ETHYL ACETATE EXTRACT OF *HELICTERES HIRSUTA* SUPPRESSES MCF-7 HUMAN BREAST CANCER CELL MOBILITY

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ABSTRACT

Objective: To investigate the anticancer activity of *Helicteres hirsuta (H. hirsute*) extract against a breast cancer cell line-MCF7.

Methods:*H. hirsute*was extracted in absolute methanol. Further, the crude extract was further partitioned in n-hexane, chloroform and ethyl acetate. The total phenolic and flavonoid content were determined by using spectroscopic methods with gallic acid and quercetin standard compounds, respectively. Anticancer activities of *H. hirsuta* extract was elucidated by MTT, wound headling, and transwell invasion assays.

Results: Total phenolic compounds in *H. hirsute* extracts reached 22.07 ± 2.54, 58.57 5.54 and 235.56 ± 7.54 mg GAE/g in each n hexan, chloroform, ethyl acetate fractions, respectively. Whereas the ethyl acetate fraction showed the greatest phenolic contents with 235.56 ± 7.54 mg GAE/g. Moreover, the flavonoid contents of H. hirsute extracts reached 5.76 ± 0.94, 9.25 ± 1.84 and 19.37 ± 2.57 mg quercetin equivalent/g in each n hexan, chloroform, ethyl acetate fractions, respectively, in which, the ethyl acetate fraction also showed the highest amounts of flavonoid contents. Further, the ethyl acetate of H. hirsute significantly decreased the viability of breast cancer MCF7 cells after 48 h treatment with IC50 value of 95 ± 2.54 µg/mL compared to control. In addition, the ethyl acetate extract of H. hirsute suppressed the invasion and migration of MCF7 cell lines in a dose-dependent manner at non-toxic concentrations.

Conclusions: The ethyl acetate of *H. hirsute*suppressedthe growth and motility of breast cancer MCF7 cells.

KEYWORDS: *Helicteres hirsute*, Anticancer, Hepatocellular Carcinoma, Motility, DNA Barcoding, EMT.

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1. INTRODUCTION

Breast cancer that is known as the second common cancers worldwide, causes the deaths of females-gender after lung cancer. In 2020, the number of new cases and deaths globally from breast cancer were recorded 2,261,419 and 684,996, respectively[1].The treatment efficiency can go up to 90% or higher, when breast cancer cases are identified at early stages. Therefore, the death ratio of breast cancer in developed countries is lower than in developing countries due to early detection and screening[2]. Generally, surgery is the first type ofbreast cancer treatment. However, approximately 20–30% of these patients suffer from distant relapse with cancer cells spreading from the primary site to other body organs (eg, bones, brain, distant nodal, liv)[3]. Therefore, there is an urgent need to identify and develop more effective treatments for breast cancer[4].

Natural products have been received an attention over the past few decades as an important source for a variety of biological activities. Anti-cancer activity that provide long-term cancer control with few side effects and show the safety[5]. Several compounds have been extracted from medicinal plants for cancer treatment such as paclitaxel from Pacific yew trees and vincristine from the leaves of Madagascar periwinkle plants.

Helicteres hirsutea (H. hirsuta) has been traditionally used in Vietnam with treat liver cancer.*Helicteres hirsuta*is known as a member of the *Helicteres* genus Steculiaceae family, also found in other Southeast Asian countries [6–8]. Previous studies showed that triterpenoids, flavonoids, and lignans are the major components of *Helicteres* species. Cucurbitacin derivatives isolated from *Helicteres angustifolia* possessed cytotoxicity on several cancer cell lines [5,9]. In this study,we focused on screening second metabolite compounds for anticancer activities from the Vietnamese medicinal plant Helicteres hirsuta such as potential cytotoxicity, wound healing and invasion activity to identify potential novel anti-metastasis agents.

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2. MATERIALS AND METHODS

2.1Plant materials

*H. hirsuta*was collected in Nghean province, Vietnam in July 2020. Plant samples were identified by Dr Thuy Thi Bich Le, Department of plant cell genetics at the Institute of Biotechnology. Plant samples were also dried to constant weight and stored at -20 °C until further use.

2.2Extraction and preparation of the powdered crude extracts

The dried *H. hirsuta* were grounded into fine powder (< 1.40 mm). The sample was extracted in absolute methanol and placed in an ultrasonic bath for 30 minutes at 40 °C (three replicates), and further incubated at room temperature overnight. The filtrates were concentrated with a rotary vacuum evaporator at 45°C. The crude extract was further partitioned in n-hexane, chloroform and ethyl acetate. All the fractions were stored at -20°C until further use. The extracts were coded as HEHH, DCLHH, EtHH, MeHH respectively with n-hexane, dichloromethane, ethyl acetate, and methanol extract.

2.3 Determination of total phenolic contents

The total phenolic content of each fraction was determined using spectroscopic method as described by Ainsworth et al. (2007)[10]. Briefly, the reaction mixture was prepared by mixing 100 µl plant extracts (1mg/mL), add 200 µlof 10% Folin-Ciocalteu's reagent dissolved in 2 mL of methanoland vortex thoroughly. Thereafter, 800 µl of 700 mM Na2CO3 was added into each tube. The mixture was vortex thoroughly and incubated at room temperature for 2 h. The blank solution was also prepared in the same conditions. The absorbance was read at 570 nm using spectrometer. Total phenolic content was calculated by gallic acid extrapolating calibration curve. The total phenolic content was expressed as gallic acid equivalent (mg GAE) per gram dry weight of each fraction. Experiments were repeated at least three times.

2.4 Determination of total flavonoid contents

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The total flavonoids content of the *H. hirsuta*was determined using aluminium chloride calorimetric method as previously described[11]. Breifly, 0.5 ml of sample was mixed with 1 ml of 2% AlCl3 ethanol solution. After 1 h at room temperature, the absorbance was read at 420 nm by using UV-spectrophotometer. Total flavonoid contents were calculated as quercetin from a calibration curve (mg QE/g of sample). All the analyses were repeated three times and the mean value of absorbance.

2.5Cell lines and culture

The breastATCC-MCF7cancer cells was used in this study. Cellswere maintained in Dulbecco's modified Eagle's minimal medium (DMEM - PAN-Biotech)supplemented with 10% fetal bovine serum,1% Penicillin-Streptomycin solution under a humidified 5% CO2 atmosphere at 37°C in anincubator.

2.6Methyl thiazolyl tetrazolium (MTT) assay and morphological observations

The MTT assay was performed as previously described. Briefly, MCF7 cells were seeded into 96-well plates at a density of 2.5 × 10^3 cell/well, incubated overnight, and treated with the indicated concentrations of extracts for 48 h, DMSO was used as control. Next, 15 µl MTT (Sigma-Aldrich) was added and cells were incubated for an additional 4 h. Thereafter, the culture medium was carefully removed and formazan crystals were dissolved in 150 µL DMSO. The absorbance was read at 570 nm using a microplate reader (BioTek, Winooski, VT, USA) to determine the growth. The percentage of viable cells was calculated using the following formula:

$$\%\ \text{viability} = \frac{(Optical\ density\ of\ the\ treated\ sample - Optical\ density\ of\ blank)x100}{Optical\ density\ of\ the\ control\ sample - Optical\ density\ of\ blank}$$

Morphological observation, cells were seeded at 2.5 × 10^4cells/well in 6-well plates, and incubated overnight to adhere. The different concentrations of extracts were added to each well for 24 h, DMSO was used as control.The morphology of cells was directly observedby using a phase-contrast inverted microscope fitted with digital camera (Digital sight DS-L1, Nikon, Japan).Experiments were repeated at least three times.

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2.7Wound healing assay

MCF7 cells were seeded at a density of 2.5~3 × 10^5 cells/well on 6-well tissue culture plates (SPL Life Sciences, Gyeonggi-do, Korea) and grown for 24 h to confluence. Monolayer cells were scratched with a sterile pipette tip to create a wound. The cells were then washed twice with serum-free DMEM to remove floating cells and incubated in DMEM containing 2% FBS with 50 μg/mL of *H. hirsuta* extracts, DMSO was used as control. Photographs of cells were taken at 0, 24, 48, and 72 h after wounding to measure the width of the wound. For each sample, an average of five wound assays was taken to determine the average rate of migration. Experiments were repeated at least three times.

2.8Invasion assay

Cell invasion was performed byusing Boyden chambers (Corning, NewYork, NY, USA) pre-coated with 1% gelatin. A total of 1 × 10^6MCF7 cells in100 μl DMEM containing 0.2% bovine serum albumin (BSA) were addedin the upper chamber and incubated with 50 μg/mL*H. hirsuta* extracts or DMSO for 24 h. The lower chamber was added with 400 μlDMEM supplemented with 2% FBS containing 0.2% BSA and 10 μg/ml fibronectin as a chemotactic agent. After incubation for 24h, cellsin the upper chamber were fixed and stained using a Diff-Quick kit(Sysmex, Kobe, Japan). The numbers of cells adhering to the under-side of the filter in five fields per chamberwere counted using a phase-contrast inverted microscope fitted with digital camera (Digital sight DS-L1, Nikon, Japan). Each invasion assay was repeated inthree independent experiments.

2.9Statistical analysis

All experiments were performed in triplicate. Data were expressed as ± standard error of the mean. Significance between two groups determined in this study was test by the Student's t-test, and analysis of variance was utilized between three or more groups. P-values less than 0.05 are considered statistically significant. All statistical analyses were performed by Sigma Plot software.

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3. RESULTS

3.1 Total phenol and flavonoid contents

In order to determine total phenolic compounds, folin Ciocalteu method was used. Total phenolic compounds were calculated based on a calibration curve of gallic acid and expressed in gallic acid equivalents (GAE) per gram dry extract weight (Table 1). The results showed that phenolic contents of *H. hirsute*extracts reached 22.07 ± 2.54, 58.57 ± 5.54, 235.56 ± 7.54 mg GAE/g of n hexan, chloroform, ethyl acetate fractions, respectively. Whereas, ethyl acetate fraction showed the greatest phenolic contents, reached 235.56 ± 7.54 mg GAE/g, while the smallest phenolic contents were found in n hexan fraction (22 .07 ± 2.54mg GAE/g). The phenolic contents of ethyl acetate fraction had significantly 10 times higher than that of n hexan.

Total flavonoids content of *H. hirsute*extracts was expressed as mg quercetin equivalents/ g of extract, varied from 5.76 ± 0.94 to 19.37 ± 2.57 mg quercetinequivalent/g extract (Table 1). The ethyl acetate fraction also showed the highest amount of flavonoid contents (19.37 ± 2.57), followed by chloroform(9.25 ± 1.84) and n hexan fraction (5.76 ± 0.94). However, the flavonoidcontent was at low level in all H. hirsute extracts.

3.2Ethyl acetate extract of H. hirsute inhibits the viability of MCF7 cells

The *H. hirsute* extracts were evaluated for cytotoxic activity on breast cancer cell lines (MCF7). The results showed that n-hexane, dichloromethane and ethyl acetate extracts decreased the viability of MCF7 cells. Moreover, the ethyl acetate extract of *H. hirsute* (EtHH) potently dose-dependently decreased the viability of MCF7 cell lines for 48 h treatment with IC50 value of 95± 2.54 µg/mL (Fig. 1 A and B). Furthermore, the number of available cells treated with EtHH much less than DMSO-treated control by examining the morphology of cells under the microscope (Figure 1A). The n Hexan of *H. hirsute* (n HXHH) and the dichloromethane extract of *H. hirsute* (DCLHH) exhibited moderate cytotoxic activity to MCF7 cells after 48 h treatment with IC50 value of 176 ± 4.54 µg/mL and 145 ± 3.44 µg/mL, respectively (data not shown).

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3.3Ethyl acetate extract of H. hirsute inhibit the motility of MCF7 cells

Migration and invasion play an important role in cancer development and progression process. Wound healing assays were performed inMCF7 breast cancer cells to investigate anticancer activities of *H. hirsute* extracts. As shown in Figure 2A and B, non-toxic concentrations (50 µg/mL) of *H. hirsute* extracts suppressed wound closure much higher than those for DMSO-treated. Treatment with the ethyl acetate extract of *H. hirsute* was exhibited the highest level of inhibition. In addition, the ethyl acetate extract of *H. hirsute*significant inhibited MCF7 cell invasion in a dose-dependent manner, when compared with DMSO-treated control (Figure 3A). The numbers of invaded cells decreased 48% by treatment with 50 µg/mLthe ethyl acetate extract of *H. hirsute.* Quantitative analysis of the numbers of invaded cells showed that the differences were significant (Figure3B). These results demonstrate that treatment with non-toxic concentrations of the ethyl acetate extract of *H. hirsute*has inhibitory activity on motility of MCF7 cells.

4. DISCUSSION

Medicinal plants have been receiving an increasing attention for health promotion due to their biologically active compounds such as phenolic and flavonoid compounds. Phenolic compounds are aromatic secondary plant metabolites, which have been widely investigated in medical plants for their varied biological effects including antioxidant, anti-inflammatory, hepatoprotective, and anticancer effects[5,12–15]. The ideal structural chemistry of phenolic compounds plays an important role for their biological activities. The hydroxyl groups in phenols can act as reducing agents and free radical quenchers by donating an electron or hydrogen atom to free radicals[16,17]. In this study, the total phenolic content for *H. hirsute* extractswas determined by the Folin-Ciocalteau method using gallic acid as a standard. The ethyl acetate extract of *H. hirsute* contained the highest total polyphenols. The results are similar to other studies from *H. hirsute* root and leave [7,8]. The total phenol content from *H. hirsute* is much higher than that from *Helicteres isora* and *Helicteres vegae*[18,19].

Cytotoxicity assay is a crucial part in preclinical studiesof screening new compounds. It provides vital information about a new molecule’s interesting biological activities. MTT assay is well known to be the most common method for evaluating cytotoxicity. In this study, MTT assay was used to measure the

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effects of*H. hirsute*extractson cell viability. The results of the cytotoxicity assay indicated that the extracts of *H. hirsute* decreased the viability of breast cancer cells MCF7. Specially, the ethyl acetate extract of *H. hirsute* (EtHH) strongly suppressed the viability of MCF7 cell lines. The biological effects of the extracts depend on their components and fraction polarity [8,20–22]. *H. hirsute* was described containing various classes of other compounds such as flavonoids and saponins. These compounds have been reported to provide several biological activities including cytotoxicity in various cancer cell lines [7], antibacterial properties, and antioxidants [8,18,23]. However, the effect of individual compounds of *H. hirsute* extracts on breast cancer cellsneeds to be further investigated.

Metastasis is a leading cause of recurrence in breast cancer patients [24]. This is a complicated cellular process affected by multiple molecular factors which activate the processes of migration and invasion of breast cancer cells [25–27]. In this process, tumor cells undergo a series of events to spread from an initial or primary site to different or secondary sites. Therefore, migration and invasion are major cause of death in cancer patients [28].In ours study, scratch wound healing assays was performed to evaluate breast cancer cell migration is affected by treatment of the extracts of *H. hirsute.* The results indicated that the ethyl acetate extract of *H. hirsute* significantly inhibited in cellular migration. Moreover, the numbers of invaded cellsdecreased after treatment with ethyl acetate extract of *H. hirsute* in dose dependent manner.Metastasis is a complex process, which is related to multipleintracellular signalssuch as production of reactive oxygen species (ROS) [29,30], and contribution of microRNAs (miRNAs) [31,32]. These changes elevateEMT, leading to increases in migration and invasion of cancer cells [33,34]. In this study,the ethyl acetate extract of *H. hirsute* significantly decrease in cellular migration and invasion of breast cancer cell MCF7 in non-cytotoxic doses. However, the mechanism of these effects needs to be elucidated for next study.

5. CONCLUSIONS

In conclusion, our findings indicate that ethyl acetate of H. hirsute inhibited the growth and motility of breast cancer cell MCF7. Further study is required to investigate the mechanism of action of cell motility in breast cancer and to identify the active compounds.

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AVAILABILITY OF DATE AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHORS' CONTRIBUTIONS

TVN conceived and designed the experiments. HTH, LTT, NAH, TND, DPQ performed the experiments. TVN analyzed the data, and wrote the paper. TTD, NHN, DHN, TTBL provided critical review of the manuscript. All authors read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

PATIENT CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

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307 **Table 1. Total phenolic and flavonoid contents of *H. hirsute extracts***

No.	Fraction	Total phenolic (mg GAE/g)	Total flavonoid (mg Catechin/g)
1	n hexan	22.07 ± 2.54	5.76 ± 0.94
2	Chloroform	58.57 ± 5.54	9.25 ± 1.84
3	Ethyl acetate	235.56 ± 7.54	19.37 ± 2.57

308 *Data presents in Mean values ± Standard error*

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List of figures

List of figures

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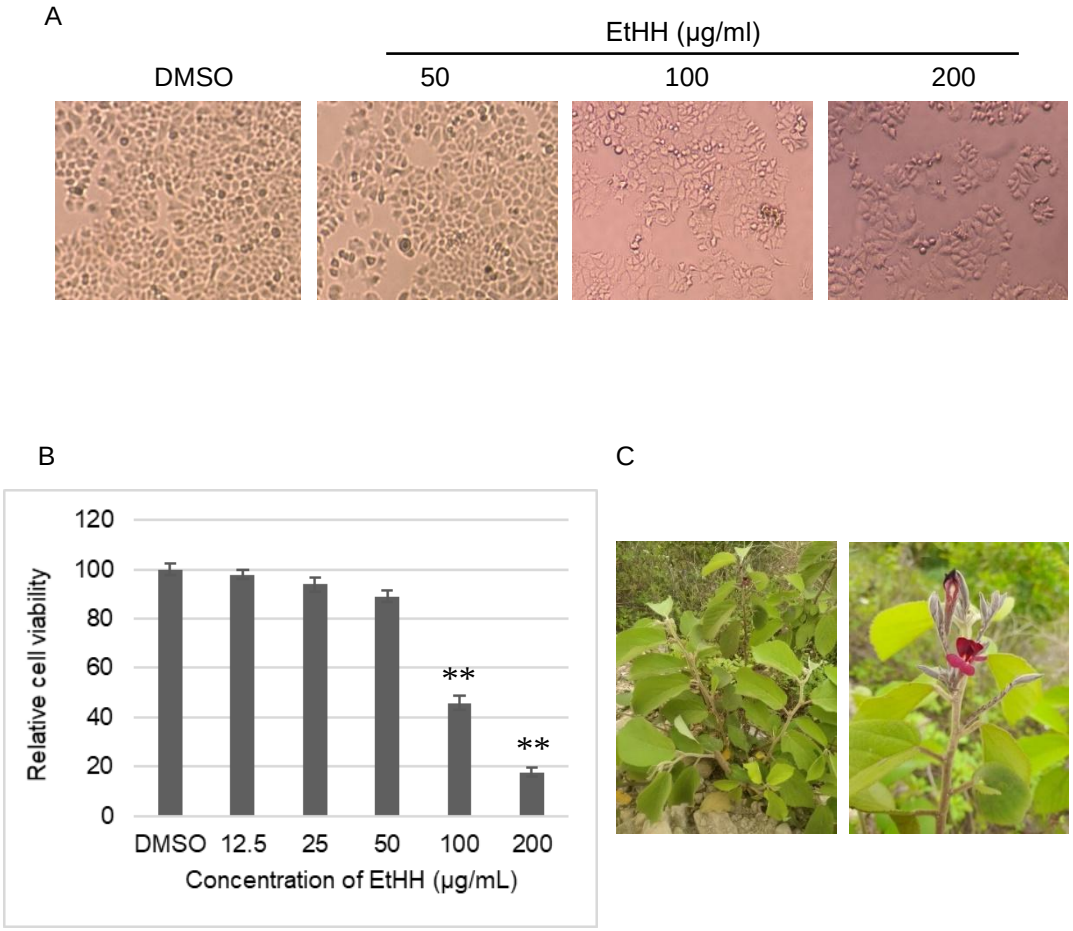
A
EtHH (µg/ml)
DMSO
50
100
200
B
C
Relative cell viability
120
100
80
60
40
20
0
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DMSO
12.5
25
50
100
200
Concentration of EtHH (µg/mL)

Fig. 1

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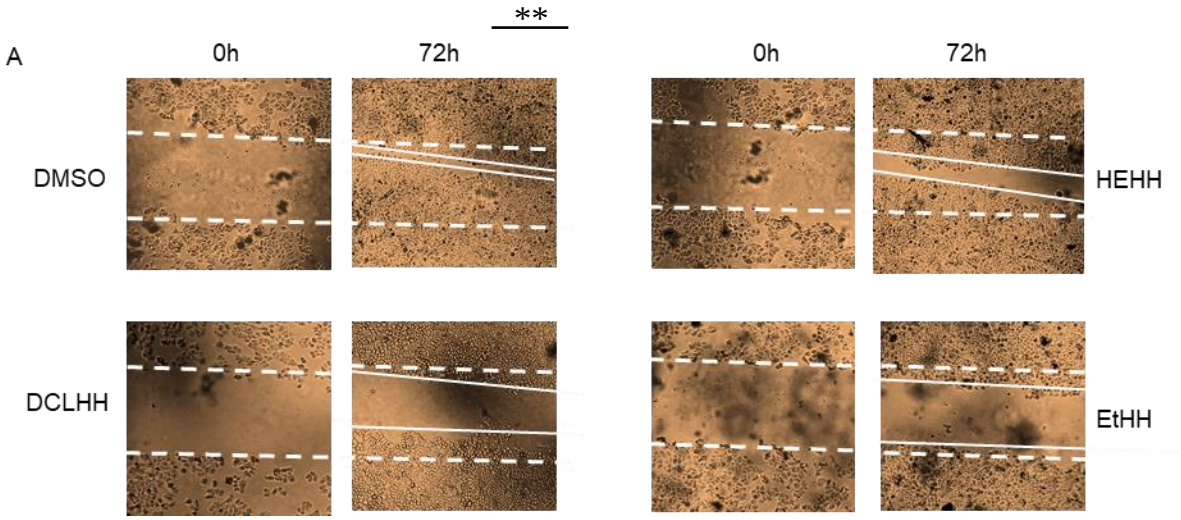
B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72h
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DMSO
HEHH
DCLHH
EtHH

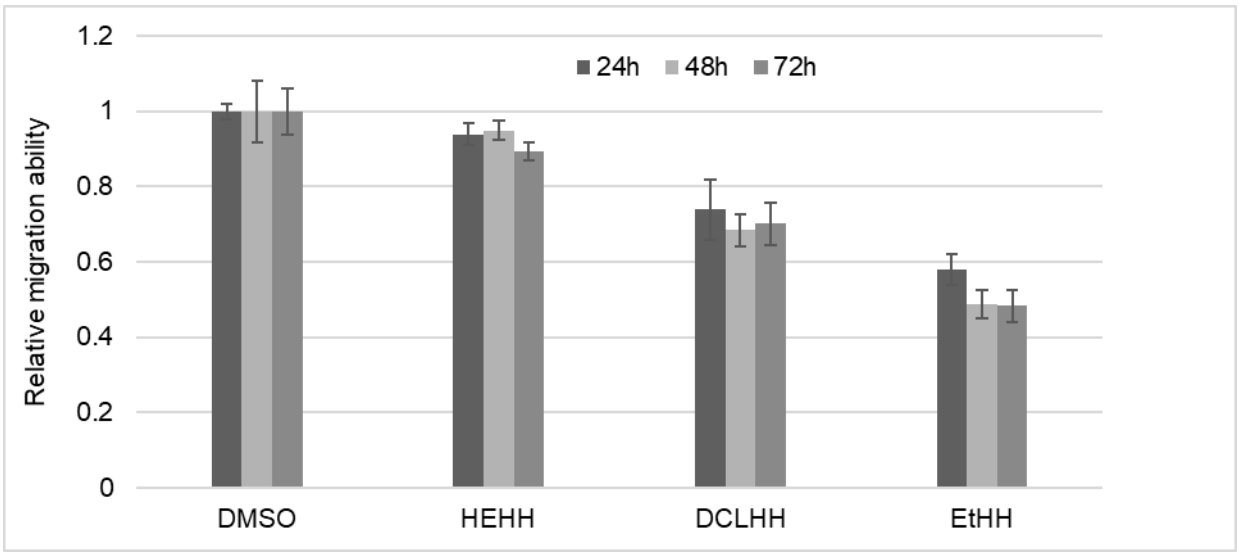
24h
48h
72h
Relative migration ability
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
HEHH
DCLHH
EtHH

Fig. 2

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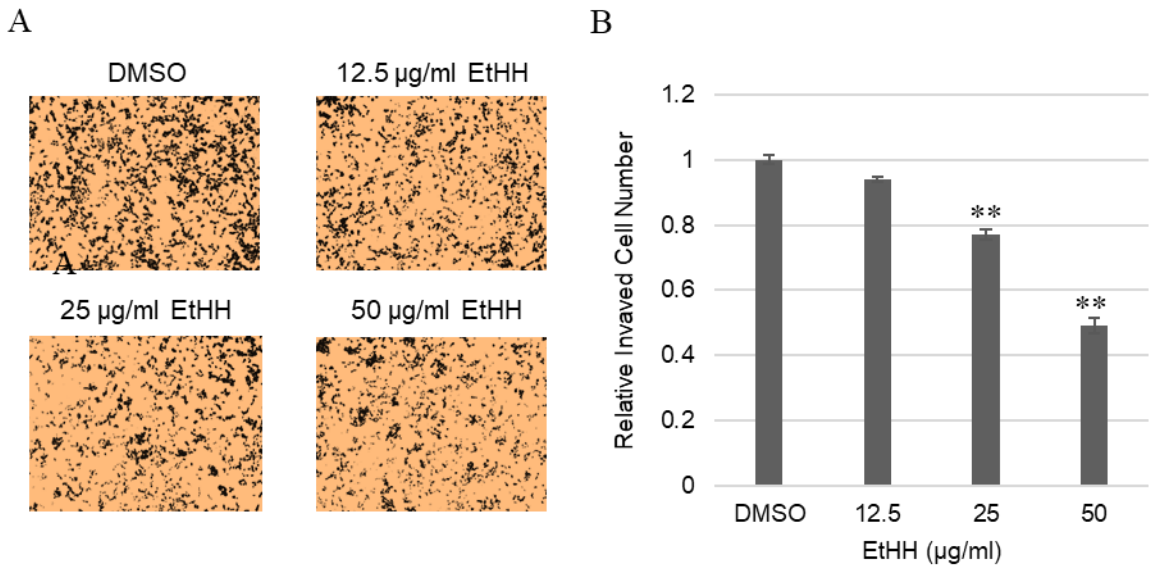
A
DMSO
12.5 µg/ml EtHH
25 µg/ml EtHH
50 µg/ml EtHH
B
Relative Invaved Cell Number
1.2
1
0.8
0.6
0.4
0.2
0
**
**
DMSO
12.5
25
50
EtHH (µg/ml)

Fig. 3

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