Essential oil extracted from *erythrina corallodendron* L. leaves inhibits the proliferation, migration, and invasion of breast cancer cells

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Abstract

*Erythrina corallodendron* L., a kind of landscape tree, has long been used as a traditional medicine. In this study, the composition of essential oil extracted from the leaves was analysed by GC-MS (gas chromatograph-mass spectrometer), with linalool identified as the main component. Its cytotoxicity against MDA-MB-231, MCF-7 and HMLE cells was examined by MTT and cloning assays. Transwell and wound-healing assays were used to examine the inhibition of migration and invasion. Western blot, qRT-PCR and immunofluorescence staining were used to measure the mRNA and protein expression of factors related to EMT (snail, slug, E-cadherin, N-cadherin and vimentin). The essential oil of *Erythrina corallodendron* leaves was found to inhibit the proliferation, migration and invasion of breast cancer cells in a dose-dependent manner. The findings of this study suggest that the essential oil of *E. corallodendron* leaves may merit further investigation as a potential clinical or adjuvant drug for treating breast cancer migration and invasion.

**Abbreviations:** DMEM = Dulbecco modified eagle medium, DMSO = dimethyl sulfoxide, ECEO = *Erythrina corallodendron* L. leaf essential oil, EMT = epithelial-mesenchymal transition, GC-MS = gas chromatography-mass spectrometry, HMLE = normal human mammary epithelial cell lines, IMC = immunofluorescence staining assay, MTT = 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2-H-tetrazolium bromide, qRT-PCR = quantitative real-time polymerase chain reaction, RPMI-1640 = Roswell Park Memorial Institute 1640 medium.

**Keywords:** breast cancer, epithelial-mesenchymal transition, *erythrina corallodendron*, essential oil, metastasis

1. Introduction

Breast cancer is one of the most harmful diseases to women worldwide, second only to lung cancer. Its features of easy metastasis and poor prognosis make it even more lethal. The distal metastasis sites of breast cancer include the bones, lungs, brain and liver, and some of the subtypes are more malignant than others. During the process of migration and invasion, the tightly integrated epithelial cells of breast cancers become loose, produce pseudopodium, and become mesenchymal cells. This process is called the epithelial-mesenchymal transformation (EMT). The EMT process occurs following alterations in a series of pathways, with characteristic features at the molecular level. Most of these processes begin with the activation of transcription factors, such as the activation of snail and slug. Once cancer cells transform into mesenchymal cells, they can easily penetrate the vascular endothelium and move from the primary site to distal secondary sites, thereby enhancing the risk of recrudescence.

The genus *Erythrina*, which includes almost 200 species, is mainly distributed in tropical and subtropical areas. One of them, *Erythrina corallodendron* L., the national flower of Argentina, is widely used for urban landscaping due to its attractive flowers. Recent years, researchers have put their attention into the chemical composition of *E. corallodendron*. Many studies have reported the function and molecular dynamics of EcorL, which is the lectin isolated from *E. corallodendron*. The proteinase inhibition of *E. corallodendron* seeds had also been investigated in early evidences. Essential oils are always present in high amounts in odorous leaves, seeds and flowers, especially in colourful organs. In addition, essential oils are widely used in treating lung cancer and metastatic cancers, such as breast cancer and leukemia. In traditional medicine, the bark of *E. corallodendron* has been used for anesthesia and acesodyne. However, the action and molecular mechanisms of *E. corallodendron* on cancer cells remains unknown. In this study, we analyzed the chemical composition of *E. corallodendron* leaf essential oil (ECEO) and investigated the underlying anti-breast cancer properties.

2. Materials and methods

2.1. Chemical reagents

DMSO and MTT (3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromide) were purchased from Sigma (Darmstadt,
2.2. Preparation of essential oil and GC-MS analysis

Erythrina corallodendron L. specimens were collected from Zhangzhou, Fujian, China in August 2018 and identified by Prof. Yu-Xin Ma (Zhejiang Ocean University). A type specimen was deposited in the specimen room of Zhejiang Ocean University. Essential oil was obtained from the fresh leaves of *E. corallodendron* (500 g) by a hydrodistillation method using a Clevenger apparatus for 3 hours. The highest essential oil yield reached 8.01%. The GC-MS analysis was performed by Disha Corporation using a ThermoFisher TSQ 8000 Evo GC-MS system.

2.3. Cell culture

All breast cancer cells were purchased from the Shanghai Institute for Biological Sciences (SIBS) and the Chinese Academy of Sciences (Shanghai, China). Human breast cancer cells (MDA-MB-231 and MCF-7) were cultured in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, UT) and non-cancerous mammary epithelial cells (HMME) were cultured in 1:1 DMEM-F-12 medium (Hyclone Laboratories, Inc.). All media contained 10% fetal bovine serum (FBS) supplemented with 100 units/mL streptomycin. All cells were seeded into 96-well plates at a concentration of $5 \times 10^3$ cells/mL. Micropipette tips were used to scratch the surface to create linear gaps. When the cell destiny reached 90%, 3 washes with 3 mL of PBS were performed to flush out any suspended cells. The cells left on the plate were starved for 12 hours to eliminate the interference of proliferation. The experiment was independently repeated 3 times. The rate of wound healing (%) was calculated as $[1 - \text{(scratch width of the ECEO group / scratch width of the control group)}] \times 100\%$ to evaluate the migration ability of cells.

2.4. MTT assay

In vitro cytotoxicity was determined by the MTT assay (Sigma). All cells were seeded into 96-well plates at a density of $5 \times 10^3$ cells/well. After 24 hours of culture, ECEO was added at various concentrations (0.25, 0.5, 1, 2, 4, 8, or 16 μg/mL). Cells were subsequently treated with 10 μL MTT (5 g/L) for 4 h, then the medium was replaced by 150 μL DMSO. The absorbance was determined at 570 nm using a microplate reader. Cell viability was calculated as the percentage absorbance relative to the absorbance of the control group.

2.5. Cell cloning assay

The cell cloning assay was performed on MDA-MB-231 cells. Cells were seeded in a growth disk at a density of 250 cells per disk. After 24 hours of growth, ECEO was added to the disks at different concentration. Cells were cultured in 5% CO₂ at 37°C for 1 week. Cells were stained with 4% methanol for 30 minutes, washed, then stained with crystal violet (Sigma). Cells clones were captured with an Olympus CKX31 microscope (Tokyo, Japan) and counted the number of clones.

2.6. Wound-healing assay

The migration capacity of cells in vitro was measured using a wound-healing assay. MDA-MB-231 and MCF-7 cells were cultured with RPMI-1640 (10% FBS) in a 24-well plate at a concentration of $5 \times 10^3$ cells/mL. Micropipette tips were used to scratch the surface to create linear gaps. When the cell destiny reached 90%, 3 washes with 3 mL of PBS were performed to flush out any suspended cells. The cells left on the plate were starved for 12 hours to eliminate the interference of proliferation. The experiment was independently repeated 3 times. The rate of wound healing (%) was calculated as $[1 - \text{(scratch width of the ECEO group / scratch width of the control group)}] \times 100\%$ to evaluate the migration ability of cells.

2.7. Transwell assay

Transwell assays were performed to measure the invasion capacity of cells. Matrigel was mixed with serum-free RPMI-1640 (1:8), then spread onto the upper chamber membrane of the insert. The solution was kept at 37°C for 1 hour to allow it to solidify into a gel. Cells with a density of $5 \times 10^3$ cells/mL were suspended in 200 μL serum-free RPMI-1640, then the suspension was poured into the upper chamber of the insert, and the lower chamber was filled with 600 μL RPMI-1640 containing 20% FBS. Cells were treated with ECEO for 24 hour, with 3 replicate inserts for each concentration. For each concentration, treated cells were incubated with 5% CO₂ at 37°C for 24 hours. The medium was discarded, then the chamber was washed twice with PBS. Cells remaining in the upper chamber were cleaned using cotton swabs. Cells in the lower chamber were fixed with methanol for 15 minutes, stained with 0.5% cresyl violet, washed 3 times with double distilled water, then dried. Cells were counted in 5 fields (100 x magnification) that were randomly caught by a Nikon TE2000 microscope. For the Transwell migration assay, Matrigel was not added into the wells, but the remaining procedure was the same as the invasion assay.

2.8. Western blot assay

The cell expression of EMT-related proteins was measured by western blot analysis. Cells treated with ECEO were incubated with 5% CO₂ at 37°C for 24 hours. Cells were then lysed using RIPA buffer (Beyotime Biotech, Shanghai, China) at 4°C for 30 minutes. The total cell protein content was separated by SDS-polyacrylamide gel electrophoresis, then proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBS-T buffer for 1 hour, then the membrane was incubated with primary antibodies against GAPDH, E-cadherin, N-cadherin, vimentin, snail and slug (diluted 1:800, Cell Signaling Technology) overnight at 4°C. After washing 3 times with TBS-T buffer and once with TBS buffer for 6 minutes, the membranes were incubated with the secondary HRP-conjugated goat anti-mouse IgG secondary antibody for 1.5 hours at room temperature. Membranes were washed using the same process described above. Proteins on the membranes were visualized using the enhanced chemiluminescence detection system (ECL, Amersham Biosciences). Protein expression was quantified by gray analysis using Image J 2.0 software.

2.9. Quantitative real-time PCR

Total RNA was extracted from cells using the ReverTra Aoe qPCR RT Kit, and RNA was converted into cDNA using the ReverTra Aoe qPCR RT Kit. The mRNA sequences were
downloaded from NCBI and primers were designed using primer premier 5. Pair of primers were listed as below:

| Components   | CAS     | RT    | Percent  |
|--------------|---------|-------|----------|
| GAPDH        | 5'-CATCAAGAAGGCTGGAGGAGCAGG-3'; 5'-TCAAGGTTGAGGAAGTGTTCTG-3'; | 9.885 | 0.847%   |
| E-cadherin   | 5'-CGAGATCTCACTCTTACGGG-3'; 5'-GGGTTGAGAGGAAAAATTAGG-3'; | 10.684 | 1.254%   |
| N-cadherin   | 5'-TCCTTGACAGACCAAGACAGCG-3'; 5'-TCTTTGAGGTCTGCTGGATG-3'; | 11.272 | 0.99%    |
| Vimentin     | 5'-GACGCACTAACACCGAGTT-3'; 5'-CTTGTGTGTTGTTACGGT-3'; | 11.670 | 1.008%   |
| snail        | 5'-CTTCCAGCAGCCCTACGAC-3'; 5'-GGGTGAGGATCGCT-3'; | 11.937 | 1.254%   |
| Slug         | 5'-TCATTTCCTGACTCCCGAAC-3'; 5'-TGATTTCCATGCTCTTGCAG-3'. | 13.162 | 0.401%   |

The GAPDH gene was used as a control for mRNA. For each PCR reaction, a master mix that included SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan), forward primer, reverse primer, and 10 ng of template cDNA was prepared. The PCR conditions were 45 cycles of sequential denaturation (95°C for 2 minutes), annealing (60°C for 15 seconds), and extension (72°C for 20 seconds). The ΔACT value was used to analyse the qPCR data. All samples were measured in triplicate in independent experiments.

### 2.10. Immunofluorescence staining (IMC) assay

For IMC staining assays, cells were seeded in 24-well plates and cultured with particular conditions for 6 hours. Then, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde at room temperature for 20 minutes. All cells were washed and permeated by 0.5% Triton-X-100 3 times at room temperature for 20 minutes and incubated with 3% BSA blocking for 30 minutes. Primary antibodies were added to each sample and incubated at 4°C overnight. After that, samples were washed using PBS-Triton 3 times, and IMC secondary antibodies were added in the dark room for 1 hour. DAPI was used for locating the nuclei. Then, images were taken by Nikon TE2000 (Tokyo, Japan) microscope.

### 2.11. Statistical analysis

Data are shown as mean ± standard deviation from triplicate experiments. All data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL), and 1-way ANOVA was used for multiple comparisons. Significant differences are indicated as follows: ∗P < .05; ∗∗P < .01.

### 3. Results

#### 3.1. Composition of Erythrina corallodendron L. leaf essential oil

The essential oil of *Erythrina corallodendron* L. leaves was a clear, pale yellow liquid. The essential oil yield was 8.01% and the density was 0.78 g/mL. The identified components are listed in Table 1. In total, 27 compounds of 99% of essential oil were identified by GC-MS analysis. Among the 27 compounds, linalool, 1,4-cineole, cyclodeca[b]furan-6-carboxylicacid,4,7,8,11-tetrahydro-3,10-dimethyl-, methyl ester, (5Z,9E)- and

| Components                              | CAS     | RT    | Percent  |
|-----------------------------------------|---------|-------|----------|
| 1,4-cineole                             | 98-55-5 | 17.039| 2.596%   |
| linalool                                | 78-70-6 | 13.080| 50.362%  |
| Menthone                                | 89-80-5 | 16.079| 5.203%   |
| Terpinen-4-ol                           | 962-74-3| 16.433| 1.703%   |
| alpha-Terpinol                          | 98-55-5 | 17.039| 2.596%   |
| alpha-cubebene                          | 17699-14-8| 20.169| 1.094%   |
| isocadin                                | 6009-89-8| 21.228| 1.048%   |
| phenol, 2-methoxy-3-(2-propenyl)-       | 1941-12-04| 21.348| 0.347%   |
| Tricyclo2.2.1.02,6,heptane, 1,7-dimethyl-7-(4-methyl-3-pentenyl)- (-) | 512-61-8| 21.641| 1.200%   |
| 2,6-DIMETHYL-6-(4-METHYL-3-PENTENYL)[BICYCLO[3.1.1]HEPT-2-ENE | 17699-05-7| 21.921| 0.565%   |
| (1R,4Z,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]unc-4-ene (-) | 118-65-0| 22.114| 3.114%   |
| (5Z,7E,10E)-eudesma-4 (14),11-diene | 511-59-1| 22.600| 0.534%   |
| 1-ethyl-1-methyl-4-propen-2-ylidene-2-prop-1-en-2-ylcyclohexane (+) | 6753-98-6| 22.933| 0.847%   |
| (+)-DELTA-CADINENE                      | 483-76-1| 25.245| 0.718%   |
| Epaotulanol                             | 6750-60-3| 27.269| 1.008%   |
| Caryophyllene Oxide                     | 1139-30-6| 27.642| 1.277%   |
| Cyclocdeca[b]furan-6-carboxylicacid,4,7,8,11-tetrahydro-3,10-dimethyl-, methyl ester, (5Z,9E)- | 29292-34-0| 31.033| 5.861%   |
α-terpineol were the top 6 compounds, each of which was present at a percentage higher than 2.5%. It is undeniable that the compounds contained in ECEO differ greatly from essential oils of other species of Erythrina, meaning it may have unique uses or mechanisms.

3.2. *Erythrina corallodendron* L. leaf essential oil inhibits the proliferation of breast cancer cells

In order to examine the proliferation-inhibiting effects of ECEO, we performed a MTT assay and compared the results with drugs that have a proven positive effect in breast cancer cells (MDA-MB-231 and MCF-7) and non-cancerous mammary epithelial cells (HMLE). The results are presented in Table 2. We found that ECEO could inhibit the proliferation of breast cancer cells in a dose-dependent manner. In addition, compared with the normal human breast cell line, HMLE, we found that ECEO had greater cytotoxicity to breast cancer cells than normal cells, which is interesting from a clinical perspective. The proliferation-inhibiting effect of ECEO was not the same as the positive drugs, but ECEO still possesses the potential for treating breast cancers. Furthermore, in order to eliminate the influence of apoptosis, we performed a cell cloning assay. As shown in Figure 1, ECEO could, in part, inhibit the proliferation of breast cancer, not only due to reduced cell viability.

3.3. *Erythrina corallodendron* L. leaf essential oil inhibits the migration and invasion of breast cancer cells

Migration and invasion are generally considered crucial factors in the process of breast cancer metastasis. When migration and invasion of cancer cells occurs, cells undergo significant morphological changes. After treating breast cancer cells with ECEO, we observed that cells showed a clear change in morphology, where mesenchymal cells became round and grew tightly, as shown in Figure 2. Such changes indicate that an EMT process occurred, which is discussed below. We then performed a wound-healing assay to check whether ECEO could inhibit the migration ability of breast cancers. As shown in Figure 3, even low doses of ECEO could significantly inhibit the migration rate of breast cancer cells. We subsequently performed a Transwell assay to simulate the extracellular environments and investigate the invasion-inhibiting ability of breast cancer cells by ECEO. The results presented in Figure 4 indicate that treatment with ECEO decreased both the migration and invasion ability of breast cancer cells in a dose-dependent way.

These results suggest that treatment with ECEO could be an efficient clinical adjuvant drug, in combination with positive drugs, for controlling the metastasis of breast cancer.

3.4. *Erythrina corallodendron* L. leaf essential oil inhibits the EMT process of breast cancer cells

In order to examine whether ECEO inhibited the migration and invasion of breast cancer cells through EMT pathways, we examined the mRNA and protein expression of EMT markers. First, as shown in Figure 5, the mRNA expression levels of snail and slug were dose-dependently inhibited by ECEO, which could directly result in lower expression of E-cadherin and suppression of the EMT process.

Furthermore, the mRNA expression of vimentin and N-cadherin was decreased by ECEO in a dose-dependent manner.

### Table 2

| Test Drugs | IC50 of Cytotoxic Activity Against Cells (μg/mL) |
|------------|-----------------------------------------------|
| ECEO       | MDA-MB-231: 3.44±0.18<sup>a</sup> MCF-7: 4.91±0.26<sup>b</sup> HMLE: 25.90±2.35<sup>a</sup> |
| Doxorubicin | MDA-MB-231: 0.56±0.03<sup>c</sup> MCF-7: 0.86±0.12<sup>a</sup> HMLE: 12.71±1.49<sup>a</sup> |
| Capecitabine| MDA-MB-231: 1.09±0.08<sup>b</sup> MCF-7: 1.47±0.09<sup>a</sup> HMLE: 21.14±3.06<sup>a</sup> |

Data are average of a duplicate (mean±SD). <sup>a</sup>, <sup>b</sup>, <sup>c</sup>: Values with different superscripts are significantly different in line (P<.05).

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Figure 1. Cell cloning assay performed on MDA-MB-231 and MCF-7 cells treated with *Erythrina corallodendron* L. leaf essential oil (ECEO). Left, photo captured after culture with ECEO and subsequent staining. Right, quantification of the number of clones.
Naturally, the protein expression showed a similar decrease, as shown in Figure 6. As the essential marker of EMT, the expression of E-cadherin represents direct evidence to determine the occurrence and degree of the EMT process. Therefore, we used IMC to further assess the expression level and site of E-cadherin expression. The results of IMC are shown in Figure 7. With an increasing concentration of ECEO, expression of E-cadherin in breast cancer cells increased and changed location into the peripheral cytoplasm. All of the results discussed above suggest that ECEO could
inhibit the migration and invasion of breast cancer through suppressing the EMT process.

4. Discussion

_Erythrina coralloidendron_ L. has been used as a traditional Chinese medicine for a long time. Some alkaloids and lectins have been isolated from this plant.[28,29] Moreover, there is emerging evidence of glycosylation effects.[30,31] However, the essential oil of _E. coralloidendron_ L. leaves and its anti-cancer effects had never been studied before now. First, our study reported the composition of ECEO and identified 27 compounds. Among these compounds, linalool, a chain terpene alcohol, was the predominant compound of ECEO. Regarding its traditional use, linalool has been used as an anti-bacterial compound, and also has a sedation effect. Researchers have reported that linalool could be used as a treatment for oral cancers by acting through the PI3K/Akt pathway.[32] Meanwhile, α-terpineol, another compound identified in ECEO, has been studied in hepatocellular carcinoma cells, and was found to have an effect on the inhibition
on cancer migration. Menthone, commonly present in essential oils, has not been reported to have anti-cancer effects so far, instead, it has a slight toxicity. These major compounds may play a critical role in treating breast cancer, and we believe that traces of these compounds might also act through unique mechanisms; however, this requires further research.

Breast cancer, due to its poor prognosis and easily metastasis, is one of the most harmful cancers worldwide. One of the initial reasons for its poor prognosis is its rapid proliferation. There are now several drugs that are used clinically to treat breast cancer, especially capecitabine and doxorubicin. These drugs, although they have a strong effect on cancer cells, possess a series of side effects including injury to normal tissues and cells. In our research, ECEO inhibited the proliferation of breast cancer with a much lower injury to normal cells, showing maximum inhibition on MDA-MB-231 cell lines. Therefore, the cytotoxic activity of ECEO merits further research.

The migration and invasion activities of cancer cells are the leading reason for poor prognosis. During the process of metastasis, the epithelioid cancer cells are transformed into mesenchymal cells, which can then separate from the primary tumor tissue and pass through the blood vessels to secondary tissues. One way to treat the metastasis of breast cancer is to suppress this process by inhibiting migration and invasion. The migration and invasion inhibition activities of ECEO were tested using a series of in vitro experiments, and the results showed a positive inhibitory tendency. Among the numerous causes of breast cancer migration and invasion, EMT is one of the most important. There are a series of notable phenomena related to EMT. First, slug and snail, the activators of EMT, were obviously decreased by ECEO treatment. Some of these EMT factors are known to change significantly, especially the overexpression of E-cadherin and the decreased expression of N-cadherin and vimentin. By investigating the changes in expression of EMT markers after ECEO treatments, we found that ECEO could inhibit the EMT process, and thereby inhibit the migration and invasion of breast cancer.

For now, researchers only have superficial knowledge about the EMT process, and none of the drugs that specifically target EMT have been used clinically. Due to a lack of specific noninvasive markers, attempts to target EMT always cause more damage to healthy cells or lead to recrudescence, even drugs that target EGFR, PDGFR, or FGFR. Essential oils are natural products with a lower cytotoxicity to healthy cells, which could be used as adjuvant drugs for cancer treatments. Our results suggest that ECEO inhibits proliferation and EMT-mediated migration and invasion in vitro, which supports its potential use as an adjuvant therapy for the treatment of breast cancer. However, further research on the in vivo toxicity and action is required.
5. Conclusion

We reported the composition of ECEO and found that linalool is the main compound, which merits further investigation. Subsequent assays demonstrated that ECEO has a high cytotoxicity toward breast cancer cells, and only causes minor damage to normal cells. Through a cloning assay, we found that the inhibition effects of ECEO are, at least in part, due to an effect on proliferation. Furthermore, the Transwell and wound-healing assays demonstrated that ECEO inhibits the migration and invasion of breast cancer cells. By measuring expression levels with qRT-PCR, IMC and western blot assays, it was found that this inhibition effect is mediated by suppression of the EMT process through the slug/snail pathways. Based on the findings discussed above, we can conclude that ECEO could be used as a clinical drug or adjuvant drug to help treat breast cancer metastasis.

Acknowledgments

We would like to thank Disha Corporation and Professor Yu-xin Ma for their generous help.

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