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CYTOTOXIC AND ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS FROM THE FRUITS OF *ZANTHOXYLUM RHETSA* GROWN IN THANH HOA PROVINCE, VIETNAM

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Summary

Cytotoxic and Antimicrobial Activities of Essential Oils from the Fruits of *Zanthoxylum rhetsa* Grown in Thanh Hoa Province, Vietnam

The essential oils from the fresh and dried fruits of *Zanthoxylum rhetsa* grown in Thanh Hoa province were obtained by hydrodistillation and analyzed by gas chromatography (GC-FID) and gas chromatography/mass spectrometry (GC/MS). The major constituents of fresh fruit essential oil (FF) and dried fruit essential oil (DF) were sabinene, limonene, β-phellandrene, and α-pinene, but their contents in two essential oils were not the same, these values are 51.95%, 7.84%, 7.69%, 3.82% in FF and 32.88%, 20.95%, 9.20%, 4.22% in DF, respectively. In addition, α-phellandrene presents at high content in the DF (10.23%) but it is only the minor component in the FF (0.65%). The EOs were also tested for their cytotoxic and antimicrobial activities. The IC_{50} values revealed that these two essential oils exhibited a moderate activity against two tested cancer cell lines (MCF-7 and HeLa). For the antimicrobial activities, the FF showed its inhibitory effect on *E. coli* and *B. subtillis* (MIC values of 100 µg/mL), while the DF exhibited its potential antimicrobial activity against *E. coli, F. oxysporum* (MIC values of 100 µg/mL) and weak activity against *P. aeruginosa* (MIC value of 200 µg/mL).

Keywords: *Zanthoxylum rhetsa, Essential oil, Rutaceae, Thanh Hoa province, Vietnam.*

1. Introduction

Zanthoxylum rhetsa (Roxb.) DC, a flowering plant of the Rutaceae family, is found in India, Myanmar, Thailand, Laos and Vietnam. This is the medium-sized trees about 14 - 18 meter-height, straight body, thorny bark of trunk and

branches, leaflets lanceolate with 10 - 15 cm long, infloescence pubescent, follicles, seeds black, bloom in June and July with clusters of gray-white flowers, fruit in October and November [1],[2]. *Z. rhetsa* called "Mắc khén" in Vietnam, is a indigenous plant in Thanh Hoa province. Since long time ago, the fruits are used as a popular spice in ethnic minorities especially Thai, Dao and H'Mong people, most of them use the seeds in their daily meals [3],[4].

There are some papers that showed the chemical composition of essential oil (EO) from the fruits of *Z. rhetsa* growing in India, Thailand and Jordan [5],[6],[7],[8],[9],[10],[11]. For the *Z. rhetsa* collected from the Kerala district, southern India, the main components of fresh green coloured seed (before ripening) EO were sabinene (47.12%), α-terpineol (7.73%), terpinen-4-ol (6.61%), β-pinene (5.99%), limonene (4.06%), α-pinene (3.87%), γ-terpinene (3.64%), α-terpinene (3.45%) and *p*-cymene (3.08%) [5], while the major constituents of fresh greenish black seed EO were sabinene (66.3%), α-pinene (6.6%), β-pinene (6.3%) and terpinen-4-ol (3.5%) [6]. Influence of pH on chemical composition of fresh greenish-black seed EO was also tested: sabinene (66.7% and 72.7%), β-pinene (6.5% and 6.6%), α-pinene (6.1% and 6.1%) and myrcene (1.5% and 1.6%) were the major components obtained from aqueous and alkaline media, respectively, while α-terpinene (23.7%), γ-terpinene (23.1%), terpinolene (5.7%) and limonene (4.7%) were the main components from acid medium [7]. For the *Z. rhetsa* collected from the Senapati district, northeast India, terpinen-4-ol (32.1%), α-terpineol (8.2%), sabinene (8.1%), β-phellandrene (7.4%) and 2-undecanone (7.1%) were the major constituents of dried seed coat EO [8]. For the *Z. rhetsa* collected from some areas of Thailand, limonene (27.10% - 59.68%) and α-phellandrene (10.88% - 19.40%) were the major components in dried fruit EOs, while sabinene (25.03% - 31.21%) is the main component of fresh fruit EOs, dependently on the geographic area (Nan, Phayao and Chiang Rai) [9]. For the fresh fruit EO of *Z. rhetsa* collected from Phayao of Thailand, terpinene-4-ol (32.33%) and sabinene (22.51%) were the major components [10]. This is similar to that of pericarp EO of *Z. rhetsa* collected from Jordan with terpinen-4-ol (25.43%) and sabinene (16.50%) were the main components [11].

In Vietnam, the chemical composition and biological activity of fruit essential oil of *Z. rhetsa* are also of great interest to study. Some results have shown that its chemical composition depends on the geographical location of *Z. rhetsa*. The EO from the fruits of *Z. rhetsa* collected from Son La province has sabinene as the major component (31.08%) [18], while the main ingredient of EO from the fruits of *Z. rhetsa* collected from Hoa Binh province was benzene-1-methoxy-4 (1-propenyl) (48.96%) [19].

In our previous reports [20], the major constituents of fresh fruits and dried fruits EOs of *Z. rhetsa* collected from Thuan Chau district, Son La province were sabinene (41.13% and 33.71%), terpinolene (27.05% and 30.37%), limonene (7.30% and 8.29%), and terpinen-4-ol (5.35% and 7.73%). In this publication, we continue to study on the chemical composition and biological activity of the fruit essential oils of *Z. rhetsa* collected from Quan Son district, Thanh Hoa province.

2. Methodology

2.1. Materials

The fresh green-coloured fruits of *Zanthoxylum rhetsa* (Roxb.) DC were collected from Quan Son, a district of Thanh Hoa province in Vietnam (November 2019). The plants were identified by Dr. Nguyen Quoc Binh, Vietnam Museum of Nature (VMN), Vietnam Academy of Science and Technology (VAST). The dried fruits of *Z. rhetsa* were obtained by drying the fresh green-coloured fruits in the shade. A voucher specimen (MK.11.2019) of the plant are kept at the Institute of Natural Products Chemistry (INPC), VAST.

2.2. Isolation of essential oils

The EOs of fruits of *Z. rhetsa* (fresh green-coloured fruits - FF and dried fruits - DF, 200 gram each), were obtained by hydrodistillation using a clevenger-type apparatus for 3 hours at normal perssure. The EOs were dried with anhydrous sodium sulphate, stored in dark glass vial in the refrigerator until analysis.

2.3. Gas chromatography combined with mass spectrometry and flame ionization detection

The chemical compositions of essential oils were analyzed by Gas chromatography (GC) Agilent Technologies HP7890A equipped with a mass spectrum detector (MSD) Agilent Technologies HP5975C and a HP5MS column. The dimensions of the column are 60 m x 0.25 mm, film thickness 0.25 µm. The injector was set at 250°C. The temperature program was 60°C ramp of 4°C/min up to 240°C. The carrier gas was Helium at a flow rate of 1 $mL.min^{-1}$. The

split ratio was 100:1 and the volume inject was 1 µL of essential oils. The MSD conditions include full scan modes under electron impact ionization voltage 70 eV, emission current 40 mA, acquisitions scan mass range 35-350 amu.

Retention time indice RI of each component was determined relative to the retention times of a homologous *n*-alkane series with the same GC program. The relative amounts of individual components were calculated based on the GC-FID peak areas.

The identification of the constituents was performed by comparing their RI and their mass spectrum with those from HPCH1607, W09N08 libraries and NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/) database.

2.4. Antimicrobial assays

Antimicrobial activity assay was performed on a 96-well microplate to determine the minimum inhibitory concentration (MIC) against 4 bacterial strains, i.e. Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 25923), Gram-positive bacteria (*Bacillus subtilis* ATCC27212, *Staphylococcus aureus* ATCC12222), and three strains of fungi (*Aspergillus niger* ATCC 9763, *Fusarium oxysporum* ATCC 48112, *Candida albicans* ATCC 10231). The fresh microorganisms were diluted with the growth medium broth to a final inoculum size of about 10^5 colony-forming units (CFU) per mL. The samples were dissolved in 5% DMSO at various concentrations and then were loaded into 96-well microplates with test microorganisms. The positive references were used such as Gentamycin (16 - 8 - 4 IU/mg), doxycycline (0,4 - 0,2 - 0,1 IU/mg) and nystatin (12 - 6 - 3 IU/mg). A blank control was treated in the same way using 5% DMSO instead of the test samples [12].

2.5. Cell proliferation assay

The Hep-G2 (Hepatocellular carcinoma), HeLa (Cervical cancer), MCF-7 (Human breast adenocarcinoma), A-549 (Human lung adenocarcinoma epithelial), HGC-27 (Human stomach carcinoma) cell lines were obtained from ATCC (Manassas, VA, USA) and maintained at 37°C in 5% CO_2 in suitable media (RPMI 1640, MEM, DMEM; Sigma Aldrich Inc., Saint. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 UI/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM).

Cell viability was assessed through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Dilute cells in 96-well microplates to a density of 5×10^4 cells per well of 200 µL mixture. The samples at different concentrations ranging from 0.63 to 5 µg/mL, DMSO and ellipticine used as controls were added to the cells and incubated at 37 °C and 5% CO_2 for 48 h. At the end of incubations, 20 µL of MTT (Sigma-Aldrich) was added to the wells and incubated for at 37 °C for 4 h. Absorbance was recorded at 540/720 nm by using a Spark multimode reader (Tecan, Männedorf, Switzerland). All the experiments were repeated at least thrice independently. The growth inhibition was assessed using the following formula: Inhibition rate (%) = $(1 - OD_{saml}/OD_{con}) \times 100\%$ where OD_{sampl} and OD_{con} are the optical densities of the experimental sample groups and control, respectively [13],[14].

3. Results and Discussion

3.1. Chemical composition of Zanthoxylum rhetsa essential oils

Essential oils from the fresh fruits and dried fruits of *Z. rhetsa* were obtained by hydrodistillation. The chemical composition of EOs were analyzed by GC-MS/GC-FID. The results are shown in Table 1, Fig. 1.

Table 1. Chemical composition of essential oils from the fresh fruits and dried fruits of *Z. rhetsa* collected in Thanh Hoa province

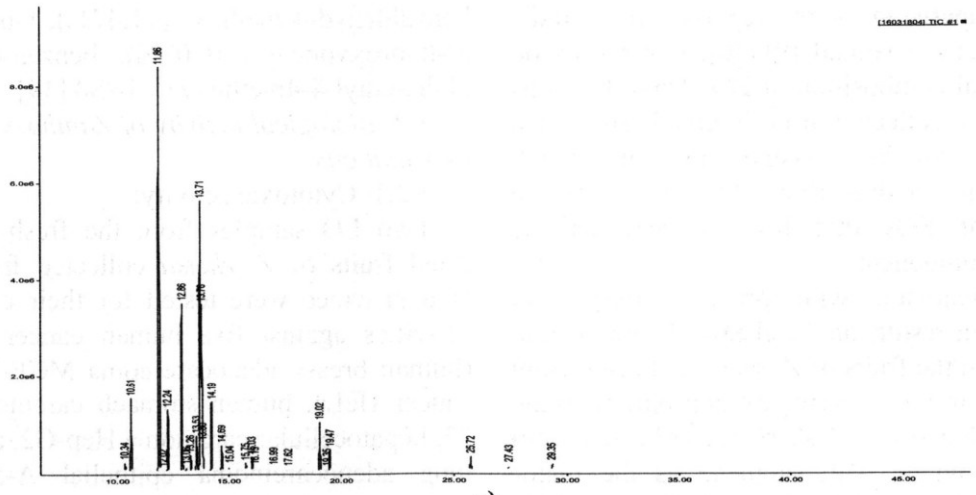
N^O	Compound name	RI$^{a/b}$	RI	FF	DF
1	α-Thujene	930	930	0.48	0.54
2	α-Pinene	939	939	3.82	4.22
3	Sabinene	975	978	51.95	32.88
4	β-Pinene	979	984	0.19	0.15
5	Myrcene	991	991	3.41	3.42
6	α-Phellandrene	1003	1010	0.65	10.23
7	δ-3-Carene	1031	1016	0.27	0.30
8	α-Terpinene	1017	1021	-	0.94

9	*o*-Cymene	1026	1029	6.15	2.25
10	Limonene	1029	1034	7.84	20.95
11	*β*-Phellandrene	1030	1035	7.69	9.20
12	*(Z)*-*β*-Ocimene	1037	1037	0.69	1.06
13	*(E)*-*β*-Ocimene	1050	1048	0.88	3.86
14	*γ*-Terpinene	1060	1063	-	1.48
15	*cis*-Sabinene hydrate	1070	1072	0.37	0.33
16	Terpinolene	1089	1094	-	0.48
17	Linalool	1097	1101	1.39	0.83
18	*trans*-Sabinene hydrate	1098	1104	0.28	0.26
19	*cis*-*p*-Menth-2-en-1-ol	1122	1128	0.26	0.21
20	*trans*-*p*-Menth-2-en-1-ol	1141	1145	0.14	0.14
21	Terpinen-4-ol	1177	1186	2.51	2.99
22	Cryptone	1183	1190	0.82	0.17
23	*α*-Terpineol	1189	1197	2.39	1.31
24	Decanal	1202	1206	0.16	-
25	Octyl acetate	1214	1210	0.11	-
26	unknown (43, 150, RI 1258)		1258	1.59	-
27	unknown (43, 152, RI 1328)		1328	1.50	-
28	Geranyl acetate	1381	1383	1.16	0.83
29	*β*-Caryophyllene	1419	1437	-	0.35
30	Germacrene D	1485	1498	-	0.49
	Total			96.70	99.87
	Monoterpene hydrocarbons			84.02	91.48
	Oxigenated monoterpenes			9.59	7.55
	Sesquiterpene hydrocarbons			-	0.84
	Unknown			3.09	-

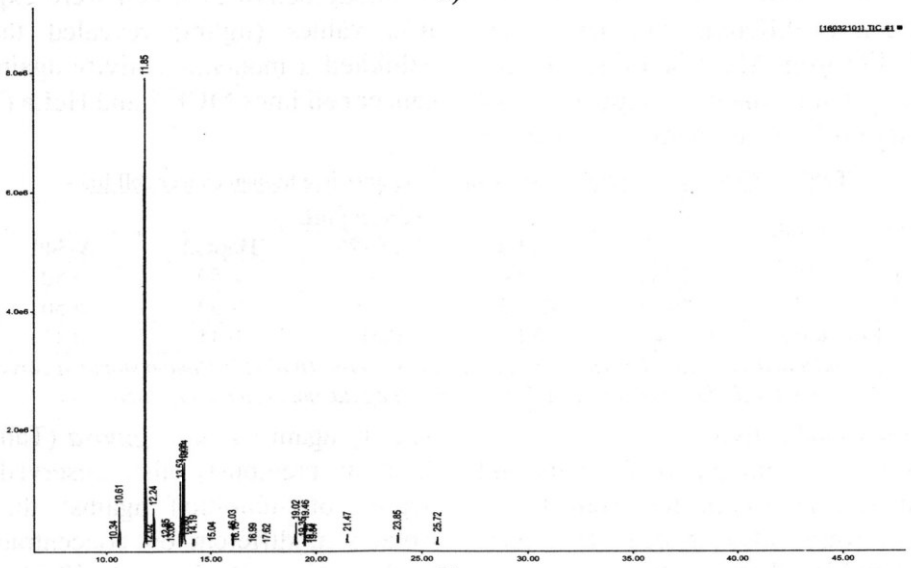
$RI^{a/b}$: Retention index compared between software prediction [15],[16],[17], DF: dried fruit; FF: fresh fruit.

For the fresh fruit EO (FF), 26 components were identified, amounting to 96.70% of the total oil. The monoterpene hydrocarbon fraction (84.02%) was the most plentiful in the EO, with sabinene (51.95%) as the ascendent component, followed by limonene (7.84%), *β*-phellandrene (7.69%), *α*-pinene (3.82%) and myrcene (3.41%). The oxygenated monoterpene fraction had a lower content (9.59%) in which the main components were terpinen-4-ol (2.51%), *α*-terpineol (2.39%), linalool (1.39%) and geranyl acetate (1.16%). An aromatic hydrocarbon compound, *o*-cymene, presents in relatively high concentrations of this EO (6.15%). The unknown compounds were 3.09% of the total of EO, however, sesquiterpene hydrocarbons and oxygenated sesquiterpenes were absent in the EO.

In 25 components identified from the dried fruit EO (DF) (amounting to 99.87% of the total oil), monoterpene hydrocarbons were mainly constituents (91.48%) with sabinene (32.88%) being the most abundant compound, followed by limonene (20.95%), *α*-phellandrene (10.23%), *β*-phellandrene (9.20%), *α*-pinene (4.22%) and myrcene (3.42%). Oxigenated monoterpene formed 7.55% of the DF, with terpinen-4-ol (2.99%) and *α*-terpineol (1.31%) being the major components of this fraction. As the FF, oxigenated sesquiterpenes were absent in the DF. However, in contrast to FF, sesquiterpene hydrocarbons present in the DF, but with low content (0.84%). In the others, *α*-terpinene, *γ*-terpinene, terpinolene, *β*-caryophyllene, and germacrene D were only identified in DF, while decanal and octyl acetate were presented in FF. Especially, *α*-phellandrene presents at hight content in the DF (10.23%) but it's only the minor component in the FF (0.65%). The aromatic hydrocarbon compound, *o*-cymene, presents with relatively high amount in FF (6.15%) but only in a smaller amount in the DF (2.25%) (Fig. 2).



a)



b)

Fig. 1. Chromatography of a) DF essential oil and b) FF essential oil

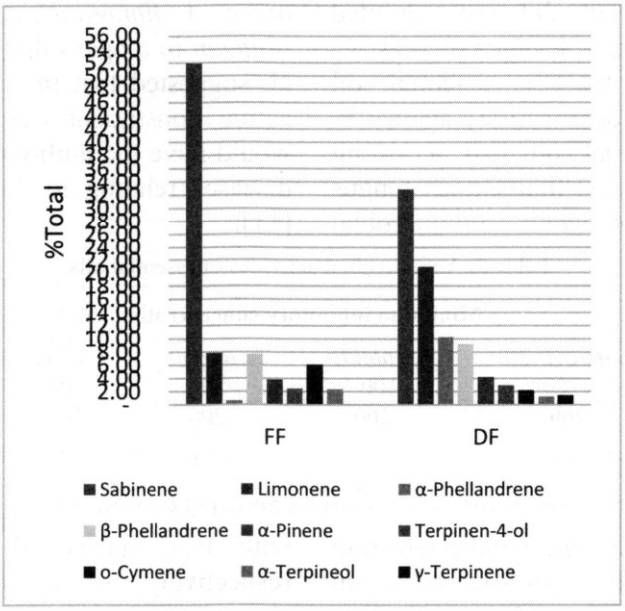


Fig. 2. The main components of EOs

In comparision with reports from India [5],[6],[7] and Thailand [9],[10], our results on the chemical composition of EOs from the fruits of *Z. rhetsa* collected from Vietnam showed a group of similar components in which monoterpene hydrocarbon fraction was an essential of EOs that had sabinene as the dominent component.

In comparision with previous reports in Vietnam, our results on the chemical composition of EOs from the fruits of *Z. rhetsa* collected from Thanh Hoa province were also appropriate to the EOs from the fruits of *Z. rhetsa* collected from Son La province with sabinene as the major component (31.08%) [18] and 33.71 - 41.13% [20]. However, it is different than that of the results of fruit EO from Mai Chau district, Hoa Binh province with main components of benzene-1-methoxy-4 (1-propenyl) (48.96%), benzaldehyd-4-methoxy (11.47%), 1-butanone-1-(4-hydroxyphenyl) (6.07%), benzen-emethanol, alpha-ethyl-4-4methoxy (5.16%) [19].

3.2. Biological activity of Zanthoxylum rhetsa essential oils

3.2.1. Cytotoxic activity:

Two EO samples from the fresh fruits and dried fruits of *Z. rhetsa* collected from Thanh Hoa province were tested for their cytotoxicity activities against five human cancer cell lines (human breast adenocarcinoma MCF-7, cervical cancer HeLa, human stomach carcinoma HGC-27, hepatocellular carcinoma Hep-G2, and human lung adenocarcinoma epithelial A-549). The cytotoxic activities which were expressed under IC_{50} values (µg/ml) revealed that two EOs exhibited a moderate activity against two tested cancer cell lines MCF-7 and HeLa (Table 2).

Table 2. Cytotoxic activity of essential oils against five human cancer cell lines

N°	Samples	IC_{50}, µg/mL				
		MCF-7	HeLa	HCG-27	HepG2	A-549
1	DF	29.41	46.94	> 50	> 50	> 50
2	FF	30.51	47.25	> 50	> 50	> 50
Ellipticine		0.42	0.36	0.51	0.34	0.35

MCF-7: Human breast adenocarcinoma cells; HeLa: Cervical cancer cells; HGC-27: Human stomach carcinoma cell; Hep-G2: Hepatocellular carcinoma; A-549: Human lung adenocarcinoma epithelial cells.

3.2.2. Antimicrobial activity:

Two EO samples from the fresh fruits and dried fruits of *Z. rhetsa* collected from Thanh Hoa province were also tested for their antimicrobial activities. The results demonstrated that the two EOs showed a strong antimicrobial activity against *E.coli* but did not inhibited bacteria *S. aureus* and mold *A. niger* or yeast *C. albicans*. Furthermore, the FF essential oil showed a strong antimicrobial activity against *B. subtillis*; the DF essential oil had a strong antimicrobial activity against filamentous fungus *F. oxysporum* and moderate antimicrobial activity against *P. aeruginosa* (Table 3). Kro H. J. et al previously also observed the various degree of inhibition against the test fungal isolates at different oil concentration from the fresh leaves of *Z. rhetsa*. At 12.5% concentration it showed highest activity against *Aspergillus niger*, *A. fumigatus*, *A. flavus* and *Penicillium italicum* in a agar dilution test [21]. Naik R.R. et al. suggested that the pericarp EO and its main active constituent (terpinen-4-ol) of *Z. rhetsa* would have the ability of handling the stress and diseases relating to the stomach and intestines [11].

Table 3. Antimicrobial activities of essential oils

Samples	Minimum inhibitory concentration (MIC, µg/mL)*						
	E. coli	*P. aeruginosa*	*B. subtillis*	*S. aureus*	*A. niger*	*F. oxysporum*	*C. albicans*
FF	100	>200	100	>200	>200	>200	>200
DF	100	200	>200	>200	>200	100	>200
Positive control*	5.9	11.0	9.1	17.8	6.7	3.3	2.1

** Positive control: doxycycline for Gr(-) and gentamicin for Gr(+) bacteria, nystatin for fungi, respectively.*

The fresh fruit EO of *Z. rhetsa* collected from Phayao, Thailand showed a cytotoxic effect on breast cancer cells (MCF-7 and MDA-MB-231) with IC_{50} values of 1.96 and 1.98 µg/mL, respectively, so could be applied as food preservatives and suggested to develop into

anticancer drug. This EO also exhibited broad spectrum antibacterial activity with the MIC/MBC values of 256/256 mg/mL against Gram-positive bacteria (*Listeria monocytogenes* DMST 17303, *Bacillus cereus* DMST 5040, *Staphylococcus aureus* DMST 8840) and Gram-negative bacteria (*Salmonella typhi* DMST 5784, *Shigella enteritidis* group B, *Escherichia coli* DMST 4212). For antioxidant activity, this EO sample demonstrated DPPH (25 mg/mL) and ABTS radical scavenging activities (16.35 mg/mL) [10].

4. Conclusions

Essential oils of fresh and dried fruits of *Zanthoxylum rhetsa* from Quan Son, Thanh Hoa were obtained by hydrodistillation and analyzed the chemical constituents by GC-FID and GC/MS. The major components of fresh fruits EO were monoterpene hydrocarbons composed of sabinene (51.95%), limonene (7.84%) and *β*-phellandrene (7.69%); sesquiterpene hydrocarbons were absent. Monoterpene hydrocarbons were also the major components of dried fruits EO with sabinene (32.88%), limonene (20.95%), and *β*-phellandrene (10.23%) were the main constituents; meanwhile, sesquiterpene hydrocarbons were present in this EO sample (0.84%). Two EOs exhibited cytotoxic activity against two tested cancer cell lines, MCF-7 and HeLa. For antimicrobial activity, both EOs showed a positive activity against *E. coli*; on the other hand, the fresh fruits EO showed activity against *B. subtillis*; the dried fruits EO showed activity against *F. oxysporum* and *P. aeruginosa.*

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