Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(5): 67-70
Received: 10-07-2020
Accepted: 26-08-2020

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Inhibitory effect on nitric oxide production of essential oil from *Zanthoxylum rhetsa* (Roxb.) dc. fruit

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DOI: https://doi.org/10.22271/phyto.2020.v9.i5a.12747

Abstract
Genus *Zanthoxylum* L. comprises about 200 species distributed in Asia, Africa, Australia and North America [3]. In Vietnam, there are about 12 species belonging to genus *Zanthoxylum* [1]. Many species in this genus are used as medicine, as spices or sources of essential oils. The fruit of *Zanthoxylum rhetsa* (Roxb.) DC. (Vietnamese name: "Sẻn hôi") was collected in Ha Quang district (Cao Bang province). Microscopical characteristics of the fruit were investigated using light microscope. From fruit of *Z. rhetsa*, the essential oil was obtained by hydrodistillation. The gas chromatography combined mass spectrometry (GC/MS) analyse of essential oil led to the identification 25 compounds, accounting for 98.72% of the total essential oil content. The main components are Sabinene (64.80%), Terpinen-4-ol (6.07%), β-Pinene (4.81%), α-Pinene (4.09%), 1.8-Cineol (2.49%) and β-Phellandrene (2.90%). The essential oil of *Z. rhetsa* strongly inhibited NO production, with IC_{50} value was 16.42 ng/ml. These results demonstrate that essential oils from the fruit of *Z. rhetsa* possesses excellent anti-inflammatory activity and thus have great potential in treatment of inflammation.

Keywords: *Zanthoxylum rhetsa*, microscopic characteristics, essential oil, gc/ms, anti-inflammatary

1. Introduction
Zanthoxylum rhetsa (Roxb.) DC. is a shrub plant belonging to the family Rutaceae. The different parts of the plant have been used medicinally for a long time. In more detail, stem bark and root bark of *Z. rhetsa* are used to treat malaria, rheumatism, loss of stomach tone; fruit could be used in the treatment of diarrhea and rheumatism. The essential oil from *Z. rhetsa* possessed the ability to inhibit breast cancer cell proliferation and cell viability and moderate antioxidant activity [9]. In this study, we aimed to investigate microscopical characteristics and essential oil composition of *Z. rhetsa* fruit as well as to evaluate the inhibittory effect of nitric oxide production of the essential oil. These results were useful to identify the *Z. rhetsa* fruit and use this medicinal plant more effectively.

2. Material and method
2.1 Plant material
The whole plant of *Zanthoxylum rhetsa* were collected at Hoa Muc hamlet, Truong Ha village, Ha Quang district, Cao Bang province in July 2020. The plant was authenticated by Mr. Nguyen Van Hieu and Mr. Dang Minh Tu, National Institute of Medicinal Materials. A voucher specimen (TB-15720) was deposited at Hebarium of National Institute of Medicinal Materials.

2.2 Microscopic characteristics
All microscopical investigations of fruit were done using microscope Leica. The powder characteristics of fruit were investigated, described and illustrated with pictures (Fig. 1).

2.3 Essential oil extraction
The essential oil was obtained from the dried fruits of *Zanthoxylum rhetsa* by hydrodistillation. The yield of essential oil was 1%, expressed in milliliters of obtained oil relative to 100 g of dry material. Then the essential oil was dried over anhydrus sodium sulfate and stored in a sealed vial at 10 ºC in a dark prior to analysis.

2.4 Gas Chromatography (GC)
The essential oil was dissolved in chloroform (1‰, v/v). The samples were analyzed using a GC–MS system (Agilent Gas chromatograph model 7890A equipped with MSD 5975C). Helium (1 mL/min) was used as a carrier gas. Injector and detector temperatures were 250 °C and 230 °C, respectively. Column DB-5MS (5% Phenyl Methyl Siloxane, 30 m x 0.25 mm x 0.25 µm) (Agilent) was used. The column temperature was programmed from 60 °C, hold for 15 minutes, after that gradually increase 3°C/min to 220°C and hold for 2 minutes. Injection volume is 1 µL.

2.5 Identification of Compounds
Retention indices of oil constituents on the column DB-5MS were detemined using an alkane standard solution C8-C20 (Aldrich Chemical Company, USA) analysed in the same condition. Individual compounds in the oil were identified by comparision of their mass spectra and retention indices with those in GC/MS libraries (NIST 08, Wiley 09) and/or with those reported in the libratures.

2.6 Biological assay
The anti-inflammatory activity of essential oil was tested by measuring the production of NO in lipopolysaccharide (LSP)-activated RAW 264.7 macrophage cells [8]. In the model of anti-inflammatory activity on RAW cell 264.7, RAW 264.7 macrophage was stimulated inflammation by LPS. RAW cell 264.7 responds to this stimulation of LPS by intracellular regulation and NO production. Experiments to evaluate the anti-inflammatory activity of essential oils on this macrophage line were evaluated through the ability of cells to reduce NO secretion. The anti-inflammatory activity of the essential oil was assessed by the NO concentration of the excreted macrophages [5, 2]. The controls were conducted in parallel with the dexamethasone condition.

3. Results and discussions
3.1 Microscopical characteristics of Z. rhetsa fruit powder
A very dark reddish-brown powder with an aromatic odour and spicy, rather bitter. The diagnostic characters are:

1. Fragment of epicarp with thick-wall cells
2. Fragment of mesocarp
3. The parenchyma of the mesocarp composed oil cells occur scattened.
4. Part of a group of fibro vascolar tissue
5. The endocarp composed of a layer of thin-walled, lignified cells which are elongated in surface view and arranged in groups with the long axes of the adjacent groups approximately parallel to one another.
6. The slereids vary in size and shape but are usually polygonal to restangular with strongly thickened walls and fairly numerous rounded.
7. The fiber is fairly long.
8. Crystal of calcium oxalate

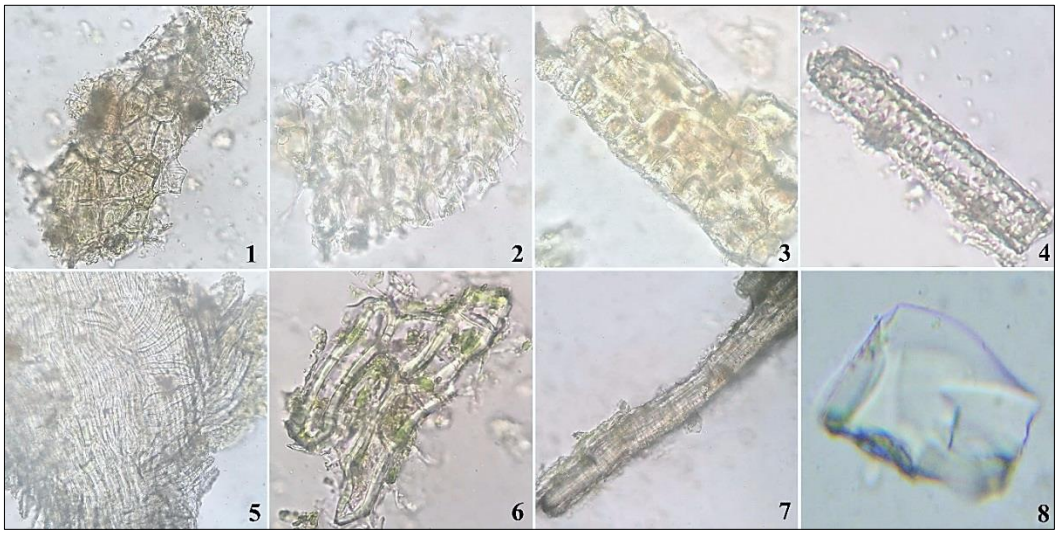

Fig 1: Powder characteristics of *Zanthoxylum rhetsa* fruit (observed at objective lens 40x) 1. Epicarp; 2. Mesocarp; 3.Sclereids and oil cells from mesocrp; 4. Part of a group of fibro vascolar tissue; 5. Endocarp; 6. Elongated sclereids from the epicarp; 7. Fiber; 8. Crystal of calcium oxalate.

3.2. Gas chromatography
The essential oil was qualitatively and quantitatively analyzed by GC-MS. About 25 compound were identified in essential oil of *Zanthoxylum rhetsa*. (Table 1). The GC-MS chromatogram was showed in Fig. 2.

Table 1: Chemical constituent of *Zanthoxylum rhetsa* essential oil

No.	Compounds	Formula	RT (min)	RI	% Area
1	α-Thujene	$C_{10}H_{16}$	6.739	918	0.62
2	α-Pinene	$C_{10}H_{16}$	7.073	924	4.09
3	Sabinene	$C_{10}H_{16}$	9.277	962	64.80
4	β-Pinene	$C_{10}H_{16}$	9.492	966	4.81
5	β-Myrcene	$C_{10}H16$	10.392	982	1.26
6	α-Phellandrene	$C_{10}H_{16}$	11.587	1002	0.32
7	α-Terpinene	$C_{10}H_{16}$	12.506	1012	1.36
8	p-Cymene	$C_{10}H_{14}$	13.211	1019	0.43
9	β-Phellandrene	$C_{10}H_{16}$	13.735	1024	2.90
10	1,8-Cineole	$C_{10}H_{18}O$	13.892	1026	2.49
11	γ-Terpinene	$C_{10}H_{16}$	16.711	1055	2.22

12	cis-Sabinenehydrate	$C_{10}H_{18}O$	17.820	1067	1.06
13	1-Octanol	$C_8H_{18}O$	18.282	1072	1.08
14	α-Terpinolene	$C_{10}H_{16}$	19.149	1081	0.49
15	p-menth-2-en-1ol	$C_{10}H_{18}O$	20.444	1094	0.84
16	Linalool	$C_{10}H_{18}O$	20.644	1096	0.76
17	trans-Sabinenehydrate	$C_{10}H_{18}O$	22.244	1118	0.27
18	cis-2-Cyclohexen-1-ol, 1-methyl-4-(1 methylethyl)	$C_{10}H_{18}O$	22.682	1125	0.19
19	Unknown monoterpene		24.811	1156	0.26
20	Terpinen-4-ol	$C_{10}H_{18}O$	26.078	1174	6.07
21	α-Terpineol	$C_{10}H_{18}O$	27.040	1188	1.68
22	Decanal	$C_{10}H_{20}O$	27.978	1202.	0.38
23	1-Decanal	$C_{10}H_{22}O$	31.735	1270	0.35
24	β-Caryophyllene	$C_{15}H_{24}$	38.506	1408	1.12
25	α-Humulene	$C_{15}H_{24}$	40.106	1444	0.13
Number (%) of constituents identified				24 (98.72%)	
Number (%) of monoterpene hydrocarbons				11 (83.3%)	
Number (%) of oxygenated monoterpenes				8 (13.36%)	
Number (%) of sesquiterpene hydrocarbons				2 (1.25%)	
Number (%) of oxygenated sesquiterpenes				0	
Number (%) of other				3 (1.81%)	
RI: retention indices determined on DB5-MS column **RT:** retention time (min)					

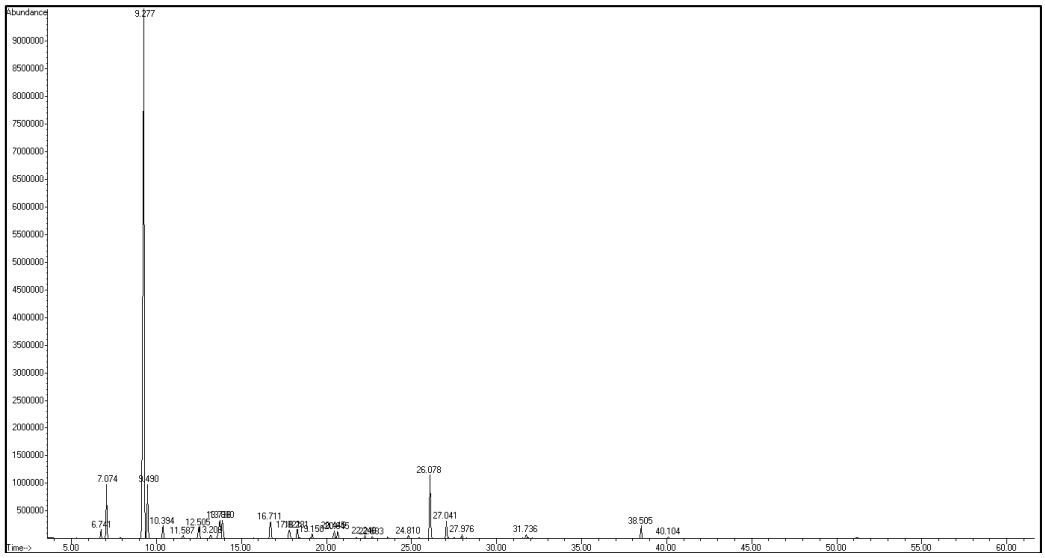

Fig 2: The GC-MS chromatogram of *Zanthoxylum rhetsa* essential oil

As shown in Table 1, Monoterpene hydrocarbons are a large propotion of all components and The major components of the essential oils are sabinene (64,80%), Terpinen-4-ol (6.07%), β-Pinene (4.81%), α-Pinene (4,09%), β-Phellandrene (2.90%) and 1,8-Cineol (2,49%). The contents of the remaining components are below 2%. The previous study reported that the major chemical constituents of the essential oil of *Z. rhetsa* collected in Thailand are sabinene (56.62%) [4, 9]. Besides, Germarene (10.10%) and Bicyclogermarene (2.94%) were also detected in the essential oil [4]. However, Germarene and Bicyclogermarene couldn't be detected in *Z. rhetsa* oil in this study. In contrast, chemical constituents of the essential oil of *Z. rhetsa* seed coat and pericarp from India and Jordan showed terpinen-4-ol as a major component (32.1%-Indian, 25.43%-Jordan) [6 7].

3.3. Anti-inflammatory activity

The essential oil of *Z. rhetsa* (ZR) showed significant inhibitory effect of NO production with the IC_{50} value was 16.42 ng/mL. In addition, MTT assay showed that concentration up to 100 ng/ml produced no significant cytotoxic effects on cells treated with *Z. rhetsa* essential oil.

Table 2: Anti-inflammatory activity of essential oil from *Zanthoxylum rhetsa*

Sample	Concentration	I% (inhibitory)	CS% (cell survival)
Cardamonin	0,3 µM	45,85 ± 2,1	86,47 ± 0,2
	3,0 µM	86,93 ± 0.9	73,8 ± 0,5
ZR	100 ng/mL	65,10 ± 1,2	85,63 ± 2,4
	30 ng/mL	54,41 ± 1,6	93,97 ± 1,0
	10 ng/mL	47,34 ± 0,6	90,10 ± 1,1

4. Conclusions

The powder characteristics of *Zanthoxylum rhetsa* (Roxb.) DC. fruit were investigated using Light microscope. The yield of essential oil in fruit was 1% (ml/g) calculated on dry material. GC-MS analyse led to identification of about 25 compounds in essential oil. Among them, sabinene (64,80%),

Terpinen-4-ol (6.07%), β-Pinene (4.81%), α-Pinene (4,09%), β-Phellandrene (2.90%) and 1,8-Cineol (2,49%) were main components. The essential oil exhibited excellent anti-inflammatory effect with IC_{50} value was 16.42 ng/mL.

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