proliferasi dan kadar migrasi sel melalui model penyembuhan luka in vitro.

Kata kunci: Derma; fibrolas; migrasi; proliferasi; penyembuhan luka; *Zingiber zerumbet*

INTRODUCTION

Non-communicable diseases are known to be the leading cause of death as the diseases contribute to 40 million mortalities globally. Diabetes has been identified as one of the highest contribution of death as it accounts for 1.6 million from overall cases (WHO 2017). It is estimated that

the cost of chronic wound healing for diabetic patients would increase gradually as the patients who are prone to develop impaired wound healing continue to grow (Wong et al. 2013). Thus, many strategies have been done to reduce the cost in response to the injury and to improve the quality of life for the patients, including the use of

traditional medicine as an alternative treatment for wound healing as it is cheap and has been traditionally used by local people (Davis & Perez 2009; Dorai 2012).

Wound healing is a process which is initiated by stimulation of the injury and followed by a series of biological events starting from the wound closure until the remodelling of the wound. There are four phases involved in wound healing process including blood coagulation, inflammation, cell proliferation and remodelling. Wound healing process requires both migration and proliferation of many cell types including endothelial, keratinocytes, neutrophils, and fibroblasts (D'souza et al. 2011). Fibroblast number will be increased during proliferation phase to synthesize substances for extracellular matrix for tissue granulation and collagen to provide skin integrity (Robson 1997). In addition, wound healing process involves cytokines such as platelet-derived growth factor (PDGF), fibroblast-derived growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor (TGF) α and β that have been found to mediate this process (Sinno & Prakash 2013).

Wound such as diabetic foot ulcers can be a major concern to the doctors and patients due to its significant implication on the quality of life of the patients. In order to cure the wound and reduce the risk of infection in minor cuts and burns, antimicrobial ointments such as silver sulfadiazine, silver nitrate, povidone-iodine, and bacitracin are applied to the wound area. Chemicals and antibiotics that have been used to cure the wound have been reported to confer a number of side effects with being only partially effective for wound healing. The rate of antibiotic resistance as anti-infection in chronic wound healing is increasing and thus researches are now focusing on natural products remedy with anti-microbe properties (Howell-Jones et al. 2005; Kon & Rai 2014; Tan et al. 2013). Because of these limitations, the use of medicinal plants have gained interest to be developed as potential treatment of skin ailments because of it is affordable and able to minimize hypersensitive reactions (Raina et al. 2008).

There are numerous ginger species (Zingiberaceae family) that are widely used in Asia due to its unique flavour and medicinal properties that can be used as a treatment for some diseases. Zingiberaceae family is considered as a moderately large genus of herbs with approximately 141 species represented by genus of Zingiber that can be found throughout Asia. *Zingiber zerumbet* (L.) Smith, from Zingiberaceae family is locally known to the Malay as 'lempoyang,' is a traditional herb found in many tropical countries, including Malaysia (Yob et al. 2011). Its rhizomes, leaf and pine cones have been widely used in traditional medicine for inflammation, antirheumatic, constipation, diarrhea, toothache, indigestion, fever, severe sprains, antispasmodic, diuretic agents and worm infection in children (Chien et al. 2008; Sulaiman et al. 2010; Yob et al. 2011).

Previously, *Z. zerumbet* has been proven to possess biological activities such as hepatoprotective,

nephroprotective and could inhibit cell proliferation and migration in multiple types of cancer cells such as leukemia or thymoma cells (Abdul Hamid et al. 2012; Hamid et al. 2018, 2017; Yan et al. 2017). *Z. zerumbet* active compound, zerumbone also has shown cytotoxic and genotoxic effects in murine thymoma cells (Hamid et al. 2017). However, its effect in normal dermal cell is still unknown and thus to be investigated in this study. The cytotoxicity of the ethyl acetate extract of *Z. zerumbet* is determined prior to proliferation and migration analyses on human dermal fibroblast adult cell line. Human dermal fibroblast adult cells (HDF-a) is used as a model to mimic the human skin wound focusing on the third phase of wound healing process which is the proliferation phase.

MATERIALS AND METHODS

PLANT MATERIALS

Fresh rhizomes of *Z. zerumbet* were purchased from a herbal farm in Temerloh, Pahang. The rhizomes was authenticated and deposited at Herbarium Universiti Kebangsaan Malaysia, Bangi, Malaysia with voucher specimen no. UKMB-29952. Prior to analysis, the rhizomes were cleaned, chopped into small pieces and air-dried at room temperature for three days. The dried rhizomes were grounded into powder form.

PREPARATION OF ETHYL ACETATE EXTRACT OF *Z. ZERUMBET*

The air-dried rhizomes of *Z. zerumbet* were sequentially soaked at room temperature in n-hexane, ethyl acetate and methanol for 72 h to produce crude extracts of hexane, ethyl acetate, and methanol. All the crude extracts were stored at 4°C until tested. Prior to experiment, the ethyl acetate extract of *Z. zerumbet* (EAZZ) was dissolved in dimethyl (DMSO) and diluted in phosphate buffer saline (PBS). EAZZ was then dissolved in DMEM without FBS, filtered and sterilized through 0.22 μ m filter (Sartorius, Germany) for experimental usage (Hamid et al. 2018).

CELL CULTURE

Human dermal fibroblast adult cells (HDF-a) were purchased from Science Cell, Team Medical & Scientific Sdn. Bhd and cultured in humidified incubator at 37°C with 5% CO₂. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, United States) supplemented with 1% Penicillin/Streptomycin (PAA Laboratories, Austria) in T-75 flask supplemented with 5% fetal bovine serum until 90% confluence before subculture.

MTT-BASED CYTOTOXICITY ASSAY AND CELL PROLIFERATION ASSAY

Prior to MTT assay, the number of cells was counted using trypan blue exclusion method. HDF-a cells were cultured at (5 \times 10⁴) and treated in 96-well culture plates

(triplicate). The cytotoxicity was assessed by MTT assay (Mosmann 1983). Briefly, 3-[4,5-dimethylthiazol-yl]-2,5-diphenyltetrazoliumbromide (MTT) was dissolved in PBS at 5 mg/mL. MTT was added to each well (10 µL per 100 µL medium), and the plates were incubated at 37°C with 5% CO₂ for 4 h. The medium was then replaced with 100 µL DMSO and the absorbance for each well was measured at 570 nm using ELISA microplate reader (Biochrom ASYS, United Kingdom). For cell proliferation assay (Mosmann 1983), 5 × 10⁵ cells were cultured in 96-well plate with different concentrations of EAZZ (500, 750, 1000, 1250, and 1500 µg/mL) of EAZZ for 24, 48 and 72 h. The range of concentrations were chosen based on MTT result that does not kill 50% of the cells.

MIGRATION RATE ANALYSIS USING SCRATCH ASSAY

HDF-a cells were grown in 6-well plates at density of 2 × 10⁵/mL for 48 h and a small linear scratch was created along the confluent monolayer of cells by gently scraping the surface using a sterile pipette tips (Harishkumar 2013). Cells were rinsed with PBS to remove cellular debris before being treated with EAZZ at 750 µg/mL. The images were taken using digital camera (Victor Company of Japan Limited, Japan) that was connected to the inverted microscope and analyzed by using image pro-express software (Meyer Instruments, United States). The images were captured at 24, 48, 72, and 96 h of treatment. The distance of cell travelled from the wound edge to the wound area in comparison to control was calculated using AxioVision software (Zeiss, Germany).

STATISTICAL ANALYSIS

The data was analyzed by using Statistical Package for Social Sciences (SPSS) version 20.0. Each experiment was carried out in three independents replicate. Mean values were calculated and One way-ANOVA test were used to compare the mean for more than two groups. Statistically significant values were set at the level of $p<0.05$.

RESULTS AND DISCUSSION

The result of cytotoxic assay demonstrated no IC₅₀ value recorded following the treatment with EAZZ at every concentration (Figure 1). At lower concentrations (0–500 µg/mL), EAZZ did not show any cytotoxicity towards HDF-a cell. The percentage of HDF-a cell viability showed significant ($p<0.05$) increases from 100% to 149.03 ± 4.97% at concentration of 500 µg/mL and 100% to 151.91 ± 6.66% at a concentration of 1000 µg/mL of EAZZ. However, cell viability decreased significantly at higher concentration of EAZZ from 100% to 53.46 ± 8.30% at a concentration of 2000 µg/mL. This result indicates that the extract could reduce the cell viability at higher concentration (>1000 µg/mL).

For proliferation assay, the increase of cell viability was observed during 24, 48, and 72 h of treatment of EAZZ up to 750 µg/mL before it decreased at higher concentrations (1000, 1250, and 1500 µg/mL) (Figure 2). Higher concentration of EAZZ (1250 and 1500 µg/mL) significantly ($p<0.05$) inhibited cell proliferation and displayed cytotoxic effect on HDF-a cells at 24, 48, and 72 h. However, there was no significant difference in cell proliferation between the control group (untreated) and cell treated with EAZZ at concentrations of 500, 750, and 1000 µg/mL.

The rate of cell migration increased as compared to the control group (Table 1 & Figure 3). At 24 h, the rate of cell migration for treatment was slightly slower (25.50 ± 4.40%) compared to the control group (26.45 ± 4.69%). For 48, 72, and 96 h, the rate of cell migration towards the wound area was faster in treatment groups (46.08 ± 2.89%, 52.93 ± 2.73% and 62.27 ± 2.94%, respectively) than the control group (Table 1).

Previously, the proliferation study indicated some inhibitory effects in the proliferation of breast cancer cells using EAZZ extract (Hadjikakou et al. 2009). However, the proliferation effect of EAZZ on normal skin focusing on fibroblast cell has not been conducted and thus becoming

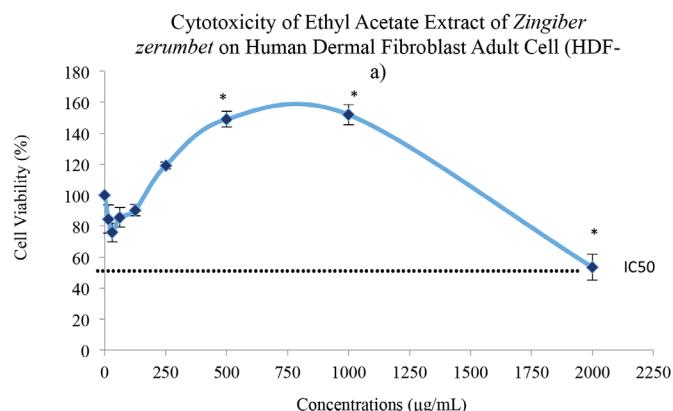


FIGURE 1. MTT assay of human dermal fibroblast adult (HDF-a) cells were treated with different concentrations of ethyl acetate extract of *Zingiber zerumbet* (15.6, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µg/mL) for 24 h. Each value is the mean ± SD of three independent experiments.

Asterisks indicate values which are significantly different ($p<0.05$) as compared to control

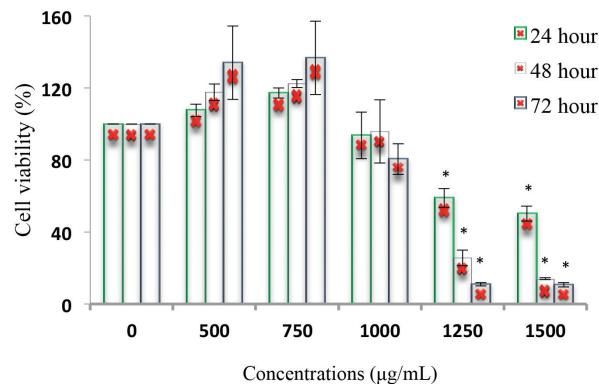


FIGURE 2. Proliferation assay of human dermal fibroblast adult cells (HDF-a) treated with ethyl acetate extract of *Zingiber zerumbet* (500, 750, 1000, 1250 and 1500 $\mu\text{g}/\text{mL}$) for 24, 48 and 72 h. Each value is the mean \pm SD of three independent experiments. Asterisks indicate values which are significantly different ($p<0.05$) from the control

TABLE 1. The rate of cell migration of human dermal fibroblast adult cells (HDF-a) treated with ethyl acetate extract of *Zingiber zerumbet* at 24, 48, 72, and 96 h in comparison to the control groups ($n=3$)

Incubation time (h)	Rate of cell migration for control (%)	Rate of cell migration for treatment (%)
0	0	0
24	26.45 ± 4.69	25.50 ± 4.40
48	37.90 ± 4.55	46.08 ± 2.89
72	48.17 ± 2.96	52.93 ± 2.73
96	56.53 ± 3.64	62.27 ± 2.94

the main focus of this study. MTT assay demonstrated no IC_{50} value recorded at the time of treatment of less than 1000 $\mu\text{g}/\text{mL}$ for 24 h. This finding is consistent with a previous study done on traditional plant extract of *Infectoria quercus* (manjakani) which did not give any IC_{50} values on human fibroblast cells (Rahman et al. 2013). Based on the findings of cell viability for proliferation assay obtained in this study, the first IC_{50} value was achieved at $>1150 \mu\text{g}/\text{mL}$ after 48 h of treatment. For 72 h, the IC_{50} values were measured at concentrations $>1100 \mu\text{g}/\text{mL}$. A compound with IC_{50} values greater than 25 $\mu\text{g}/\text{mL}$ is considered not to cause toxicity to the cells, IC_{50} values of 10 to 25 $\mu\text{g}/\text{mL}$ is categorized as less toxic, IC_{50} values of 5 to 10 $\mu\text{g}/\text{mL}$ is moderately toxic substance and IC_{50} that is less than 5 $\mu\text{g}/\text{mL}$ is categorized as an active compound or very toxic to cells (How et al. 2008).

In this study, cell proliferation assay showed high viability at a concentration of 500 and 750 $\mu\text{g}/\text{mL}$ at 72 h. These results indicated that these might be the suitable concentrations to enhance cell proliferation of HDF-a. Based on the percentage of cells viability, concentration of 750 $\mu\text{g}/\text{mL}$ was chosen for scratch assay as this concentration gave the highest viability. However, it was not significant when compared with the control. This may be due to the mixture of active compounds such as zerumbone, humulene, kaempferol, verbena and caryophyllene oxide that are present in EAZZ that reduce its property (Pari et al. 2004; Singh et al. 2014). The dose

and time dependent information could provide the specific information regarding the best condition in applying the extract to the patient.

The active compounds found in the EAZZ extract can give different effects on the cells depending on the content (Demma et al. 2009; Pari et al. 2004). The active compounds identified in *Z. zerumbet* were zerumbone, kaempferol and humulene with zerumbone as the highest content in the extract (51.57%) (Ruslay 2006; Yob et al. 2011). The rhizomes of *Z. zerumbet* possess anti-inflammatory effect and this effect is believed to be due to the presence of zerumbone and kaempferol. These compounds were shown to facilitate wound healing by reducing inflammation of the wound (Chien et al. 2008). The anti-inflammatory effects of this extract was also supported by a previous finding that the extract of *Z. zerumbet* able to reduce edema in rats (Nhareet et al. 2003). In addition, another active compound found in this plant namely curcumin is also known to possess wound healing effects in rats. Curcumin increase the production of collagen on the wound area and has faster effect for wound healing (Kulac et al. 2013). Thus, curcumin can reduce the healing time for wounds that happened during inflammatory and proliferative phase in which the collagen formation occurs.

Scratch assay enables identification of fibroblasts and cell-matrix interactions between cells. Interaction between cells during the migration process can resemble

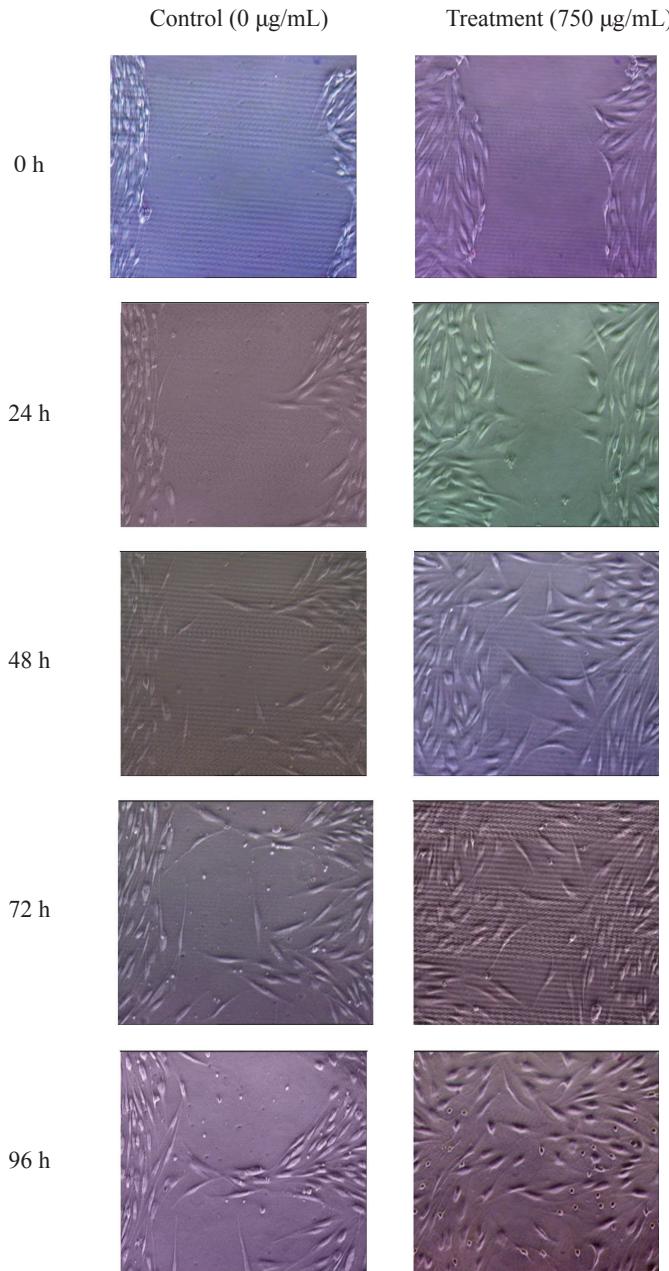


FIGURE 3. Representative images for *In Vitro* migration assay of human dermal fibroblast adult (HDF-a) cells treated with ethyl acetate extract of *Zingiber zerumbet* at 24, 48, 72 and 96 h ($\times 40$). The distance of cell travelled from the wound edge to the wound area was calculated using AxioVision software (Zeiss, Germany)

cell migration that occurs during wound healing *in vivo*. The study on scratch assay demonstrated an increase in cell migration rate using EAZZ extract. However, at 24 h post-treatment, cell migration was lower as compared to control group. At 48, 72 and 96 h post-treatment with EAZZ extract, the cells migration increased. At 24 h, the fibroblast are still in the process of adaptation to adjust to the surrounding cells containing EAZZ compounds. The cells depend on the mechanical condition of the environment by using a specific protein from the extracellular matrix (ECM) to adapt to a new environment (Rhee 2009).

Fibroblast cells produce ECM and collagen (Lodish et al. 2000). Previous study using other traditional plant, *Alocasia denudata* found that the proliferation phase starts at day 6 as the fibroblast and epithelium cells start to migrate into the wound area (Latif et al. 2015). ECM also contains polysaccharides, water and collagen that produce the unique characteristics of the skin. The unique feature is available as a result of a combination of molecules found in the ECM secreted by cells which are elastin, laminin, collagen and proteoglycans such as hyaluronan. In this study, it can be deduced that EAZZ extract increases cell proliferation of HDF-a cells thus promoting the formation

of ECM and fibroblasts that produce collagen at the wound surface.

The percentage of cell migration using EAZZ extract was higher than the control, but this increase was not significant. In most circumstances, the cell fibroblasts migrated into the wound area in response to cytokines and growth factors such as PDGF, TGF- β and FGF. After the fibroblasts have migrated into the matrix, it will start to grow and synthesize collagen, elastin, proteoglycans, and other components consisting of granulation tissue. Previous study has found that growth factor TGF- β affects the synthesis of ECM (Greg 2005). In addition, this extract is also more likely to give a better impression to other cells such as keratinocytes that are also present in the epidermis layer of the skin during the healing process.

CONCLUSION

EAZZ extract is not toxic from the concentrations up to 1000 $\mu\text{g}/\text{mL}$ and can increase cell proliferation of HDF-a cells at the concentration of 750 $\mu\text{g}/\text{mL}$. EAZZ has the potential to be developed as an alternative agent for treating wounds in the future.

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