JST: Engineering and Technology for Sustainable Development
Volume 31, Issue 4, October 2021, 019-025

Compositional Analyses and Cytotoxic Activity of Star Anise (*Illicium Verum*) Essential Oils in Vietnam

Phân tích thành phần và hoạt tính gây độc tế bào của tinh dầu Hồi (*Illicium verum*) Việt Nam

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Abstract

By means of the gas chromatography-mass spectrometry (GC/MS) method, 21 volatile components have been identified in the essential oils of fruits and the leaves of star anise (Illicium verum Hook. f., Illiciaceae) in Vietnam, in which, essential oil of star anise fruits has 18 components, essential oil of star anise leaves has 16 components, with content have been determined more than 98.60% total compounds of essential oil. The major compound was identified as trans- anethole with 87.05% in leaves essential oil and 90.12% in fruits essential oil. The in vitro anti-cancer activity of star anise essential oil was evaluated by cytotoxicity assay. The results showed that the essential oil obtained from leaves of star anise inhibited the grow of liver cancer cell (IC_{50} = 10.22 µg/ml) and colon cancer cell (IC_{50} = 6.26 µg/ml). The essential oil obtained from fruits of star anise inhibited the grow of liver cancer cell and colon cancer cell with the IC_{50} values of 5.93 and 3.20 µg/ml, respectively.

Keywords: *Illicium verum*, star anise, essential oils, anticancer, cytotoxicity assay, GC/MS.

Tóm tắt

Bằng phương pháp sắc ký khí ghép nối khối phổ (GC/MS) đã xác định được 21 cấu tử dễ bay hơi trong tinh dầu hồi (Illicium verum Hook. f., Illiciaceae) Việt Nam, trong đó, tinh dầu quả hồi có 18 cấu tử, tinh dầu lá hồi 16 cấu tử, với hàm lượng trên 98,60% tổng số hợp chất có trong tinh dầu. Cấu tử chính có trong tinh dầu quả Hồi và lá hồi là trans- anethole với hàm lượng là 87,05% trong tinh dầu lá hồi và 90,12% trong tinh dầu quả Hồi. Bước đầu xác định hoạt tính chống ung thư của tinh dầu hồi bằng phương pháp thử gây độc tế bào in vitro. Kết quả cho thấy tinh dầu lá hồi thể hiện hoạt tính ức chế dòng tế bào ung thư gan (IC_{50} = 10,22 µg/ml) và dòng tế bào ung thư đại tràng (IC_{50} = 6,26 µg/ml). Tinh dầu quả Hồi thể hiện hoạt tính gây độc đối với dòng tế bào ung thư gan và dòng tế bào ung thư đại tràng với giá trị IC_{50} tương ứng là 5,93 và 3,20 µg/ml.

Từ khóa: *Illicium verum*, đại hồi, tinh dầu, hoạt tính chống ung thư, gây độc tế bào, GC/MS.

1. Introduction

Star anise (*Illicium verum* Hook. f., Vietnamese name: hồi, đại hồi, bát giác hồi hương) belonging to the Illiciaceae family. It is an aromatic tree bearing purple-red flowers and anise-scented star-shaped fruit. It is cultivated in tropical and subtropical regions, grown almost exclusively in southern China and Vietnam. Its fruit is an important traditional Chinese medicine as well as a commonly used spice, attractive in food processing. Star anise oil is widely used in the technology of processing aperitifs, liqueurs, beverages, and confectionery. In traditional medicine of our country, China, India, Japan, Thailand, and other Asian countries..., star anise is used as a medicine that causes defecation, stimulates digestion, cures abdominal pain, reduces pain, and reduces contractions in the stomach and intestines [1,2]. According to previously published reports, main chemical constituents of star anise include monoterpenoids, sesquiterpenoids, phenylpropanoids, lignans, flavonoids, and volatile compounds. Major essential oils identified were *trans*-anethole, α-pinene, limonene, safrole, β-phellandrene, α-terpineol and farnesol [3]. Previous studies also have reported the antibacterial, anti-inflammatory, antioxidant, analgesic, anticonvulsive, insecticidal, and sedative activities of star anise essential oils [4-6]. Star anise essential oils inhibit the growth of tuberculosis bacteria and many other bacteria, should be used as an antiseptic, treatment of ringworm and scabies. Star anise is also used in the production and processing of herbicides, the destruction of lice, aphids, and some foreign parasites in cattle [7]. The main components of essential oils are considered responsible for their biological activity the objective. Moreover, star anise

ISSN: 2734-9381
https://doi.org/10.51316/jst.153.etsd.2021.31.4.4
Received: March 30, 2021; accepted: June 29, 2021

oil has been studied and tested for its biological activity, but there are not many reports on cancer cell inhibitory activity of *Illicium verum* oil in Vietnam.

Therefore, the aim of this article was to identify the chemical constituents, as well as evaluate anticancer activity *in vitro* of essential oils from star anise fruits and leaves in Vietnam.

2. Methods and Experiment

2.1. Plant Materials

Fruits of *Illicium verum* were collected from Van Quan, Lang Son province, leaves of *Illicium verum* were obtained from Bac Me, Ha Giang province, Viet Nam, in April 2019 (Fig. 1). The plant materials were identified by Dr. Tran Huy Thai (Institute of Ecology and Biological Resources, VAST, Vietnam). Voucher's specimens IC-BK01 and IC-BK02, respectively were deposited at the herbarium of SCE-HUST, Vietnam.

After removing dust and other matter, the fruits and leaves of *Illicium verum* were chopped, dried under shiny, and oven-dried at 50 ^{o}C to give dried samples.

2.2. Preparation of Illicium Verum Essential Oils

The dried fruits (100 g) and dried leaves (200 g) of *Illicium verum* were powdered and distillated for 6h using a Clevenger apparatus to give the raw essential oils. The oils were then dried with anhydrous sodium sulphate (Na_2SO_4) to remove the remaining water trace. Essential oils were kept in a closed dark glass bottle and stored at 5 ^{o}C until use.

The obtained essential oils of fruits (9.12 g) and leaves (7.00 g) were then used to analyze chemical compositions and evaluate cytotoxic activity.

2.3. Gas Chromatography - Mass Spectrometry (GC/MS) Analysis

The *Illicium verum* essential oils were analyzed by GC/MS using Agilent 7890A GC System (USA). GC column was an HP5-MS fused silica capillary (5% phenyl and 95% methyl siloxane stationary phase).

GC/MS analysis was carried out the following conditions: The carrier gas was helium and a flow rate 1 ml/min, sample injection temperature was 250 ^{o}C; the GC oven temperature program is kept at 40 ^{o}C initial temperature for 5 minutes, then increased 4 ^{o}C/min to 240 ^{o}C/min, hold on this temperature for 5 minutes; Agilent 5975C mass spectrometer conditions: source temperature is 230 °C, ionization potential 70 eV, ionization source 2A, resolution 1000.

The identification of constituents of *Illicium verum* essential oils were performed on the basic of retention indices (RI). Calculation of RI with the following formular [8,9]:

$$\mathrm{RI} = 100 \times \left[\frac{\left(t_{R_i} - v\right) - \left(t_{R_z} - v\right)}{\left(t_{R_{(z+1)}} - v\right)} - \left(t_{R_z} - v\right) + Z \right]$$

where: t_{Ri} : retention time of sample peak; v: column void time; t_{RZ}: retention time of n-alkane peak eluting immediately before sample peak; $t_{R\ (Z+1)}$: retention time of n-alkane peak eluting immediately after sample peak; Z: carbon number of n-alkane peak eluting immediately before sample peak.

Further identification was performed by comparison of their mass spectra with those from NIST [10] and the MS library built up from pure substances and components of known essential oils, as well as by comparison of their retention indices with literature values [11]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

Fig. 1. Images of *Illicium verum* plant materials
(a) Fruits were collected from Van Quan, Lang Son and (b) leaves were obtained from Bac Me, Ha Giang.

2.4. Cytotoxicity Assay

Test materials:

Two cancer cell lines include Hep-G2 (human liver hepatocellular carcinoma), and HT-29 (human colon carcinoma) were obtained from Prof. J. M. Pezzuto, Long-Island University, US and Prof. Jeanette Maier, Milan University, Italia. Fetal bovine serum (FBS) was obtained from Gibco, Life Technologies Inc., (Gaithersburg, MD, USA). Sulforhodamine B (SRB), trichloroacetic acid (TCA), tris base [tris(hydroxymethyl)aminomethane], dimethyl sulfoxide (DMSO), ellipticine and other chemicals were purchased from Sigma, and Promega companies.

SRB assay:

The *in vitro* cytotoxic method performed according to the method of Skehan *et al.* [12]. This method is suitable for ordinary laboratory purposes and for very large-scale applications, such as the National Cancer Institute's disease-oriented *in vitro* anticancer-drug discovery screen. The test carried out by SRB assay [13], following the previously described protocols [14,15].

Briefly, tumor cells were cultivated in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. Cell viability was examined by the SRB method for cell density determination, based on the measurement of cellular protein content. DMSO 10% was used as a blank sample while ellipticine was used as a positive control. Experimental cultures were plated in 96-well microtiter plates (Costar, USA), containing 10 µL of each test sample and 190 µL of growth medium (10% fetal bovine serum) per well at a density of 6000 cells/well. The duration assay was adopted as 3 days. One plate with no added samples served as 0-day control. Test plates were incubated in a humidified atmosphere of 5% CO_2, 37 °C for 72 h, while the 0-day control was incubated for 1 h. Thereafter, the medium was removed, and the remaining cell monolayers are fixed with the cold 20% (w/v) trichloroacetic acid for 1 h at 4 °C and stained by 1X SRB staining solution at room temperature for 30 min, after which the unbound dye is removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye is dissolved in 10 mM unbuffered Tris base solution for optical density (OD) determination at 515 nm on an ELISA Plate Reader (Bio-Rad).

The tests are to determine the total cell protein content based on OD measured when the protein content of the cell is stained with SRB. The measured OD value is directly proportional to the amount of SRB attached to the protein molecule, so the more protein, the larger OD value.

The cytotoxic activity was indicated as half inhibition concentration (IC_{50}) values from concentrations of 100, 20, 4, and 0.8 µg/ml. All tests were performed in triplicate. Ellipticine was used as a positive control.

Statistical analysis:

The IC_{50} values were calculated by TableCurve 2Dv4 Software (System Software Inc., San Jose, CA, USA). The inhibition rate (IR) of cell growth was calculated by the following formula:

$$IR\% = \left[100\% - \frac{(OD_t - OD_0)}{(OD_c - OD_0)}\right] \times 100$$

where: OD_t: average optical density value at day 3; OD_0: average optical density value at time-zero; OD_c: average optical density value of the blank DMSO control sample.

All data were statistically analyzed using analysis of variance and the results were considered significant at $P < 0.05$.

3. Results and Disscutions

3.1. Chemical Composition

The *Illicium verum* essential oils were prepared by hydrodistillation using a Clevenger apparatus. They were pale yellow liquids with strong odor and taste of star anise characteristics. The yields on the dry weight basis of samples of *Illicium verum* fruits and leaves essential oil were 11.07% and 4.19%, respectively.

Chemical analysis of the components of star anise fruits and leaves essential oils by GC/MS led to identification of 21 components, in which, essential oil of star anise fruits has 18 components, essential oil of star anise leaves has 16 components. The difference in the number of components in star anise fruits and leaves essential oils can be explained the plant materials collected from two different places (Lang Son and Ha Giang) in Vietnam, as well as different parts of trees.

The results in Table 1 showed that the contents of compounds have been determined more than 98.60% total compounds of essential oils. The major components were *trans*-anethole (90.12% in fruit oil and 87.05% in leaves oil), limonene (3.46% in fruit oil), *p*-anisaldehyde (3.37% in leaves oil), α-pinene (1.39% in leaves oil) and linalool (1.18% in leaves oil). Chemical structures of the main compounds of star anise essential oil in Fig. 2.

The results in Table 1 and the GC chromatograms in Fig. 3 also showed that *trans*-anethole from both essential oils of fruits and leaves star anise were higher than Vietnam Standard 8853:2001 result (86% *trans*-anethole) [16] and report of Soher E. Aly *et al.* (86% *trans*-anethole) [17]. Our results are in consistent with the report of Wichtl M. [18], that the major compound of star anise oil is *trans*-anethole, which ranged from 86.0 to 93.0%.

Table 1. Volatile compounds of star anise fruits and leaves essential oils from Lang Son and Ha Giang, Vietnam

No	Compounds	RI[a]	Percent peak area[b] (%)	
			Star anise fruits in Lang Son	Star anise leaves in Ha Giang
1	α-Pinene	939	0.49	1.39
2	Myrcene	992	0.10	-
3	α-Phellandrene	1010	0.14	0.11
4	3-Carene	1016	0.32	-
5	*o*-Cymene	1030	-	0.19
6	Limonene	1034	3.46	0.21
7	β-Phellandrene	1036	0.27	0.14
8	1,8-Cineole	1038	0.44	0.11
9	Linalool	1103	0.66	1.18
10	Terpinen-4-ol	1187	0.23	0.11
11	α-Terpineol	1200	0.18	-
12	Estragole	1206	0.56	0.28
13	*cis*-Anethole	1261	-	0.25
14	*p*-Anisaldehyde	1265	0.30	3.37
15	***trans*-Anethole**	**1298**	**90.12**	**87.05**
16	α-Copaene	1389	0.11	-
17	Anisyl acetone	1394	-	0.29
18	α-*cis*-Bergamotene	1425	0.11	-
19	β-Caryophyllene	1437	0.17	-
20	α-*trans*-Bergamotene	1446	0.31	0.35
21	Foeniculin	1688	0.41	1.09
Total identified			98.60	98.67

[a] RI: Retention indices on HP-5MS capillary column; [b] Raw percentages based on total without standardization

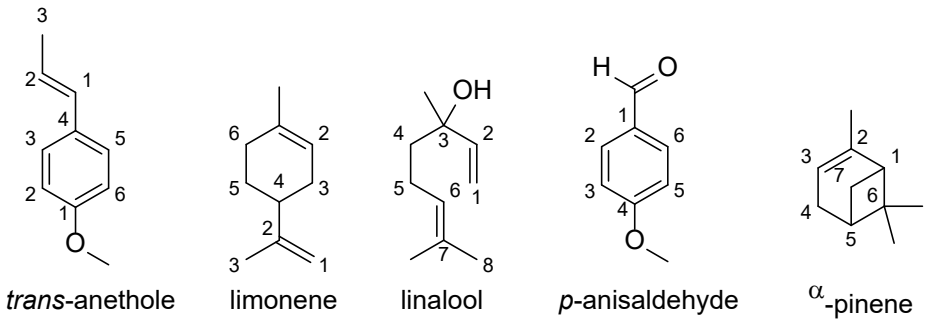
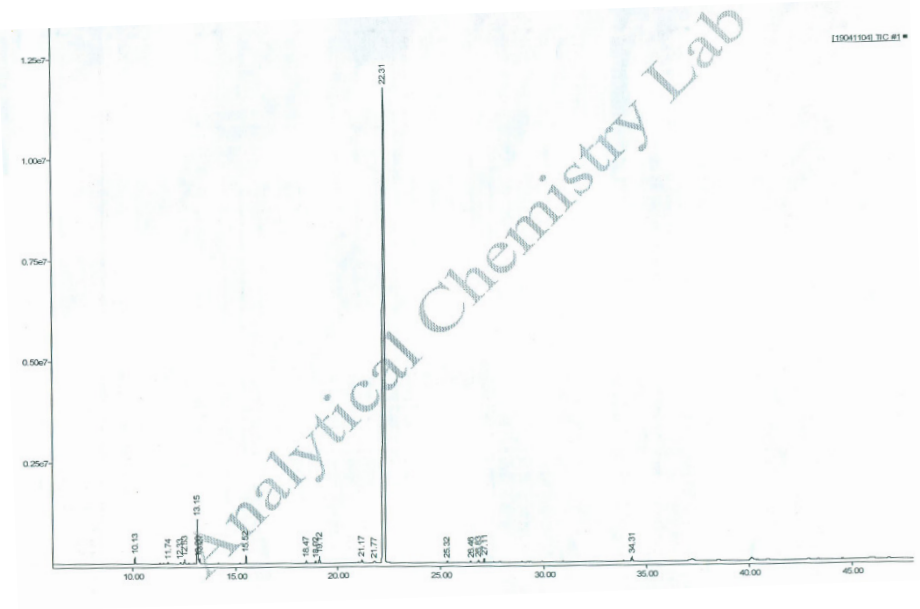
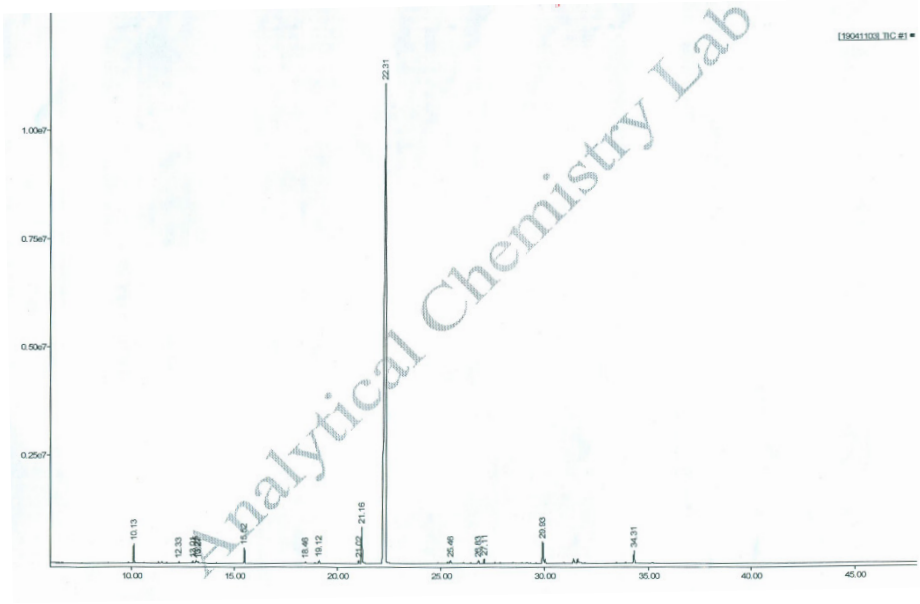


Fig. 2. Chemical structures of main compounds of *Illicium verum* essential oils



(a) essential oils of fruits star anise



(b) essential oils of leaves star anise

Fig. 3. GC chromatograms of the essential oils of *Illicium verum* fruits and leaves

Trans-anethole (IUPAC name: 1-methoxy-4-((1*E*)-prop-1-en-1-yl)benzene) is a phenylpropanoid synthesized via the shikimic acid-phenylpropanoid pathways. This compound occurs naturally as a major component of essential oils from star anise (*Illicium verum*), fennel (*Foeniculum vulgare*) and is also present in numerous plants such as basil (*Ocimum basilicum*), dill (*Anethum graveolens*), and tarragon (*Artemisia dracunculus*). It is also used as flavor agents in foods in the many other countries in the world [19].

On the other hand, *trans*-anethole had a cytotoxic effect on breast cancer, fibrosarcoma tumor, cervical carcinoma, hepatocytes, and Ehrlich ascites tumor [20,21]. It is considered responsible for biological activity of the star anise essential oil and its cytotoxic potential. Consequently, the high content of *trans*-anethole can determine the quality of the star anise essential oils.

Moreover, our study is a good rationale for recommendation for anise leaves development in different regions, to shorten harvesting time, and to reduce the risk of environmental pollution when insects and diseases. Branches and leaves are included as raw materials for distillation of essential oil, reducing the risk of labor accidents when people harvest anise fruits at high altitudes.

Table 2. Cytotoxic activity of star anise fruits and leaves essential oils

Concentration (µg/ml)	Inhibition (%)				Concentration (µg/ml)	Inhibition (%)	
	Leaves star anise essential oil		Fruits star anise essential oil			Ellipticine (Positive control)	
	Hep-G2	HT-29	Hep-G2	HT-29		Hep-G2	HT-29
100	83.03	92.91	99.52	105.22	10	108.40	96.46
20	75.63	82.03	80.38	89.35	2	77.50	71.36
4	22.19	30.27	34.01	44.71	0.4	51.29	50.94
0.8	15.45	19.31	19.14	22.25	0.08	27.40	26.37

3.2. Evaluation of Cytotoxic Activity

Both essential oils were screened for *in vitro* cytotoxic activity against human colon carcinoma cells (HT-29) and human liver hepatocellular carcinoma cells (Hep-G2).

The study samples which have potential effects with the percentage of cell survival less than 50% at tested concentrations of 50 and 100 µg/ml was further studied to determine IC_{50} values (Table 2).

The results in Table 3 showed that the essential oil obtained from leaves of star anise inhibited the grow of liver cancer cell and colon cancer cell with the IC_{50} values of 10.22 and 6.26 µg/ml, respectively. The essential oil of star anise fruits inhibited the grow of liver cancer cell and colon cancer cell with the IC_{50} values of 5.93 and 3.20 µg/ml, respectively.

Table 3. The IC_{50} values of *Illicium verum* fruits and leaves essential oils against cancer cell lines

Cell lines	IC_{50} (µg/ml)		
	Leaves essential oils	Fruits essential oils	Ellipticine (positive control)
Hep-G2	10.22±1.13	5.93±0.48	0.34±0.02
HT-29	6.26±0.65	3.20±0.18	0.38±0.04

Comparison with the research results of Muhammad Asif *et al.* the cytotoxic activity of both star anise leaves and fruits essential oils in Vietnam were stronger than that of star anise oil in Malaysia [22]. The reason for the difference of obtain results is due to the origin, source, and growing conditions of star anise plants. On the other hand, the difference of harvesting time and methods to obtain essential oils also has effect on chemical constituents and cytotoxic activity of star anise essential oils. Therefore, star anise fruits and leaves in Vietnam could be considered a good source of natural compounds with significant anticancer activity, which can be attributed to the high percentage of the main constituents or to synergy among the different oil constituents.

4. Conclusion

The chemical constituents of volatile oils from fruits and leaves of star anise (*Illicium verum* Hook. f.) in Vietnam has been studied. The essential oils of fruits and leaves of star anise were obtained by distillation using a Clevenger apparatus and analyzed by GC/MS. 21 volatile components of the essential oils were identified, accounting for more than 98.6% of the total oil. The main component was *trans*-anethole, which include 90.12% in fruits oil and 87.05% in leaves oil.

Both *Illicium verum* fruits and leaves essential oil samples showed quite strong cytotoxic activity on the test cell lines with IC_{50} values of 3.20 - 10.22 µg/ml. The cytotoxic activity of the star anise essential oils can be attributed to high content of *trans*-anethole, which was confirmed as the main active component among the volatile compounds in the star anise essential oils. However, further studies about the *in vivo* anticancer activity of star anise essential oils against steps in tumor apoptosis and metastasis are encouraged and developed.

Altogether, our study contributes more scientific evidence about *Illicium verum* in Vietnam, also indicates that the essential oil of fruits and leaves of star anise have potential for the development of natural products with anticancer activity.

Acknowledgment

This work was supported by the research project of Hanoi University of Science and Technology (Grant No: T2020-PC-050).

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