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Research Article

Zanthoxylum rhetsa (Roxb.) DC Oil Suppresses the Proliferation, Activates Apoptosis, Inhibits Migration of Human Cervical Cancer Cells

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Abstract

Background and Objective: *Zanthoxylum rhetsa* (Roxb.) DC are spices for Thai food and it has been used for the treatment of several diseases. However, the activity of *Z. rhetsa* oil against cancer has not been fully investigated. In this study, the constituents of *Z. rhetsa* oil were characterized and explored the cytotoxic effects in cervical cancer cells. **Materials and Methods:** *Zanthoxylum rhetsa* oil was attained by hydro-distillation and was considered the active compound by Gas Chromatography-Mass Spectrometry (GCMS). The oil was evaluated for antioxidant, cytotoxicity, colony formation, apoptosis and migration in the human cervical cancer HeLa cell line. **Results:** Twenty-eight known compounds were identified in oil, including monoterpenes, sesquiterpenes and hydrocarbon. The oil inhibited the HeLa cell proliferation with a low IC₅₀ value and decreased colony formation. Significantly, the induction of early apoptotic cell death was induced by the oil and a higher percentage was indicated at 250 µg mL⁻¹. The cell number was reduced after incubating with the oil for 24 hrs and morphological change was observed at 250 µg mL⁻¹. The mechanism of cell death and apoptosis was detected in the induction of ROS formation and were significant at the dose of 100 and 250 µg mL⁻¹. Finally, the oil stimulated anti-migratory activities of the cervical HeLa. **Conclusion:** *Zanthoxylum rhetsa* oil was demonstrated to induce cancer cell death, activate early apoptosis and suppress the migration ability of cervical cancer cells through stimulating ROS formation.

Key words: *Zanthoxylum rhetsa* oil, proliferation, apoptosis, cervical cancer, migration, cytotoxicity, HeLa

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cervical cancer is the most common cancer that is reported in the fourth woman cancer worldwide¹. In the early process of diagnosis, the surgical method is started with anticancer drugs which allow patients to receive highly effective treatment. However, the prognosis of early cervical cancer is still poor². The surgical procedure is effective for the treatment of this cancer following the active anticancer drugs and leads the patient to a longer survival rate. Nevertheless, the standard anticancer drug treatment is still toxic in normal cells and death and serious complications take place. Cervical cancer death rates may be due to invasion, angiogenesis, metastasis and drug resistance³. Therefore, we immediately require to discover new natural compounds with less toxicity and high efficacy for treating or preventing cervical cancer.

Reactive oxygen species (ROS) lead to a variety of human cancers and antioxidants or scavenging agents are used to negate them⁴. Plants and other natural sources are significant origins of the encounter of anticancer remedies. *Zanthoxylum rhetsa* (Roxb.) DC belong to the Rutaceae family found in the Northern part of Thailand, locally called "Makhwaen". Various parts and many extracts of *Z. rhetsa* were reported in numerous actions. The stem bark has been shown to possess anti-inflammatory activity⁵, sunscreen⁶, anticancer⁷, antinociceptive and antidiarrheal activity⁸. The part of the thorn that is rubbed with the water can be applied to the chest area to reduce pain and increase breast milk in lactating women⁹. The seed had photoprotective property¹⁰, antioxidant¹¹, antibacterial¹² and anticancer¹³. The fruit had anti-inflammatory¹⁴, antibacterial and anticancer¹⁵⁻¹⁷ and the volatile from the pericarp of fruit had antioxidant, antibacterial, antidiarrheal and non-selective spasmolytic activity¹⁸. For cytotoxicity, *Z. rhetsa* oil in Thailand was studied only against lung¹⁶ and breast cancer cell lines¹⁷.

From the data obtained, lignans and alkaloids from *Z. rhetsa* bark play a crucial role to activate melanoma B cell death with the highest activity from methanol extraction⁷. For breast cancer demonstrated that purified proteins from the pericarp of *Z. rhetsa* inhibited cell proliferation with a low IC₅₀ value, decreased cell colonies and activated apoptosis¹⁹. The apoptotic induction was observed by detecting DNA fragmentation correlating with the arrested cell cycle¹⁹. Next, *Z. rhetsa* oil had a high ability to suppress human breast cancer cell proliferation and cell viability in both estrogen-positive MCF-7 cells and estrogen-negative MDA-MB-231 cells with equally IC₅₀ values¹⁶.

Nonetheless, so far *Z. rhetsa* oil has been little tested for its anti-cancer effects. In present study aimed to evaluate the

Z. rhetsa oil effects from fresh fruit on the growth, apoptosis and migration in human cervical cancer HeLa cells with the underlying mechanism of action through investigation of intracellular ROS formation.

MATERIALS AND METHODS

Study area: Fresh fruit was gathered from Nan Province on December, 2017 which was identified and deposited at Forest Herbarium, Thailand with a voucher specimen (BKF No. 194816).

Chemicals: The DPPH and L-ascorbic acid were obtained from Sigma Chemical Co., USA. All other chemicals used were obtained from local suppliers.

Plant extraction: Six hundred grams of fresh fruits were ground and distilled with water for 6 hrs. The *Z. rhetsa* oil was then added with anhydrous sodium sulfate to draw out the water and stored at 4 °C before analyses.

Antioxidant assay: Antioxidant activity was tested using DPPH as previously reported²⁰. Various concentrations of the oils were incubated with methanolic DPPH solution. After 30 min in dark at room temperature, the absorbance was measured at 515 nm. Blank and positive control (ascorbic acid) were also measured. All the determinations were performed in triplicate. The scavenging ability of DPPH was expressed as 50% of the inhibitory concentration (IC₅₀).

GCMS assay: *Zanthoxylum rhetsa* oil was performed on a GC-MS using a GC 7890A Agilent joined to a mass spectrometer MSD5975C (EI) Agilent. Capillary column 5MS (30 m × 0.25 mm × 0.25 μm) was used. The conditions were as follows: Injection inlet was 250 °C, injection volume was 0.5 μL in the split ratio (50:1), helium was the carrier gas, flow rate was 1.0 mL min⁻¹. The column oven was programmed from 60-240 (4 °C min⁻¹). The total run time was 60 min. The temperature of the ion source and quadrupole were 230 and 150, respectively. The electron ionization was set at 70 eV with a mass scan of 30-500 amu. The mass of each component was done using the database of the National Institute Standard and Technology (NIST).

Cell lines and cytotoxicity assay: HeLa human cervical cancer cell was purchased from the ATCC and grown in DMEM (Gibco, Thermo Fisher Scientific, Inc with 10% fetal bovine serum (BSA) and 1% antibiotics. For the cytotoxic assay, cells were cultured onto 96-well culture plates with 1 × 10⁴ cells per

well for 24 hrs and then treated with the *Z. rhetsa* oil with various concentrations (0-250 $\mu\text{g mL}^{-1}$) for 24, 48 and 72 hrs. After incubation periods, the cells were fixed, stained with 0.4% sulforhodamine B (SRB), washed with PBS buffer and solubilized with 10 mM Tris base buffer. The optical density was read by spectrophotometry at 540 nm.

Colony formation assay: The 500 cells per well were cultured in 6-well culture plates for 24 hrs and then treated with the *Z. rhetsa* oil at indicated doses (0-250 $\mu\text{g mL}^{-1}$) for 24 hrs. After 10 days, cells were fixed and stained with 0.25% crystal violet. The cells in culture plates were washed, captured and colonies.

Apoptosis by flow cytometry: The 2×10^5 cells per well were cultured in 6-well culture plates for 24 hrs and incubated with various concentrations (0-250 $\mu\text{g mL}^{-1}$) of *Z. rhetsa* oil for 24 hrs. Next, cells were collected and exposed to Annexin V-FITC and PI solution (BD Biosciences, San Diego, CA, USA) for 15 min. The cells were measured the apoptotic cells by flow cytometer (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Acridine Orange and Ethidium Bromide (AO/EB) staining assay: The 2×10^5 cells per well were cultured in 6-well culture plates for 24 hrs and incubated with various concentrations (0-250 $\mu\text{g mL}^{-1}$) of *Z. rhetsa* oil for 24 hrs. After that, cells were collected and exposed to AO/EB dye (1 μL AO/EB) for 15 min at room temperature. Then, the apoptotic cells were captured by an inverted fluorescence microscope (20 \times magnification, Olympus Corporation, USA).

Reactive oxygen species assay: The HeLa cells approximately (2×10^5 cells per well) were plated in 6-well plates for 24 hrs. Next, the cell was exposed to various concentrations of *Z. rhetsa* oil (0-250 $\mu\text{g mL}^{-1}$) for 24 hrs. Then cells were harvested and incubated with DCF-DA (25 μM) in DMEM medium for 30 min in dark at 37 $^\circ\text{C}$ incubator. The fluorescent intensity was analyzed (488 nm excitation and 525 nm emission) by flow cytometry (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Wound healing assay: The 2×10^5 cells per well were plated in 24-well culture plates for 24 hrs, made the wound by 0.2 mL sterile pipette tips and incubated with various concentrations (0-250 $\mu\text{g mL}^{-1}$) of *Z. rhetsa* oil for 24 hrs. Wound size was measured at 0 and 48 hrs using a microscope imaging system ($\times 4$ magnification, Olympus Corporation, USA).

Statistical analysis: Results were expressed as Mean \pm SE and statistical analysis was conducted using GraphPrism version 5 software. The significance of the difference between control and treatment groups was examined with the student's t-test, * $p < 0.05$.

RESULTS

Active compounds in *Z. rhetsa* oil and antioxidant activity:

The percentage yield of *Z. rhetsa* oil obtained by hydrodistillation displayed 9.5% v/w with pale-yellow colour. The GC-MS analysis was illustrated in Table 1. The oil contained twenty-nine known compounds and one unknown. The major components were limonene, beta-phellandrene and alpha-phellandrene with 26.61, 25.96 and 12.86%, respectively.

The DPPH assay of the *Z. rhetsa* oil was represented as IC_{50} values and compared to L-ascorbic acid. The results indicated that the IC_{50} values of L-ascorbic acid ($10.70 \pm 0.78 \mu\text{g mL}^{-1}$) act as a standard for DPPH assay was significantly almost 1,745 times greater than the *Z. rhetsa* oil ($76.45 \pm 6.93 \text{ mg mL}^{-1}$).

Table 1: Chemical composition of *Z. rhetsa* oil

Retention time (min)	Compounds	Relative area (%)
5.294	Alpha-thujene ^{a,c}	0.17
5.512	Alpha-pinene ^{a,c}	5.37
6.547	Sabinene ^{a,c}	1.00
6.713	Beta-pinene ^{a,c}	0.13
7.022	Beta-myrcene ^{a,c}	8.17
7.491	Pseudolimonene ^{a,c}	0.13
7.606	Alpha-phellandrene ^{a,c}	12.86
7.652	3-Carene ^{a,c}	0.45
7.909	Alpha-terpinene ^{a,c}	0.21
8.201	o-Cymene ^{a,c}	5.84
8.447	Beta-phellandrene	25.96
8.510	Limonene ^{a,c}	26.61
8.910	Trans-beta-ocimene ^{a,c}	3.45
9.334	Gamma-terpinene ^{a,c}	0.19
9.792	1-Cyclopropylpentane ^c	0.21
10.335	Alpha-terpinolen ^{a,c}	0.53
10.942	Linalool ^{b,d}	3.83
11.892	p-Menth-2-en-1-ol ^{a,d}	0.18
14.186	1-Terpinen-4-ol ^{a,d}	0.45
14.444	Crypton ^{a,d}	0.52
14.815	Alpha-terpineol ^{b,d}	0.76
15.279	Decanal ^{c,d}	0.29
15.456	1,2-Methyleneheptane ^c	0.33
17.150	Trans-geraniol ^{a,d}	0.22
18.272	Phellandral ^{a,d}	0.20
22.574	Lavandulyl acetate ^{a,d}	0.58
23.902	Unknown	0.18
24.148	Beta-caryophyllen ^{b,c}	0.37
26.654	Germacrene D ^{b,c}	0.82

^aMonoterpene, ^bSesquiterpene and ^cHydrocarbon

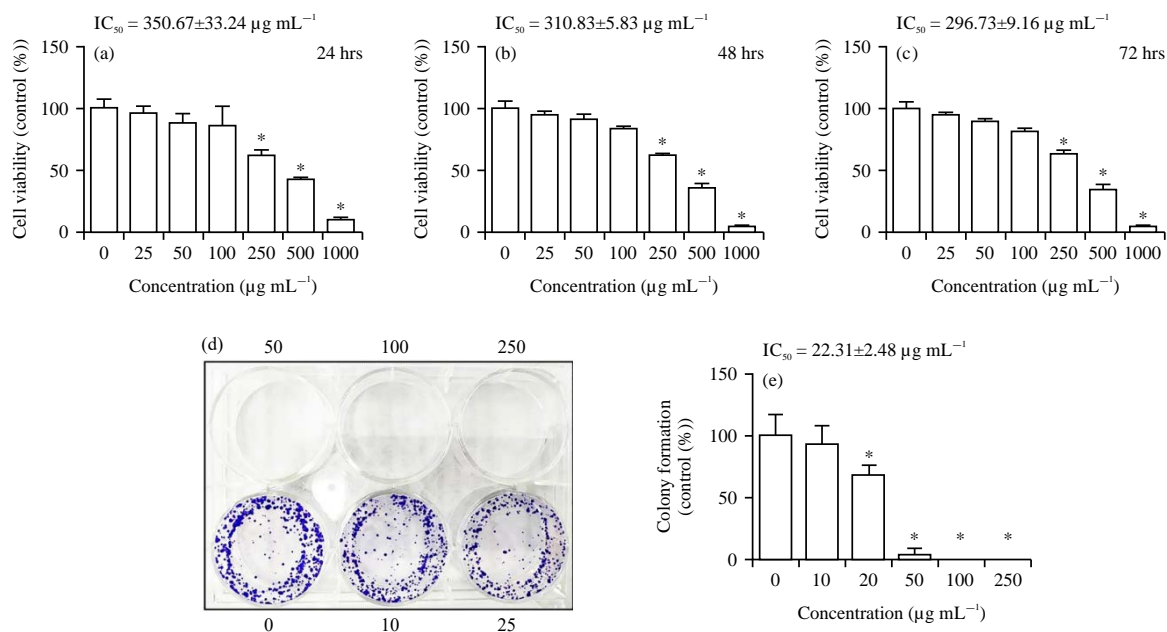


Fig. 1(a-e): *Zanthoxylum rhetsa* oil effects on HeLa cells proliferation and colony formation, (a-c) Cells were incubated with the extract for 24-72 hrs and measured the cell growth by SRB assay and (d-e) Cells were treated with the oil extract for 24 hrs, further cultured the cancer cells for 10 days and examined the cell colony formation

*p<0.05

Effects of the *Z. rhetsa* oil on cell growth and colony formation:

The antiproliferative activities of *Z. rhetsa* oil against human cervical HeLa cancer cell line SRB assay were shown in Fig. 1a-c. The extract exhibited antiproliferative activities against cancer cells in a concentration and time-dependent manner with the IC₅₀ values of 350.67 ± 33.24, 310.83 ± 5.83, 296.73 ± 9.16 µg mL⁻¹ for 24, 48 and 72 hrs, respectively.

The colony formation assay of *Z. rhetsa* oil was shown in Fig. 1d-e. The extract concentration-dependently reduced the number of colonies in HeLa cells with a low IC₅₀ value (22.31 ± 2.48 µg mL⁻¹) than the data of cell viability. These results demonstrated that the *Z. rhetsa* oil significantly suppressed the cell viability and colony formation of human cervical cancer cells.

Effects of the *Z. rhetsa* oil on cell number and cell apoptosis:

To investigate whether the *Z. rhetsa* oil decreased cell growth by modulating apoptosis, cell apoptosis was conducted in HeLa cancer cells. The percentages of early and late phase apoptosis were significantly increased by the *Z. rhetsa* oil in a concentration-dependent manner (Fig. 2a-g). Especially late phase apoptosis induction, the oil stimulated late apoptosis in a dose-dependent manner with 3.0, 5.7, 24.2 and 47.8%, respectively. These results demonstrated that

the *Z. rhetsa* oil significantly induced the cell apoptosis of human cervical cancer cells.

To confirm apoptosis induction by the oil, morphological changes were conducted with AO/EB staining. As shown in Fig. 3a-d, growth reduction was induced by the *Z. rhetsa* oil and viable cell number was greatly reduced in a dose-dependent manner and the number of apoptotic bodies was found at a high dose (250 µg mL⁻¹) of the *Z. rhetsa* oil. Similarly, a 250 µg mL⁻¹ dose of oil activated apoptosis on HeLa cells after detection with flow cytometry and AO/EB staining. These data indicated that the apoptosis of cells induced by the *Z. rhetsa* oil contributed to their antiproliferative effects.

Effects of the *Z. rhetsa* oil on ROS formation:

Intracellular ROS formation was measured using a DCF-DA fluorescent probe in HeLa cervical cancer cells. After the cells were incubated with the *Z. rhetsa* oil for 24 hrs, the data from flow cytometry revealed that the oil significantly stimulated ROS production in a concentration-dependent manner (Fig. 3e-i). At the dose of 250 µg mL⁻¹, the *Z. rhetsa* oil showed higher levels of ROS formation approximately 20.5% correlated with growth inhibition and apoptosis induction in HeLa cells. The results demonstrated that there is a reduction in cell growth and an increase in apoptosis when the level of ROS was increased with the *Z. rhetsa* oil.

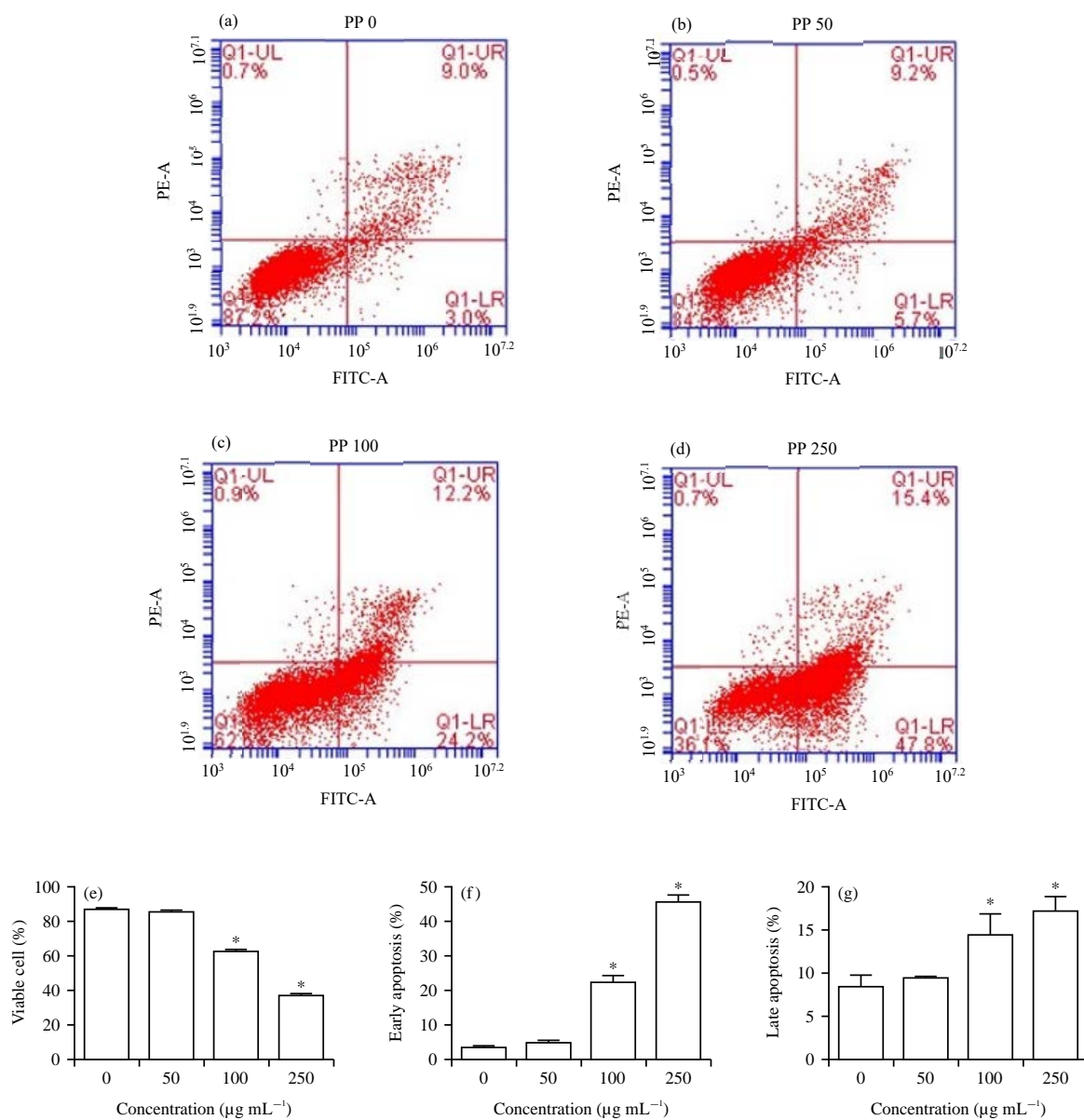


Fig. 2(a-g): *Zanthoxylum rhetsa* oil effects on HeLa cells apoptosis, (a-g) Cells were treated with the oil extract for 24 hrs and examined the apoptosis by flow cytometry

* $p < 0.05$

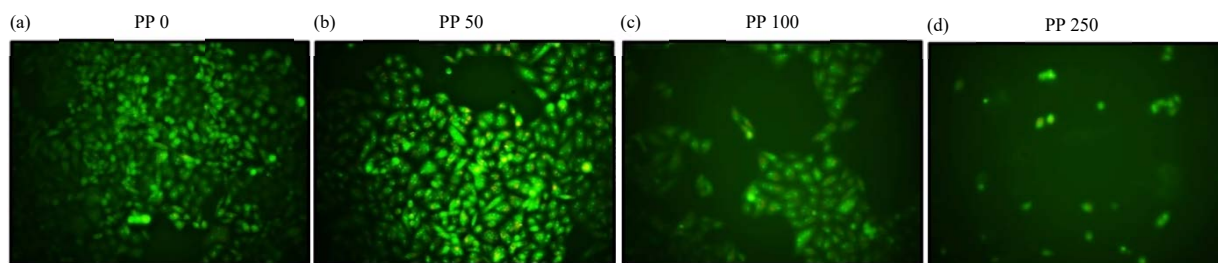


Fig. 3(a-i): Continue

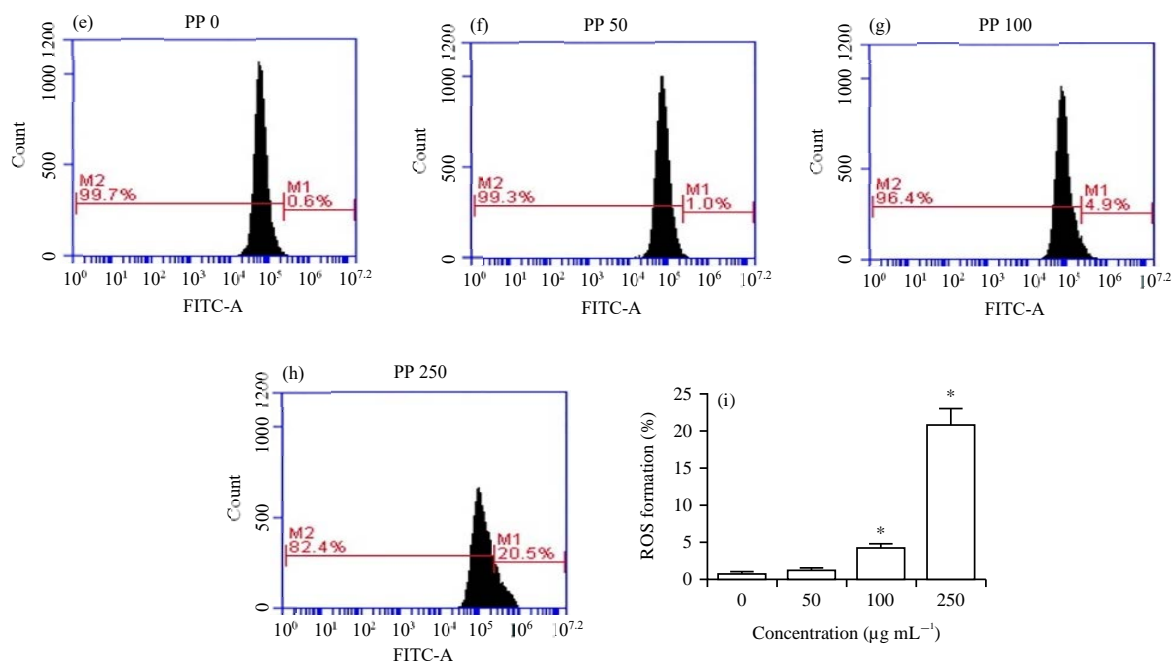


Fig. 3(a-i): *Zanthoxylum rhetsa* oil effects on HeLa cell morphology and ROS formation, (a-d) Cells were treated with the oil extract for 24 hrs, loaded with AO/EB solution and photographed the cell morphology by inverted fluorescence microscopy and (e-i) Cells were treated with the oil extract for 24 hrs, loaded with DCF-DA fluorescent-probe and examined the ROS formation by flow cytometry
*p<0.05

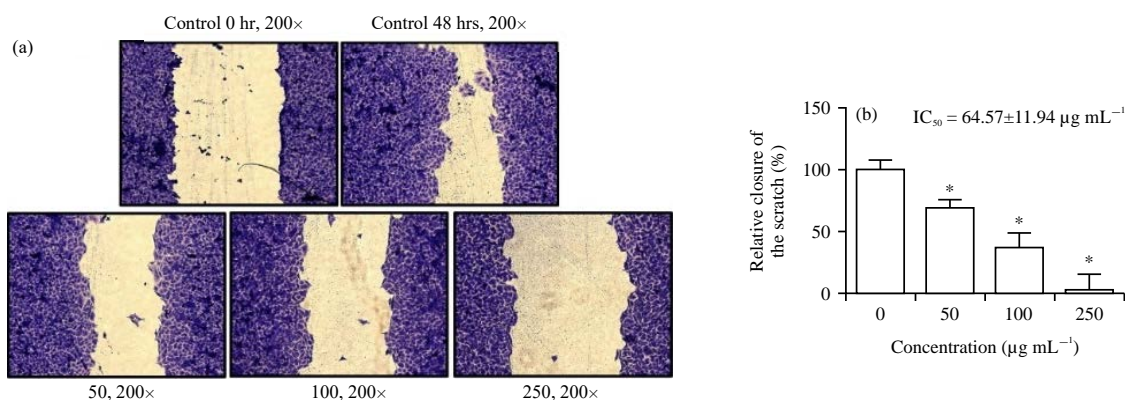


Fig. 4(a-b): *Zanthoxylum rhetsa* oil effects on HeLa cell migration, (a-b) Cells were made a wound, treated with the oil extract for 48 hrs and examined the cell migration
*p<0.05

Effects of the *Z. rhetsa* oil on cell migration: To determine the effect of the *Z. rhetsa* oil on the HeLa cells migration by wound healing method. As shown in Fig. 4a-b, the percentage of cell migration rate was significantly reduced by the *Z. rhetsa* oil in HeLa cells in a concentration-dependent manner. Taken together, the results indicated that the *Z. rhetsa* oil had an inhibitory effect on cervical cancer cell migration.

DISCUSSION

This work demonstrated that *Z. rhetsa* oil significantly inhibited cervical cancer cells by attenuating proliferation, activating apoptosis and inhibiting migration in a dose-dependent manner. The standard anticancer drugs are the most frequent agents for treating cervical cancer,

nevertheless, the resistance and toxic ability correlated with the agents are a matter of concern. Therefore, several compounds or natural products are discovered for exploring and evolving better than anticancer agents which exhibit high efficacy and low toxicity. *Zanthoxylum rhetsa*, a Thai vegetable with a spicy taste, is commonly found in the Northern part of Thailand and has been demonstrated with antioxidative activity and antiinflammation, however, the anticancer effects of *Z. rhetsa* oil is still less information.

Monoterpenes, hydrocarbon and sesquiterpenes were the main constituents found in *Z. rhetsa* oil. The leading compounds were limonene and beta-phellandrene (26.61 and 25.96%, respectively). *Zanthoxylum rhetsa* oil from northern Nan with high content of limonene exhibited strong cytotoxic properties against lung cancer H460 cells¹⁶. Recently, the leaf petiole extract of *Z. rhetsa* with a high percent of limonene showed strong inhibitory activity against MCF-7 (human breast adenocarcinoma cells)¹⁷. *Zanthoxylum rhetsa* oil grown in Vietnam with high content of sabinene showed an inhibitory effect against cervical cancer cells with the IC₅₀ of more than 100 µg mL⁻¹¹⁷.

The effects of *Z. rhetsa* oil were investigated on cervical cancer HeLa cell growth by SRB and colony formation assay. Other *Zanthoxylum* species have also been demonstrated to be non-toxic to normal cell lines²¹. *Zanthoxylum rhetsa* oil inhibited cervical cancer cell growth with the IC₅₀ value of 296.73 µg mL⁻¹ and reduced the cancer cells in replication in a dose-dependent manner. As previous data found that several parts of *Z. rhetsa* had numerous actions on diseases and an anticancer effect was observed. Consistently, *Z. rhetsa* stem bark extract significantly suppressed melanoma B cells growth and the strongest growth inhibitory effects were demonstrated by chloroform, hexane, ethyl acetate, methanol and butanol fractions with IC₅₀ values 156, 132.7, 174.3, 168 and 263.1 µg mL⁻¹, respectively⁷. Additionally, the purified proteins from the pericarp caused inhibition of cell growth, reduction of cell colonies and activation of apoptosis¹⁹. The extract of *Z. rhetsa* showed the strongest anticancer effects in several cancer cells including cervical cancer cells.

For apoptosis in HeLa cells indicated that *Z. rhetsa* oil extract induced cancer cell early apoptosis in a dose-dependent manner from 50-250 µg mL⁻¹. The observation of apoptosis was reported in human breast cancer MCF-7 cells by increasing DNA fragmentation and arresting the cell cycle at G2/M²⁰. Several natural compounds from South-East Asian countries including Thailand, significantly induced cervical cancer cell apoptosis and suppressed the

cell cycle distribution^{22,23}. The *Careya arborea* leaf extract indicated that this extract induced cervical cells apoptosis at the high rate of late apoptosis and arrested the cell cycle at G0/G1 phase²². Confirmation with one of the most Thai vegetables, *Oroxylum indicum*, showed the anticancer effects in several cancer cell types including cervical cancer cells. At 250 µg mL⁻¹ of seed, *Oroxylum indicum* demonstrated that it reduced viable cancer cells and stimulated late apoptosis by inducing ROS formation and mitochondrial dysfunction²³. Finally, Thai vegetables had the strongest anticancer activities including *Z. rhetsa* oil extract.

Furthermore, this study indicated that *Z. rhetsa* oil extract significantly suppressed HeLa cell migration in a dose-dependent manner (50-250 µg mL⁻¹) in *in vitro* study. Consistently, *in vivo* studies showed angiogenic properties suppression in the angiogenesis in the treated animal as compared to the control animal peritoneum, through reduction in the Vascular Endothelial Growth Factor (VEGF) level as compared to the control groups¹⁹. In conclusion, *Z. rhetsa* oil extract suppressed cervical cell migration.

The mechanism of *Z. rhetsa* oil extract-induced cell death and apoptosis was focused on ROS formation. The data demonstrated that these oil extracts stimulated the production of ROS formation in intracellular HeLa cells. Importantly, intracellular ROS act as messengers that play role in normal physiological functions. Nevertheless, ROS overproduction can cause damage to DNA, lipids, carbohydrates and proteins, leading to normal and cancer cell death²⁴. Thai plant extract significantly activated cancer cells apoptosis which reduced the mitochondrial function and induced ROS formation²². The four Thai extracts of *Careya sphaerica*, *Azadirachta indica*, *Piper nigrum* and *Oroxylum indicum* stimulated ROS overgeneration with increasing caspase 3 activity and then lead to induced cancer cell apoptosis as *Z. rhetsa* oil-stimulated ROS²⁵. Finally, *Z. rhetsa* oil may be useful for treating or preventing cervical cancer.

CONCLUSION

Monoterpenes were the predominant compound in *Z. rhetsa* oil from fresh fruits with a mild antioxidant effect. In addition, *Z. rhetsa* oil stimulates cervical cancer HeLa cell death, induces early apoptosis and inhibits migration. The mechanism of action indicated that *Z. rhetsa* oil activates ROS formation significantly at a high dose of the oil. The data showed that *Z. rhetsa* oil has potential effects for treating cervical cancer.

SIGNIFICANCE STATEMENT

This study explored the mechanism of *Z. rhetsa* oil in the cervical cancer HeLa cell line. The study reported that the oil-induced cell death via apoptosis and stimulated ROS system. This study will help the researcher to discover a natural compound for treating cervical cancer.

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