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ORIGINAL ARTICLE

Molecular Mechanisms of *Dolichandrone serrulata* Flower Ethanolic Extract on Antimigration of Human Cervical Cancer Cell Line

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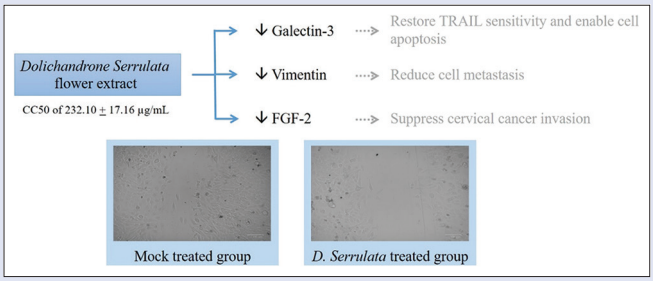
ABSTRACT

Background: *Dolichandrone serrulata* flower has been known for its antioxidation and anti-inflammation. Its ability to induce cell death and reduce migration on cancer cell lines has been previously demonstrated. **Aim:** The present study aimed to investigate the molecular mechanisms involved in the antimigration effect of *D. serrulata* flower ethanolic extracts of HeLa cell line. **Materials and Methods:** HeLa cell line viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The antimigration of the extract was investigated using a wound healing assay. Its possible molecular mechanism for the migration of HeLa cell line was investigated using a protein array assay. **Results:** *D. serrulata* extract caused cell death with CC_{50} of 232.10 ± 17.16 µg/mL. The result from the wound healing assay demonstrated that *D. serrulata* extracts at concentrations of 62.5, 125, and 250 µg/mL caused the reduction of the narrowing in the wound site, corresponding to the progress over time. To eradicate the possibility of the cytotoxic effect of the herb, *D. serrulata* at a concentration of 125 µg/mL was further observed for protein expression using a protein array assay. The result showed the reduction of galactin-3, vimentin, and fibroblast growth factor-2 expression compared with the control group. **Conclusion:** Antimigratory effect of *D. serrulata* flower ethanolic extracts might be attributable to the reduction of vimentin and fibroblast growth factor-2 gene expression, but not galactin-3-induced apoptosis. Further study is needed to confirm and investigate a more in-depth pathway on its antimigration effect in the cervical cancer cell line.

Key words: Antimigration, cervical cancer cell line, *Dolichandrone serrulata*, wound healing assay

SUMMARY

- *Dolichandrone serrulata* flower extract provided IC_{50} of 171.06 + 8.32 µg/mL.
- At a low concentration (62.5 µg/mL), *D. serrulata* flower extract could progress wound healing process.
- *D. serrulata* flower extract is proposed of providing an antimigration effect on cervical cancer cell line without inducing cell death through galectin-3, vimentin, and FGF-2.



Abbreviations used: AGS: A human gastric adenocarcinoma cell-line; DMSO: Dimethyl sulfoxide; DS: *Dolichandrone serrulate*; DSF: *Dolichandrone serrulata* (Wall. ex DC.) Seem. Flower; EMEM: Eagle's minimum essential medium; FGF-2: Fibroblast Growth Factor-2 or Fibroblast Growth Factor -basic; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; T2DM: Type 2 diabetes mellitus.

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INTRODUCTION

Cervical cancer is one of the world's most common cancer found in women for both incidence and mortality rate.[1,2] The estimated number of newly diagnosed patients with cervical cancer has increased from 569,847 cases in the year 2018 to 604,127 cases in the year 2020. It was also found that the number of deaths from this cancer has increased from 311,365 cases in the year 2018 to 341,831 cases in the year 2020. Of these, approximately 90% of cases were found in low- and middle-income countries.[3] In a middle-income country, surprisingly, cervical cancer is the second most common cancer found in Thai women.[4] Despite the decreasing incidence rate worldwide, this cancer is still troubling Thai women with approximately 10% out of all cancer incidents found in the year 2020 with an age-standardized incidence rate and age-standardized mortality rate of 16.4 per 100,000 population and 7.4 per 100,000 population, respectively.[4] Currently, with the increasing trend of herbal medicine usage in several illnesses, many herbs were introduced and their effects were studied on cervical cancer. Of those, *Dolichandrone serrulata* is one of the magnificent herbal medicine.

Dolichandrone serrulata (Wall. ex DC.) Seem. flower (DSF) (Thai name: Khae Na) belongs to the Bignoniaceae family. It is commonly consumed as a vegetable and has a bitter taste. In Thai traditional medicine, the flower *D. serrulata* has been used as a mucolytic and to increase blood flow.[5] Yannasithinon *et al.*[5] also suggested that *D. serrulata* flower can improve reproductive damages in male type 2 diabetes mellitus (T2DM) rats and can provide antioxidant activity.[6,7] It also has long been used as a member of the Ben-Cha-Moon-Yai remedy for its antipyretic and anti-inflammatory activities.[8] A recent study has suggested the antiproliferation and antimigration effects of *D. serrulata* extract in

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the cervical cancer cell line.[7] Previously, Phanthong *et al.*[6] have demonstrated the chromatographic separation of *D. serrulata* flower ethyl acetate extract with two new identified compounds; hallerone and protocatechuic acid. Hallerone has been reported as one of the markers of *Phyla nodiflora*,[9] that was previously investigated for its inhibitory effect on breast cancer cell lines by inducing apoptosis and cell cycle arrest.[10-12] Protocatechuic acid also has been demonstrated to have an apoptotic effect in human breast, lung, liver, cervix, and prostate cancer cell lines.[13] and antimetastasis effect in lung tumor cells and human gastric carcinoma gastric adenocarcinoma cell line (AGS) cells.[14,15] However, there is no current evidence on how *D. serrulata* affects cervical cancer. Thus, this study is aimed to investigate the effect of *D. serrulata* flower extract in cervical cancer invasion *in vitro*.

MATERIALS AND METHODS

Plant preparation and extraction

Flower parts of *D. serrulata* were collected from a cultivation area in Mahasarakham province, Thailand. Briefly, the flowers were cleaned and dried using a hot air oven at 50°C. They were grounded into powder and followed by the extraction process using 95% ethanol. The collection was processed after 7 days of fermentation. The solution was filtered, evaporated, and lyophilized in a freezer dryer before the further extraction phase in ethyl acetate using a separatory funnel. The ethyl acetate fraction was dried at 50°C and kept at 4°C until used.

Cell culture

Human cervical cancer cell line, HeLa was grown in Eagle's minimum essential medium (EMEM) modification with l-glutamine supplement (Hyclone) with 10% fetal bovine serum (Gibthai), 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were cultured in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 until it reaches 80%–90% confluent of a well plate before the experiment. Morphology of cells was observed under a cell imager using ×20 objective lens and light microscope.

Cell proliferation assay

MTT assay is a colorimetric assay for assessing cell viability and proliferation. Briefly, HeLa cells were placed in 96-well plates. Five concentrations (25, 50, 100, 200, and 400 μg/mL) of *D. serrulata* flower extract were coincubated in HeLa cells in parallel with the control group. The cells were incubated at 37°C with 5% CO_2 for 24 h prior to the MTT assay. The formazan product was dissolved with dimethyl sulfoxide (DMSO) (Sigma) and quantified using a microplate reader at 570 nm. Cytotoxic value of cells was expressed as a percent of cell viability.

$$\text{Percent of cell viability} = ([OD_{sample} - OD_{blank}]/[OD_{control} - OD_{blank}]) \times 100$$

Wound healing assay

HeLa cells were cultured in 24-well plates until they reached 90%–100% confluence. The wound was generated using a scratcher to the size of 0.5 mm. The scratcher was pressed against the top of the tissue culture plate and generated a vertical wound down through the cell monolayer according to the protocol reported previously.[7,16] The scratched cells were exposed to normal media used as a control group, Trolox, DMSO, *D. serrulata* flower extracts at a concentration of 62.5, 125, and 250 μg/mL. Migration of the cells was observed at 0, 3, 6, 12, and 24 h under the cell imager. The width of the wound was measured using the ImageJ program and the length of the control group and treatment groups were compared according to their corresponding times.

Proteome profiler analysis

Proteome Profiler Human XL Oncology Array Kits (R&D Systems) contains 84 human cancer-related proteins spotted in duplicate onto a nitrocellulose membrane. The array kits allowed the comparison between control and 125 μg/mL of *D. serrulata* flower extract-treated groups to determine the changes in oncological profile. The lysates were prepared using lysis buffer supplied with the kit and measured the quantities by BCA Protein Assay Kit (Boster Biological Technology). Briefly, the cells were rinsed with PBS according to the manufacturer's protocol. The oncology array membranes were blocked in 2 mL of Array Buffer 6 for 1 h on a rocking platform shaker prior to the addition of the sample containing 150 μg of protein for incubation overnight at 4°C. Each array membrane was washed three times with wash buffer in a separate container and incubated with antibody cocktail for 1 h at room temperature. Furthermore, three washes were applied before the incubation with 2 mL of 1 × Streptavidin-HRP (R&D Systems) for 30 min at room temperature. Another three were washed and the signal was developed by Clarity™ Western ECL substrates (Bio-Rad) according to the manufacturer's protocol. The result was observed under ChemiDoc™ Touch system. The mean values were compared between control and treated array membranes.

Statistics analysis

All variables were summarized using means and standard deviations. Statistical analysis was carried out to compare groups by Brown–Forsythe test followed by Bonferroni correction for multiple comparisons. Statistical significance was considered for $P < 0.05$ (*R* program).

RESULTS

Cell morphology and cell viability

HeLa cell lines were cultured in EMEM with l-glutamine supplement, 10% fetal bovine serum, penicillin, and streptomycin in an incubator, as previously mentioned in the methods. The cells were then treated with cultured media, as a control group, DMSO, Trolox, or different concentrations of *D. serrulata* flower extract (62.5, 125, and 250 μg/mL) before being observed under a cell imager 24 h after treatment. The result was as shown in Figure 1. To observe the survivability of HeLa cells treated with *D. serrulata* flower extract, MTT assay was introduced. After being treated with different products, HeLa cell lines were incubated in a 96-well plate for 24 h, and 100 μL of 5 mg/mL MTT were further applied and incubated for 4 h in a CO_2 incubator. Cell survival percentages were measured for the formazan product using a microplate reader at an absorbance of 570 nm. The result showed the CC_{50} of 232.10 ± 17.16 μg/mL.

Wound healing assay

HeLa cell migration was observed *in vitro* under ZOE™ fluorescent cell imager using a wound-healing assay. The protocol is to introduce the "wound" using a sterile scratcher with a tip size of 0.5 mm as described in the method previously. The migration rate of HeLa cells in control, Trolox, DMSO, and different concentrations of *D. serrulata* flower extract-treated group was shown in Figures 2 and 3. After being treated, HeLa cells were observed at 0, 3, 6, and 24 h [Figures 2 and 3]. It could be seen that there was a slight movement in *D. serrulata* treated group at a concentration of 62.5 and 125 μg/mL, and a significant increase in the width of the wound at 250 μg/mL.

Protein expression in *D. serrulate*-treated cervical cancer cell line

Proteome Profiler Human XL Oncology Array kits were performed to observe 84 human cancer-related protein expressions. The protocol

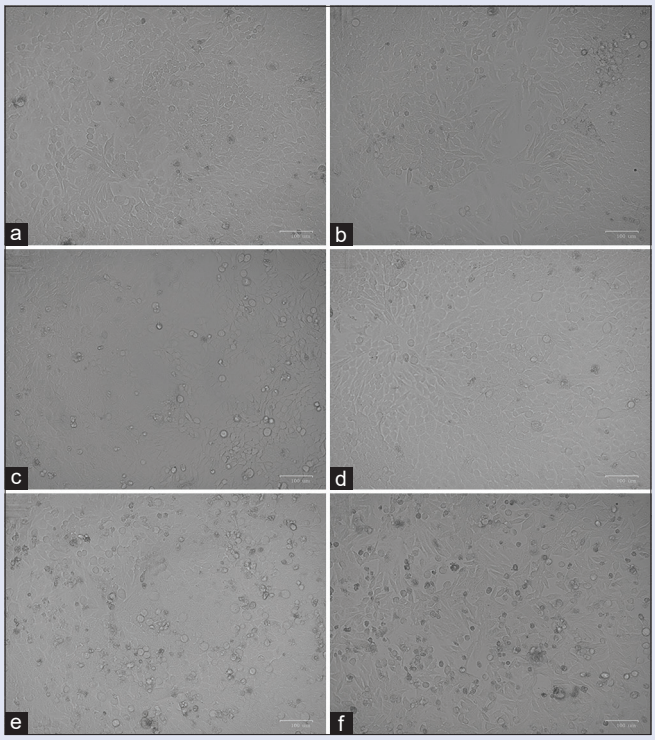

Figure 1: Morphology of HeLa cells observed under fluorescent cell imager using brightfield mode with ×20 objective lens (ZOE™ fluorescent cell imager) after 24 h treatment. HeLa cells treated with cultured media (a), DMSO (b), Trolox (c), and *D. serrulata* flower extract (62.5 µg/mL (d), 125 µg/mL (e), and 250 µg/mL (f)

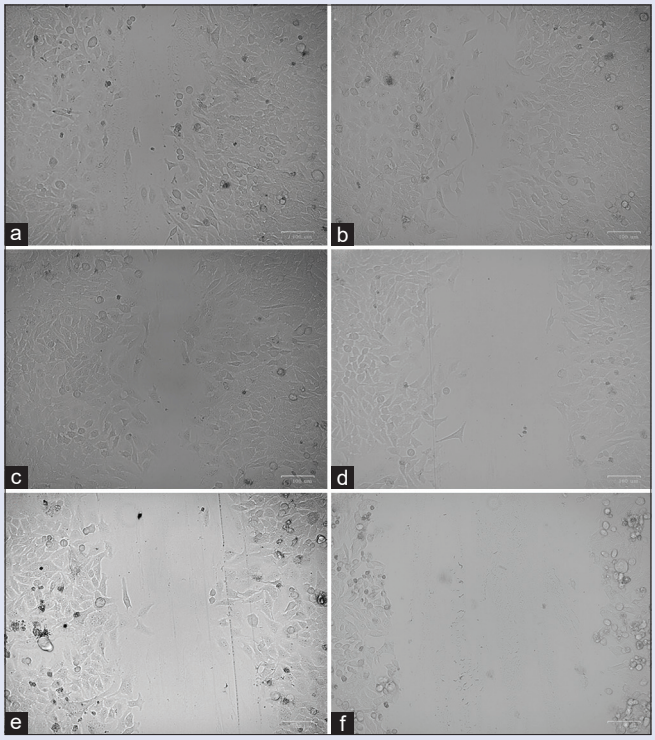

Figure 2: Migration of HeLa cell observed under fluorescent cell imager using brightfield mode with ×20 objective (ZOE™ fluorescent cell imager). HeLa cells treated with cultured media (a), DMSO (b), Trolox (c), and *D. serrulata* flower extract (62.5 µg/mL (d), 125 µg/mL (e), and 250 µg/mL (f))

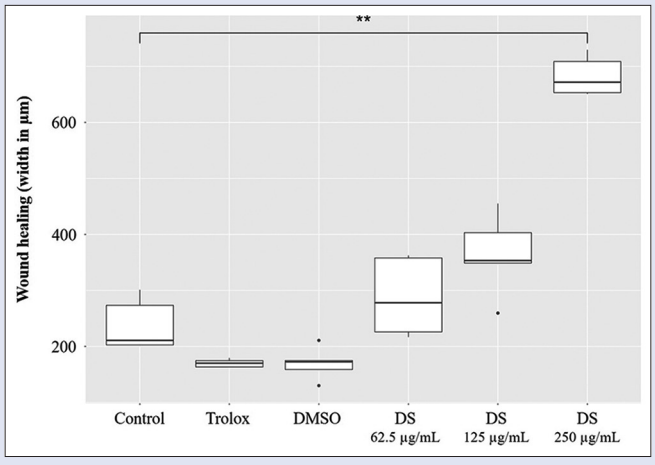

Figure 3: Distance of the wound (µm) generated by 0.5 mm scratcher at different time points measured by ImageJ program. Statistical analysis was performed in *R* 3.4.0 using Brown–Forsythe test/Bofferroni. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control. DS: *Dolichandrone serrulata*

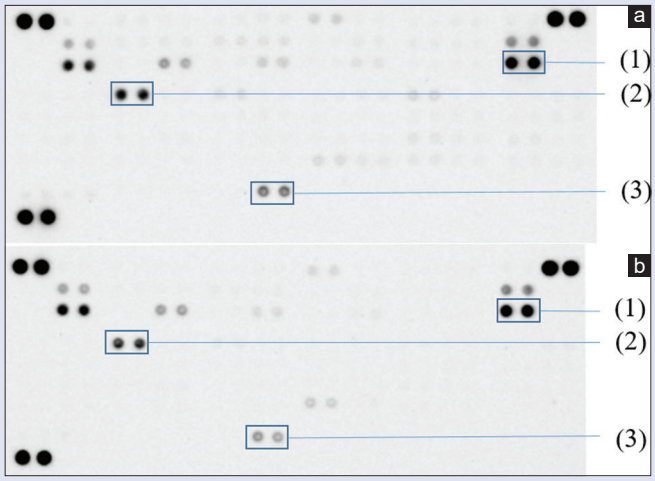

Figure 4: Human oncology antibody array after incubation with mock-treated (a) and *D. serrulate*-treated lysates (b). (1) FGF-2; (2) Galactin-3; (3). Vimentin

was according to the manufacturer's instructions. Quantification of the antibody array signal allowed comparison of relative protein levels in *D. serrulate*-treated group (125 µg/mL) compared with the mock-treated group in the cervical cancer cell line. The changes in protein level were as shown in Figures 4 and 5. Of all proteins, three proteins showed a dramatic reduction in expression, namely, galectin-3, vimentin, and fibroblast growth factor 2 (FGF2), compared with the control group [Figures 4 and 5].

DISCUSSION

Cancer is a disease involving an atypical cell that can invade the body. One of the most recognized cancers found in women is cervical cancer and it is found to be the second most common cancer in Thai women.[4] Several developing types of research are inquired for more information about novel therapeutic methods. In the current study, the researcher aimed to further investigate the possible underlying

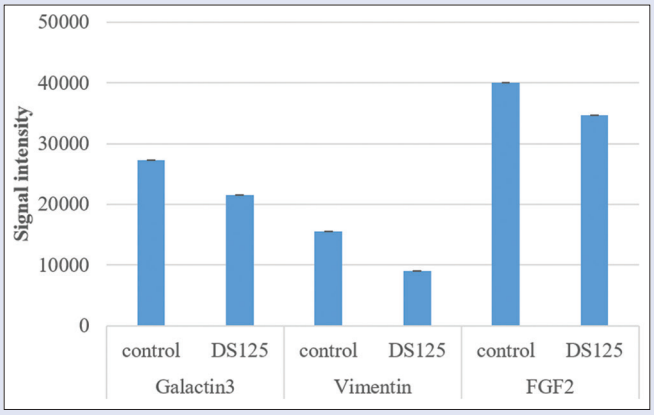

Figure 5: Proteins from antibody array with more than 5% changes in expression after treated with *D. serrulata*

mechanism of *D. serrulata* flower extract on cell migration of cervical cancer cell line.

Different concentrations of *D. serrulata* flower extract were treated with HeLa cells and the result showed a CC_{50} of 232.10 ± 17.16 µg/mL. This finding is consistent with the reported data previously,[7] that the cell characteristic was altered according to the concentration of the extract. Interestingly, the rat models of recent studies showed no subchronic toxicity on the vital, however, the results showed a slight alteration in some liver and kidney functions of male rats.[17,18]

Cell migration assay was performed by introducing the "scratch" to HeLa cell line in a 24-well plate with 90%–100% confluence. The migration of HeLa cell line in control, Trolox, DMSO, and different concentrations of *D. serrulata* flower extract (62.5, 125, and 250 µg/mL)-treated group were observed under fluorescent cell imager using brightfield mode with × 20 objective after 24 h of incubation [Figures 2 and 3]. The result showed that the width of the introduced wound was significantly increased in the concentration of 250 µg/mL of *D. serrulata* flower extract while *D. serrulata* at a concentration of 62.5 and 125 µg/mL were slightly increased in comparison with the control. The result was in parallel with the previous study that *D. serrulata* flower extract could widen the width introduced by the scratcher at a concentration of 125 µg/mL.[7] One of the possible mechanisms of how the extract could maintain or widen the "wound" might be due to the influence of *D. serrulata* flower extract on the reduction of cell vitality [Figure 1]. Phanthong *et al.*,[6] previously identified two compounds from ethyl acetate fraction collected from chromatographic separation, which was hallerone and protocatechuic acid. While hallerone was previously identified as a marker of *Phyla nodiflora*,[9] which is the medicinal plant proposed of having antiproliferative and apoptotic effects against breast cancer cell lines,[10-12] protocatechuic acid was also proposed of inducing apoptosis in various cancer such as human breast, lung, liver, cervix, and prostate cancer cell line,[13] as well as and antimetastasis effect in lung tumor cells and human gastric carcinoma AGS cells.[14,15] Also in the previous study of Chatchanayeunyong,[7] demonstrated the inhibitory effect of *D. serrulata* ethanolic extract against cell migration in HeLa cell line. According to the result of the cell survivability test, the CC_{50} of *D. serrulata* flower extract was 232.10 ± 17.16 µg/mL. To confirm whether or not the impact on cell migration of *D. serrulata* flower extract was the result of cell death, the concentration of 125 µg/mL was applied for further investigation.

To observe the possible underlying mechanism of *D. serrulata* flower extract on cervical cancer cell migration, the protein array was introduced to this study. The antibody array signal was compared between mock- and *D. serrulata*-treated group from Proteome Profiler Human XL Oncology Array kits. The result showed that, of all proteins, only galectin-3, vimentin, and FGF2 proteins have at least 5% changes in protein expression [Figures 4 and 5]. Galectin-3 is the protein proposed of being a potential target to prevent cancer cell metastasis.[19] It has been proposed earlier that galectin-3 correlated with the development of cervical cancer and its overexpression could be a predictor of poor prognosis in patients with cervical cancer.[20] Galectin-3 has been reported to translocate either from cytosol or from the nucleus to mitochondria, inhibiting cytochrome c release from mitochondria, thus preventing apoptosis.[21,22] It is also proposed of its effect inhibits the expression of TRAIL protein.[23] By upregulating galectin-3 protein, it could enhance cervical cancer cell invasiveness via the interaction with vascular endothelial growth factor C (VEGF-C).[24] A previous study has reported that silencing galectin-3 expression could restore TRAIL sensitivity and promote TRAIL-mediated endocytosis of TRAIL/death receptors complexes.[25]

Overexpression of vimentin in cervical carcinomas was also found to be associated with the invasive and migratory potential of the cervical cancer cells.[26] It was proposed for serving as an independent prognostic marker for cervical cancer patients with primary surgery.[27] As an epithelial-to-mesenchymal transition (EMT) marker, a recent study showed that by blocking vimentin, the volume of the tumor and the number of lung metastases are markedly decreased in a mouse model.[28]

Fibroblast growth factor 2 (FGF2) can be produced by various cell types and is closely related to the activation of fibroblasts. It plays a role in neoangiogenesis by influencing other growth factors and chemokines such as PDGF, HGF, and MCP-1, contributing to angioblast differentiation, cell growth, and invasion.[29] FGFR2 is reported to be strongly correlated with cervical cancer and could play important role in carcinogenesis and cervical cancer cell growth.[30,31] A recent study showed that targeting FGF2, could suppress cervical cancer invasion.[32]

CONCLUSION

D. serrulata ethanolic extract has been proposed here for its antimigration effect on the cervical cancer cell line. From Figures 2 and 3, *D. serrulata* ethanolic extract could stabilize the width of the wound introduced by the scratcher compared with the mock- and Trolox-treated group. Observing the protein expression using a human oncology antibody array suggested that *D. serrulata* could reduce the expression of galectin-3, vimentin, and FGF2 protein. Thus, it is possible that *D. serrulata* ethanolic extract could be a therapeutic target for cervical cancer by inducing apoptosis and suppressing cervical cancer cell invasion. However, the in-depth pathway still needs to be further investigated. Further study is needed to adjust the plausible dose of *D. serrulata* ethanolic extract and further investigate the possible pathway in suppression of cervical cancer cell migration and metastasis.

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Conflicts of interest

There are no conflicts of interest.

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