Supporting Information

***Rec. Nat. Prod*. 16:6 (2022) 657-662**

Integrated Analysis of Vietnamese *Illigera trifoliata* ssp. *cucullata* (Merr.) Kubitzki), Leaf and Stem Essential Oils by GC-FID/GC-MS and ^{13}C NMR

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S.1. Photo and Drawings of Species

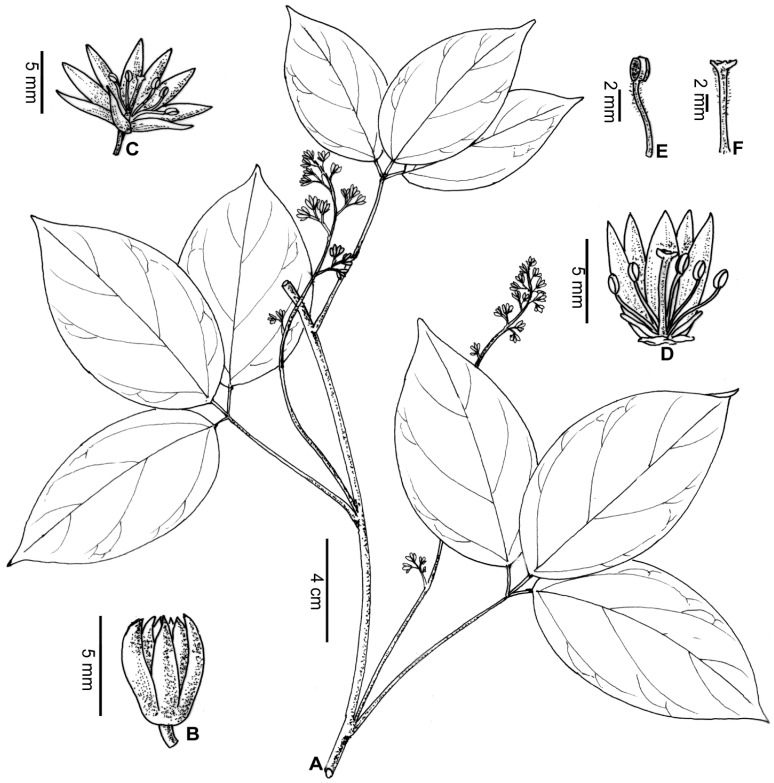

FIGURE 1. *Illigera trifoliata* ssp. *cucullata* (Merr.) Kubitzki
A. Habitat, **B.** Bud, **C.** Flower, **D.** Flower open showing stamens and style; **E.** Stamen, **F.** style and stigma
(Drawing by Nguyen Quang Hung, IEBR)

FIGURE 2. *Illigera trifoliata* ssp. *cucullata* in the wild
(Photo by Tran Huy Thai, IEBR)

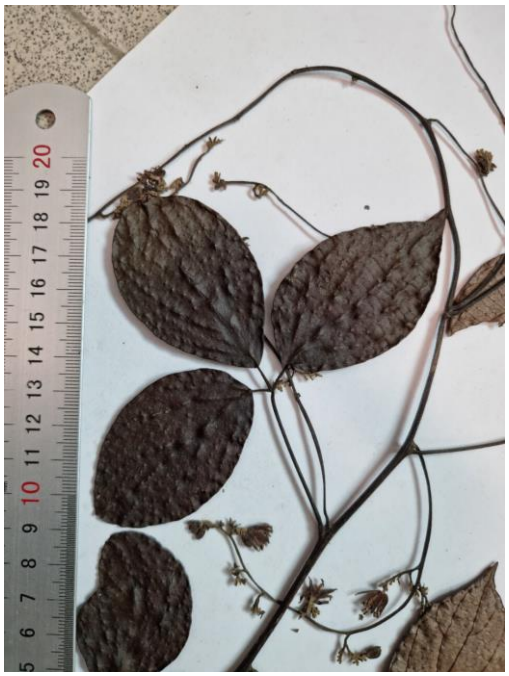

FIGURE 3. *Illigera trifoliata* ssp. *cucullata* specimen
(Photo by Tran Huy Thai, IEBR)

S.2. Hydrodistillation of Essential Oils

Briefly, 1000 g each of the air-dried pulverized samples were carefully introduced into a 5 L flask and distilled water (5 L) was added until it covers the sample completely. Hydrodistillation was carried out with a Clevenger-type distillation unit designed according to the specification (Vietnamese Pharmacopoeia, 1997) [1]. The distillation time was 4-5 h and conducted at normal pressure. The volatile oils distilled over water and were collected separately into clean weighed sample bottles. The oils were kept refrigerated (4ºC) until they were tested and analysed.

S.3. Analysis of Essential Oils

Gas Chromatography (GC) Analysis

Analyses were carried out using a Clarus 500 Perkin Elmer Chromatograph (Courtaboeuf, France), equipped with flame ionization detector (FID) and two fused-silica capillary columns (50 m x 0.22 mm, film thickness 0.25 µm), BP-1 (polydimethyl siloxane) and BP-20 (polyethylene glycol). The oven temperature was programmed from 60-220°C at 2°C/min and then held isothermal at 220°C for 20 min; injector temperature: 250°C; detector temperature: 250°C; carrier gas: helium (0.8 mL/min); split: 1/60; injected volume: 0.5 ìl. Retention indices (RI) were determined relative to the retention times of a series of *n*-alkanes (C8-C29) with linear interpolation (« Target Compounds » software from Perkin Elmer) [5].

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Samples were analyzed with a Perkin Elmer TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem XL (Courtaboeuf, France), equipped with a Rtx-1 (polydimethylsiloxane) fused-silica capillary column (60 m x 0.22 mm i.d., film thickness 0.25 µm). The oven temperature was programmed from 60 to 230 °C at 2°/min and then held isothermal for 45 min; injector temperature, 250 °C; ion-source temperature, 250 °C; carrier gas, He (1 ml/min); split ratio, 1:80; injection volume, 0.2 µL; ionization energy, 70 eV. The electron ionization (EI) mass spectra were acquired over the *m/z* range 35 - 350 Da [5].

Nuclear Magnetic Resonance (NMR) Analysis

All ^{13}C-NMR spectra were recorded on a Bruker AVANCE 400 Fourier transform spectrometer (Bruker, Wissembourg, France) operating at 100.623 MHz for ^{13}C, equipped with a 5 mm probe, in $CDCl_3$, with all shifts referred to internal TMS. The following parameters were used: pulse width = 4 µs (flip angle 45°); relaxation delay D1 = 0.1 s, acquisition time = 2.7 s for 128K data table with a spectral width of 25 000 Hz (250 ppm); CPD mode decoupling; digital resolution = 0.183 Hz/pt. The number of accumulated scans was 3000 for each sample or fraction (40 mg, when available, in 0.5 mL of $CDCl_3$) [6].

Identification of Individual Components

Identification of the individual components was carried out: (i) by comparison of their GC retention indices (RI) on polar and apolar columns, with those of reference compounds;[1,2] (ii) on computer matching against commercial mass spectral libraries;[1,3,4] (iii) on comparison of the signals in the ^{13}C-NMR spectra of the mixtures with those of reference spectra compiled in the laboratory spectral library, with the help of a laboratory-made software.[5] This method allows the identification of individual components of the essential oil at content as low as 0.4-0.5% [2-4,6].

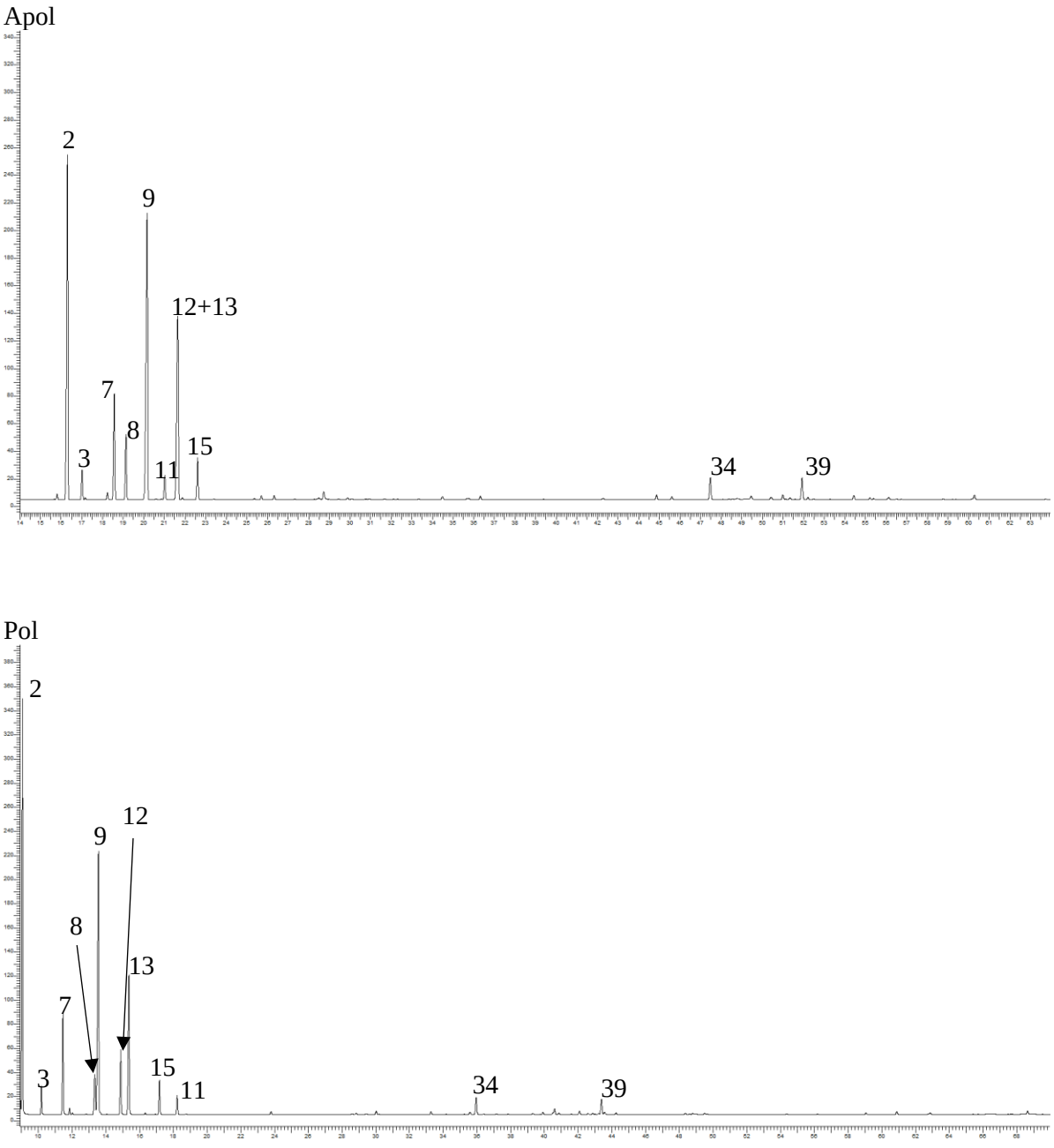

Figure S1: GC Chromatogram of Leaf essential oil of species

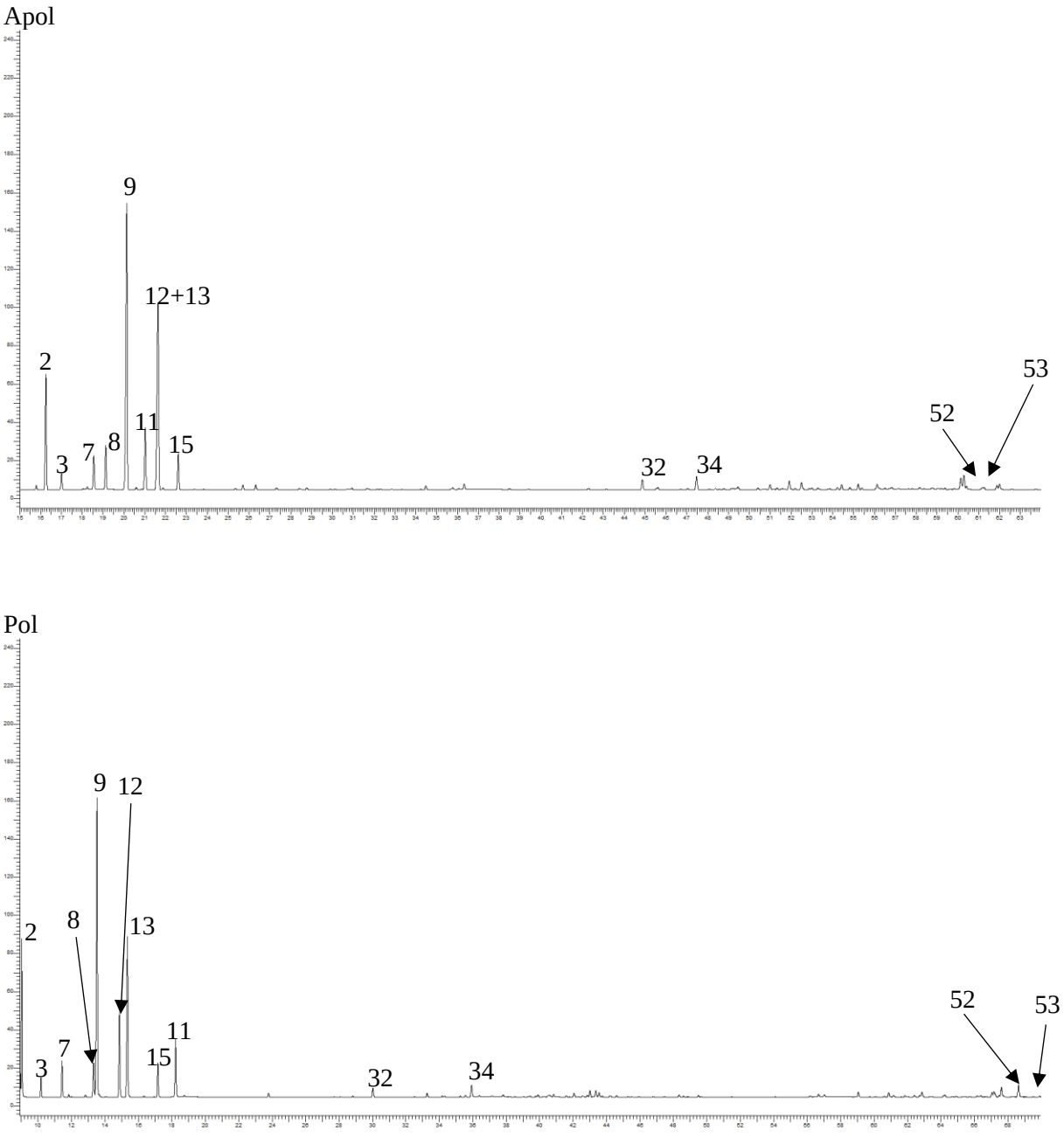

Figure S2: GC Chromatogram of stem essential oil of species

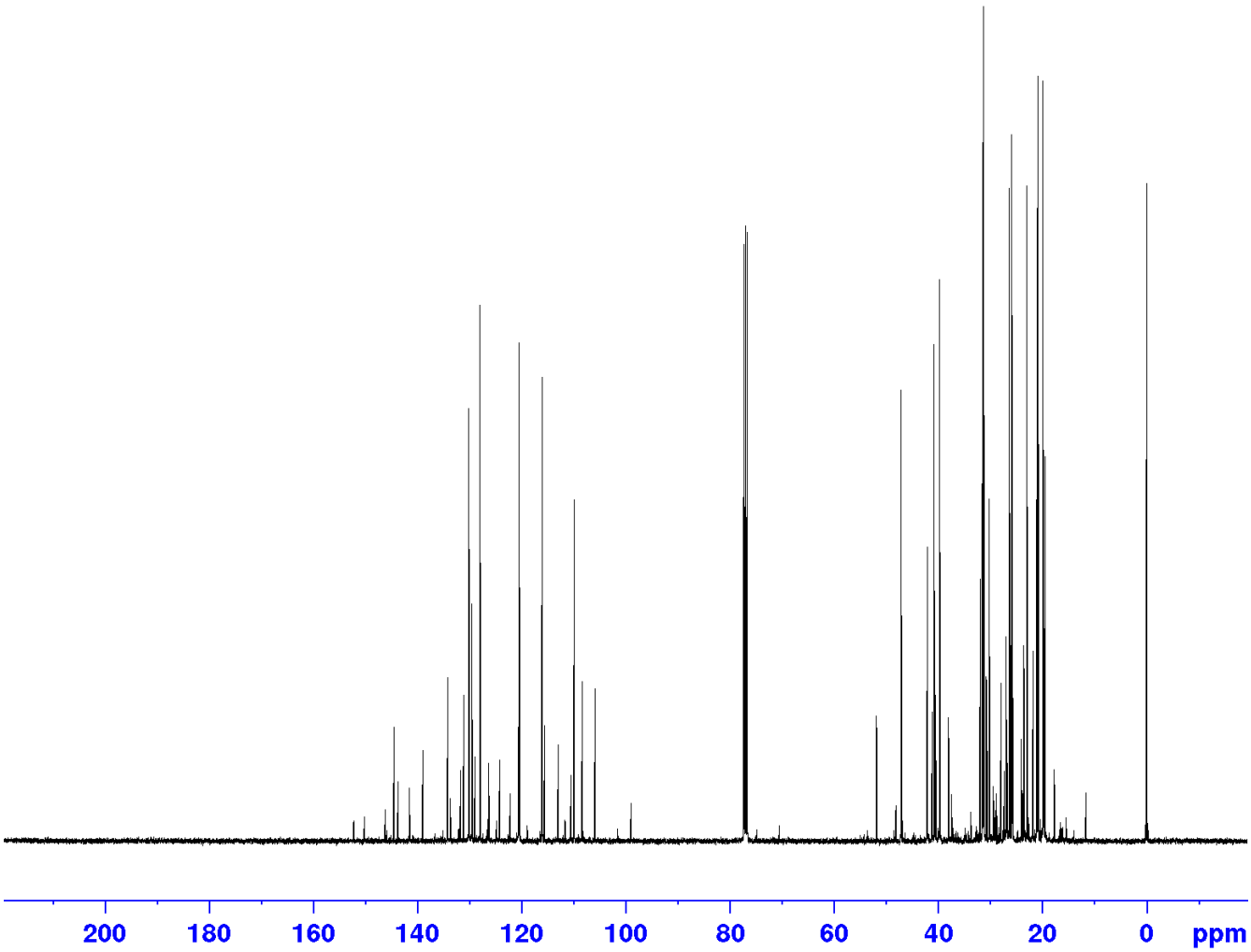

Figure S3: ^{13}C NMR spectrum of leaf essential oil of species

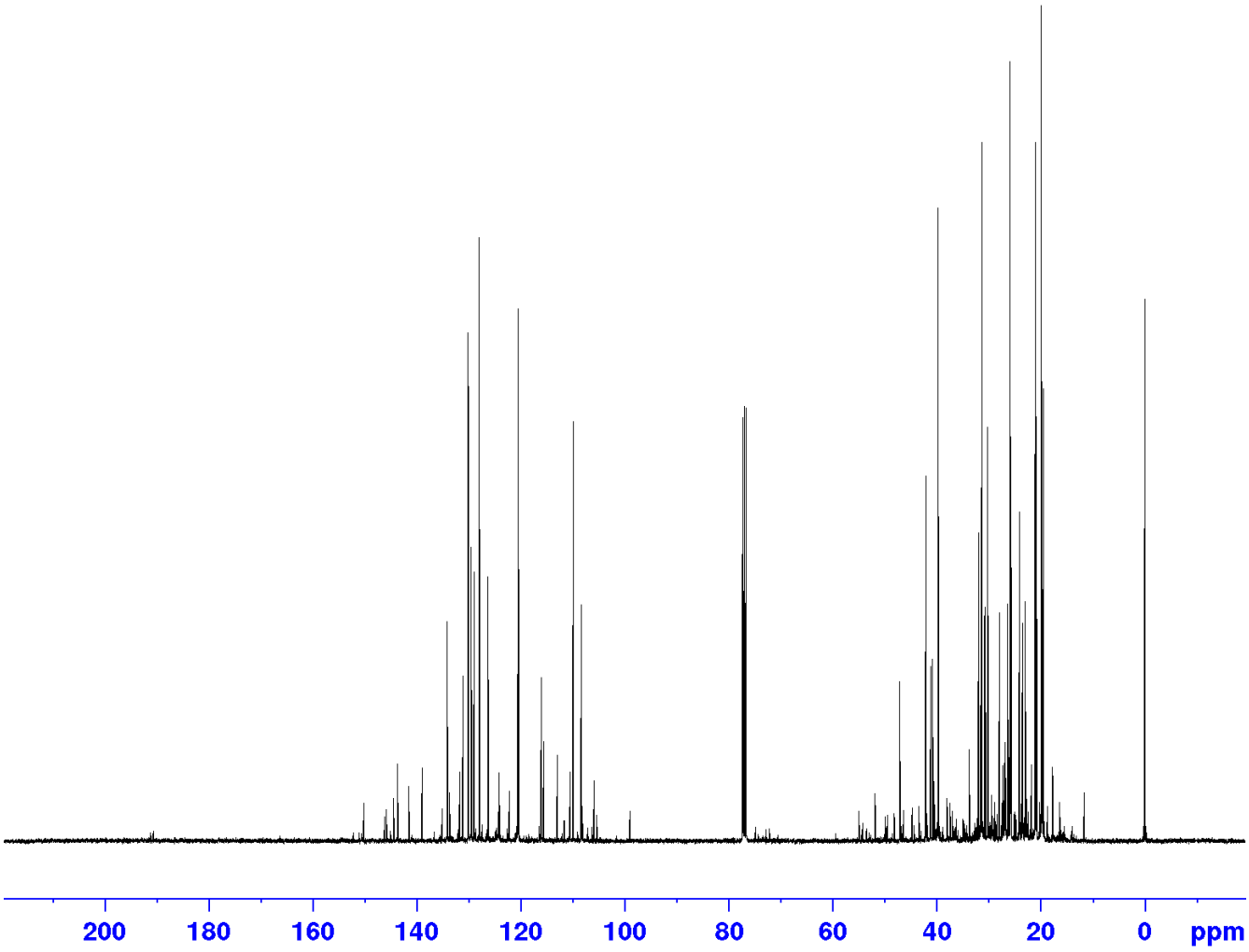

Figure S4: ^{13}C NMR spectrum of stem essential oil of species

Table S3. The Composition of *Illigera trifoliata* ssp. *cucullata* leaf and stem essential oils

No	Compound[a]	Ria[litt]	Rip[litt]	RIa	RI p	%L	%S	Identification mode
1	α-Thujene	916-938	1012-1039	924	1022	0.4	0.4	RI, MS
2	α-Pinene	924-951	1008-1039	932	1022	26.0	9.5	RI, MS, ^{13}C NMR
3	Camphene	936-965	1043-1086	945	1066	2.0	1.5	RI, MS, ^{13}C NMR
4	Thuja-2,4(10)-diene	937-957	1109-1137	948	1128	0.1	t	RI, MS
5	Oct-1-en-3-ol	958-980	1411-1465	963	1449	t	0.1	RI, MS
6	Sabinene	958-981	1098-1140	967	1123	0.5	0.2	RI, MS, ^{13}C NMR
7	β-Pinene	962-987	1085-1130	972	1112	7.5	3.0	RI, MS, ^{13}C NMR
8	Myrcene	975-991	1140-1175	982	1162	4.4	3.7	RI, MS, ^{13}C NMR
9	α-Phellandrene	990-1009	1148-1186	1000	1168	25.8	29.2	RI, MS, ^{13}C NMR
10	δ-3-Carene	997-1027	1122-1169	1007	1149	0.1	0.2	RI, MS
11	*p*-Cymene	1004-1029	1246-1291	1013	1273	1.8	5.7	RI, MS, ^{13}C NMR
12	Limonene*	1018-1026	1178-1219	1023	1203	*5.5**	*8.1**	RI, MS, ^{13}C NMR
13	β-Phellandrene*	1018-1026	1188-1233	1023	1212	*12.8**	*15.8**	RI, MS, ^{13}C NMR
14	(*Z*)-β-Ocimene	1017-1040	1211-1251	1026	1233	0.1	0.1	RI, MS
15	(*E*)-β-Ocimene	1027-1050	1232-1267	1038	1250	2.9	3.2	RI, MS, ^{13}C NMR
16	γ-Terpinene	1035-1062	1222-1266	1050	1246	0.1	0.1	RI, MS
17	Terpinolene	1064-1091	1261-1300	1080	1283	0.1	0.1	RI, MS
18	Linalool	1074-1098	1507-1564	1085	1547	0.3	0.4	RI, MS, ^{13}C NMR
19	3-Acetoxyoct-1-ene	1083-1100	1365-1402	1095	1378	0.3	0.4	RI, MS, ^{13}C NMR
20	*cis*- *p*-Menth-2-en-1-ol	1108^{+}	1559^{+}	1110	1563	t	0.2	RI, MS
21	*trans*-*p*-Menth-2-en-1-ol	1124^{+}	1624^{+}	1126	1616	0.2	0.1	RI, MS
22	*trans*-Verbenol	1114-1157	1665-1691	1131	1678	0.7	0.2	RI, MS, ^{13}C NMR
23	*p*-Cymen-8-ol	1148-1184	1813-1865	1162	1848	0.0	0.1	RI, MS
24	Terpinen-4-ol	1148-1180	1564-1630	1164	1602	0.1	0.2	RI, MS
25	α-Terpineol	1159-1191	1659-1724	1175	1696	0.1	0.2	RI, MS
26	Estragole	1169-1190	1652-1690	1182	1671	t	0.1	RI, MS
27	Neral	1211-1240	1641-1706	1217	1682	0.3	0.4	RI, MS, ^{13}C NMR
28	Geraniol	1231-1256	1795-1865	1237	1845	0.1	0.2	RI, MS
29	Geranial	1242-1252	1680-1750	1245	1733	0.3	0.6	RI, MS, ^{13}C NMR
30	Carvacrol	1272-1300	2140-2246	1278	2195	0	0.1	RI, MS
31	δ-Elemene	1322-1381	1447-1481	1337	1468	0.1	0.1	RI, MS
32	α-Copaene	1360-1392	1462-1522	1377	1489	0.4	1.1	RI, MS, ^{13}C NMR
33	β-Elemene	1372-1403	1565-1608	1389	1587	0.3	0.3	RI, MS
34	(*E*)-β-Caryophyllene	1400-1442	1569-1632	1419	1594	1.8	1.4	RI, MS, ^{13}C NMR
35	*trans*-α-Bergamotene	1417-1440	1560-1590	1434	1581	t	0.1	RI, MS
36	α-Humulene	1430-1466	1637-1689	1452	1666	0.3	0.3	RI, MS
37	Germacrene D	1458-1491	1676-1726	1477	1705	0.4	0.5	RI, MS, ^{13}C NMR
38	β-Selinene	1463-1498	1686-1743	1483	1714	0.1	0.1	RI, MS
39	Bicyclogermacrene	1474-1501	1692-1757	1493	1729	1.8	0.9	RI, MS, ^{13}C NMR
40	(*E*,*E*)-α-Farnesene	1484-1509	1714-1763	1497	1745	0.2	0.1	RI, MS
41	β-Bisabolene	1485-1511	1698-1748	1502	1722	0.1	0.8	RI, MS, ^{13}C NMR
42	Calamenene#	1492-1528	1802-1844	1509	1828	0	0.1	RI, MS
43	δ-Cadinene	1498-1526	1722-1774	1511	1752	0	0.2	RI, MS
44	β-Elemol	1518-1555	2043-103	1536	2076	0.4	0.5	RI, MS, ^{13}C NMR
45	*cis*-7-*epi*-Sesquisabinene hydrate	1543^{+}	1991^{+}	1543	1995	0.0	0.2	RI, MS
46	(*E*)-Nerolidol	1538-1565	1995-2055	1550	2034	0.2	0.5	RI, MS, ^{13}C NMR
47	Spathulenol	1549-1580	2074-2150	1565	2118	0.2	0.6	RI, MS, ^{13}C NMR
48	Caryophyllene oxide	1549-1587	1936-2023	1572	1978	0.1	0.1	RI, MS
49	Globulol	1561-1595	2049-2104	1577	2082	0	0.2	RI, MS
50	Zingiberenol I	1599^{+}	2107^{+}	1601	2107	0	0.2	RI, MS
51	10-*epi*-γ-Eudesmol	1595-1617	2089-121	1623	2108	0	0.1	RI, MS
52	β-Eudesmol	1611-1655	2196-2272	1637	2223	0	1.3	RI, MS, ^{13}C NMR
53	Selin-11-en-4α-ol	1632-1656	2207-2274	1639	2246	0.5	1.6	RI, MS, ^{13}C NMR
54	α-Eudesmol	1619-1668	2186-2250	1642	2220	t	0.2	RI, MS
55	β-Bisabolol	1644-1674	2090-2189	1655	2148	0	0.2	RI, MS
56	*epi*-β-Bisabolol	1651-1690	-	1657	-	0	0.2	RI, MS
57	α-Bisabolol	1649-1686	2178-2234	1668	2210	0	0.4	RI, MS, ^{13}C NMR
58	*epi*-α-Bisabolol	1651-1690	2186-2256	1671	2213	0	0.6	RI, MS, ^{13}C NMR
	Total					99.4	97.2	

[a] Order of elution and percentages are given on apolar column (BP-1), excepted those whose names are followed by an asterisk (*), percentages on polar column (BP-20) ; #correct isomer not identified; RI[litt] (apolar and polar), except components 20 and 21, component 45 and component 50.

S.4. Microbial Strains

The antimicrobial activity of the essential oils was evaluated using 1 strain of Gram-positive test bacteria *Staphylococcus aureus* (ATCC 13709), 1 strain of Gram-negative test bacteria *Escherichia coli* (ATCC 25922), and 1 strain of yeast *Candida albicans* (ATCC 10231). Minimum inhibitory concentration (MIC) and median inhibitory concentration (IC_{50}) values were determined using 3 strains of Gram-positive test bacteria including *Staphylococcus aureus* (ATCC 13709), *Bacillus subtilis* (ATCC 6633), and *Lactobacillus* fermentum (VTCC N4), 3 strains of Gram-negative test bacteria including *Salmonella* enterica (VTCC), Escherichia coli (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 15442), and 1 strain of yeast *Candida albicans* (ATCC 10231). The ATCC strains were obtained from American Type Culture Collection, The VTCC strains were obtained from Vietnam Type Culture Collection – Vietnam National University, Hanoi.

S.5. Screening of Antimicrobial Activity

The agar disk diffusion method was performed to test the antimicrobial activity of essential oil [7-8]. Testing media included Mueller-Hinton Agar used for bacteria and Sabouraud Agar used for fungi. Microorganisms were stored at −80°C and activated by culture medium prior to testing to reach concentration of 1.0 × 106 CFU/mL. A 100 µL inoculum solution was taken and spread evenly over the surface of the agar. Four holes were made on agar plates (about 6 mm in diameter each hole) using an aseptic technique. A total of 50 µL of each of original essential oil and two-fold diluted oil was put into each two holes using a pipette. The petri dishes were kept at room temperature for 2 to 4 hours and then incubated at 37°C for 18 to 24 hours. The presence or absence of growth around each antimicrobial disk on each plate culture was observed. The diameters of inhibition growth zones values were measured using a ruler with millimeter markings. The zone of inhibition is the point at which no growth is visible to the unaided eye. An inhibition zone of 14 mm or greater (including diameter of the hole) was considered as high antibacterial activity [9-10].

Minimum inhibitory concentration (MIC) and median inhibitory concentration (IC_{50}) values were measured by the microdilution broth susceptibility assay [10-11]. Stock solutions of the oil were prepared in dimethylsulfoxide (DMSO). Dilution series were prepared from 8192 µg/mL to 2 µg/mL (2^{13}, 2^{12}, 2^{11}, 2^{10}, 2^{9}, 2^{7}, 2^{5}, 2^{3}, 2^{1} µg/mL) in sterile distilled water in micro-test tubes, from where they were transferred to 96-well microtiter plates. Bacteria grown in double-strength Mueller-Hinton broth or double-strength tryptic soy broth, and fungi grown in double-strength Sabouraud dextrose broth were standardized to 5×10^5 and 1×10^3 CFU/mL, respectively. The last row, containing only the serial dilutions of sample without microorganisms, was used as a negative control. Sterile distilled water and medium served as a positive control. After incubation at 37°C for 24 h, the MIC values were determined at well with the lowest concentration of agents completely inhibit the growth of microorganisms. The IC_{50} values were determined by the percentage of microorganisms inhibited growth based on the turbidity measurement data of EPOCH2C spectrophotometer (BioTeKInstruments, Inc Highland Park Winooski, USA) and Rawdata computer software (Belgium) according to the following equations:

$$\%\ \text{inhibition} = \frac{OD_{control(+)} - OD_{test\ agent}}{OD_{control(+)} - OD_{control(-)}} \times 100\%$$

$$IC_{50} = High_{Conc} - \frac{(High_{Inh\%} - 50\%) \times (High_{Conc} - Low_{Conc})}{(High_{Inh\%} - Low_{Inh\%})}$$

Where:
OD: optical density; control (+): only cells in medium without Antimicrobial agent; test agent: coresponds to a known concentration of Antimicrobial agent; control (-): culture medium without cells.

$High_{Conc}$/Low_{Conc}: Concentration of test agent at high concentration/low concentration;
$High_{Inh\%}$/$Low_{Inh\%}$: % inhibition at high concentration/% inhibition at low concentration.

Reference materials: Ampicillin for Gram-positive bacterial strains with MIC values in the range of 0.004 to 1.2 µg/mL, Cefotaxime for Gram-negative bacterial strains with MIC values in the range of 0.07-19.23µg/mL, Nystatine for fungal strains with MIC value of 2.8 µg/mL.

S.6. Antibacterial and Anti-yeast Activity

Essential oil extracts of *Illigera trifoliata* ssp. *cucullata* (Merr.) Kubitzki) at concentrations of 100% and diluted 50% were used to screen the antimicrobial activity. The standard agar disk diffusion method [7-8] was performed against three test microorganisms including *Staphylococcus aureus* (Gr(+) bacteria), *Escherichia* coli (Gr(-) bacteria), and *Candida albicans* (yeast).

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