SUPPLEMENTARY MATERIAL

Studies on chemical constituents and anti-hepatoma effects of essential oil from *Annona squamosa* L. pericarps

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Abstract

Annona squamosa L. fruit played great anti neoplastic activities. Its pericarps were discarded as waste. In this study, essential oil extracted from *A. squamosa* L. pericarps (APEO) was obtained by hydrodistillation and analyzed by GC–MS. Furthermore, the anti hepatoma activities and the underlying mechanism of the oil were firstly described.

A total of 59 compounds were identified by GC-MS. The major compound in the oil was (-)-spathulenol (32.51%). The APEO demonstrated anti hepatoma activity against SMMC-7721 hepatoma cell line with IC₅₀ lower than 55 µg/mL. At the same time, nucleus shrinkage or broken were found in cells incubated with APEO through fluorescent microscope. In addition, pro-apoptosis and cell cycle arrest effects were confirmed by flow cytometry analysis.

Keywords: *Annona squamosa* L.; essential oil; GC-MS; apoptosis; cell cycle

Supporting information

Table S1. Chemical compounds of the essential oil of *Annona squamosa* L. pericarps(APEO)

Figure S1. Total ion chromatogram (GC-MS TIC) from APEO

Figure S2. Inhibition of APEO on proliferation of SMMC-7721 cells. APEO inhibited the proliferation of 7721 cells in a concentration-dependent manner.

Figure S3. Apoptosis was investigated by flow cytometry. D was the control group. E was the low concentration group. F was the high concentration group.

Figure S4. Cell phase distribution was analyzed by flow cytometry. H was the control group. I was the low concentration group. J was the high concentration group. K was a cell phase distribution histogram of different groups.

Experimental section

1.1 Supplies and chemicals

Bovine calf serum was purchased from Zhejiang Tianhang Biotechnology (Hangzhou, China). Hoechst Staining Kit was purchased from Beyotime Biotechnology (Shanghai, China). Dimethylsulfoxide was purchased from Cell Signaling Technology (Beverly, MA, USA). DMEM medium, MTT, PI staining kit and AnnexinV-FITC/PI apoptosis detection kit were purchased from KeyGen Biotechnology (Nanjing, China).

1.2 Plant materials and APEO isolation

The fruit of *A. squamosa* L. was collected from lincang, Yunnan Province in October 2015 and identified by Prof. Jian-wei Chen (Nanjing University of Chinese Medicine, Jiangsu, China). The pericarps were dried in the shade of the nature A voucher specimen (No. 20151022zh) was deposited in the Pharmaceutical College.

Dried pericarps of *A. squamosa* L. were powdered and subjected to hydrodistillation for 4 h using a modified clevenger type apparatus (Liu et al., 2016) . The oil was collected, dried over anhydrous sodium sulphate and stored at 4 °C in the dark until analysis.

1.3 Gas chromatography–mass spectrometry (GC–MS) analysis

Qualitative analyzes of APEO were carried out using an Agilent 6890-5975 GC-MS with an Agilent HP-5ms capillary column. Helium was the carrier gas at 1.0 mL/min flow rate. The oven temperature program was at 60 °C for 4 minutes, at a rate of 5 °C/min to 170 °C, at 170 °C for 4 min, then at 5 °C/min to 250 °C, and at 250 °C for 5 min. The volume injected was 1 µL with a 1:20 split ratio.

1.4 Cell culture

The human hepatoma cell line SMMC-7721 were purchased from KeyGen Biotechnology (Nanjing, China) and cultured in DMEM medium supplemented with 10 % bovine calf serum, 100 µg/mL streptomycin and 100 µg/mL penicillin at 37 °C in a wettish atmosphere of 5 % CO₂. Cells were passaged every 2-3 days.

1.5 Cell viability

To evaluate the viability of SMMC-7721 hepatoma cells, cells were seeded in 96-well flat-bottomed plates both at the density of 8×10^3 cell per well and incubated with various concentrations (200 µL) of the APEO for 48 h. Then 20 µL MTT solution (5 mg/ml) was added to each well, and the cells were further incubated for 4 h. After removing the medium, 150 µL of dimethylsulfoxide was added to solubilize the MTT formazan salt. The absorbance of solution was measured on a microplate reader (Spectra MAX190, Molecular Devices) at 490 nm. According to the absorbances of control wells and medicated wells, the value of IC₅₀ was calculated by SPSS. Cell viability assay was repeated 3 times at least.

1.6 Apoptosis analysis

To investigate the mechanism of APEO involved in the anti-proliferative activity on SMMC-7721 cells, the above hepatoma cells was seeded onto 6-wells plate at a density of 2×10^5 cells per well. 12h later, cells were treated with APEO at two different concentrations (20 and 40 µg/mL) for 24 h. Then the cells were gathered

respectively and washed with PBS.

For apoptosis analysis, some of the cells were observed by fluorescent microscope (Axio Imager A2, Carl Zeiss Jena, Germany), after dyed with Hoechst Staining Kit. Some were dyed with Annexin V/PI and analyzed using a flow cytometer (Accuri C6, Becton & Dickson, San Jose, CA, USA).

1.7 Cell cycle analysis

For analysis on the cell cycle distribution, the washed cells were fixed in cold 70% ethanol at -20 °C and attached with PI according to the manufacturer's recommendation.

Table S1. Chemical compounds of the essential oil of *A. squamosa* L. pericarps(APEO)

Retention time	Compounds	Molecular formula	Molecular weight	Peak area %
9.993	4,4-dimethyl-Tetracyclo[5.2.1.0(2,6).0(3,5)]decane	C ₁₂ H ₁₈	162	0.30
10.611	Nonanal	C ₉ H ₁₈ O	142	0.28
11.275	2,2,3-trimethyl-3-Cyclopentene-1-acetaldehyde	C ₁₀ H ₁₆ O	152	0.31
11.675	[1S-(1 α ,3 α ,5 α)]-6,6-dimethyl-2-methylene-Bicyclo[3.1.1]heptan-3-ol	C ₁₀ H ₁₆ O	152	1.02
11.790	(1S)-1,7,7-trimethyl-Bicyclo[2.2.1]heptan-2-one	C ₁₀ H ₁₆ O	152	0.76
11.898	[1S-(1 α ,2 β ,5 α)]-4,6,6-trimethyl-Bicyclo[3.1.1]hept-3-en-2-ol	C ₁₀ H ₁₆ O	152	0.69
12.007	3-Methylenecyclohexene	C ₇ H ₁₀	94	0.44
12.373	6,6-dimethyl-2-methylene-Bicyclo[2.2.1]h	C ₁₀ H ₁₄ O	150	0.73

	eptan-3-one			
12.505	(1 <i>S</i> -endo)-1,7,7-trimethyl-Bicyclo[2.2.1]heptan-2-ol	C ₁₀ H ₁₈ O	154	3.17
12.837	(<i>R</i>)-4-methyl-1-(1-methylethyl)-3-Cyclohexen-1-ol	C ₁₀ H ₁₈ O	154	0.44
13.363	6,6-dimethyl-Bicyclo[3.1.1]hept-2-ene-2-carboxaldehyde	C ₁₀ H ₁₄ O	150	1.11
13.787	4,6,6-trimethyl-Bicyclo[3.1.1]hept-3-en-2-one	C ₁₀ H ₁₄ O	150	0.56
15.858	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-ester	C ₁₂ H ₂₀ O ₂	196	3.70
16.270	(-)-trans-Pinocarvyl acetate	C ₁₂ H ₁₈ O ₂	194	0.24
17.541	α -Cubebene	C ₁₅ H ₂₄	204	0.55
18.244	Copaene	C ₁₅ H ₂₄	204	0.46
19.028	4,4,11,11-tetramethyl-7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol	C ₁₅ H ₂₄ O	220	0.22
19.366	Caryophyllene	C ₁₅ H ₂₄	204	0.23
19.864	[1 <i>aR</i> -(1 <i>a</i> α ,4 <i>a</i> α ,7 <i>a</i> ,7 <i>a</i> β ,7 <i>b</i> α .)]-decahydro-1,1,7-trimethyl-4-methylene-1H-Cyclopropene]azulene	C ₁₅ H ₂₄	204	0.20
20.241	Caryophyllene oxide	C ₁₅ H ₂₄ O	220	0.32
20.808	[1 <i>S</i> -(1 <i>a</i> ,7 <i>a</i> ,8 <i>a</i> α)]-1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-Naphthalene	C ₁₅ H ₂₄	204	1.07
21.122	2-methyl-9-(prop-1-en-3-ol-2-yl)-Bicyclo[4.4.0]dec-2-ene-4-ol	C ₁₅ H ₂₄ O ₂	236	0.50

21.277	(S)-6-ethenyl-6-methyl-1-(1-methylethyl)- 3-(1-methylethylidene)-Cyclohexene	C ₁₅ H ₂₄	204	0.53
21.786	(1 α ,4 $\alpha\alpha$,8 $\alpha\alpha$)-1,2,3,4,4a,5,6,8a-octahydro- 7-methyl-4-methylene-1-(1-methylethyl)- Naphthalene	C ₁₅ H ₂₄	204	0.74
21.952	(1S-cis)-1,2,3,5,6,8a-hexahydro-4,7-dimet hyl-1-(1-methylethyl)-Naphthalene	C ₁₅ H ₂₄	204	1.55
22.450	5,8-dimethyl-Quinoline	C ₁₁ H ₁₁ N	157	0.69
22.736	trans-Longipinocarveol	C ₁₅ H ₂₄ O	220	1.73
22.999	Aromadendrene oxide-(2)	C ₁₅ H ₂₄ O	220	0.85
23.343	(-)-Spathulenol	C ₁₅ H ₂₄ O	220	32.51
23.646	Aristolene epoxide	C ₁₅ H ₂₄ O	220	1.68
23.880	Isoaromadendrene epoxide	C ₁₅ H ₂₄ O	220	0.54
24.086	2-methylene-6,8,8-trimethyl-Tricyclo[5.2. 2.0(1,6)]undecan-3-ol	C ₁₅ H ₂₄ O	220	2.22
24.424	1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1- methylethyl)-Naphthalene	C ₁₅ H ₂₄	204	0.67
24.733	2-isopropyl-5-methyl-9-methylene-Bicycl o[4.4.0]dec-1-ene	C ₁₅ H ₂₄	204	1.45
25.110	(6,8-Bis-hydroxymethyl-4-isopropyl-7-me thylene-bicyclo[3.2.1]oct-1-yl)-methanol	C ₁₅ H ₂₆ O ₃	254	3.20
25.568	Murolan-3,9(11)-diene-10-peroxy	C ₁₅ H ₂₄ O ₂	236	2.67
25.774	1,3,4,6,7,8a-hexahydro-1,1,5,5-tetramethy l-2H-2,4a-Methanonaphthalen-8(5H)-one, Acetic acid,	C ₁₅ H ₂₄ O	220	1.28
26.141	(1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethyl naphth-2-yl)methyl ester	C ₁₆ H ₂₆ O ₂	250	0.44

27.583	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-en-1-yl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	C ₂₃ H ₃₂ O	324	0.61
27.783	1-(2,4-dichlorophenyl)-Ethanone	C ₈ H ₆ Cl ₂ O	189	0.27
28.687	Aromadendrene oxide-(1)	C ₁₅ H ₂₄ O	220	0.36
29.402	9-Octadecyne	C ₁₈ H ₃₄	250	1.86
30.878	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	296	1.04
31.725	(5 α)-Pregnan-3-one	C ₂₁ H ₃₄ O	302	0.44
33.402	6-Chrysenamine	C ₁₈ H ₁₃ N	243	0.65
33.768	Androst-2,16-diene	C ₁₉ H ₂₈	256	0.66
33.888	13,13-dimethyl-8 β -Podocarpin-7 α -ol	C ₁₉ H ₃₄ O	278	0.68
34.357	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	9.41
34.832	[3R-(3 α ,4 α ,6 α ,10 α ,10 β)]-3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-1H-Naphtho[2,1-b]pyran	C ₂₀ H ₃₄ O	290	0.62
35.416	Kaur-16-ene	C ₂₀ H ₃₂	272	0.35
36.566	7-methoxy-6-(3-methyl-2-butenyl)-2H-1-Benzopyran-2-one	C ₁₅ H ₁₆ O ₃	244	0.69
37.567	14-Oxatricyclo[9..2.1.0(1,10)]tetradecane, 2,6,6,10,11-pentamethyl	C ₁₈ H ₃₀ O	262	1.03
38.019	(3 β ,5 α ,11 β)-Androstane-3,11-diol	C ₁₉ H ₃₂ O ₂	292	1.18
38.683	Oxymetazoline	C ₁₆ H ₂₄ N ₂ O	260	1.71
39.009	Androst-5-ene-3 β -ol	C ₁₉ H ₃₀ O	274	1.21
39.576	(+/-)-7,7-dimethyl-2-oxo-Bicyclo[2.2.1]heptane-1-methanesulfonic acid	C ₁₀ H ₁₆ O ₄ S	232	0.92
40.594	2,4-Dichloro-5-nitrobenzotrifluoride	C ₇ H ₂ Cl ₂ F ₃ NO ₂	260	2.31

40.995	7-Tetradecyne	$C_{14}H_{26}$	194	1.02
	(1 α ,4 $\alpha\beta$,5 α ,8 $\alpha\alpha$)-decahydro-5-(hydroxyme			
43.713	thyl)-5,8a-dimethyl- ζ ,2-bis(methylene)-1-	$C_{20}H_{34}O_2$	306	2.90
	Naphthalenepentanol			

Peak area %: relative percentage (peak area relative to the total peak area)

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Figure S2. Inhibition of APEO on proliferation of SMMC-7721 cells. APEO

inhibited the proliferation of 7721 cells in a concentration-dependent manner.

Figure S3. Apoptosis was investigated by flow cytometry. D was the control group. E was the low concentration group. F was the high concentration group.

Figure S4. Cell phase distribution was analyzed by flow cytometry. H was the control group. I was the low concentration group. J was the high concentration group. K was a cell phase distribution histogram of different groups

References

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