Chemical components as a potential resource for synthesis of organic compounds and anti-inflammatory activity of essential bark oil of *Illicium verum*

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Abstract

In this work, the essential bark oil (EBO) of Illicium verum cultivated at Cao Bang in Vietnam obtained by hydro-distillation was investigated for its chemical composition by gas chromatography-mass spectrometry (GC-MS). Trans-anethole (78.6%), estragole (13.7%), linalool (2.1%) and eucalyptol (2.0%) were determined to be the main components among 14 identified compounds, which accounted for 96.4% of the total EBO. Anti-inflammatory activity of EBO was evaluated by inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

EBO exhibited an interesting inhibitory effect on NO production in LPS-stimulated RAW246.7 cells with an inhibitory value at 93.87 µg/mL. From the starting material, trans-anethole isolated from EBO, there are two new amine-derivatives that were synthesized. The structure elucidation of these analogues of trans-anethole was carried out by 1D (^{1}H NMR and ^{13}C NMR) and IR spectral analysis.

Keywords: *Illicium verum*, Bioactive compounds, Bio-activities, Essential oil, Potential resources.

Introduction

The *Illicium verum* is one of the most abundant aromatic evergreen trees with star-framed fruits native to Northeast Vietnam and Southwest China, also commonly known as star anise or Chinese star anise[2]. This plant plays an important role not only in the food industry used as a flavor, but also used in traditional Chinese and Vietnamese medicines for its antiviral effects[8]. Traditionally, the fruits have been frequently used as a well known spice in cooking and in preparation of a unique Vietnamese noodle soup (Pho) and biryani and masala chai in Indian food, and widely Chinese cuisine[8].

Moreover, in terms of Eastern Asian traditional medicine, Chinese star anise has been used in treatment of various common diseases for thousands of years such as flu, fever and digestive diseases, stomach aches, flatulence, spasmodic colonalgia, dysentery, cough, asthma, rheum arthritis, facial paralysis, sepsis and so forth[8,12]. The main chemical constituents of star-framed fruits have been reported which included monoterpenoids, sesquiterpenoids, phenylpropanoids, lignans, flavonoids, and essential oil (EO)[9,10,13]. The major components of EO identified were trans-anethole, *α*-pinene, limone, *β*-phellandrene, *α*-terpineol, farnesol, and safrole[5,8]. Trans-anethole has been reported to have various antitumor activities such as breast cancer, cervical carcinoma, fibrosarcoma, and Ehrlich ascites tumor[1-4,7]. The shikimic acid in the fruit of *Illicium verum* is a significant precursor in the pharmaceutical synthesis of the anti-influenza medication oseltamivir (Tamiflu), an antiviral remedy for influenza A and B[11].

In present work, the essential bark oil (EBO) of *Illicium verum* cultivated at Cao Bang in VietNam obtained by hydro-distillation was investigated for its chemical composition by gas chromatography-mass spectrometry (GC-MS) and trans-anethole isolated from EBO was used as a potential resource for synthesis of new organic compounds.

Material and Methods

Plant material: The dry brak of *Illicium verum* was collected in October from Cao Bang Province in North Vietnam, and was identified by Asso. Prof. Dr. Vu Tien Chinh of Vietnam National Museum of Nature, Vietnam Academy of Science and Technology. The voucher specimen (TNUS-HCB-102021) was deposited in the Laboratory of Medical Chemistry, Thai Nguyen University of Sciences. The bark was stored at 4 ºC before use.

Preparation of EBO: The dry brak of *Illicium verum* was broken into pieces, ground and then subjected to hydrodistillation in a Clevenger-type apparatus. The distilled oil was extracted with hexane, and then dried over anhydrous sodium sulfate and preserved in a dark glass vial at 4 ºC until required. Yield of the essential bark oil (EBO) was 5.26% (w/w at dry weight basis).

Essential oil analysis: The composition of essential bark oil (EBO) of *Illicium verum* was determined by using GC (FID) and GC/MS technique. The total neutral essential oil from *I. verum* bark was analyzed by an Agilent 7890A Network GC (gas chromatograph) system with an Agilent 5975C Network mass selective detector. The machine was equipped with an HP-5MS (mass spectroscopy) column [30 m × 0.25 mm (5%-phenyl)-methylpolysiloxane capillary column, film thickness × 0.25 µm], a split-splitless injector at 250 ºC, and a flame ionization detector (FID) at 280 ºC.

The oven temperature was programmed as follows: initial temperature 60 ºC for 1 min, increase 8 ºC /min up to 280 ºC. The carrier gas was helium. The amount of sample injected was 1.0 µL (split ratio 1:20) and the ionization energy was 70 eV. The components were identified by comparison of their mass spectra with those of NIST20 library data of the GC-MS system. The relative amounts (RA) of individual component of the essential oil was expressed as percentages of the peak area relative to the total peak area. RI value of each component was determined relatively to the retention times (RT) of a series of C_8-C_{40} *n*-alkanes (Sigma) with linear interpolation on the HP-5MS column according to the Van den Dool approach.

Nitric oxide assay: Nitric oxide assay was carried out as previous described[6]. The RAW264.7 cells were received from Perugia University, Italy and were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES and 1 mM sodium pyruvate. The cells were dispensed into a 96-well plate (2×105 cells/well) and incubated at 37°C in a humidified atmosphere (5% CO_2 and 95% air). After 24h incubation, the culture medium was replaced with DMEM without FBS and continuously incubated for 3h. The cells were treated with either compounds or vehicle solution and then stimulated with LPS (1 µg/mL) in the next 2h. After an additional 24 h incubation, the cell culture medium (100 µL) was mixed with an equal volume of Griess reagent (Promega, Fitchburg, WI, USA) for 10 min and the absorbance was read at 540 nm.

The amount of nitrite, an indicator of NO production in the medium, was obtained from a standard curve, which was constructed by $NaNO_2$ serial dilution. NG-monomethyl-L-Arginine (L-NMMA) was used as a positive control. Cell viability was determined by adding 10 µL MTT solution (5 mg/mL) and incubating for 4h. Formazan crystals were dissolved in 50 µL of DMSO. Absorbance was read at 540 nm and compared with the vehicle group. Experiments were performed in triplicate and data are expressed as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software.

Synthesis of new compounds

General experimental procedures*:* All reactions were performed in appropriate oven-dried glass apparatus under nitrogen atmosphere. Unless otherwise stated, solvents and chemicals were obtained from commercial sources and used without further purification. Flash column chromatography was performed using silica gel as adsorbent. The ratio between the amount of silica gel and fraction was 20/1 (w/w). A fraction collector was set by volume per tube (3 mL/tube). Fractionation was monitored by thin-layer chromatography (TLC) to combine test tubes showing a similar TLC pattern. TLC was carried out on precoated silica gel 60 F_{254} plates. Compounds were visualized by ultraviolet irradiation (254 nm) and by spraying with a sulfuric acid solution (5%) followed by heating with a heat gun. NMR spectras of pure compounds were recorded on a Bruker Advance I spectrophotometer (500 MHz). Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz). IR spectras were recorded on a Perkin Elmer Spectrum Two.

Isolation of trans-anethole: The EBO (5.0 g) was chromatographed on a silica gel column, eluting with hexane/ethyl acetate (99/1, v/v), to give five subfractions (EBO1-EBO5). The EBO2 (3.7 g) fraction was chromatographed on a silica gel column eluting with *n*-hexane to give pure trans-anethole (3.5 g): 70% yield, colorless liquid. ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 1.82 (3H, dd, *J*=1.5; 5.5 Hz, C<u>H</u>$_3$); 3.72 (3H, s, OC<u>H</u>$_3$); 6.01-6.07 (1H, m, olefin); 6.30 (1H, dd, *J*=1.5; 13.0 Hz, olefin); 6.79 (2H, dd, *J*=2.0; 8.5 Hz, H-3, H-5); 7.21 (2H, dd, *J*=2.0; 8.5 Hz, H-2, H-6). ^{1}H NMR ($CDCl_3$, 125 MHz) δ (ppm): 18.4 (<u>C</u>H$_3$); 55.2 (OCH$_3$); 113.9 (C-3, C-5); 123.4 (C olefin); 126.9 (C-2, C-6); 130.5 (C-4); 130.9 (C olefin); 158.7 (C-1).

Synthesis of compound TDH.V1-Br: A solution of trans-anethole (TDH.V1) (1.0 equiv.) and NBS (5.0 equiv.) in dry CH_3CN (10 mL) was stirred at room temperature for 5h and the progress of the reaction was monitored by TLC using 2% ethyl acetate in hexane. Afterwards, the reaction mixture was added to water (30 mL) and extracted three times with dichlomethane (30 mL each time). The organic phase was washed with water and saturated brine. Drying of the organic phase ($MgSO_4$), filtration of the drying agent, and evaporation of the solvent in vacuo afforded crude compounds TDH.V1-Br purified by silica gel column chromatography (*n*-hexane-EtOAc, 99:1) to obtain pure compound TDH.V1-Br. 85% yield, colorless liquid.

General procedure for the synthesis of compounds TDH-A1 and TDH-A5: A solution of TDH.V1-Br (1.0 equiv.) and benzene-1,2-diamine (1.0 equiv.) or indole (1.0 equiv.) in C_2H_5OH (15 mL) was stirred at room temperature for 12h. After stopping the reaction, the mixture was added to water (50 mL) and extracted with ethyl acetate three times, each time 30 mL. The organic phase was washed with water and saturated brine. Drying of the organic phase ($MgSO_4$), filtration of the drying agent, and evaporation of the solvent in vacuo afforded crude compounds TDH-A1 or TDH-A5 purified by silica gel column chromatography (*n*-hexane-EtOAc, 97:3 or 90:10) to obtain pure compounds TDH-A1 or TDH-A5.

Compound TDH-A1: 75% yield, yellow solid. ^{1}H NMR ($CDCl_3$, 500 MHz): 7.83-7.80 (2H, m); 7.48-7.50 (2H, m); 7,5 (2H, dd, *J*=2.0, 8.5 Hz); 6.95 (2H, d, *J*=2.0, 8.5 Hz); 6.86 (1H, dd, *J*=1.5; 12.5 Hz); 6.46 (1H, s); 3.7 (3H, s); 2.50-2.49 (2H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): 158.6; 146.5; 144.4; 139.7; 137.5;130.2; 130.0; 127.4; 126.1; 125.9(2xC); 113.7; 113.6(2xC); 55.2; 55.1. IR (KBr, d cm^{-1}): 3400; 3221; 3055; 2920; 2812; 1651; 1606; 1510; 1425; 1213; 1028; 800; 750.

Compound TDH-A5: 89% yield, white solid. ^{1}H NMR ($CDCl_3$, 500 MHz): 8.21 (1H, d, *J*=6.5 Hz); 7.73 (1H, d, *J*=

6.5 Hz); 7.63 (1H, d, *J*=7.0 Hz); 7.42 (1H, t, *J*=6.0 Hz); 7.30 (2H, dd, *J*=2.0, 8.0 Hz); 7.26- 7.20 (2H, m); 7.15 (2H, dd, J=2.0, 8.5 Hz); 6.84 (1H, t, *J*= 6.0 Hz); 6.68 (1H, d, *J*= 7.0 Hz); 3,90 (3H, s), 2.64 (2H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): 158.2; 139.3; 132.6; 131.2 (2xC); 126.7; 124.7; 124.3; 123.8; 121.1; 120.7; 119.0; 118.7; 118.2 (2xC); 113.9; 111.3; 55.1. IR (KBr, d cm^{-1}): 3072; 2835; 1654; 1604; 1558; 1514; 1240; 1176; 738.

Results and Discussion

GC-MS analysis: Fourteen compounds were confirmed by searching NIST-EPA-NIH mass spectral library (NIST 20) in ChemData (Fig. 1 and table 1) which accounted for 99.95% of the total EBO. There are four main components of EBO including trans-anethole (78.6%), estragole (13.7%), linalool (2.1%) and eucalyptol (2.0%). The comparison of this result with the other GCMS analysis results for star anise essential oil corroborates with previous reports[1,3,4] in terms of the main component. The trans-anethole is always the highest concentration in all types of *Illicium verum* essential oil.

Inhibition of NO production: Anti-inflammatory activity of essential bark oil was evaluated by its ability to inhibit NO production in LPS- stimulated RAW 264.7 cells.

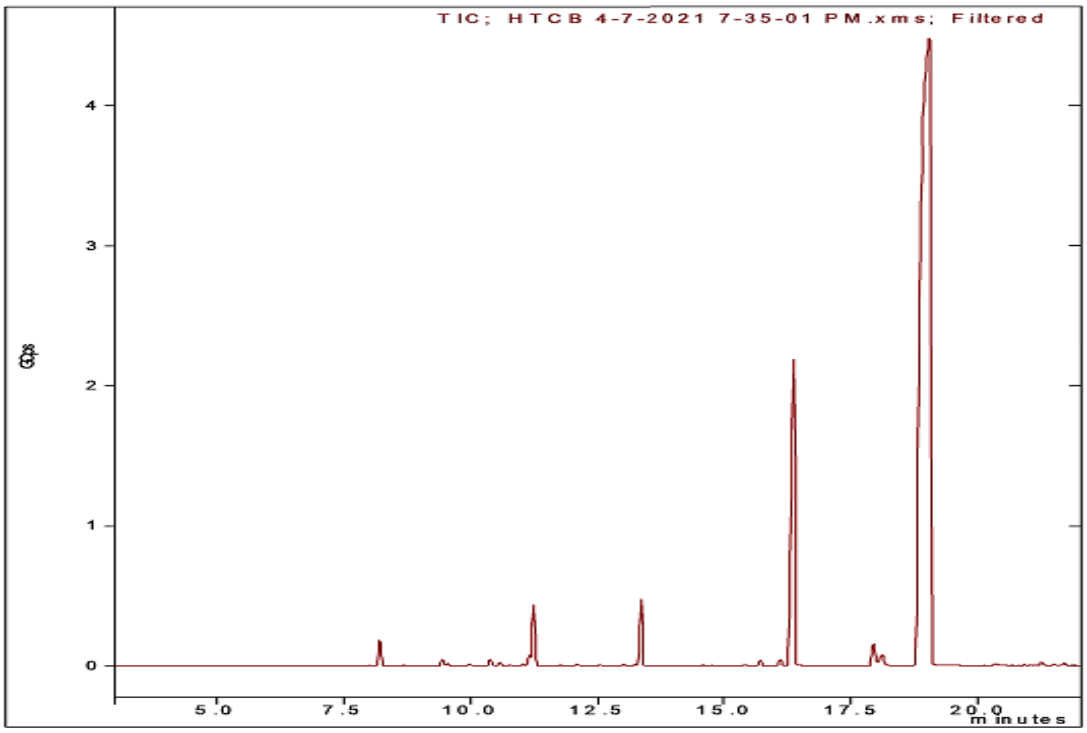
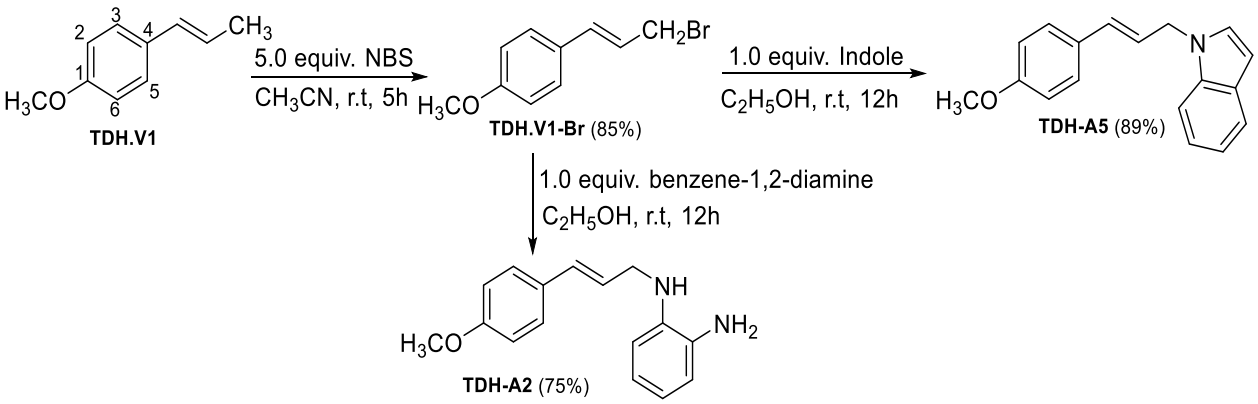


Fig. 1: GC-MS chromatogram of Illicium verum essential bark oil

Table 1
The compositions of Illicium verum essential bark oil

S.N.	Compounds	Molecules	R_t	%
1	*α*-Pinene	$C_{10}H_{16}$	8.22	0.74
2	4-methylene-1-(1-methylethyl)-Bicyclo[3.1.0]hexane	$C_{10}H_{16}$	9.45	0.11
3	*α*-Phellandrene	$C_{10}H_{16}$	10.39	0.19
4	3-Carene	$C_{10}H_{16}$	10.58	0.11
5	D-Limonene	$C_{10}H_{16}$	11.16	0.33
6	Eucalyptol	$C_{10}H_{18}O$	11.24	1.97
7	Linalool	$C_{10}H_{18}O$	13.35	2.10
8	Terpinen-4-ol	$C_{10}H_{18}O$	15.70	0.17
9	*α*-Terpineol	$C_{10}H_{18}O$	16.09	0.22
10	Estragole	$C_{10}H_{12}O$	16.35	13.72
11	*Cis*-anethole	$C_{10}H_{12}O$	17.93	0.84
12	4-methoxy Benzaldehyde	$C_8H_8O_2$	18.10	0.64
13	*Trans*-Anethole	$C_{10}H_{12}O$	19.03	78.63
14	1,3-Benzenediol, monobenzoate	$C_{13}H_{10}O_3$	21.24	0.11



Scheme 1: The synthesis of new compounds TDH-A2 and TDH-A5

This essential oil was assessed at a concentration of 100 µg/mL. EBO demonstrated an interesting inhibition of NO producion with inhibitory action at 93.87± 2.2 µg/mL. This result will contribute significantly to medical database of *Illicium verum*.

Isolation of trans-anethole: *Trans*-anethole (TDH.V1) was isolated from EBO by using chromatography columns with mobile phase hexane-ethyl acetate. The pure compound TDH.V1 is colorless liquid and its structure was confirmed by 1D NMR. The ^{1}H nuclear magnetic resonance spectrum of pure compound exhibited the signals at 6.79 ppm (2H, dd, *J*=2.0; 8.5 Hz) and 7.21 ppm (2H, dd, *J*=2.0; 8.5 Hz) corresponding to H-3, H-5 and H-2, H-6 of 1,4-phenyl respectively. Beside, the singlet signal of three protons at 3.72 ppm is methoxy group. The double-doublet at 1.82 ppm with constant corrections at 1.5 and 5.5 Hz is confirmed for the methyl group linked to the methylene segment.

There are two signals of protons olefin at 6.05 ppm and 6.30 ppm with large constant *J*=13.0 Hz as the resonance of two proton olefin having trans-configuration. The ^{13}C NMR spectrum of TDH.V1 indicated the signals of nine carbons, including one carbon of methoxy group at 55.2 ppm, six carbons of 1,4-phenyl group and one prop-1-en-1-yl segment. Detailed analysis of the ^{1}H, ^{13}C NMR confirmed that the structure of the isolated compound (TDH.V1) is trans-anethole.

Synthesis of new compounds from trans-anethole: Two new compounds TDH-A1 and TDH-A5 were synthesized successfully through two steps from trans-anethole as a starting material. The first step is bromide reaction of trans-anethole with *N*-Bromosuccinimide (NBS) at room temperature in the presence of acetone nitrile as a solvent to get bromine derivative TDH.V1-Br with 85% of yield. Then, the bromide compound TDH.V1-Br continued to react with 1.0 equivalent of benzene-1,2-diamine for 12h at room temperature in ethanol to form TDH-A2 with high yield. Meanwhile, the reaction of TDHV1-Br with Indole has the same condition to get TDH-A5 (89%) (Scheme 1). The structures of these compounds were confirmed by using spectral NMR and IR (Supplemental Data).

Conclusion

The present study confirmed the composition and anti-inflammatory activity of essential bark oil (EBO) of *Illicium verum* cultivated at Cao Bang in Vietnam by using gas chromatography-mass spectrometry (GC-MS) and nitric oxide assay respectively. There are 14 natural products in this essential oil. The main components of EBO are trans-anethole (78.6%), estragole (13.7%), linalool (2.1%) and eucalyptol (2.0%) which accounted for 96.4% of the total essential oil. This essential oil showed an interesting inhibitory effect on NO production in LPS-stimulated RAW246.7 cells at 93.87 µg/mL.

From trans-anethole isolated from EBO, there are two new amine-derivatives that were synthesized successfully. The synthesis in this work, in particular transformation of trans-anethole, showed powerfully potential resources for synthesis of organic compounds.

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