Ethanol Extract of *Artocarpus odoratissimus* (Tarap) Bark Inhibit Migration of MDA-MB-231 Triple Negative Breast Cancer Cells

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Abstract

Breast cancer is the most diagnosed cancer in the world, with triple negative breast cancer (TNBC) being one of its subtypes that exhibits an aggressive clinical course, a tendency for early metastasis, and a poor prognosis. Tarap (*Artocarpus odoratissimus Blanco*) is an endemic plant of East Kalimantan, which possesses anticancer potential. This study aimed to explore the ability of ethanol extract of Tarap bark (EETB) in reducing cell viability, inducing apoptosis and inhibiting migration of MDA-MB-231 cells. Cell viability was observed by 3-(4.5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. EETB -induced apoptosis was analyzed by double staining allophycocyanin (APC) Annexin V with PI kit through flow cytometry. Cell migration was determined through scratch assay. EETB was found to decrease cell viability with an IC_{50} value of 1607.302 µg/ml (R=0.369). EETB's ability to induce total apoptosis did not show significant differences across various concentrations. EETB was able to inhibit cell migration, especially at concentrations of 800 mg/ml, both at 24-h and 48-h observations. This finding demonstrates that EETB has a weak effect on reducing the viability and inducing the apoptosis of MDA-MB-231 cells. EETB induced morphological cells type change in to globular type and significantly inhibits two-dimensional migration of MDA-MB-231 cells.

Keywords: *Tarap, Artocarpus odoratissimus, triple negative breast cancer.*

INTRODUCTION

Breast cancer is the most diagnosed cancer in the world. The burden of breast cancer is predicted to increase to more than 3 million new cases per year by 2040, representing an increase of approximately 40% compared to 2020. Deaths from breast cancer are also predicted to increase by more than 50% in 2040 compared to 2020 (Arnold, *et al*., 2022). Breast cancer is not a homogeneous disease, but comprises several subtypes that determine the therapy and prognosis. Data from Indonesia

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for the 2014-2019 period shows that the most common subtype is luminal (64.9%), followed by triple-negative (18.1%) and HER2-positive (17%) (Widiana & Irawan, 2020). This pattern aligns with the incidence rates observed in other Asian countries (Pandit, *et al*., 2020; (Chuaychai & Sriplung, 2022) and the United States of America (Acheampong, *et al*., 2020).

Triple negative breast cancer (TNBC) is a molecular subtype of breast cancer that express neither estrogen, progesterone, nor human epidermal growth factor receptor 2 (HER2) receptors. This subtype is characterized by aggressive clinical course, early metastasis and poor prognosis (Baranova, *et al*., 2022). The treatment of TNBC is aimed at inhibiting the growth of the primary tumor and metastasis to distant organs (Baranova, *et al*., 2022). TNBC therapy relies on surgical modalities and neoadjuvant/adjuvant chemotherapy (Kepmenkes RI, 2018; Koppikar, *et al*., 2023).

The risk of metastases correlates with the primary tumor progression. However, 0.3-1% of the total incidence of breast cancer is clinically recognized metastatic carcinoma from an undetectable primary breast tumor or occult breast cancer (Barbieri, *et al*., 2020). One of the characteristics of cancer cells that support metastasis is the ability to migrate through epithelial-mesenchymal transition (Welch & Hurst, 2019). Therefore, migratory ability of cancer cells become a promising target for inhibiting metastasis (Wells, *et al*., 2014), which led to the emergence of a new class of therapy called migrastatics (Raudenská, *et al*., 2023). The challenge in developing migrastatics for breast cancer is its multiple metastatic targets organs, particularly in bone, brain, liver, lung, and distant lymph nodes. Each organ has unique environmental pressures, thus in order to succeed, metastatic clones require specific characteristics suitable for colonizing the organ (Vitos & Gerlee, 2022). Identification of the best targets and development of multiple migrastatic agents is necessary to provide personalized migration inhibition (Raudenská, *et al*., 2023).

Tarap (*Artocarpus odoratissimus Blanco*) is a Kalimantan native plant, part of the Artocarpus genus along with jackfruit, cempedak, and breadfruit. Traditionally, Tarap is utilized in the treatment of diabetes, antimalarial, and antitoxin for centipede and scorpion bites. Tarap contains several active compounds such as terpenoid, phenolic, and stilbenoid groups, which have bioactivity properties as anti-hypertensive and antitumor and antioxidants (Abu Bakar, *et al*., 2009; Erwin, *et al*., 2015). Artosimmin, a secondary metabolite isolated from Tarap, potentially inhibits the growth of leukemia cells (HL-60) and breast cancer (MCF-7) (Ee, *et al*., 2010). Tarap's migratory inhibition had not been tested, but xanthones isolated from various Artocarpus species inhibited H460 non-small cell lung cancer (NSCLC) cell migration in vitro and metastasis in vivo in mice (Bailly, 2021). Based on this premise, the research was aimed at exploring the potential of Tarap in inhibiting the growth and migration ability of TNBC subtype breast cancer, which is an aggressive and metastatic type of breast cancer.

METHODS

The research was an experimental study with post-test only control group design. Bark was collected from a Tarap tree growing in a yard at Samarinda, Kalimantan Timur, Indonesia during May 2022. The sample was authenticated by "Herbarium Mulawarman" of Tropical Forest Ecology and Biodiversity Conservation Laboratory, Mulawarman University, Indonesia.

The triple negative breast cancer cells used were MDA-MB-231 human breast cancer cells (ATCC® HTB-26™, USA). The culture medium consisted of used were Dulbecco's Modified Eagle's Medium (DMEM) high glucose (GIBCO, Invitrogen, USA) and fetal bovine serum (SangonBio, Shanghai, China). The chemicals were trypsin-EDTA 0,25% (GIBCO), allophycocyanin (APC) annexin-V apoptosis kit with propidium iodide (PI) (Biolegend, California, USA), 3-(4.5-Dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit-colorimetric (Biovision, USA), ethanol 96%, and dimethyl sulfoxide (DMSO) (Merck, USA).

Tarap Bark Extraction

Crude extract of Tarap bark was prepared using the modified method of Yadav, *et al*. (2015). The Tarap bark (500 grams) was dried in an oven at 50°C for 5 days and then mashed with an electric blender. The powder was submerged in 96% ethanol with a ratio of 1:4 (v/v), and left at room temperature for 72 h. The crude extract was filtered using 125 mm Whatman filter paper under sterile condition. This process was repeated 3 times and then the solvent was evaporated using rotary evaporator at temperature below 50°C. The residue was further dried by incubating for 5 days in the oven at 50°C. The extract was kept at 4°C until use. The yield of the extract was 10 % (w/w). Preparation of Tarap ethanol extract for testing The ethanol extract of Tarap bark (EETB) was dissolved in DMSO to obtain a stock solution with concentration of 105 µg/ml. During testing, each treatment well was added stock solution and culture medium to make final concentration of 25, 50, 100, 200, 400, and 800 µg/ml.

Cell Viability Assay

The MDA-MB-231 cell viability was determined by MTT assay. The cell line was grown to 90% confluency in DMEM high glucose medium with 10% FBS. After the cells were harvested, it plated in 96-well plates ($2x10^4$ cells/well) together with one of 6 concentrations of EETB or control without treatment (3 wells for each concentration and control). After 24 h incubation, the medium was removed. 50 µl of serum-free media and 50 µl of MTT Reagent was added to each well, then incubated at 37°C for 3 h. MTT Solvent (150 µL), that provided in the Biovision MTT kit, was added afterward, and the well was shaken on an orbital shaker for 15 minutes. Finally, the plate was read using the microplate reader at 590 nm. A linear regression analyzis of EETB concentration versus cell viability percentage was made to predict 50% inhibition concentration (IC_{50}).

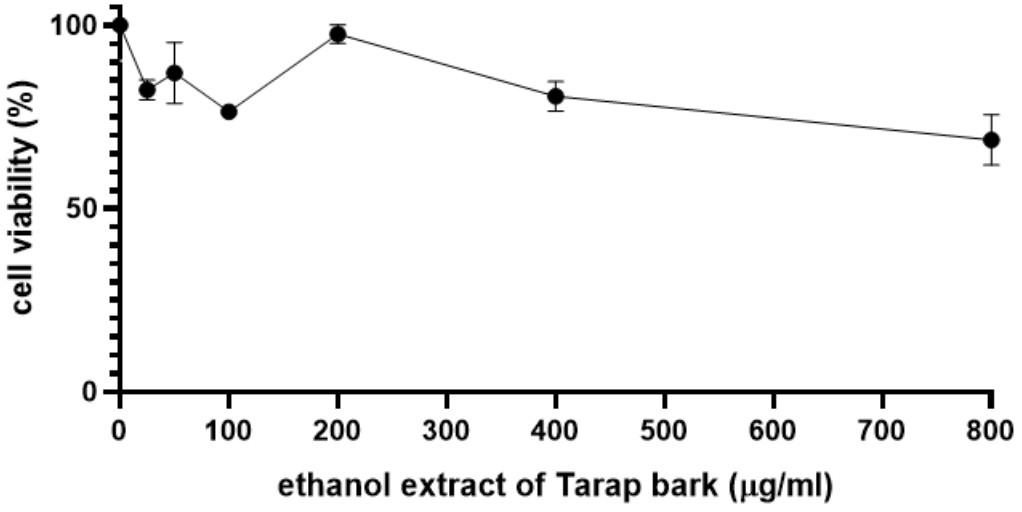

Figure 1. Cell viability assay of EETB in MDA-MB-231 cancer cell after 24 h treatment. The relative amount of viable cells was estimated by measuring cell suspension absorbance after MTT assay. Calculation of IC_{50} value was predicted through the trend line equation (y=0.0252X-90.50, R=0.369) based on graph of cell viability versus EETB concentration. Data was shown as mean +SEM.

Apoptosis Assay

MDA-MB-231 cell line were grown in 24-well plates until 60% confluent. A series of EETB concentrations (100, 200, 400, and 800 µg/ml) and control were added to each well, and incubated for 24 h. The apoptosis assay was carried out according to Biolegend APC-Annexin-V apoptosis kit with PI protocol. The cells were harvested, centrifuged, and washed twice with cell staining buffer. Annexin-V binding buffer was added to the cells at a concentration of $0.25x10^7$ cells/ml. The cell suspension (100 µL) was stained with APC-Annexin V (5 µL) and PI solution (10 µL). After incubation for 15 minutes at room temperature in the dark, Annexin-V binding buffer was added to each tube. The percentage of non-viable, apoptotic cells was assessed using flow cytometry (BD FACSMelody™; BD Bioscience).

Migration Assay

The effect of EETB on MDA-MB-231 cell migration was studied using *in vitro* scratch assay (Rachmi, *et al*., 2020). The MDA-MB-231 cells ($5x10^4$ cells) were plated in 24-well palate and incubated until 90% confluence. Then, the culture was scratched with a p200 pipette tip, and EETB in medium with 5% FBS was added immediately after wound scratching. The wells were evaluated within 0, 24, and 48 h prior. The wounded area was photographed serially in three field of view for each well, under an inverted microscope at 400x. Cell migration was determined by the numbers of migrated cell to the wounded area and cell free area. The cell counting and area measurement were assisted by ImageJ software. The number of migrated cells was the average of the number of cells residing at the scratch area in the 3 fields of view.

Cell free area=(cell free area at 24- or 48-h observation/cell free area at 0 h) x 100%

Statistical Analysis

The results were reported as average±standard error mean (SEM). Analysis of variance (ANOVA) followed by post-hoc–Tukey analysis was used to explore comparisons between treatment groups. A P value of less than 0.05 was considered statistically significant.

RESULTS

EETB exposure caused a decrease in cell viability in proportion with the increase in EETB concentration. The IC_{50} value after 24 h of exposure was estimated based on the trend line and the dose-response linear regression equation (Figure 1). Based on this equation, the concentration of EETB that is thought to cause a decrease in cell viability by up to 50% is 1607,302 µg/mL.

Based on the result of cell viability assay, EETB concentration that favored the decreasing trend of cell viability was determined. EETB increased total apoptosis compared to the negative control. The percentage of end-stage apoptotic cells increased significantly at 800 µg/ml of EETB, compared to the negative control, 100, 200, and 400 µg/ml of EETB, However, the ability in inducing total apoptosis was not significantly different between all EETB concentrations (Figure 2).

Based on the results of the scratch wound healing test, EETB was able to inhibit the two-dimensional migration of MDA-MB-231 cells (Figure 3A). The inhibition of cell migration was demonstrated by decreasing the number of cells migrating to the cell-free area as the EETB concentration increased. The suppression of cell migration mainly occurred at EETB concentration of 800 µg/ml after 48 h of treatment. The number of cells moving to the cell-free area at the 48^{th} h of observation was not significantly different from the 24^{th} h at all EETB concentrations (Figure 3B). The effect of EETB on the migration of MDA-MB-231

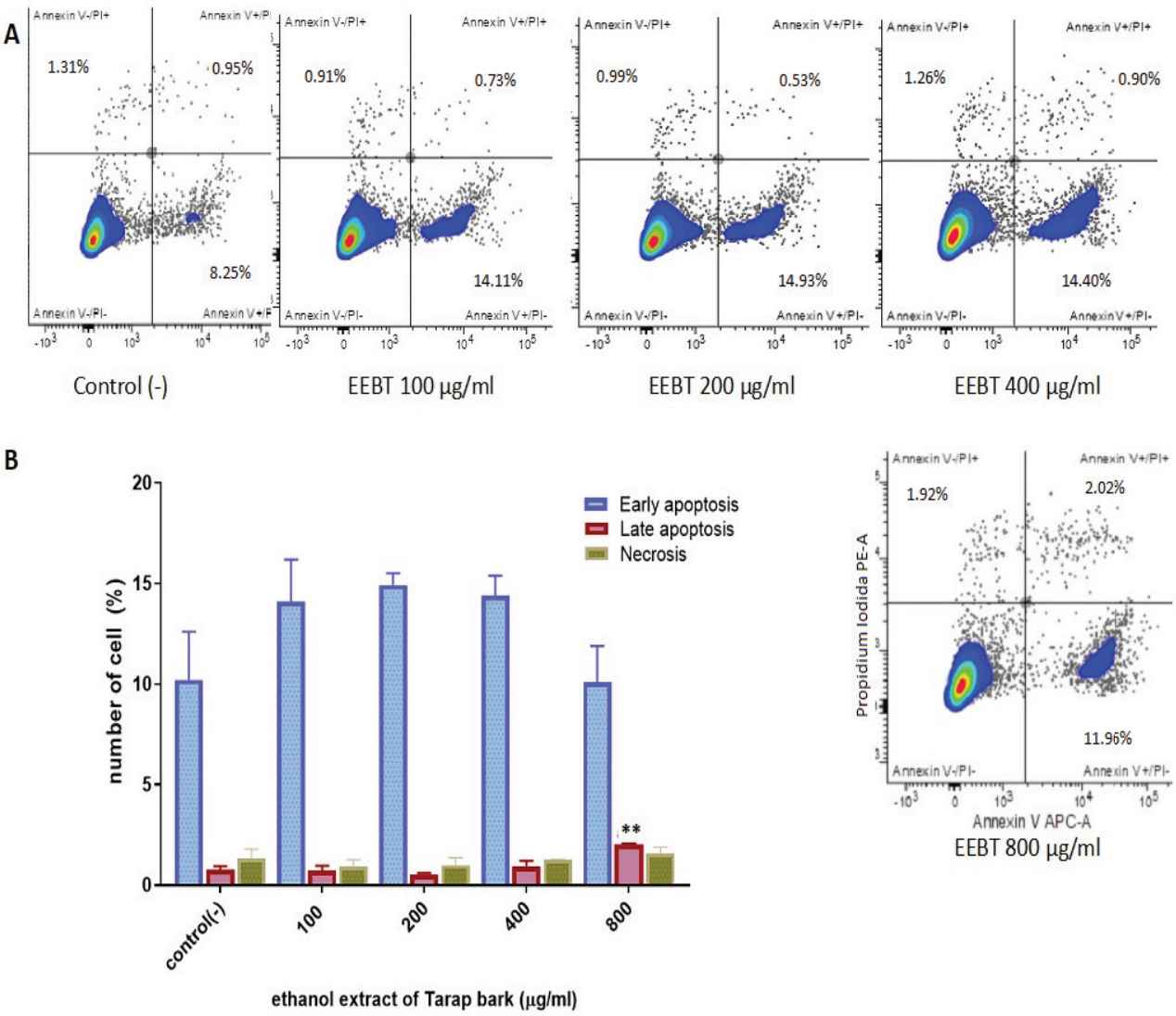

Figure 2. A. The flowcytometry result of EETB apoptotic effect on MDA-MB-231 cell was assayed using AnnexinV/PI. The color scheme represented a density plot that convey information about the number of events that were in the same location. B. Summary of the apoptosis and necrosis percentage in the histogram form. **P<0.05 vs. untreated cells.

cells was also analyzed based on the percentage of cell-free area. The analysis showed a significant increase in the percentage of the cell-free area due to 800 µg/ml EETB exposure for 48 h, in a similar pattern to the decrease in migrating cells (Figure 3C).

The decrease in the number of cells migrating to the cell-free area was accompanied by a decrease of cell density in the cell-occupied area and shrinking of the cells. The decrease of cell density in the cell-occupied area was observed at 24- and 48- h exposure to EETB of 400 and 800 µg/ml. Upon closer observation, cells morphology became more dominated by globular-shaped along with the increase of EETB concentration. This phenomenon could be seen since the exposure of 400 µg/ml EETB for 24 h (Figure 4).

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DISCUSSION

The results of this study showed that EETB had a weak effect in reducing cell viability, as indicated by the value of the correlation coefficient (R) of 0.369 between EETB concentration and cell viability in the MTT assay, as well as an increase in apoptosis that did not differ significantly between EETB concentrations. Different results were shown in exposure MCF-7 with Artosimmin (a pyranoflavone derivative) isolated from Tarap bark. Artosimmin reduced MCF-7 cells viability with IC_{50} 3.4 µg/ml based on the results of MTT assay (Ee, *et al*., 2010). The difference in the results of these two studies is related to two possibilities. This study used crude extract with polar solvents with the aim of obtaining phenolic and stilbenoid content that have anticancer potential. The results showed that polar compounds in Tarap bark have weak cytotoxic abilities compared to Artosimmin which is non-polar. Another possibility is the different types of cell cultures used in the two studies. MCF-7 expresses estrogen receptors and epidermal growth factor (EGF) whereas MDA-MB-231 is

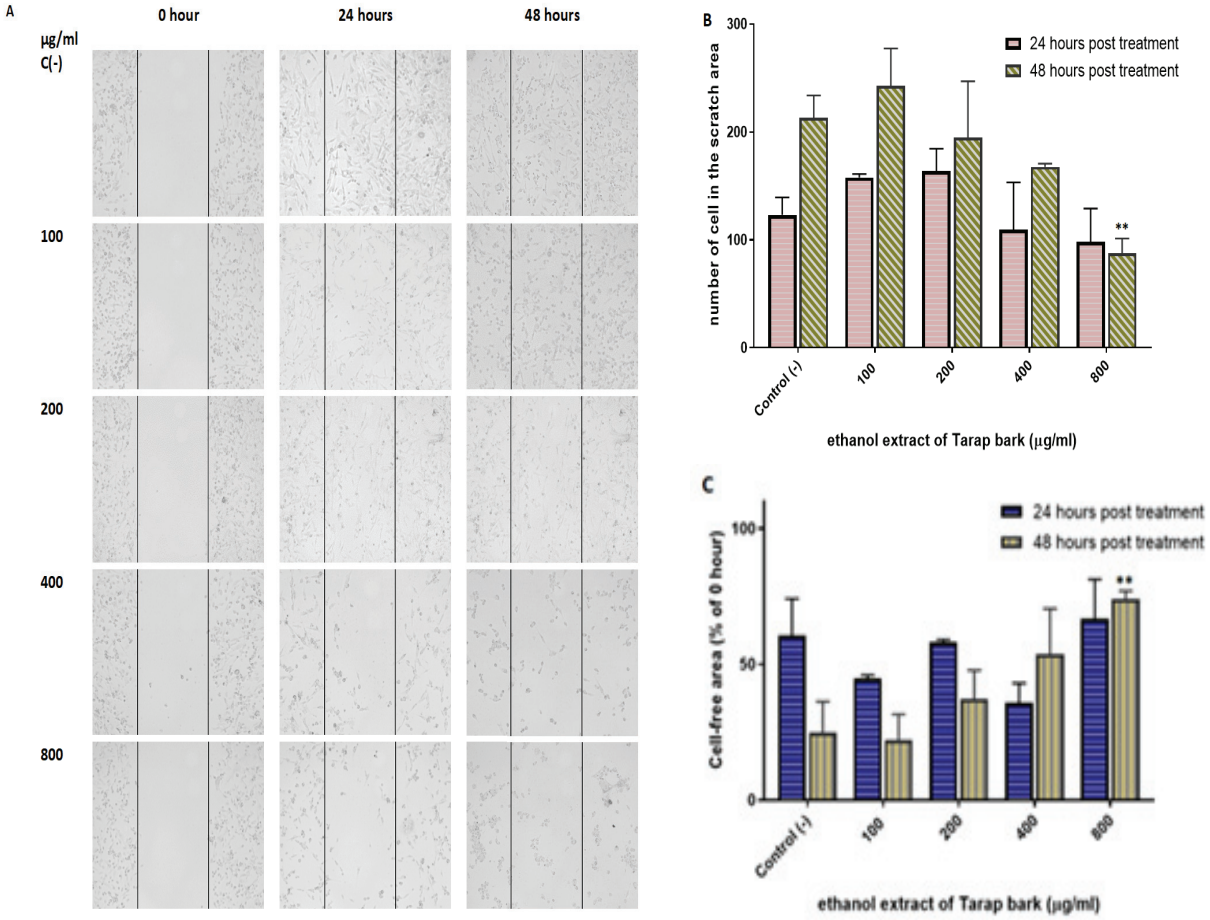

Figure 3. Effect of EETB on MDA-MB-231 cell migration. (A) The migration was inhibited after EETB treatment, determined by wound scratch assay. EETB suppression on cell migration was quantified based on the number of cells that migrated to the cell-nude area (B) and percentage of cell-free area (C). Data were shown as mean + SEM. ***P*<0.05 vs. untreated cells. No significant difference between 24- and 48- h observation of each EETB concentration.

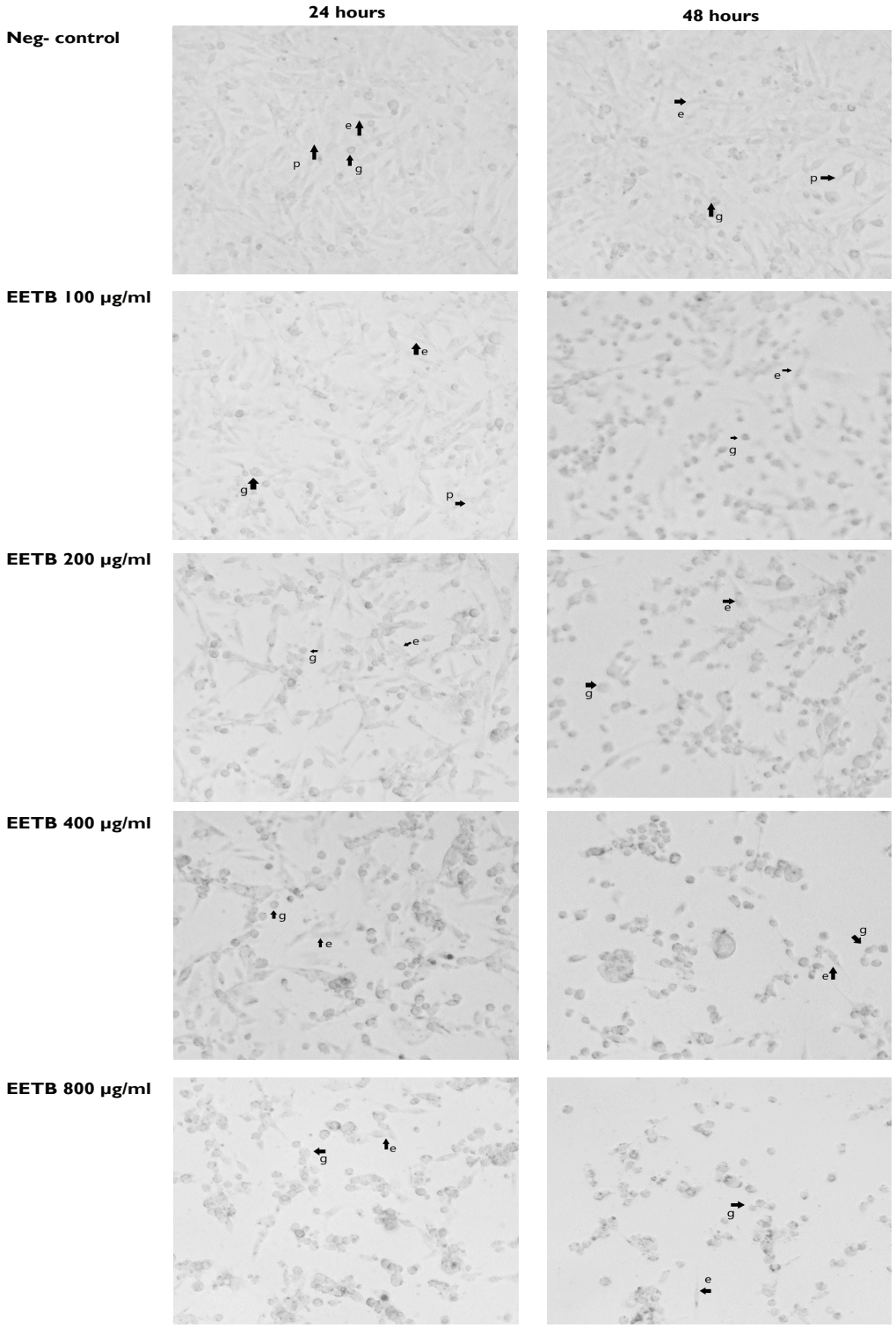

Figure 4. Effect of EETB on MDA-MB-231 cell morphology and density after 24- and 48- h. The cell morphology was observed under an inverted microscope at 400x, Cell types were categorized as elongated (e), flattened polygonal (p), and globular (g). As the concentration increases, the cell density decreases and the globular cell type predominates.

more hormone independent. These differences in characteristics can lead to different outcomes in exposure to proliferative or cytotoxic inhibitory agents (Aumsuwan, *et al*., 2016; Garbar, *et al*., 2017; Kocak, *et al*., 2020).

EETB's ability to suppress MDA-MB-231 cell migration was proven through scratch tests that modeled cell movement in two dimensions. Scratch wound healing serve as a model of directional migration because cells form protrusion and migrate towards the scratch area (Molinie dan Gautreau, 2018). The possibility of pseudo-migration as a proliferative effect was minimized by serum starvation during EETB treatment (Grada, *et al*., 2017). Two-dimensional migration testing can be a model of mesenchymal movement in MDA-MB-231 cells that tend to migrate individually or multicellular streaming (non-adherent cells moving through the same path) *in vivo* (Clark & Vignjevic, 2015). Analysis of EETB effect on cell movement to cell-free areas showed that exposure 800 µg/ml EETB caused significant inhibition of cell-free area closure as well as reduction of migrating cells at 24 h and 48 h. Inhibition of MDA-MB-231 cell migration by EETB observed through scratch assay might be an indicator of EETB's potential as an anti-metastatic *in vivo* (Choi, *et al*., 2014; Li, *et al*., 2017). The effect of MDA-MB-231 cell migration inhibition at sufficiently large EETB concentrations (800 ug/ml) may be related to small concentrations of mygrastatic active metabolites in crude extracts. Therefore, further research is needed to explore the fractions that have the optimal migrastatic ability.

In the present study, EETB induced phenotype changes in MDA-MB-231 cells from predominantly elongated cells exhibiting lamellipodia at one or both end to globular shaped cells. The cells shape transformation became apparent along with increasing EETB concentration. In 2D flask cultures, MDA-MB-231 cells phenotypes correlate with the epithelial or mesenchymal state of the cells. The elongated shaped cells are the morphological expression of epithelial to mesenchymal transition (EMT) and correlate with increased invasive potential. The polygonal flattened cells are comparable to an epithelial phenotype (Franchi, *et al*., 2020). On the other hand, the globular or round phenotype is the slowest migrated cells and they might be arise from the absence of a mechanical compliance, such as adhesion, confinement or extracellular matrix stiffness (Geiger, *et al*., 2019; Mohammed, *et al*., 2020).

The decrease in cell density and an increase in globular cell type was observed at 24 h EETB exposure, whereas the incidence of total cell death remained below 20% up to the highest concentration. Thus, the increase of globular phenotype might correlate with the decrease of motility ability rather than cell death processes. This phenotype might also contribute to decreased cells density because of its smaller size due to minimal cell spreading, and possible cells detachment due to weaker adherence. The MDA-MB-231 cells have high resistance to anoikis which is a form of programmed cell death when they are detached from the surrounding extracellular matrix (ECM). Thus, the detached MDA-MB-231 cells might be viable and have the ability to proliferate when the environment is suitable (Bizjak, *et al*., 2017). The EETB effect on MDA-MB-231 should be explored of its effect on the detach cells. Although, the detach cells in this study are suspected to be in a non-motile form, cancer cells can have direct access to the bloodstream through vessel-like passage, thus the detach cells can easily intravasate (Zavyalova, *et al*., 2019). The EETB effect to MDA-MB-231 cells migration needs to be followed up with further research regarding the effect of EETB on apoptosis at more than 24 h of exposure. In this way, it can be ascertained whether EETB is able to inhibit migration with minimal effect on cell death, thus it can be combined with other therapeutic modalities.

CONCLUSION

EETB had a weak ability to reduce cell viability and induce apoptosis of MDA-MB-231 cells. EETB inhibited MDA-MB-231 cells migration in two-dimensional model, and increase globular phenotype cells. EETB migration inhibition capabilities need to be further explored in terms of obtaining fractions with the most effective migrastatic ability in two and three-dimensional migration .

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