

Comparison between Volatile Oil from Fresh and Dried Fruits of *Zanthoxylum rhetsa* (Roxb.) DC. and Cytotoxicity Activity Evaluation

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ABSTRACT

Introduction: *Zanthoxylum rhetsa* is a tree found in northern Thailand. It has been used as a traditional herb with medicinal properties. However, the comparison of composition in volatile oil from fresh and dried fruit is still not fully investigated. In study, we evaluated the constituents in oil from fruits of *Z. rhetsa* and evaluated the cytotoxicity in non-small lung cancer cells.

Methods: The hydro distillation was applied to afford the oil. The obtained oil was analyzed by GC-MS and evaluated the cytotoxicity and apoptosis in non-small lung cells by using MTT method and flow cytometry respectively. **Results:** Fresh and dried fruits provided a higher yield of volatile oil by approximately 10% and 20% respectively, compared to other studies. Twenty-eight compounds were identified and the major components of fresh and dried fruits were not distinctly different. The major component, α -limonene, was found in dried fruits from Phayao, southern Nan and Chiang Rai province. A high content of β -phellandrene was found in dried fruits from northern Nan province and the (+)-sabinene was found in high content of fresh fruits from southern Nan, Phayao and Chiang Rai province. Furthermore, the cytotoxicity tests displayed that all of oil products were active against lung cancer cells. Among the components investigated, fresh and dried fruits from southern Nan province showed to be the most potent ($EC_{50} = 1.91 \pm 0.53, 1.79 \pm 0.43 \mu\text{L/mL}$, respectively). **Conclusion:** Volatile oil of *Z. rhetsa* exhibited a strong cytotoxic properties against cytotoxic may be potentially used as natural anticancer agents.

Key words: *Zanthoxylum rhetsa*, Local variation, Volatile oil, Chemical composition, Cytotoxic activity.

INTRODUCTION

The Rutaceae family is distributed throughout the tropical areas of the world and it is composed of more than 1500 species.¹ Most species of Rutaceae are fragrant plants, medium-sized trees at 5-10 meter-height. Many extracts from Rutaceae have been shown to possess biological activities such as antioxidant,² antimicrobial³ and antifungal.⁴ The *Zanthoxylum*, a member of genus in Rutaceae, is used as traditional medicine, seasoning, perfume and other purposes. *Zanthoxylum rhetsa* is one species found in northern Thailand, locally called "Makhwaen". Numerous research papers have reported interesting pharmacological activities from various parts of *Z. rhetsa* such as toothache,⁵ inhibitory activity against leukemia cells (HL-60).⁶ The petroleum ether extract and essential oil from the fruit has been reported as green mosquito repellent and larvicidal agent.⁷⁻⁸ In addition, other researchers described the activity of this volatile oil as antioxidant,² antimalarial,⁹ antimicrobial¹⁰ In addition, Some constituents of essential oil had been reported to possess anticancer activities such as limonene, carvacrol,

sabinene, α -pinene, myrcene, γ -terpinene, thujones and etc. The anticancer mechanisms were considered through the antioxidant, antimutagenic, anti-proliferative and enhancement of immune system.¹¹

Recently, it was reported a hydrodistillation approach to afford essential oil from *Z. rhetsa* analyzed with GC-MS.¹² However, there are no detailed studies which compared the essential oils from fresh and dried fruits of *Z. rhetsa*. In this research, we performed eco-friendly extraction by using hydro distillation to obtain the volatile oil, conducting from plant samples selection from four different areas in northern Thailand, an investigation into the compositions of fresh and dried fruits. In addition, we have evaluated the cytotoxicity against a non-small lung cancer cell line.

MATERIALS AND METHODS

Plant Material

The fruits were gathered from four different regions in Thailand; northern and southern Nan province,

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Phayao province and Chiang Rai province. The collection period was from October to December in 2014. The plant sample was identified as *Zanthoxylum rhetsa* (Roxb.) DC. A voucher specimen (No.039023) was referenced at CMU Herbarium, Faculty of Science, Chiang Mai University, Thailand.

Fruits were dried in an oven with a constant temperature of 45°C for 24 h until constant weight. Then the products were finely grounded before tested. Even fresh fruits were finely grounded to use.

Chemical and Solvents

Sodium chloride, potassium chloride, disodium phosphate and potassium dihydrogen phosphate were of analytical grade. *N, N*-Dimethyl sulfoxide were of biological reagent grade.

Extraction and isolation

Fresh and dried fruits (500 g) were put into hydrodistillation for 6 h. The volatile oil was dried over anhydrous sodium sulfate. The oil was kept at 4°C in a refrigerator before analyses.

Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectrum of the volatile oil was obtained by ATR technique using IRTracer-100 Shimadzu FTIR spectrometer. The spectrum of volatile oil was taken from 4000 cm⁻¹ to 650 cm⁻¹ wave number range. The spectrum obtained was compared among different sources.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS analysis of volatile oil was performed using a GC 7890A Agilent interfaced to a mass spectrometer MSD5975C (EI) Agilent. The experiment was conducted on a 5MS capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness). The temperature of GC injection inlet was 250°C. Then the column oven was programmed at 60°C (0 min), then increased by 3°C per min to 240°C (0 min). The total run time was 60 min. The injection volume was 0.5 µL and split ratio injection was 50:1. Helium was used as the carrier gas at constant flow-rate of 1.0 mL/min. The temperature of MS quadrupole was 150°C after injection. The ion source temperature was set at 230°C. The electron impact ionization mode was operated at 70 eV; fragment mass range, 30-500 amu. The mass of each compound was compared with mass spectra of references or Wiley libraries or database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components were compared with the spectrum of known components stored in the NIST library.

Biological test

Cell culture

H460 cells (Human large cell lung cancer cell line) were cultured by using Roswell Park Memorial Institute (RPMI) 1640 Medium with L-glutamine (Biowest, France). Whereas MRC-5 cells (Human fibroblast cell lung normal cell line) were cultured by using Eagle's Minimum Essential Medium (EMEM) with sodium bicarbonate, non-essential amino acids, L-glutamine, and sodium pyruvate (Corning Inc., USA). To prepare testing cells, both media were supplemented with 10% fetal bovine serum (Biowest, France), 100 U·mL⁻¹ Penicillin and 100 µg/mL Streptomycin (Gibco, Life Technologies Inc., USA). The cells were cultured under 5% CO₂ humidified incubator at 37°C.

Assessment of cytotoxic activity

All the volatile oils were determined for cytotoxicity in both H460 and MRC-5 cell line. Each cells were seeded in separated 96 well microtiter plates at a concentration of 10,000 cells/well in suitable medium as above

mention. After 24-h incubation, cells were treated with presence or absence of different concentration of volatile oil and incubated for 24 h at 37°C with 5% CO₂. On the experimental day, cells were washed with phosphate buffer solution and 90 µL of PBS containing 0.5 mg/mL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added to each well and incubated for 4 h at 37°C. Then the solution was removed and 100 µL of DMSO were added. The plates were shaken well and measured with spectrophotometric microplate reader at 570 nm within 30 min. The optical density (OD) was obtained using Infinite M200 Pro Nano quant Absorbance Micro plate Reader obtained from Tecan Group Ltd. (Switzerland) at wavelength of 570 nm. The relative cell viability was expressed as a percentage relative to the untreated control cells as following equation:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{\text{treated}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{untreated}} - \text{OD}_{\text{blank}}} \times 100$$

After the percentage of cell viability was calculated, then the mean growth-inhibitory concentration (IC₅₀) values were plotted and fitted using the Prism software version 5.01.

Morphology of treated cancer cell

Morphology of H460 cells were determined after treatment with volatile oil of *Zanthoxylum rhetsa* in three separated 96 well microtiter plates at a concentration of 10,000 cells/well in RPMI supplemented with 10% FBS, penicillin (100 µg/mL), and streptomycin (100 µg/mL). After 24-h incubation, cells were treated with presence or absence of a fixed concentration of extracted volatile oils. After 18-h incubation, the medium was discarded and was washed the cells twice with cold phosphate buffer. The resulting cells were added with binding buffer (50 µL) and stained with Annexin V-FitC and propidium bromide (FitC Annexin V apoptosis detection kit I, BD Bioscience). The cells were examined by using IN cell Analyzer 2000 (GE healthcare company, USA)

Flow cytometric analysis (FACS)

Some active volatile oils were chosen to investigate the apoptosis mechanism of H460 cell by using flow cytometer. After 18-h incubation, cells were stained by FitC Annexin V apoptosis detection kit I and followed BD Bioscience protocol. The stained cells were then analyzed by using BD FACSVerse (BD Bioscience, USA)

RESULTS

Extraction and isolation

The results showed that the volatile oil obtained from dried fruits from various areas displayed higher yields than those obtained from fresh fruits. The highest yield of volatile oil was obtained from dried fruits of Southern Nan (Table 1).

The GC-MS analysis of volatile oil obtained from fresh and dried fruits and the mass spectra of all compounds are illustrated in Table 2. The chromatogram shows twenty seven known compounds and one unknown as noted no attempts to identify the unknown compound were performed. The major component of oil obtained from fresh fruits from Phayao and

Table 1: Yield of volatile oil from Fresh and dried fruits.

Type of fruit	Yield of volatile oil (% v/w)			
	NN	NS	PY	CR
Fresh fruit	11.80	13.60	8.10	8.30
Dried fruit	14.17	15.33	13.17	13.83

(NN: oil derived from northern Nan, NS: oil derived from southern Nan, PY: oil derived from Phayao and CR: oil derived from Chiang Rai)

Table 2: The composition of oil obtained from fresh and dried fruits via GC-MS method.

No.	RT	Compounds	% Relative area							
			NN		NS		PY		CR	
			Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried
1	4.3	Thujen	0.38	0.34	0.65	0.14	0.60	0.63	0.47	0.22
2	4.5	a.Pinene	8.83	8.68	5.89	5.88	5.68	5.99	1.46	7.36
3	5.4	Sabinene	0.63	0.68	26.89	0.42	25.03	25.11	31.21	0.87
4	5.5	b.Pinene	0.23	0.22	-	-	-	-	-	0.17
5	5.9	b.Myrcene	7.42	6.96	4.40	4.56	4.74	4.55	2.41	5.59
6	6.3	a.Phellandrene	22.45	19.40	12.45	10.88	11.87	11.06	2.32	16.65
7	6.4	3-Caren	1.45	1.42	0.71	0.67	0.79	0.83	-	-
8	6.6	a.Terpinene	-	-	0.40	-	0.36	0.38	-	-
9	6.9	o-Cymene	2.11	4.09	2.91	-	2.77	3.49	-	-
10	7.0	b.Phellandrene	28.75	29.70	21.61	-	-	-	-	-
11	7.1	Limonene	-	-	-	59.68	27.10	28.90	49.09	52.19
12	7.2	trans-arOcimene	5.02	4.70	2.69	3.03	2.70	2.67	1.00	3.44
13	7.6	(E)-bEOcimene	9.84	9.23	9.56	11.30	9.53	8.41	8.86	7.84
14	7.9	g.Terpinen	-	-	0.87	-	0.70	0.82	0.49	0.95
15	8.9	a.Terpinolen	0.35	0.28	-	-	0.31	0.29	-	-
16	9.3	Linalool	1.83	1.40	1.03	0.46	0.97	0.99	-	-
17	10.5	Limonene oxide	-	-	-	-	-	-	-	0.23
18	12.2	1-Terpinen-4-ol	-	-	2.11	-	1.98	2.17	0.92	-
19	12.7	b2Fenchyl alcohol	1.02	1.05	1.83	-	1.87	1.80	-	-
20	13.4	Decanal	1.17	1.26	-	-	-	-	-	-
21	13.6	2-Ethyl hexyl acetate	4.33	4.87	-	-	-	-	-	-
22	19.8	1-Methoxy-2-methylthiobenzene	-	-	0.80	-	-	-	-	-
23	19.8	Unknown	-	-	-	0.73	0.73	-	-	-
24	20.7	Lavandulyl acetate	2.41	3.35	-	-	0.66	0.28	-	-
25	21.8	b1Caryophyllen	1.23	1.82	1.45	1.01	0.69	0.71	0.40	1.05
26	24.3	Germacrene d	0.54	0.55	3.10	1.09	0.92	0.90	1.37	2.83
27	24.9	Bicyclogermacrene	-	-	0.65	-	-	-	-	0.42
28	28.0	Spathulenol	-	-	-	0.14	-	-	-	0.21

(NN: oil obtained from northern Nan, NS: oil obtained from southern Nan, PY: oil obtained from Phayao and CR: oil obtained from Chiang Rai), RT = retention time (min)

Chiang Rai was α -limonene (27.10 and 49.09%, respectively). Similar results were also obtained dried fruits (28.90 and 52.19%, respectively).

The major component of the oil obtained from both fresh fruits and dried fruits from northern Nan was β -phellandrene. However, the major components of the oil obtained from both fresh fruits and dried fruits from southern Nan were (+)-sabinene and α -limonene.

Fourier transform infrared spectroscopy (FTIR) analysis

All volatile oils were analyzed by FTIR instrument. The results were displayed spectrum from different sources provide same figure print pattern. (Figure 1)

Biological test

All volatile oils were tested for cytotoxicity against non-small lung cancer cells (H460 cell-line) and lung normal cells (MRC-5 cell line). The results indicated that all volatile oils obtained from fresh fruits and dried fruits were cytotoxic against lung cancer. The cytotoxicity results of all oil

samples obtained from both fresh fruits and dried fruits showed slightly different activity against lung cancer cell. Among all samples, it was found the oil obtained from dried fruits of southern of Nan exhibited the best inhibitory effect on the growth of H460 cells (EC_{50} 1.79 μ L/mL). For cytotoxicity against MRC-5 cells, the oil samples revealed wide range of EC_{50} value, ranging from 2.03 μ L/mL to 7.07 μ L/mL. Our research group also calculated the selectivity index (SI) of oil samples. The SI index was determined by the EC_{50} ratio of tested oil against cancer cells and normal cells. Among the oil samples obtained fresh fruits and dried fruits, the SI_s of oil obtained from fresh fruits from northern of Nan, southern of Nan and Chiang Rai were shown the high selectivity with 2.86, 1.92 and 1.81 μ L/mL, respectively (Table 3, Figure 2).

In the addition, the morphology of untreated H460 cells and treated cell with oil samples at concentration of 2 μ L/mL was also examined. After 18 h of the treatment, the cell was stained with Annexin V-FitC and propidium bromide to investigate the change of membrane in early or late apoptosis stage and nuclei in cell lysis stage, respectively. The images

exhibited the untreated cells, showed a high confluency of monolayer cells, typical growth patterns and a smooth, flattened morphology with normal nuclei. The cells that were treated with oil samples displayed the abnormal morphology changing from round shapes to deformed cell membrane as shown in bright filed view and fluorescent view (Figure 3). Furthermore, we also examined apoptosis via Annexin V-Fit C and PI staining by FACS analysis (Figure 4). Cells were treated with oil obtained from fresh fruits from Chiang Rai. The H460 cells grown in the presence or absence of oil sample for 18 h were washed once in PBS trypsinize to harvest the cells and centrifuge. The resulting cells were washed once

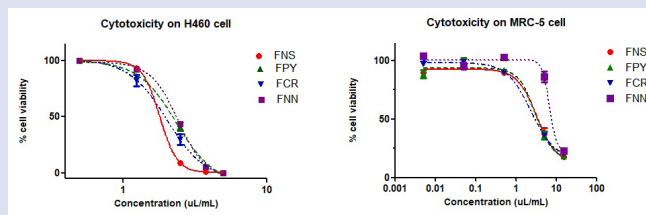


Figure 2: Concentration-response curve of the oil derived from fresh fruit on a) cancer cell line, H460 (b) on normal lung cell line (MRC-5). Values are mean of at least three determinations from separate experiments. (FNN = fresh fruit of northern of Nan, FNS = fresh fruit of south of Nan, FPY = fresh fruit of Phayao, FCR = fresh fruit of Chaing Rai).

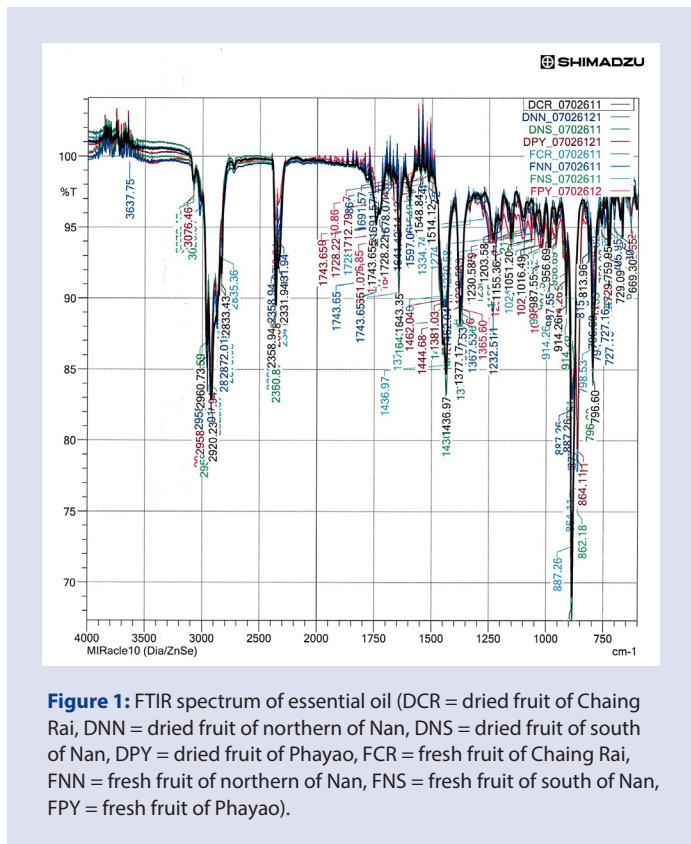


Figure 1: FTIR spectrum of essential oil (DCR = dried fruit of Chaing Rai, DNN = dried fruit of northern of Nan, DNS = dried fruit of south of Nan, DPY = dried fruit of Phayao, FCR = fresh fruit of Chaing Rai, FNN = fresh fruit of northern of Nan, FNS = fresh fruit of south of Nan, FPY = fresh fruit of Phayao).

Table 3: EC₅₀ of volatile oil from fresh and dried fruit against H460 cells and MRC-5 cells and selectivity index (SI).

Sample	H460		MRC-5		SI**	
	EC ₅₀ (µL/mL) ± SD		EC ₅₀ (µL/mL) ± SD		Fresh	Dried
	Fresh	Dried	Fresh	Dried		
NN	2.47 ± 0.09	2.83 ± 0.41	7.07 ± 0.72	2.38 ± 0.36	2.86	0.84
NS	1.91 ± 0.53	1.79 ± 0.43	3.66 ± 1.29	2.06 ± 0.17	1.92	1.15
PY	2.37 ± 0.13	2.00 ± 0.47	2.41 ± 0.17	2.03 ± 0.22	1.02	1.02
CR	2.09 ± 0.40	2.09 ± 0.42	3.78 ± 1.10	2.45 ± 0.64	1.81	1.17
Doxorubicin	0.15 ± 0.02 µM		1.92 ± 0.02 µg/mL***			-

*EC₅₀ values represent the calculated EC₅₀ values for volatile oil. Data presented EC₅₀ values in mean ± SD of at least 3 determinations from separate experiments.

**SI is the selectivity index equal to EC₅₀ of tested compound in a normal cell line /EC₅₀ of the same tested compound in cancer cell line

***The EC₅₀ value from research reference¹⁵

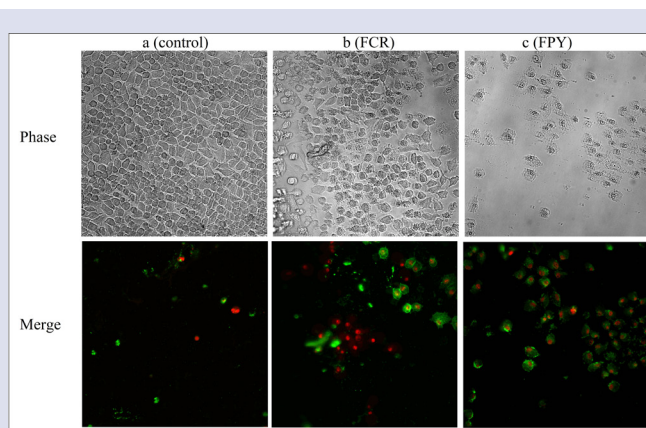


Figure 3: Phase-contrast images and fluorescence image (x40) of H460 cells at approximate logarithmic phase after 18-h treatment with a) vehicle, b) oil obtained from fresh fruits of Chiang Rai (FCR), c) oil obtained from fresh fruits of Phayao (FPY). The cancer cell was exposed to Annexin V-Fit C and propidium iodide and appear green- and red-staining, respectively.

with cold PBS and finally re-suspend cells with binding buffer to obtain approximate 1x10⁶ cells/mL. The harvested cellular DNA were stained with Annexin V-Fit C/PI.

From FACS analysis, the dual parametric dot plots of oil derived from Chiang Rai-treated cells displayed the mainly late apoptotic cells in the upper right quadrant (UR) and low population in the early apoptotic cells in the lower right quadrant (LR), whereas the control showed the viable cell population in the lower left quadrant. These preliminary results show the oil may induce apoptosis in tumor cells.

DISCUSSION

The oil derived from various areas displayed different chemical constituents. The major composition was monoterpenes and the others were oxygenated monoterpene, oxygenated sesquiterpene and hydrocarbon. It was found that monoterpenes such as limonene and sabinene have been report to have antioxidant.² Antioxidants are agents that protect the body from oxidative stress which lead to molecular damages, cardiovascular diseases and cancer.¹³ From southern Nan, the constituents in oil from fresh fruits and dried fruits were distinctly different. The GC-MS results showed nineteen analytes in fresh fruit but only fourteen analytes in dried fruit. The reason was possibly due to the evaporation during the drying process.

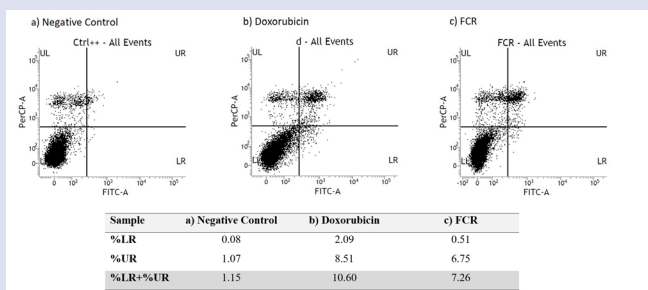


Figure 4: Flow cytometric analysis (FACS) of the distribution of H460 cells treated with the a) negative control b) doxorubicin c) oil sample derived from FCR, Annexin V-PI analysis in H460 cells, following 18 h of compound treatment by Annexin-V-FITC method at IC_{50} concentrations, which are 0.15 μ M and 2 μ L/mL, respectively. Results are representative of one of three independent experiments

SI value displayed the differential cytotoxic activity performance of the tested sample against cancer and normal cells.¹⁴ The high SI value indicates a higher selectivity for cytotoxic activity against H460 cells than MRC-5 cells.

CONCLUSION

In this manuscript, we collected oil from fresh and dried fruits of *Zanthoxylum rhetsa* from different part of Thailand. We determined the composition of the oil, showing that the major components are α -limonene (Phayao, southern Nan and Chiang Rai), β -phellandrene (northern Nan) and (+)-sabinene (southern Nan).

In addition, the volatile oil showed considerably cytotoxic activity against lung cancer cell, H460 and some oil samples obtained from fresh fruit had tendency to be more selectivity on cancer cell. The cytotoxic activity possibly from some essential oil possessing anticancer properties and this is an interesting outcome which will lead us to further study in deep the biological activity of individual components.

The principle of Annexin V-Fit C staining is based on the change of membrane. When the cell are undergo apoptosis, the lipid phosphatidylserine is translocate and lead to bind with Annexin V-Fit C and show the green staining. In addition, propidium iodide (PI) is a popular red fluorescent to distinct between viable and necrosis cell. PI is able to stain DNA nucleus or DNA-containing organelles in which late apoptosis or dead cells.

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CONFLICT OF INTEREST

There is no conflict of interest.

ABBREVIATIONS

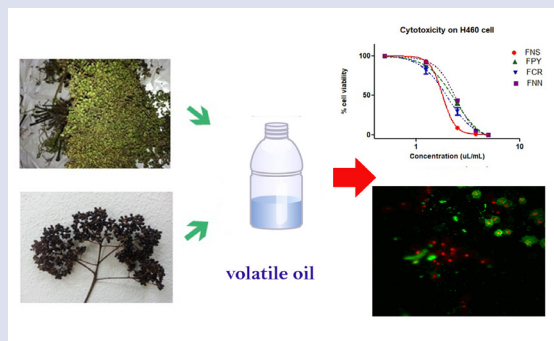
CR: Oil derived from Chiang Rai; **FCR:** Fresh fruit of Chaing Rai; **FNN:** Fresh fruit of northern of Nan; **FNS:** Fresh fruit of south of Nan; **FPY:** Fresh fruit of Phayao; **NN:** Oil derived from northern Nan; **NS:** Oil derived from southern Nan; **PY:** Oil derived from Phayao; **RT:** Retention time.

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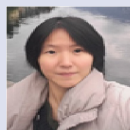
GRAPHICAL ABSTRACT



SUMMARY

- Twenty-eight compounds were identified from the volatile oil of *Zanthoxylum rhetsa* (Roxb.) DC. fruits.
- The oil was analyzed by GC-MS technique and found that limonene, β -phellandrene and sabinene were the major components.
- The volatile oil exhibited a strong cytotoxicity against H460 human large cell lung cancer cell line.

ABOUT AUTHORS



Dr. Sewan Theeramunkong Working as a Assistant Professor in Division of Pharmaceutical Science, Faculty of Pharmacy, Thammasat University, Thailand. Her research area is the synthesis of bioactive compounds and discovery of novel scaffold for antitumoral agents. In addition, she also examines the anticancer properties of some traditional medical plants in Thailand. Recently, she has been interested in synthesis of compounds with antimalarial activity.



Dr. Maleeruk Utsintong Working as a Assistant Professor and Researcher at the School of Pharmaceutical Sciences, University of Phayao, Thailand. Her research involves drug design, synthesis of compounds, bioactivity and phytochemical studies resulting over 10 publications, 3 books and a petty patent.