SUPPLEMENTARY MATERIAL

Chemical composition and anti-inflammatory activity of the essential oil from the leaves of *Limnocitrus littoralis* (Miq.) Swingle from Vietnam

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Abstract: The oil from the leaves of *Limnocitrus littoralis* (Miq.) Swingle, was obtained by hydrodistillation and investigated by gas chromatography combined with mass spectrometry (GC/MS), the anti-inflammatory ef[1]fect of the oil was examined on a LPS-induced RAW264.7 cells. An aggregate of forty components were identified, representing 93.0% of the oil. This oil was subjugated by monoterpene hydrocarbons (27.7%), sesquiterpene hydrocarbons (32.3%) and oxygenated sesquiterpenes (4.6%). The significant constituents of *L. littoralis* essential oil were determined as follows; myrcene (24.9%), γ–muurolene (11.0%), and oleic acid (10.3%). The essential oil of *L. littoralis* showed activity against the nitric oxide (NO) generation with the IC_{50} values to 12.50±1.19 µg/L. The anti-inflammatory effect of essential oil from the leaves of *L. littoralis* is reported for the first time.

Keywords: *Limnocitrus littoralis* (Miq.) Swingle; *essential oil; anti-inflammatory activity*

Experimental

Plant material

The leaves of *Limnocitrus littoralis* (Miq.) Swingle (3 × 40 g), were collected from Quang Ngai province, Vietnam in September 2018 (15°25'55.6"N 109°04'56.1"E) and were identified by Dr. Nguyen Tien Chinh, Vietnam National Museum of Nature. A voucher specimen (QNG-T112) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

Hydrodistillation of the essential oils

Air-dried leaves of *L. littoralis* (3 × 40 g) were shredded and their oils obtained by hydrodistillation using a Clevenger apparatus for three and half hours at normal pressure, according to the Vietnamese Pharmacopoeia (1997). The essential oil was then collected, dried by sodium sulfate, and stored in sealed vials at 4^{o}C. The experiments were performed in triplicate.

Analysis of the essential oil

The chemical composition of the essential oil (Table S1) was analysed using the gas chromatography combined with mass spectrometry (GC/MS) QP2010 system (Shimadzu, Kyoto, Japan) equipped with Inert CAP 5 column (30 m × 0.25 mm, film thickness 0.25 µm). The column oven temperature was gradually increased from 60˚C to 280˚C at 2˚C/min. Helium was used as the carrier gas at 1.8 mL/min, and the sample (1 µL) was injected in the split ratio at 1:17; injector temperature was 280˚C. The essential oil was dissolved with *n*-hexane before injection. The MS conditions were as follows: ionization voltage 70 eV; acquisitions scan mass range of 40-500 amu; ion source temperature: 230˚C at a sampling rate of 1.0 scan/s. Identification of the constituents of essential oil was carried out on the basis of retention index (RI determined with reference to homologous series of *n*-alkanes C_8 - C_{31}) under identical experimental conditions. The identification of individual constituents was performed by mass spectra library search (NIST 08 matching the database) and by comparision of the retention indexes (RI) of the separated constituents with data reported in the literature (Stenhagen et al 1974; Adams 2017). The relative percentages of particular components in the essential oil were calculated on the basis of area percent report (uncalibrated calculation procedure) generated by GC software.

Biological material

Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sodium nitrite, sulfanilamide, N-1-napthylethylenediamine dihydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were purchased from Grand Island Biological Company (GIBCO, Invitrogen). RAW 264.7 cell lines were gotten from University of Perugia, Perugia, Italy. Cell culture flasks and 96-well plates were from Corning Inc. (Corning, USA). The ELISA Plate Reader (Bio-Rad, California, USA) was utilized to measure the absorbance of cells in the MTT cell viability assay.

Cell culture

RAW264.7 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM L-Glutamine, 10mM HEPES, 1mM sodium pyruvate and 10% fetal bovine serum (FBS). The cells were maintained at 37ºC with 5% CO_2 in a humidified chamber. The cells were sub-cultured every 3-5 days with the ratio of (1:3) and incubated at 37°C under humidified 5% carbon dioxide atmosphere (Tsai et al. 2007; Joo et al. 2014).

Inhibition of nitric oxide (NO) production

The authors decided to chose nitric oxide (NO) method because NO is well known proinflammatory mediator. The over-production of NO inducing tissue damage associated with acute and chronic inflammation (Bogdan et al., 2000). Besides, inhibition of NO production in LPS-stimulated RAW 264.7 cells is widely used to screen the anti-inflammatory activity from plant sources (Joo et al., 2014; Ng et al., 2015; Soonthornsit et al., 2017). Although cells are expensive, we studied this experiment to get reliable results.

Various concentrations of the essential oil of *L. littoralis* (100, 20, 4, and 0.8 µg/mL) were utilized in evaluating the inhibition of NO produced (Table S2). The RAW264.7 cells were seeded at a density of 2×10^5 cells/well in 96-well plates and incubated for 24h at 37°C and 5% CO_2. Then, the media of each well were aspirated and fresh FBS-free DMEM media were replaced for 3h. Test samples were added carefully into each well of 96-well plates and the cultivation was continued under the same conditions. After 2h treatment, cells were stimulated with 1µg/mL of LPS for 24 h. The presence of nitrite was determined in cell culture media using commercial NO detection kit Griess Reagent System (Promega Cooperation, WI, USA). Protocols supplied with the assay kit were used for the application of the assay procedure. Briefly, 100 µL of cell culture medium with an equal volume of 100 µL Griess reagent: 50µL of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 50 µL 0.1% (w/v) N-1-naphthyl ethylenediamine dihydrochloride in a 96-well plate was incubated at room temperature for 10min. At that point, the absorbance was measured at 540nm in a microplate reader (Bio-Rad, California, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve. FBS-free DMEM media was used as a blank sample while L-N^G-monomethyl arginine citrate (L-NMMA) was used as positive control and macrophages stimulated with LPS at 1 µg/mL and untreated was used as a negative control. The ability to inhibit the nitric oxide (NO) was measured at doses of 100, 20, 4, and 0.8 µg/mL respectively

and estimated as a half maximal inhibitory concentration (IC_{50}), which was calculated by the program TableCurve Version 4.0 (Tsai et al. 2007; Joo et al. 2014).

MTT cell viability assay

RAW 264.7 cells were seeded in 96-well plates in the presence of various concentrations of essential oil of *L. littoralis*. After 24 h of incubation, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h at 37°C and 5% CO_2. Finally, the supernatant was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm. The percentage of dead cells was determined relative to the control group (Tsai et al. 2007; Joo et al. 2014).

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Table S1. Chemical composition of the essential oil from the leaves of *Limnocitrus littoralis*

N_o	**Compounds**	**RT**	**RI[a]**	**RI[b]**	**Percentage (%)**
1	β-Pinene	6.84	974	972	0.5
2	**Myrcene**	**7.43**	**988**	**992**	**24.9**
4	*(Z)*-β-Ocimene	9.21	1032	1034	1.1
5	*(E)*-β-Ocimene	9.67	1044	1044	0.4
6	Linalool	12.18	1095	1099	0.6
7	Citronellal	15.02	1148	1149	0.3
8	*(2E)*-Nonenol	15.99	1163	1166	0.3
9	δ-Elemene	26.18	1335	1332	0.8
10	β-Elemene	29.67	1389	1387	0.8
11	*(E)*-Caryophyllene	31.20	1417	1411	1.4
12	*(E)*-α-Bergamotene	32.42	1432	1431	0.5
13	α-Humulene	33.30	1452	1445	0.8
14	Geranyl acetone	33.61	1453	1450	1.3
15	*(E)*-9-epi-Caryophyllene	33.75	1464	1452	0.8
16	Sesquisabinene	33.92	1457	1455	0.4
17	Dauca-5,8-diene	34.88	1471	1471	0.3
18	**γ-Muurolene**	**35.09**	**1478**	**1474**	**11.0**
19	Neryl isobutanoate	35.39	1490	1479	0.2
20	Bicyclogermacrene	36.03	1500	1489	8.4
21	γ-Amorphene	36.64	1495	1499	0.3
22	β-Bisabolene	36.98	1505	1505	5.3
23	δ- Amorphene	37.74	1511	1517	0.5
24	β-Sesquiphellandrene	37.84	1521	1519	0.9
25	*(E)*-iso-γ-Bisabolene	39.04	1528	1539	0.2
29	α-Acorenol	44.40	1632	1630	0.3
30	epi-α-Cadinol	44.59	1638	1633	0.9

31	α-Cadinol	45.35	1652	1647	1.1
32	Germacra-4(15),5,10(14)-trien-1-α-ol	47.10	1685	1677	0.4
33	*(2E,6Z)*-Farnesal	48.95	1713	1710	0.2
34	*(2E,6Z)*-Farnesol	49.41	1714	1719	0.5
35	*(2E,6E)*-Farnesal	50.45	1715	1738	0.2
36	Hexadecanoic acid	62.22	1959	1964	3.2
37	Linoleic acid	70.30	2133	2134	3.3
38	**Oleic acid**	70.65	2142	2141	**10.3**
39	Nezukol	70.89	2133	2146	5.8
40	Ethyl octadecanoate	71.72	2196	2164	3.2
Sesquiterpene hydrocarbons					**32.3**
Monoterpene hydrocarbons					**27.7**
Oxygenated sesquiterpenes					**4.6**
Oxygenated Monoterpenes					**0.8**
Others					**27.6**
Total					**93.0**

[a]Retention Indices on INERT CAP 5 column; [b]Retention Indices in literature (Adams 2017)

Table S2. Biological activity of the essential oil of *L. littoralis* on the NO inhibitory activity

Concentration (µg/ml)	**Essential oil of *L. littoralis***		**L-NMMA**	
	% Inhibition NO	**% Survival cells**	**% Inhibition NO**	**% Survival cells**
100	82.69	50.94	85.38	85.34
20	68.56	99.63	72.98	90.93
4	18.35	> 100	37.41	> 100
0.8	5.63	> 100	15.02	> 100
IC_{50}	**12.50±1.19**		**7.61±0.59**	