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Vol. 60 No. 10: October 2023

Published: 2023-09-27

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Indian Journal of Biochemistry & Biophysics
Vol. 60, October 2023, pp. 763-769
DOI: 10.56042/ijbb.v60i10.1293

Eleutherine bulbosa bulb extract induces apoptosis and inhibits cell migration by downregulating Sonic hedgehog in human tongue cancer cells: An *in vitro* study

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Received 09 May 2023; *revised 18 September 2023*

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards tongue cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of tongue cancer cells. Human oral squamous cell carcinoma (HSC)-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner (P <0.05). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner (P <0.05). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: Apoptosis, Cell migration, *Eleutherine bulbosa*, Sonic hedgehog, Tongue cancer

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica* [4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation *via* cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have

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also been reported to have antimicrobial[11] and antioxidant[12] activity.

Eleutherine bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4-di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

Eleutherine bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human tongue cancer cells has not been investigated. Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of tongue cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5×10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that, 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8 µm pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the 24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P < 0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared compared with that of the sham group ($P < 0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P < 0.05$) than that of Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27±0.14%. Apoptotic HSC-3 cells were significantly higher compared with the sham group ($P < 0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P < 0.05$) than that of Doxorubicin-treated HSC-3 cells (95.95±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was

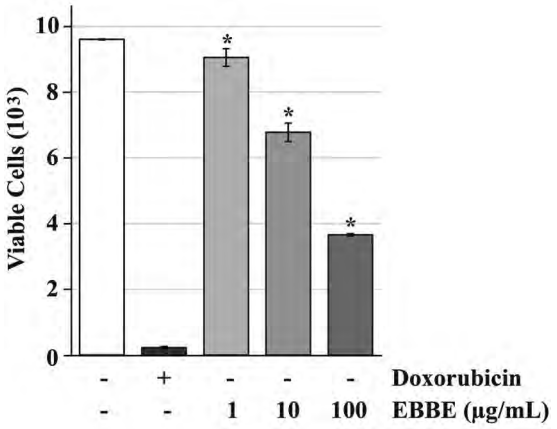

Fig. 1 — EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin or various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). *P <0.05 compared to the sham group

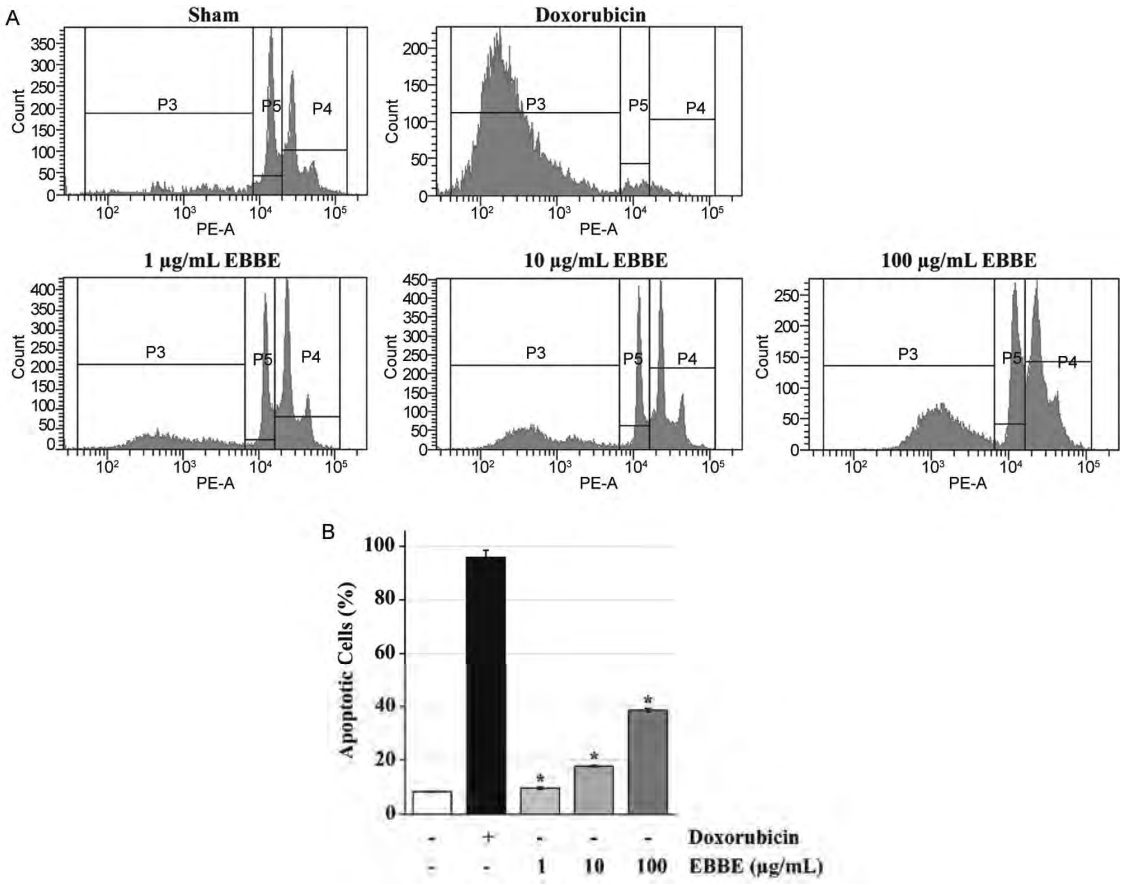

Fig. 2 — EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin or various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). *P <0.05 compared to the sham group

96.11±4.17%. After EBBE treatment, the migratory activities of HSC-3 cells were significantly lower compared with that of the sham group (P <0.05) in a concentration-dependent manner. Gap closure percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%, 76.78±4.87%, and 60.67±7.62%, respectively. These percentages were significantly higher (P <0.05) than that of cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than that of the sham group (P <0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P <0.05) than that of cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE downregulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P <0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P <0.05) compared with that in the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared with that in cyclopamine tartrate-treated HSC-3 cells (P <0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P= 0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

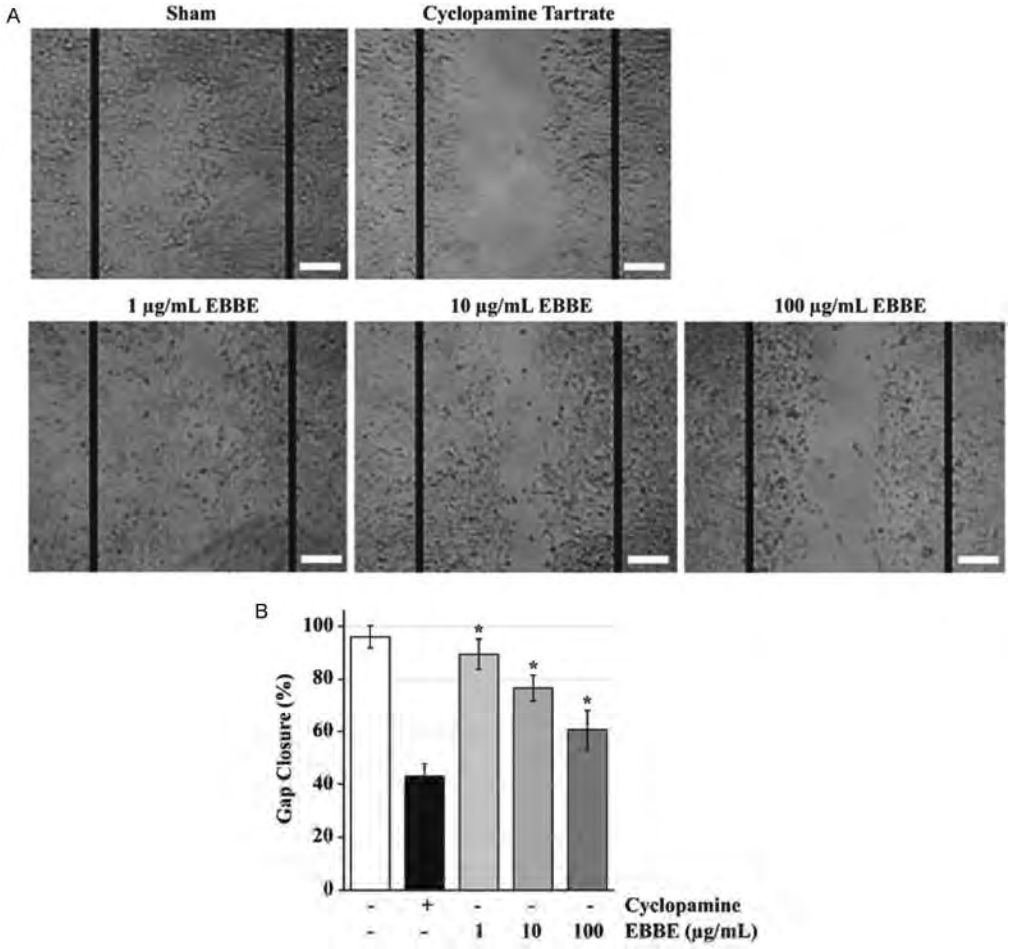

Fig. 3 — EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 µM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). **P* <0.05 compared to the sham group

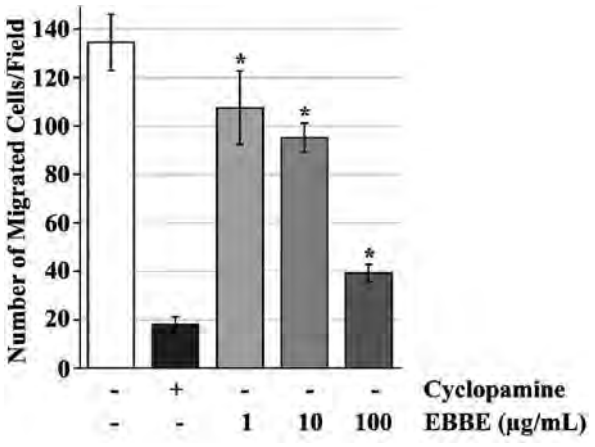

Fig. 4 — EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). **P* <0.05 compared to the sham group

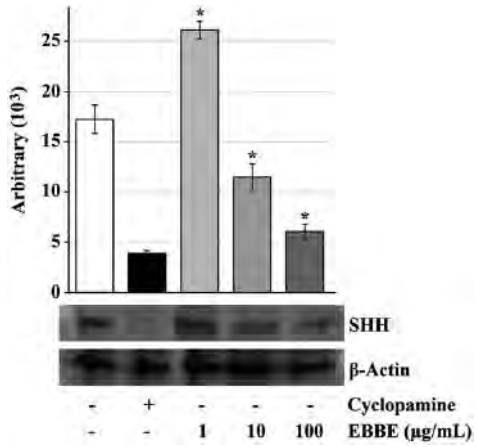

Fig. 5 — EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). **P* <0.05 compared to the sham group

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18–20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target molecules that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

Conclusion

EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an antimetastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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Submission ID:	2272330930

Indian Journal of Biochemistry & Biophysics
Vol. 60, October 2023, pp. 763-769
DOI: 10.56042/ijbb.v60i10.1293

Eleutherine bulbosa bulb extract induces apoptosis and inhibits cell migration by downregulating Sonic hedgehog in human tongue cancer cells: An *in vitro* study

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Received 09 May 2023; revised 18 September 2023

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards tongue cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of tongue cancer cells. Human oral squamous cell carcinoma (HSC-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner (P <0.05). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner (P <0.05). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: Apoptosis, Cell migration, *Eleutherine bulbosa*, Sonic hedgehog, Tongue cancer

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica* [4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation *via* cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have

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Submission date: 17-Jan-2024 11:17AM (UTC+0700)
Submission ID: 2272330930
File name: IJBB_83_auth_corr_1.pdf (9.06M)
Word count: 3960
Character count: 22117

Indian Journal of Biochemistry & Biophysics
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also been reported to have antimicrobial[11] and antioxidant[12] activity.

Eleutherine bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4-di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

Eleutherine bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human tongue cancer cells has not been investigated. Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of tongue cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5×10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that, 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8 µm pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the 24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P < 0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared compared with that of the sham group ($P < 0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P < 0.05$) than that of Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27±0.14%. Apoptotic HSC-3 cells were significantly higher compared with the sham group ($P < 0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P < 0.05$) than that of Doxorubicin-treated HSC-3 cells (95.95±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was

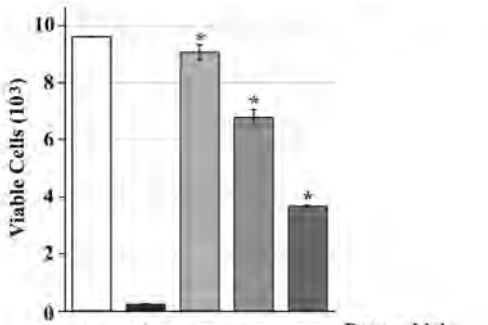

Fig. 1 — EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin or various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). *P <0.05 compared to the sham group

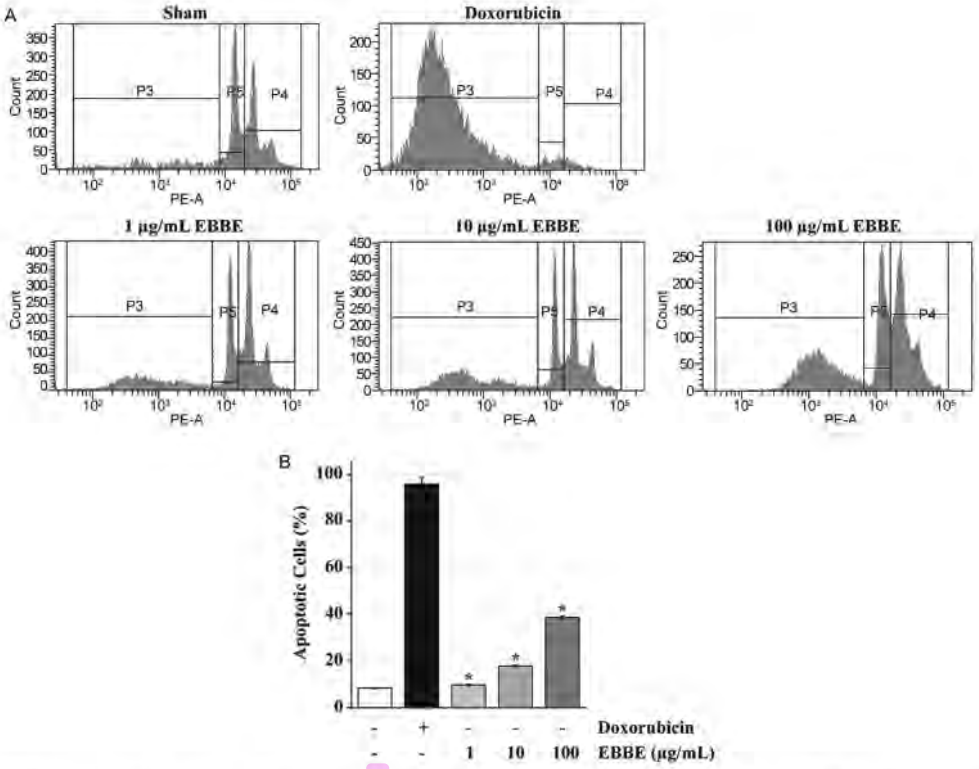

Fig. 2 — EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin or various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). *P <0.05 compared to the sham group

96.11±4.17%. After EBBE treatment, the migratory activities of HSC-3 cells were significantly lower compared with that of the sham group (P <0.05) in a concentration-dependent manner. Gap closure percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%, 76.78±4.87%, and 60.67±7.62%, respectively. These percentages were significantly higher (P <0.05) than that of cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than that of the sham group (P <0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P <0.05) than that of cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE downregulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P <0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P <0.05) compared with that in the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared with that in cyclopamine tartrate-treated HSC-3 cells (P <0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P= 0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

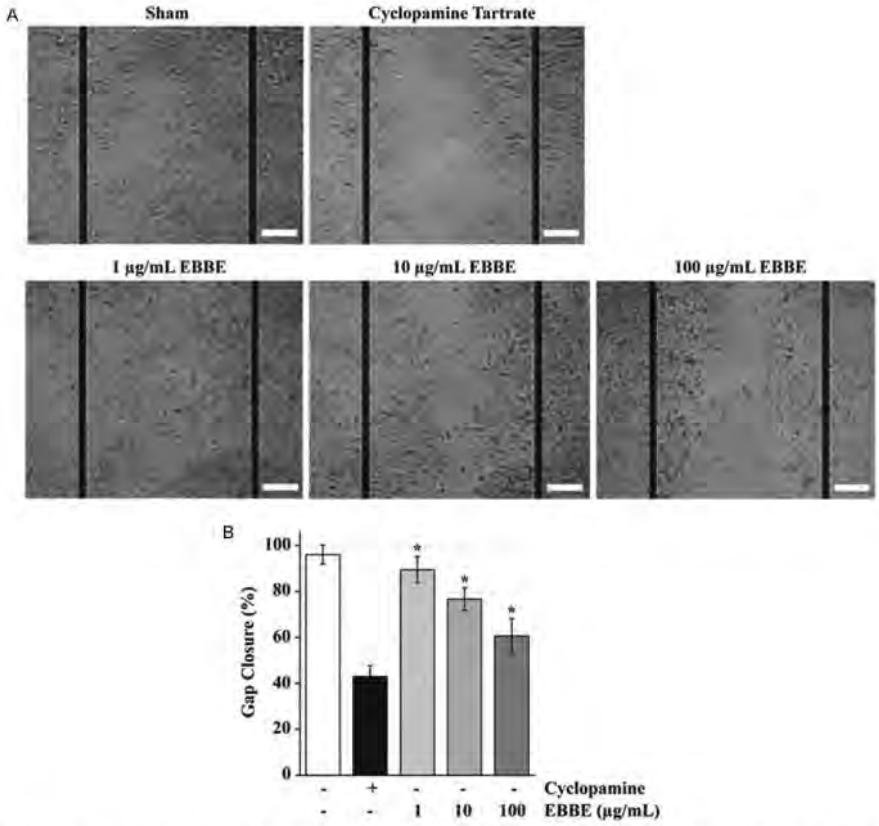

Fig. 3 — EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 µM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). *P <0.05 compared to the sham group

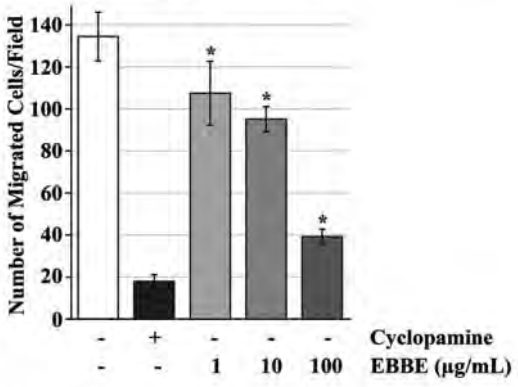

Fig. 4 — EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). *P <0.05 compared to the sham group

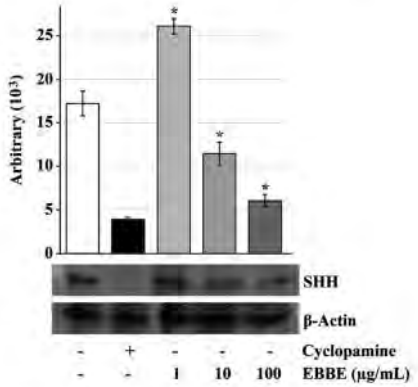

Fig. 5 — EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). *P <0.05 compared to the sham group

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18-20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target molecules that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

Conclusion

EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an antimetastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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Eleutherine bulbosa Bulb Extract Induces Apoptosis and Inhibits Cell Migration by Downregulating Sonic Hedgehog Gene in Human Tongue Cancer Cells: An *in vitro* Study

Running title: *Eleutherine bulbosa* Effect on Tongue Cancer Cells

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Abstract

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards oral cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells. Human oral squamous cell carcinoma (HSC)-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner ($P<0.05$). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner ($P<0.05$). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: HSC-3, MTT, sub-G1, transwell, immunoblotting

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica*[4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation via cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have also been reported to have antimicrobial[11] and antioxidant[12] activity.

E. bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4- di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

E. bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human oral cancer cells has not been investigated.

Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5 × 10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad,

Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8-µM pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the 24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-

G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P<0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared to the sham group ($P<0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P<0.05$) than Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27±0.14%. Apoptotic HSC-3 cells were significantly higher compared to the sham group ($P<0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P<0.05$) than Doxorubicin-treated HSC-3 cells (95.95±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was 96.11±4.17%. After EBBE treatment, the migratory activities of HSC-3 cells were significantly lower compared to the sham group ($P<0.05$) in a concentration-dependent manner. Gap closure percentage of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%,

76.78±4.87%, and 60.67±7.62%, respectively. These percentages were significantly higher (P<0.05) than cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than the sham group (P<0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P<0.05) than cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE downregulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P<0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P<0.05) compared to the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared to cyclopamine tartrate-treated HSC-3 cells (P<0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P=0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18–20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle

arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target genes that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

In conclusion, EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an antimetastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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Figure and Figure Legends

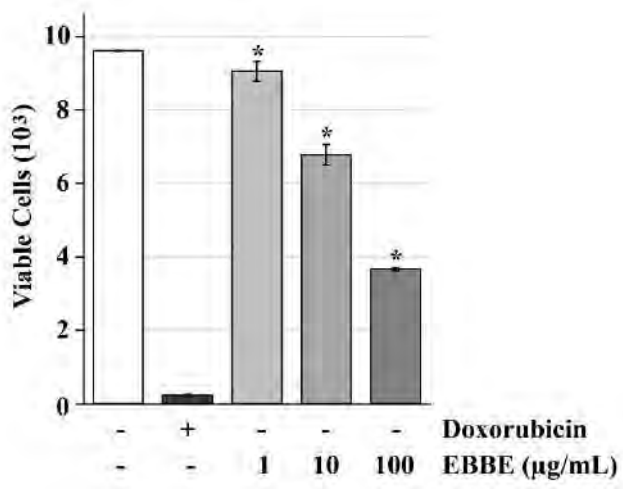

Fig. 1. EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 μM Doxorubicin and various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). **P*<0.05 compared to the sham group. EBBE: *E. bulbosa* bulb extract.

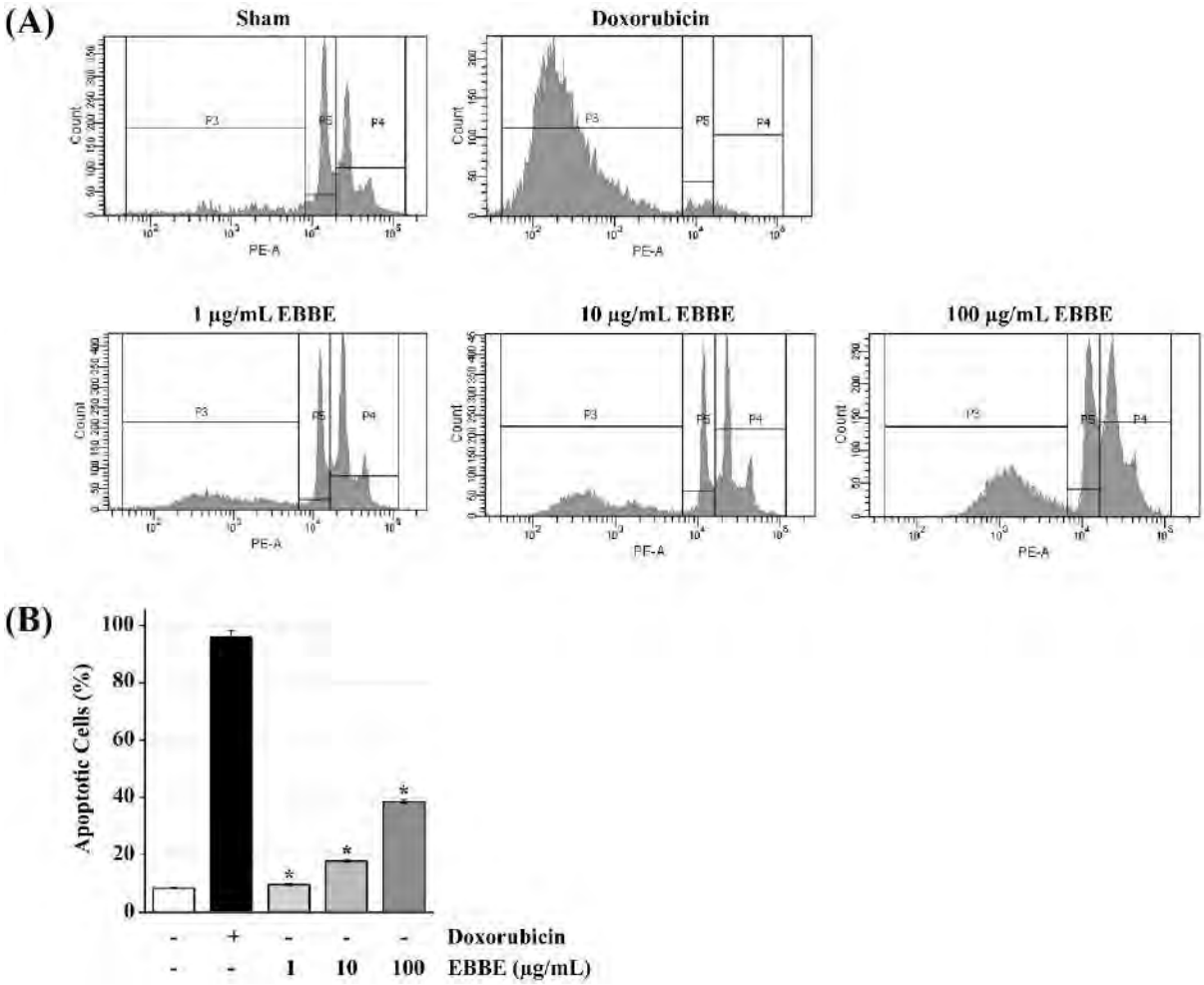

Fig. 2. EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 μM Doxorubicin and various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). **P*<0.05 compared to the sham group. EBBE: *E. bulbosa* bulb extract.

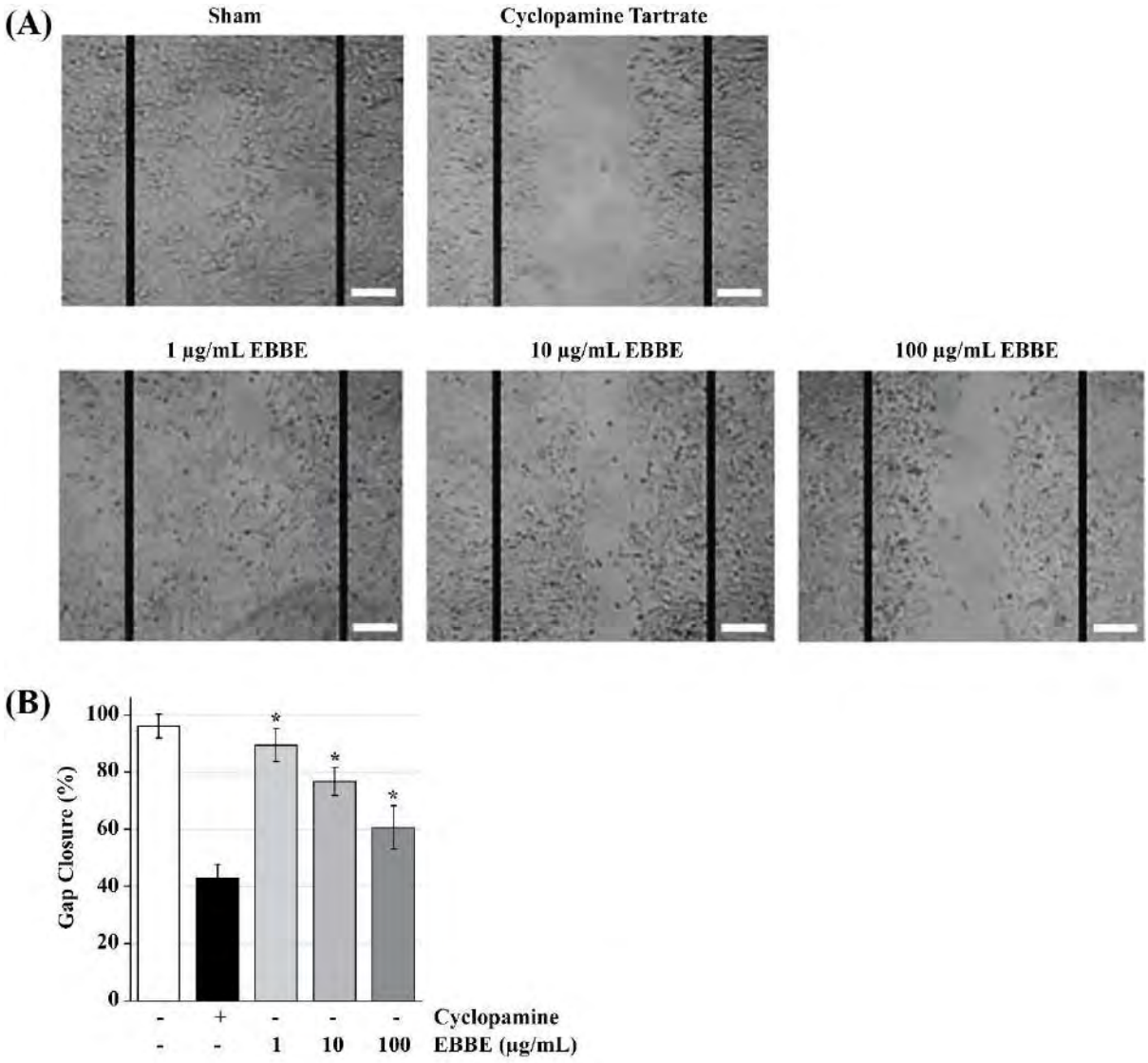

Fig. 3. EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 μM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 μM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). **P*<0.05 compared to the sham group. EBBE: *E. bulbosa* bulb extract.

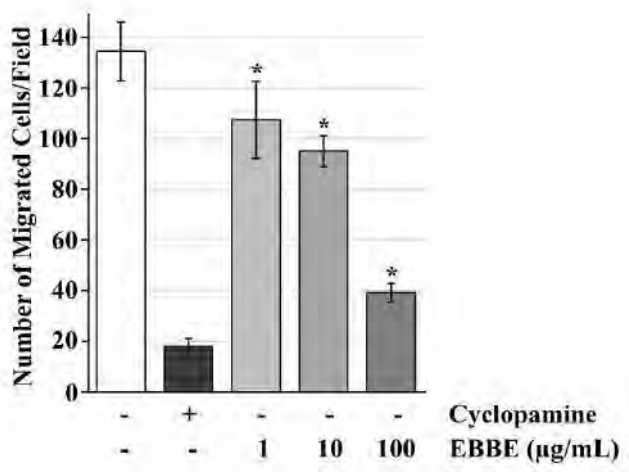

Fig. 4. EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 μM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). **P*<0.05 compared to the sham group. EBBE: *E. bulbosa* bulb extract.

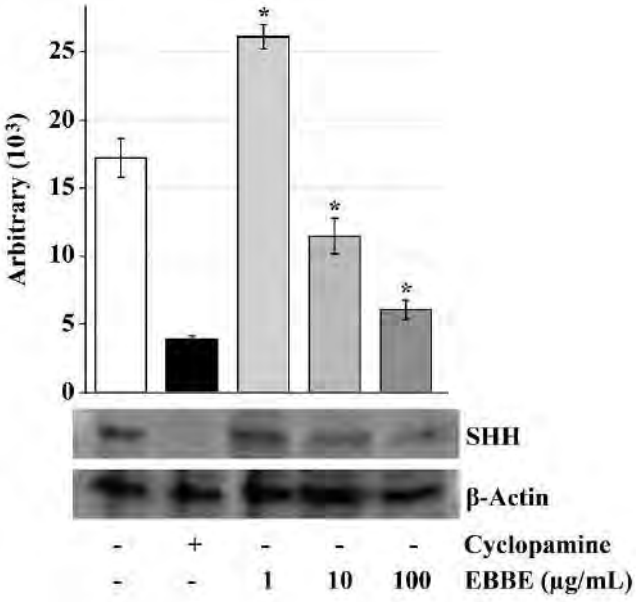

Fig. 5. EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 μM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). **P*<0.05 compared to the sham group. EBBE: *E. bulbosa* bulb extract; SHH: Sonic hedgehog.

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Indian Journal of Biochemistry & Biophysics
Vol. 60, October 2023, pp.
DOI:

Eleutherine bulbosa Bulb Extract Induces Apoptosis and Inhibits Cell Migration by Down regulating Sonic Hedgehog Gene in Human Tongue Cancer Cells: An *in vitro* Study

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Received 09 May 2023; *revised 18 September 2023*

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards oral cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells. Human oral squamous cell carcinoma (HSC)-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner ($P< 0.05$). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner ($P< 0.05$). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: Apoptosis, Cell migration, *Eleutherine bulbosa*, Sonic hedgehog, Tongue cancer

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica*[4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation *via* cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have also been reported to have antimicrobial[11] and antioxidant[12] activity.

E. bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several

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compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4- di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

E. bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human oral cancer cells has not been investigated. Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5×10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8-µM pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the

24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P < 0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared to the sham group ($P < 0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27±0.14%. Apoptotic HSC-3 cells were significantly higher compared to the sham group ($P < 0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (95.95±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was 96.11±4.17%. After EBBE treatment, the migratory activities of HSC-3 cells were significantly lower compared to the sham group ($P < 0.05$) in a concentration-dependent manner. Gap closure percentage of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%, 76.78±4.87%, and

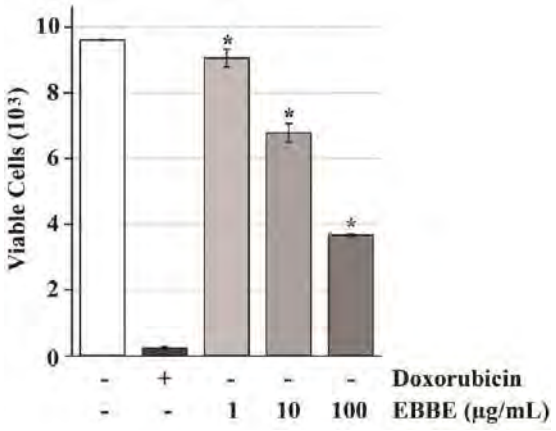

Fig. 1 — EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). *$P < 0.05$ compared to the sham group

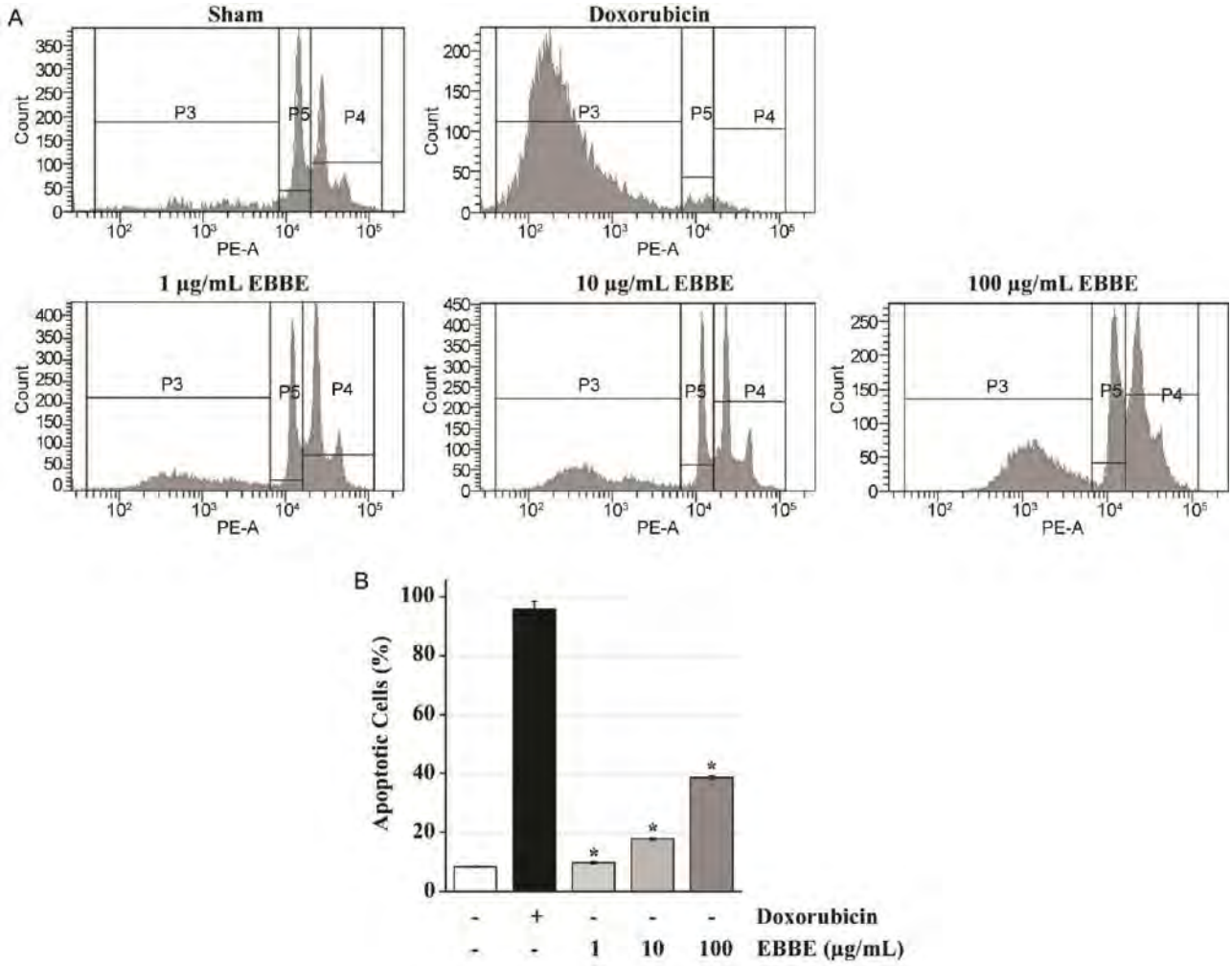

Fig. 2 — EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). *P< 0.05 compared to the sham group

60.67±7.62%, respectively. These percentages were significantly higher (P< 0.05) than cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than the sham group (P< 0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P< 0.05) than cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE down regulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P< 0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P< 0.05) compared to the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared to cyclopamine tartrate-treated HSC-3 cells (P< 0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P= 0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

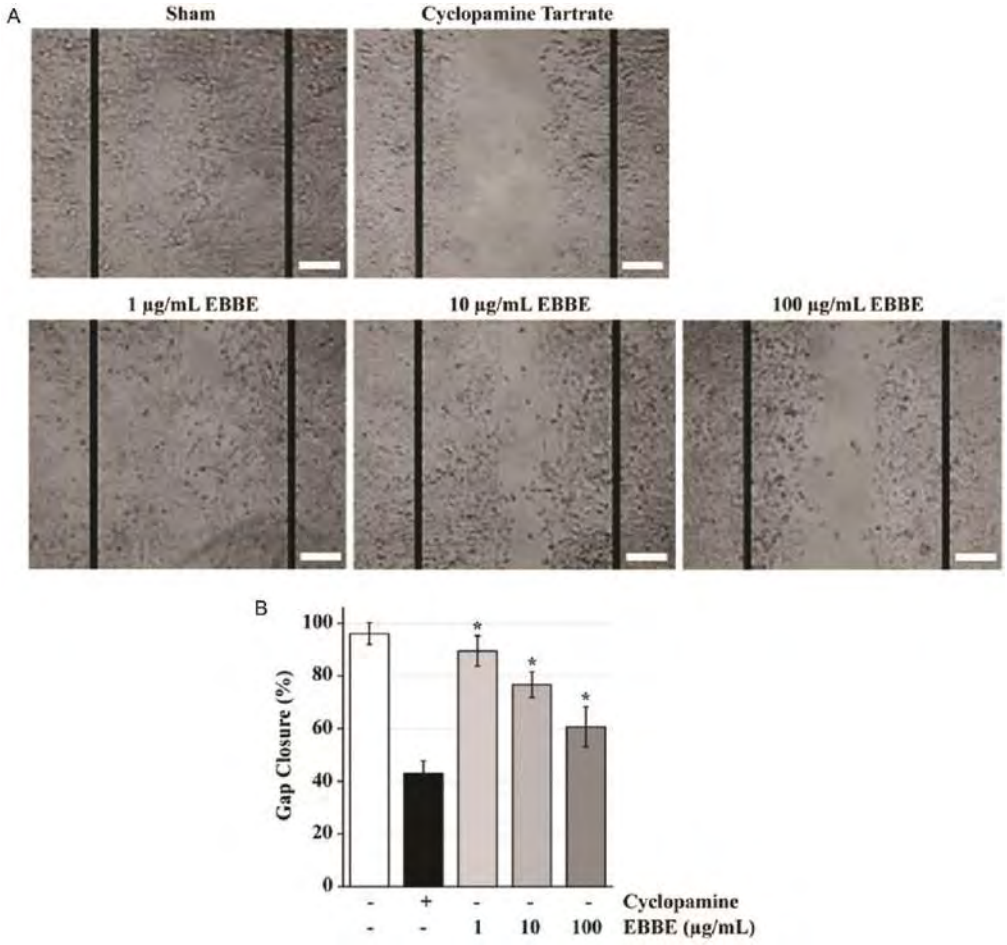

Fig. 3 — EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 µM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). **P*< 0.05 compared to the sham group

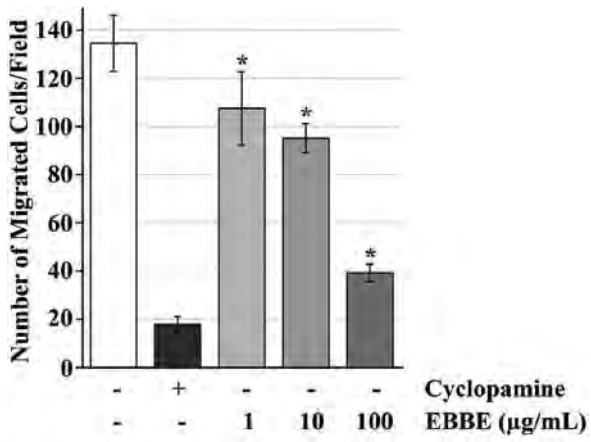

Fig. 4 — EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). **P*< 0.05 compared to the sham group

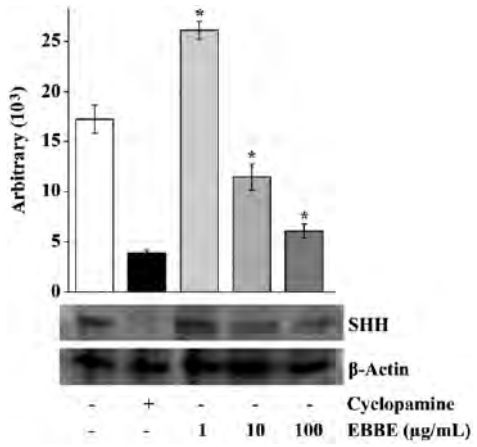

Fig. 5 — EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). **P*< 0.05 compared to the sham group

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18–20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target genes that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

Conclusion

EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by down regulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an ant metastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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There are some corrections in this PRC:
1. In the title of the manuscript, please delete the word "Gene" (highlighted in red, double strikethrough).
2. In the affiliation list, we added one more author (Kyung Hoon Lee) and his affiliation (highlighted in red).
3. In the 4th paragraph of the Discussion section, we changed the word "genes" to "molecules" (highlighted in red).
4. In Reference no. 18, please delete the 'h' in the word "Publich" (highlighted in red, strikethrough).

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Indian Journal of Biochemistry & Biophysics
Vol. 60, October 2023, pp.
DOI:

Eleutherine bulbosa Bulb Extract Induces Apoptosis and Inhibits Cell Migration by Downregulating Sonic Hedgehog ~~Gene~~ in Human Tongue Cancer Cells: An *in vitro* Study

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Received 09 May 2023; *revised 18 September 2023*

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards oral cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells. Human oral squamous cell carcinoma (HSC)-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner ($P< 0.05$). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner ($P< 0.05$). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: Apoptosis, Cell migration, *Eleutherine bulbosa*, Sonic hedgehog, Tongue cancer

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica*[4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation *via* cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have also been reported to have antimicrobial[11] and antioxidant[12] activity.

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E. bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4- di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

E. bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human oral cancer cells has not been investigated. Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5×10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously

described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8-µM pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the 24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P < 0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared to the sham group ($P < 0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27±0.14%. Apoptotic HSC-3 cells were significantly higher compared to the sham group ($P < 0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (95.95±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was 96.11±4.17%. After EBBE treatment, the migratory

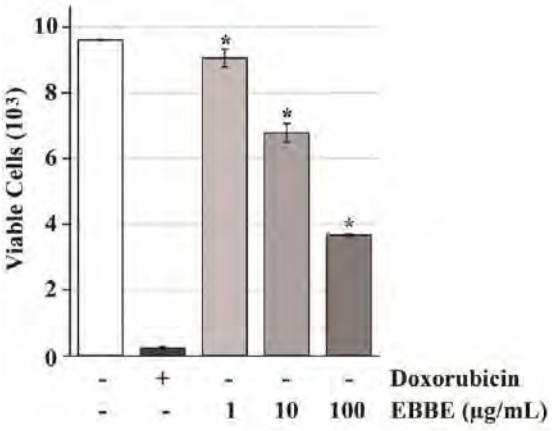

Fig. 1 — EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). *$P < 0.05$ compared to the sham group

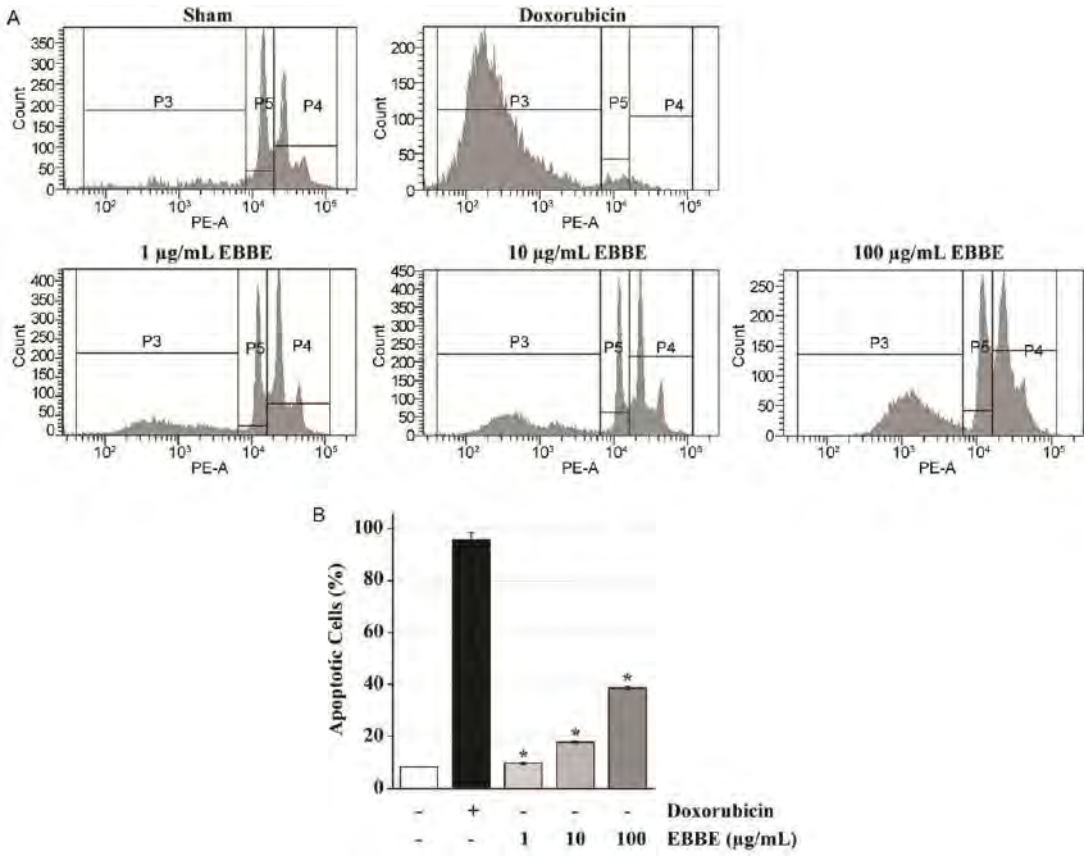

Fig. 2 — EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). *P< 0.05 compared to the sham group

activities of HSC-3 cells were significantly lower compared to the sham group (P< 0.05) in a concentration-dependent manner. Gap closure percentage of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%, 76.78±4.87%, and 60.67±7.62%, respectively. These percentages were significantly higher (P< 0.05) than cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than the sham group (P< 0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P< 0.05) than cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE downregulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P< 0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P< 0.05) compared to the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared to cyclopamine tartrate-treated HSC-3 cells (P< 0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P= 0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

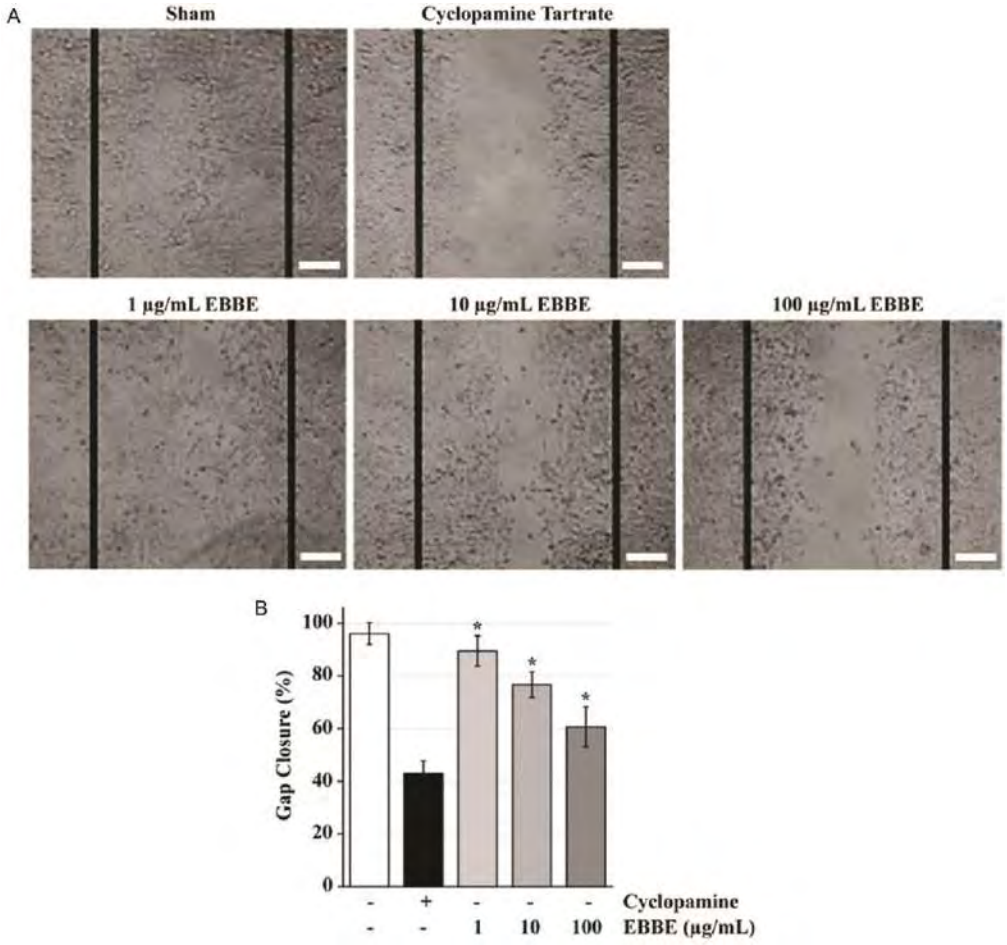

Fig. 3 — EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 µM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). **P*< 0.05 compared to the sham group

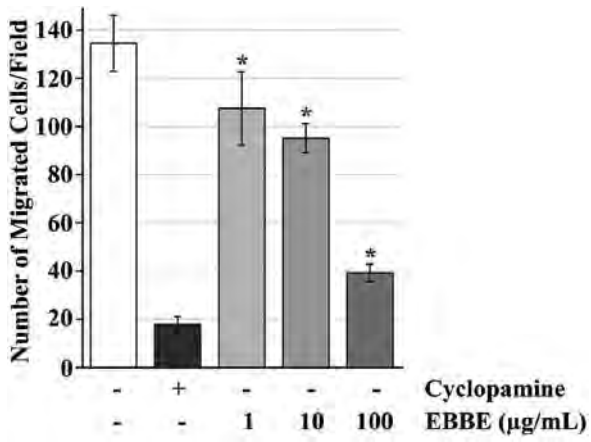

Fig. 4 — EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). **P*< 0.05 compared to the sham group

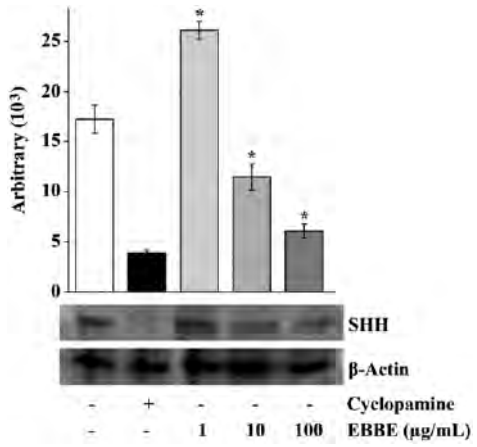

Fig. 5 — EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). **P*< 0.05 compared to the sham group

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18–20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target molecules that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

Conclusion

EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by down regulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an ant metastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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Indian Journal of Biochemistry & Biophysics
Vol. 60, October 2023, pp. 763-769
DOI: 10.56042/ijbb.v60i10.1293

Eleutherine bulbosa bulb extract induces apoptosis and inhibits cell migration by down-regulating sonic hedgehog in human tongue cancer cells: An *in vitro* study

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Received 09 May 2023; *revised 18 September 2023*

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards oral cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells. Human oral squamous cell carcinoma (HSC)-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner (P <0.05). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner (P <0.05). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: Apoptosis, Cell migration, *Eleutherine bulbosa*, Sonic hedgehog, Tongue cancer

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica* [4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation *via* cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have

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also been reported to have antimicrobial[11] and antioxidant[12] activity.

Eleutherine bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4-di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

Eleutherine bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human oral cancer cells has not been investigated. Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5×10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8-µM pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the 24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P < 0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared to the sham group ($P < 0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27±0.14%. Apoptotic HSC-3 cells were significantly higher compared to the sham group ($P < 0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (95.95±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was

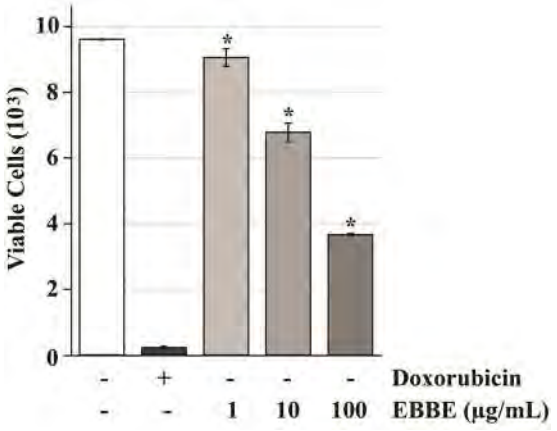

Fig. 1 — EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). *$P < 0.05$ compared to the sham group

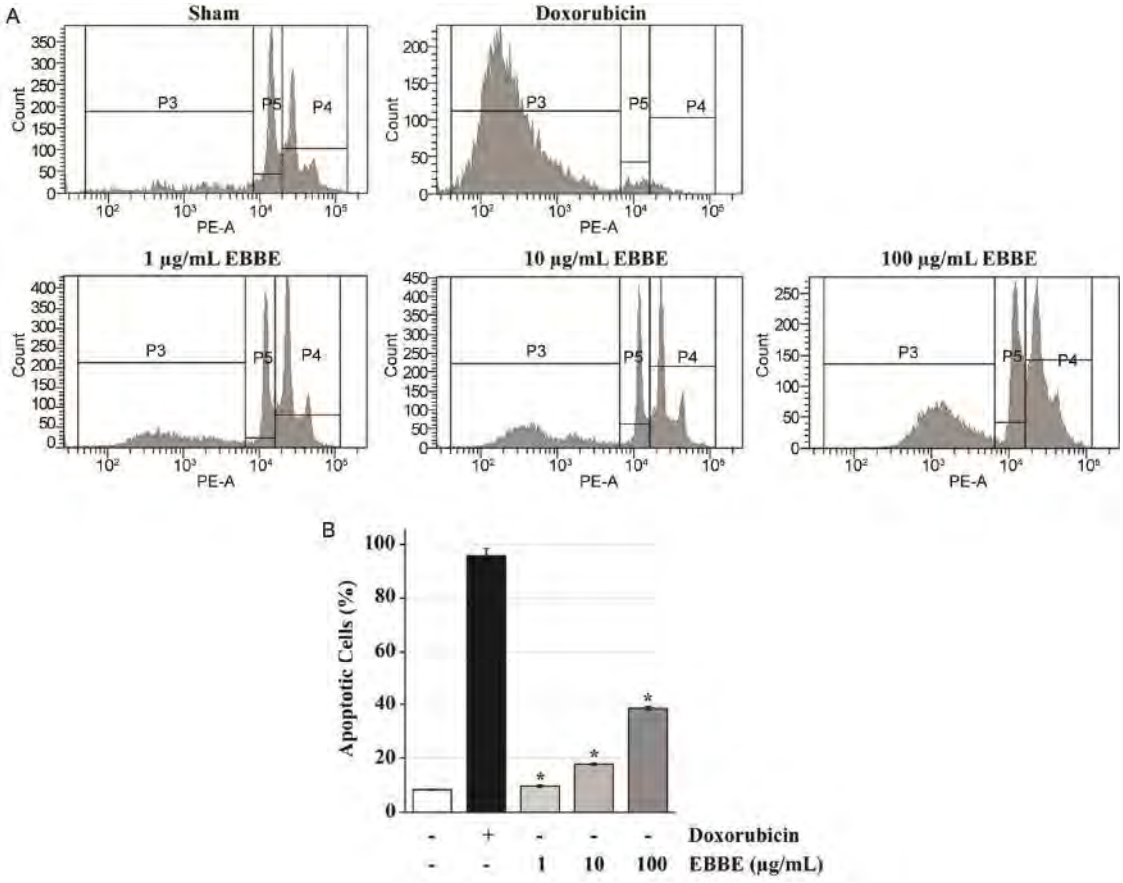

Fig. 2 — EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). *P <0.05 compared to the sham group

96.11±4.17%. After EBBE treatment, the migratory activities of HSC-3 cells were significantly lower compared to the sham group (P <0.05) in a concentration-dependent manner. Gap closure percentage of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%, 76.78±4.87%, and 60.67±7.62%, respectively. These percentages were significantly higher (P <0.05) than cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than the sham group (P <0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P <0.05) than cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE downregulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P <0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P <0.05) compared to the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared to cyclopamine tartrate-treated HSC-3 cells (P <0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P= 0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

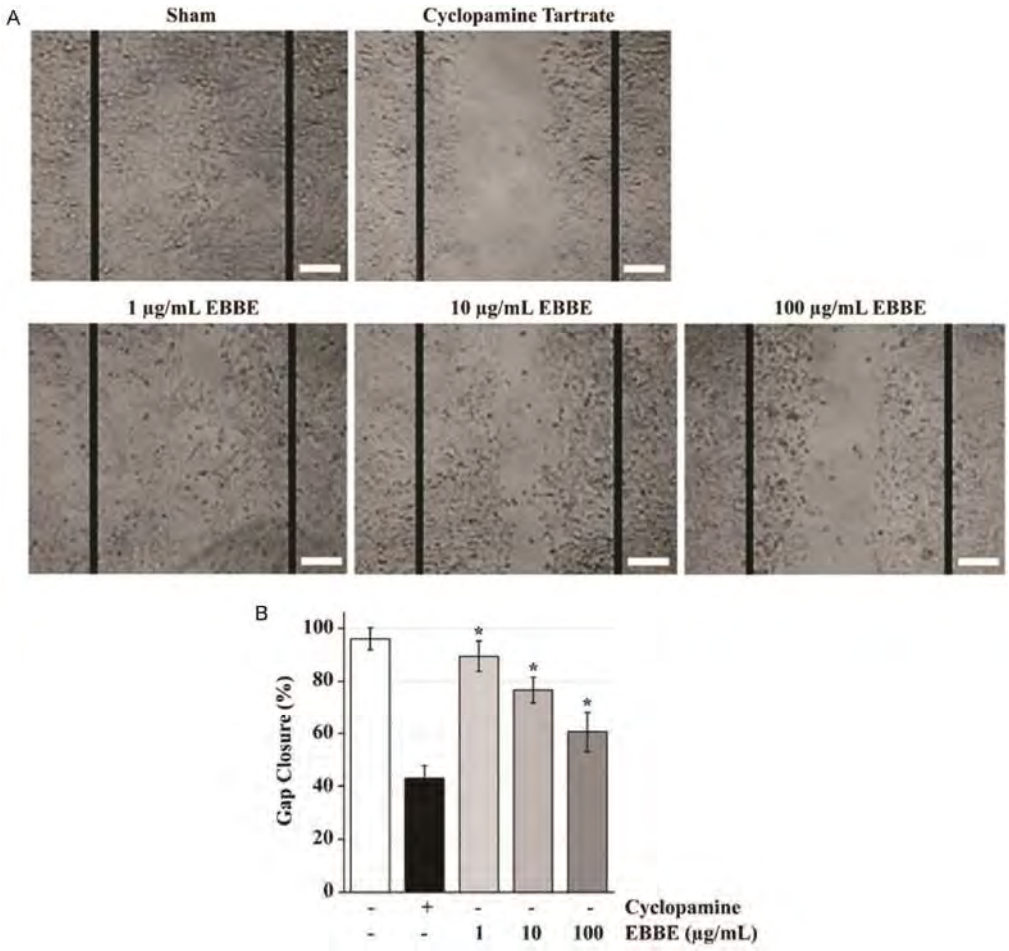

Fig. 3 — EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 µM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). **P* <0.05 compared to the sham group

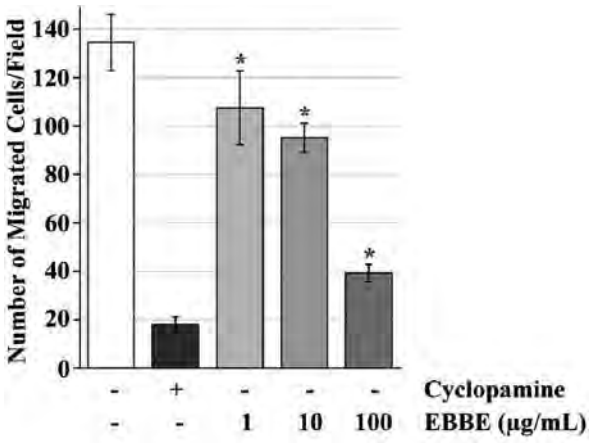

Fig. 4 — EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). **P* <0.05 compared to the sham group

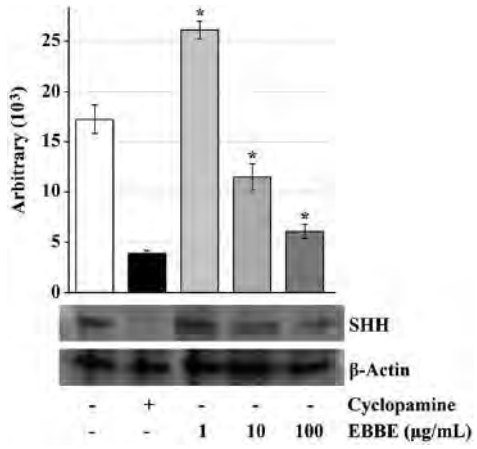

Fig. 5 — EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). **P* <0.05 compared to the sham group

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18–20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target molecules that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

Conclusion

EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by down regulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an ant metastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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Vol. 60, October 2023, pp. 763-769
DOI: 10.56042/ijbb.v60i10.1293

Eleutherine bulbosa bulb extract induces apoptosis and inhibits cell migration by down-regulating sonic hedgehog in human tongue cancer cells: An *in vitro* study

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Received 09 May 2023; *revised 18 September 2023*

Eleutherine bulbosa (Mill.) Urb. is a medicinal plant which has long been used to treat cancer. *E. bulbosa* bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer. However, the cytotoxic effect of EBBE towards oral cancer cells has not been investigated. The present study aimed to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells. Human oral squamous cell carcinoma (HSC)-3 cells were treated with various concentrations of EBBE for 24 h. The number of viable and apoptotic HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sub-G1 assay, respectively. The migratory activities of HSC-3 cells were assessed using scratch and transwell assay. Sonic hedgehog (SHH) expression was measured using immunoblotting. Upon EBBE treatment, apoptotic HSC-3 cells were significantly higher in a concentration-dependent manner (P <0.05). Meanwhile, viability, migratory activities, and migrated HSC-3 cell number were significantly lower in a concentration-dependent manner (P <0.05). The SHH expression levels in EBBE-treated HSC-3 cells were also lower in a concentration-dependent manner. EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by downregulating SHH expression.

Keywords: Apoptosis, Cell migration, *Eleutherine bulbosa*, Sonic hedgehog, Tongue cancer

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer[1] which arises from squamous epithelial cells in the oral cavity. This cancer generally affects the floor of the mouth and the tongue[2]. Various modes of treatment are developed to manage OSCC, including chemotherapy, radiotherapy, surgery, or its combination[3]. However, these treatments cause numerous side effects.

Discovery and development of new therapeutic agents derived from natural sources, particularly plants with less side effects are recently one of the main focuses on oncology research. Several plants have been reported to have anticancer effects, including *Brucea javanica* [4], *Cinnamomum cassia*[5], *Myristica fragrans*[6], and *Oroxylum indicum*[7]. Plants have been known as rich sources of various active compounds, including flavonoids, which was reported to inhibit cancer proliferation *via* cell cycle arrest, antiproliferative and antioxidative activity, apoptosis induction, promotion of differentiation, inhibition of angiogenesis and carcinogen metabolic activation, and multidrug resistance modulation[8]. In addition, several non-flavonoid phenolic compounds have also been reported to show anticancer properties, such as caffeic acid[9].

Eleutherine bulbosa (Mill.) Urb. is a traditional medicinal plant belonging to the Iridaceae family. Its bulbs are commonly used for the treatment of breast cancer, hypertension, stroke, and diabetes mellitus. It has also been used to increase the production of breast milk and treat sexual disorder[10]. *E. bulbosa* bulbs have

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also been reported to have antimicrobial[11] and antioxidant[12] activity.

Eleutherine bulbosa contains a wide variety of compounds, primarily naphthoquinones, anthraquinones, and naphthalene[13]. The main compounds found in this plant are eleutherin, isoeleutherin, and eleutherol[14]. Several compounds obtained from *E. bulbosa* bulbs have been reported to show anticancer activities, such as 6,8-hydroxy-3,4-di-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester[15], eleutherinoside C, and isoeleutherin[16].

Eleutherine bulbosa bulb extract (EBBE) has been reported to show cytotoxicity towards several types of human cancer, such as cervical cancer (line HeLa)[17], colon cancer (line SW480, HCT116, DLD1, WiDr)[16,18], breast cancer (line T47D)[19], leukemia (line K562)[15], and retinoblastoma (WERI-Rb-1)[20]. However, the cytotoxic effect of EBBE towards human oral cancer cells has not been investigated. Therefore, the present study was conducted to evaluate the effects of EBBE towards the viability, apoptosis, and migratory activities of oral cancer cells.

Materials and Methods

E. bulbosa bulb extract (EBBE) preparation

E. bulbosa bulbs were obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Indonesia. Plant identification was carried out by expert botanist at Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, Indonesia (No. B-1269/IPH.3/KS/X/2020). EBBE was extracted by maceration method[4]. Briefly, bulbs of *E. bulbosa* were minced and dried. The dried material was extracted with ethanol 70%, filtered and evaporated using a rotary evaporator. The resulting crude EBBE was then stored at 4°C.

HSC-3 cell culture

HSC-3 cell culture was performed as previously described[4] with modification. The HSC-3 cell line was purchased from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). HSC-3 cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2, 37°C incubator. HSC-3 cells were then dissociated with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) after reaching 80% confluency. Upon reaching the desired cell number, HSC-3 cells were seeded into 24-well plate and 96-well plate for further assays.

Cell viability assay

The number of viable cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[21] with modification. Briefly, 5×10^3 HSC-3 cells were seeded into 96-well plates and then treated with EBBE (1, 10, or 100 µg/mL), 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) or medium merely for 24 h. After that 100 µL of MTT (Sigma-Aldrich) in culture medium was loaded into each well. After incubating the plates for 4 h, culture medium was discarded, and the formed formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO). Results were measured at OD_{570} using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experimental group was measured in six replicates. In addition, the number of untreated cells were counted with hemacytometer and used for interpolating OD_{570} values of HSC-3 cells.

Sub-G1 assay

To investigate the cytotoxic effect of EBBE on HSC-3 cells, the number of apoptotic cells were determined using sub-G1 assay as previously described[9] with modification. Treated and untreated HSC-3 cells were harvested and suspended in 450 µL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). After that, cell suspensions were incubated in the dark for 2 h at room temperature. Fluorescence of individual nuclei was measured using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 events.

Scratch assay

Scratch assay was performed as previously described[22]. HSC-3 cells (5×10^4) were seeded into 24-well plates and incubated overnight. Scratch was made on each well with micropipette yellow tips. Then, the wells were washed with phosphate-buffered saline (PBS) (Wako) and treated with/without EBBE or 20 µM cyclopamine tartrate (BioVision, Milpitas, CA, USA) (as positive control) for 24 h. The gap closures were observed and documented under an inverted light microscope (Carl Zeiss, Jena, Germany).

Transwell assay

Transwell assay was performed as previously described[23] with modification. HSC-3 cells (5×10^4) were seeded in the 8-µM pore size chamber insert (Merck, Darmstadt, Germany). After that, 800 µL culture medium with/without EBBE or 20 µM cyclopamine tartrate was loaded into each well of the 24-well plate. HSC-3-containing chamber inserts were then placed on the 24-well plate. After incubating the chamber for 24 h at 37°C, migrated cells in the lower surface of the chamber were fixed. The cells were counted and documented under an inverted light microscope.

Immunoblotting

Expression levels of Sonic hedgehog (SHH) protein were measured using immunoblotting method as previously described[24] with modification. HSC-3 cells were lysed and homogenized with Laemmli sample buffer (Bio-Rad). The proteins from each experimental group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk solution, the sheet was incubated with rabbit monoclonal anti-SHH antibody as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as a secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblot results were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) to measure the density of each SHH band.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Differences of MTT assay, sub-G1 assay, and scratch assay results between experimental groups were analyzed using Kruskal-Wallis H with Mann-Whitney U *post hoc* analysis. Meanwhile, differences of transwell assay results and SHH band density between experimental groups were analyzed using one-way analysis of variances (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* analysis. All results were expressed as mean ± standard deviation (SD). $P < 0.05$ were considered as statistically significant.

Results

EBBE reduced the number of viable HSC-3 cells

The number of viable HSC-3 cells cultured in medium containing extract solvent (sham group) for 24 h was 9,607±13.81. Treatment with EBBE showed that the viability of HSC-3 cells was significantly lower compared to the sham group ($P < 0.05$) in a concentration-dependent manner. The number of viable HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9,055±268.69, 6,777±278.04, and 3,659±32.86, respectively. The viability of EBBE-treated HSC-3 cells was significantly higher ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (226±32.34) (Fig. 1). These findings suggested that EBBE could reduce the viability of HSC-3 cells.

EBBE induced apoptosis of HSC-3 cells

Sub-G1 assay results showed that the apoptosis percentage of the sham group was 8.27 ±0.14%. Apoptotic HSC-3 cells were significantly higher compared to the sham group ($P < 0.05$) in concentration-dependent manner after EBBE treatment. Apoptosis percentages of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 9.60±0.35%, 17.78 ±0.32%, and 38.64±0.70%, respectively. These percentages were significantly lower ($P < 0.05$) than Doxorubicin-treated HSC-3 cells (95.95 ±2.60%) (Fig. 2). Therefore, these results suggested that EBBE treatment could promote apoptosis of HSC-3 cells.

EBBE inhibited HSC-3 cell migration

The gap closure percentage of HSC-3 cells cultured in medium containing extract solvent after 24 h was

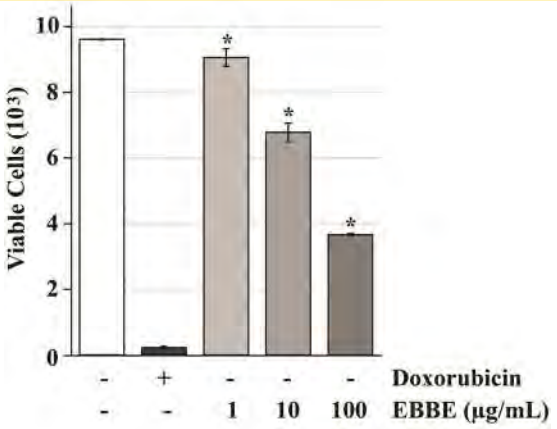

Fig. 1 — EBBE decreased the number of viable HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated in the panel. The number of viable cells were measured with MTT assay as described in Materials and Methods. The data are expressed as mean ± SD (n=6). *$P < 0.05$ compared to the sham group

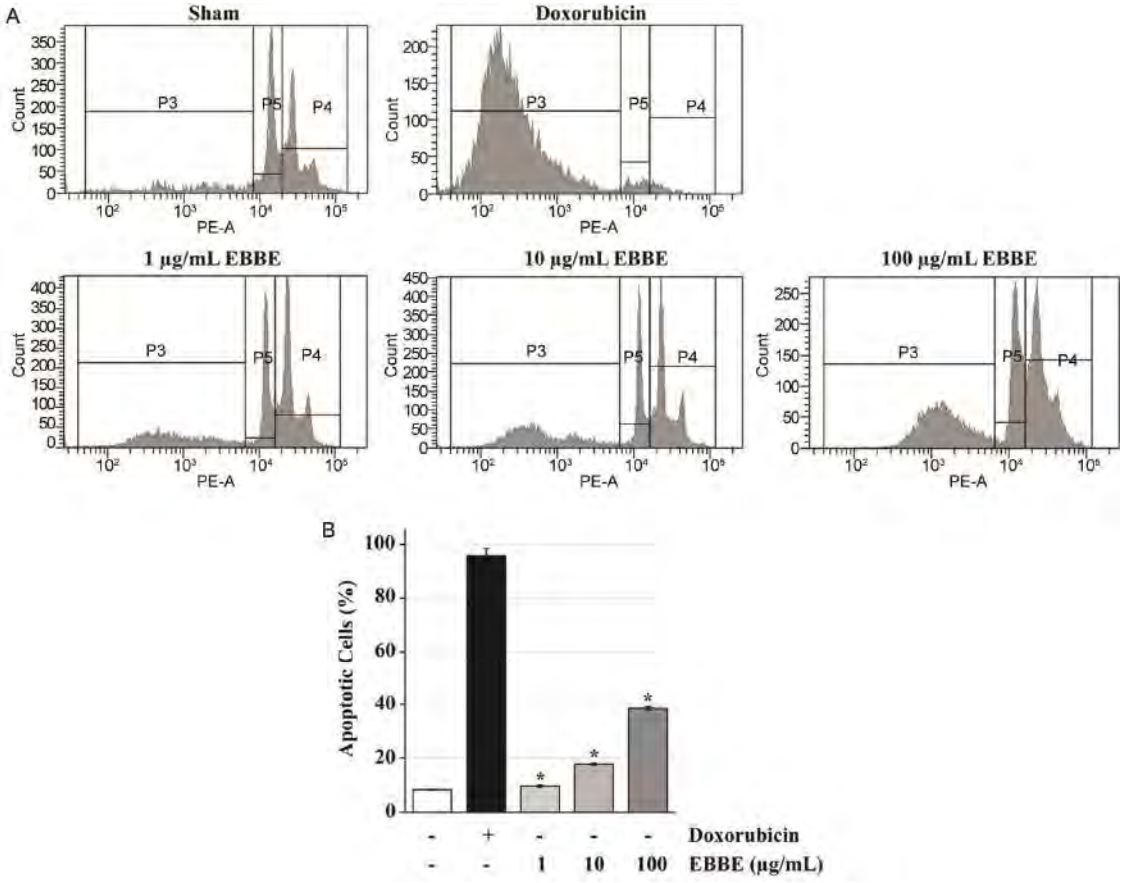

Fig. 2 — EBBE increased the percentage of apoptotic HSC-3 cells in a concentration-dependent manner. HSC-3 cells were cultured and treated with/without 1 µM Doxorubicin and various concentrations of EBBE for 24 h as indicated. Treated cells were then subjected to sub-G1 assay as described in Materials and Methods. (A) Histogram of flow cytometric results. (B) Percentage of apoptotic HSC-3 cells. The data are expressed as mean ± SD (n=18). *P <0.05 compared to the sham group

96.11±4.17%. After EBBE treatment, the migratory activities of HSC-3 cells were significantly lower compared to the sham group (P <0.05) in a concentration-dependent manner. Gap closure percentage of HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 89.56±5.77%, 76.78±4.87%, and 60.67±7.62%, respectively. These percentages were significantly higher (P <0.05) than cyclopamine tartrate-treated HSC-3 cells (43.00±4.82%) (Fig. 3).

Transwell assay also showed similar results. The number of migrated HSC-3 cells in the sham group was 134.44±11.60. The number of migrated HSC-3 cells was significantly lower than the sham group (P <0.05) in a concentration-dependent manner after EBBE treatment. The number of migrated HSC-3 cells treated with 1, 10, and 100 µg/mL EBBE were 107.44±15.21, 95.11±6.01, and 39.22±3.56, respectively. These numbers were significantly higher (P <0.05) than cyclopamine tartrate-treated HSC-3 cells (17.89±3.22) (Fig. 4). Overall, these results indicated that EBBE exhibited an anti-migratory effect towards HSC-3 cells.

EBBE downregulated the expression of SHH

Upon the addition of EBBE, the SHH expression levels in HSC-3 cells were lower in a concentration-dependent manner. Expression level of SHH in HSC-3 cells treated with 1 µg/mL EBBE was significantly higher (P <0.05), while SHH expression levels in HSC-3 cells treated with 10 and 100 µg/mL EBBE were significantly lower (P <0.05) compared to the sham group. SHH expression levels in HSC-3 cells treated with 1 and 10 µg/mL EBBE were significantly higher compared to cyclopamine tartrate-treated HSC-3 cells (P <0.05). However, no significant difference was observed in the SHH expression levels between cyclopamine tartrate and 100 µg/mL EBBE treatment (P= 0.142) (Fig. 5). Thus, these findings suggested that EBBE could decrease the expression of SHH.

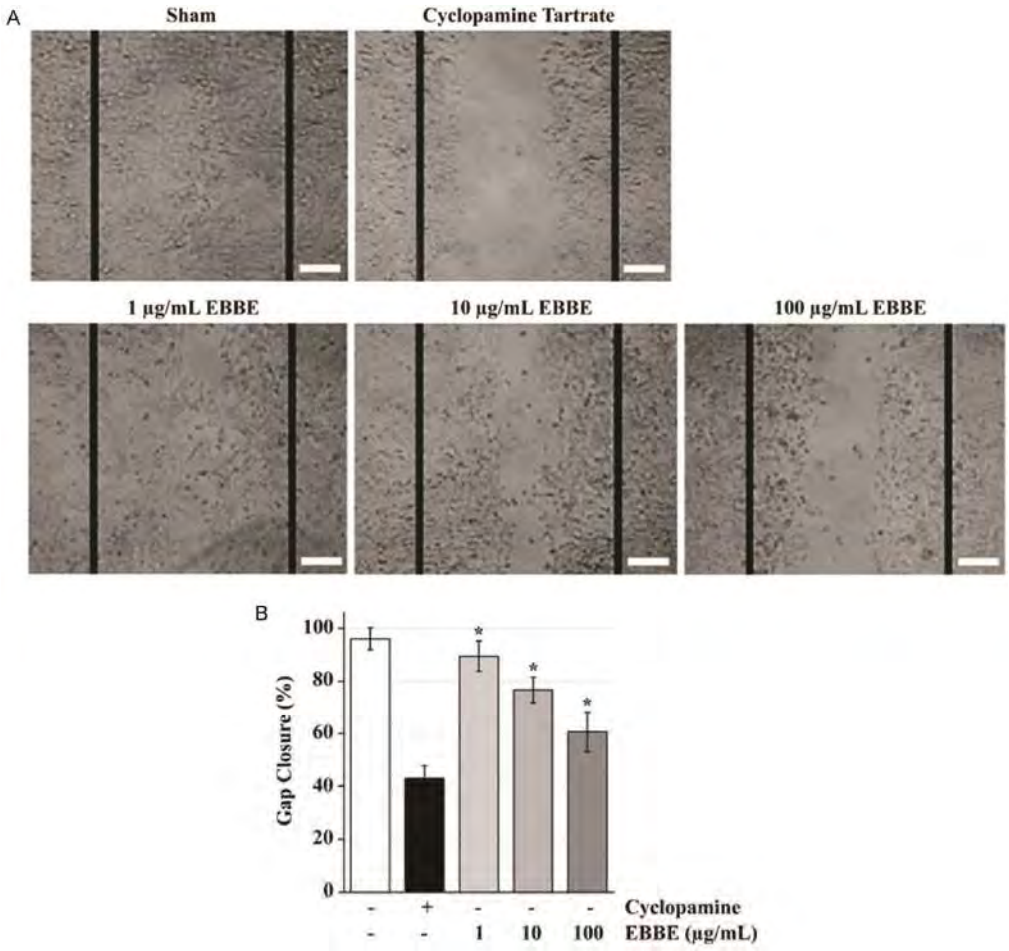

Fig. 3 — EBBE inhibited migratory activities of HSC-3 cells. HSC-3 cell culture was scratched and treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Gap closure was measured as described in Materials and Methods. (A) Scratched areas of HSC-3 cell culture. White bar: 100 µM. (B) Gap closure percentage of EBBE-treated HSC-3 cell. The data are expressed as mean ± SD (n=9). **P* <0.05 compared to the sham group

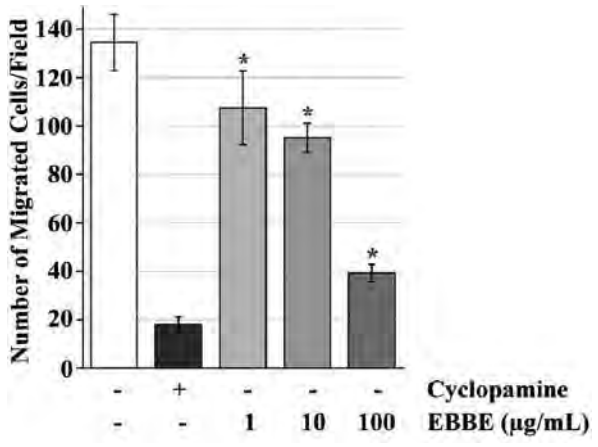

Fig. 4 — EBBE reduced the number of migrated HSC-3 cells. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. Migrated cells were measured as described in Materials and Methods. The data are expressed as mean ± SD (n=9). **P* <0.05 compared to the sham group

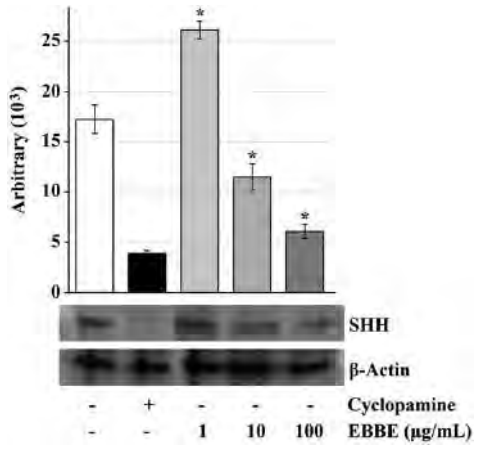

Fig. 5 — EBBE reduced the expression of SHH. HSC-3 cell culture was treated with/without EBBE or 20 µM cyclopamine tartrate for 24 h as indicated. HSC-3 cells were lysed, homogenized, and immunoblotted using anti-SHH antibody as described in Materials and Methods. The data are expressed as mean ± SD (n=3). **P* <0.05 compared to the sham group

Discussion

In the present study, we investigated the potency of EBBE as anticancer agent for OSCC treatment. EBBE showed a cytotoxic effect towards HSC-3 cells by reducing the viability of HSC-3 cells in a concentration-dependent manner. According to the sub-G1 assay results, the decrease in HSC-3 cell viability was caused by apoptosis. These results were in line with previous studies that EBBE was able to induce apoptosis of cancer cells[18–20,25]. EBBE was also reported to inhibit cell cycle in several cell lines[19,25], indicating that EBBE may induce apoptosis of HSC-3 cells through cell cycle arrest since both processes are interconnected[26]. Based on a molecular docking study, eleutherol, eleutherin, and isoeleutherin could induce apoptosis by activating caspase-8[27].

Cell migration is one of the key processes involved in cancer metastasis[28]. Scratch and transwell assays demonstrated that EBBE restrained the migration of HSC-3 cells in a concentration-dependent manner. This showed the novel activity of EBBE in impairing HSC-3 cell migration. Therefore, EBBE may have anti-metastasis activity towards tongue cancer cells.

Metastasis of tongue cancer is regulated by several proteins, including SHH. One of the SHH signaling pathways that has been reported to regulate cell migration in tongue cancer is SHH/glioma-associated oncogene homologue (SHH/GLI)[29]. SHH protein binds and inactivates its receptor, Patched 1 (PTCH1). This interaction causes the activation of transcription factors from the GLI family, which play a major role in regulating cancer migration, invasion, and proliferation[30].

The present study revealed that EBBE decreased SHH expression levels along with the increase in EBBE concentrations, suggesting that the inhibitory effect of EBBE towards the expression of SHH may be responsible for its anti-migratory property. To the best of our knowledge, this is the first study demonstrating SHH downregulation in EBBE-treated HSC-3 cells. EBBE may inhibit HSC-3 cell migration via inhibition of SHH/GLI signaling pathway. Possibly, all signaling pathways that are regulated by SHH can be inhibited by EBBE. EBBE may also restrain HSC-3 cell migration through another pathway. In SW480 colon cancer cell line, eleutherinoside C and isoeleutherin obtained from *E. bulbosa* bulbs was reported to inhibit Wnt/β-catenin pathway[16], which has been reported to play an important role in regulating cancer migration[31]. Therefore, signaling pathways involved in the inhibition of HSC-3 cell migration by EBBE still need to be explored. In addition, signaling components downstream to SHH and target molecules that are affected by EBBE should be identified. Since EBBE also contains various bioactive compounds, further research is needed to identify which compounds are responsible for apoptosis and anti-migratory activity. Thus, more investigations are required to understand the molecular mechanisms underlying EBBE-induced HSC-3 cell apoptosis and confirm which SHH signaling pathway contributes to the inhibition of cell migration by EBBE.

Conclusion

EBBE reduces HSC-3 cell viability through apoptosis and inhibits its migratory activities by down regulating SHH expression. Taken together, EBBE could be a potential anti-tongue cancer agent, particularly as an ant metastatic agent.

Conflict of interest

All authors declare no conflicts of interest.

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