SUPPLEMENTARY MATERIALS

Five new ent-kaurane diterpenes from Annona squamosa L. pericarps

Ya-yun Chen, Cheng-yao Ma, Mao-lin Wang, Jia-hui Lu, Peng Hu, Jian-wei Chen, Xiang Li*, Yong Chen*

College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China
State Key Laboratory Cultivation Base for TCM Quality and Efficacy, Nanjing 210023, China
Jiangsu Key Laboratory for Functional Substance of Chinese Medicine, Nanjing 210023, China

*Corresponding author: Xiang Li, Tel: +8613913925677, Email: lixiang_8182@163.com.
Yong Chen, Tel: +8615251825386, Email: achenyongmail@163.com

Abstract

In the present study, five new ent-kaurane diterpenes including 4α-hydroxy-17,19-dinor-ent-kaurane-16-one (1), 4β-hydroxy-16β-H-18-nor-ent-kaurane-17-oic acid (2), 4β,17-dihydroxy-16α-acetoxy-18-nor-ent-kaurane (3), Annosquamosin Z (4) and 16α-H-ent-kaurane-17,18-dioic acid, 17-methy ester (5) were isolated from Annona squamosa L. pericarp. The compounds were also evaluated for their cytotoxic activities against SMMC-7721 and HepG2 cell lines, among which compound 3 exhibited potent cytotoxicity with IC₅₀ value of less than 20 μM.

Keywords: Annona squamosa Linn; diterpene; hepatoma; SMMC-7721 cell; HepG2 cell
1. Figure S1. Key HMBC and NOESY correlations of compound 1
2. Figure S2. X-ray of compound 1
3. Figure S3. Key HMBC and NOESY correlations of compound 2
4. Figure S4. Key HMBC and NOESY correlations of compound 3
5. Figure S5. Key HMBC and NOESY correlations of compound 4
6. Figure S6. Key HMBC and NOESY correlations of compound 5
7. Table S1. $^1$H NMR and $^{13}$C NMR data of compound 1, compound 2 and compound 3
8. Table S2. $^1$H NMR and $^{13}$C NMR data of compound 4 and compound 5
9. Table S3. IC$_{50}$ values of five diterpenes against SMMC-7721 and HepG2 cell lines
10. Experimental section
11. Mass, $^1$H-NMR, $^{13}$C-NMR, HSQC, HMBC and NOESY spectrums of compound 1 to compound 5
Figure S3. Key HMBC and NOESY correlations of compound 2

Figure S4. Key HMBC and NOESY correlations of compound 3

Figure S5. Key HMBC and NOESY correlations of compound 4

Figure S6. Key HMBC and NOESY correlations of compound 5
| Position | Compound 1 (CDCl₃) | Compound 2 (DMSO-D₆) | Compound 3 (DMSO-D₆) |
|----------|---------------------|-----------------------|-----------------------|
|          | δ_H                 | δ_C                   | δ_H                   | δ_C                   |
| C-1      | CH₂, 0.80, m, 1.85, dr | 39.85                 | CH₂, 0.70, m, 1.67, m | 39.9                  | CH₂, 0.76, m, 1.81, m | 41.7 |
| C-2      | CH₂, 1.78, m         | 18.35                 | CH₂, 1.44, 1.57, m    | 18.1                  | CH₂, 1.35, 1.46, m   | 19.77 |
| C-3      | CH₂, 1.35, m, 1.75, m | 40.88                 | CH₂, 1.24, m, 1.58, m | 43.0                  | CH₂, 1.47, 1.91, m   | 41.50 |
| C-4      | -                   | 72.05                 | OH, 3.91, s           | 70.5                  | OH, 3.91, s          | 70.59 |
| C-5      | CH, 0.93             | 54.38                 | CH, 1.02, m           | 57.3                  | CH, 1.00, m          | 57.34 |
| C-6      | CH₂, 1.67, 1.54, m   | 18.42                 | CH₂, 1.01, 1.79, m    | 19.2                  | CH₂, 1.54, m         | 18.87 |
| C-7      | CH₂, 1.42, 2.37, m   | 37.65                 | CH₂, 2.42, brs        | 8                     | CH₂, 1.06, 1.47, m   | 39.90 |
| C-8      | -                   | -                     | -                     | 39.5                  | -                     | 43.77 |
| C-9      | CH₂, 2.38, m         | -                     | CH, 1.26, m           | 8                     | CH, 1.03, m          | 57.03 |
| C-10     | -                   | -                     | -                     | 44.3                  | -                     | 39.9  |
| C-11     | CH₂, 1.42, d, 1.78, m | 57.0                  | CH₂, 1.15, d, 1.44, m | 5                     | CH₂, 1.47, m         | 18.92 |
|          |                     |                       |                       | 39.7                  |                       |      |
| C-12     | CH₂, 1.53, 1.76      | 47.83                 | CH₂, 1.46, 1.48       | 19.7                  | CH₂, 1.40            | 26.88 |
|          |                     | 39.19                 |                       | 6                     |                       |      |
|          |                     | 17.79                 |                       | 27.6                  |                       |      |
|          |                     | 29.56                 |                       | 4                     |                       |      |
| C-13     | CH, 1.16, m          | 54.02                 | CH, 1.78, m           | 42.2                  | CH, 1.30, m          | 43.05 |
| C-14     | CH₂, 1.64            | 40.44                 | CH₂, 1.44, 1.54, m    | 8                     | CH₂, 0.93, 2.02, m   | 38.13 |
| C-15     | CH₂, 1.93, m         | 55.23                 | CH₂, 1.04, 1.95, m    | 41.2                  | CH₂, 1.38, m         | 53.03 |
| C-16     | -                   | 222.9                 | CH₂, 2.83, m          | 8                     | -                     | 77.23 |
|          |                     | 8                     |                       | 40.6                  |                       |      |
|          |                     | -                     |                       | 3                     |                       |      |
| C-17     |                     | 30.93                 | COOH, 12.09, s        | 45.2                  | CH₂, 3.85, d         | 71.21 |
|          |                     | 17.63                 |                       | 9                     |                       |      |
| C-18     | CH₂, 1.17, s         | -                     | -                     | -                     | -                     |      |
| C-19     | -                   | CH₃, 0.95, s          | 23.2                  | CH₃, 0.95, s          | 23.22                |
| C-20     | CH₃, 1.21, s         | CH₃, 0.91, s          | 17.3                  | CH₃, 0.91, s          | 17.28                |
| O₂COCH   | -                   | -                     | -                     | -                     | -                     |      |
| O₂COCH   | -                   | -                     | -                     | -                     | CH₂, 2.03, s         | 21.35 |
Table S2. $^1$H NMR and $^{13}$C NMR data of compound 4 and compound 5

| Position | Compound 4 (DMSO-D$_6$) | | | Compound 5 (CDCl$_3$) | | |
|----------|------------------------| | | | | |
|          | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| C-1      | CH$_2$,0.81,1.84,m     | 41.35       | CH$_2$,0.81,1.85,m | 40.78     |
| C-2      | CH$_2$,1.23,1.65,m     | 18.59       | CH$_2$,1.41,1.86,m | 18.66     |
| C-3      | CH$_2$,0.95,2.03,m     | 37.91       | CH$_2$,0.99,1.21,m | 37.94     |
| C-4      | -                      | 43.22       | -                      | 43.75     |
| C-5      | CH,1.02,m              | 56.34       | CH,1.05,m              | 56.99     |
| C-6      | CH$_2$,1.32,1.38,m     | 19.40       | CH$_2$,1.84,1.77,m     | 22.43     |
| C-7      | CH$_2$,1.38,2.14,m     | 34.73       | CH$_2$,1.44,1.55,m     | 41.06     |
| C-8      | -                      | 38.52       | -                      | 45.39     |
| C-9      | CH,1.00,m              | 52.98       | CH,1.00,m              | 55.08     |
| C-10     | -                      | 39.69       | -                      | 39.60     |
| C-11     | CH$_2$,1.70,1.73,m     | 20.42       | CH$_2$,1.51,1.64,m     | 19.14     |
| C-12     | CH$_2$,1.37,1.74,m     | 29.31       | CH$_2$,1.50,1.50,m     | 31.14     |
| C-13     | CH,2.35,m              | 30.38       | CH,2.45,m              | 41.06     |
| C-14     | CH$_2$,1.18,1.59,m     | 43.94       | CH$_2$,1.86,2.14,m     | 38.01     |
| C-15     | CH$_2$,1.93,2.13,m     | 59.59       | CH$_2$,1.67,m          | 44.63     |
| C-16     | -                      | 212.39      | CH,2.63,m              | 45.09     |
| C-17     | CH$_2$,2.14,2.49,m     | 47.46       | -                      | 177.94    |
| C-18     | CH$_3$,1.13,s          | 29.10       | -                      | 183.88    |
| C-19     | COOH,11.97,s           | 179.2       | CH$_3$,1.22,s          | 29.03     |
| C-20     | CH$_3$,0.91,s          | 16.98       | CH$_3$,0.92,s          | 15.57     |
| COOCH$_3$| -                      | -                       | CH$_3$,3.66,s              | 51.66     |

Table S3. IC$_{50}$ values of five diterpenes against SMMC-7721 and HepG2 cell lines

| Compound | IC$_{50}$ (μM, Mean±SD, n≥3) |
|----------|-------------------------------|
|          | SMMC-7721 | HepG2 |
| 1        | >200         | >200 |
| 2        | 141.9±2.55   | 161.32±15.51 |
| 3        | 17.28±4.98   | 11.56±1.72 |
| 4        | >200         | >200 |
| 5        | >200         | >200 |

Experimental section

1. Supplies and chemicals

Fetal calf serum was purchased from AnJieYou Biotechnology (Nanjing, China). DMEM medium and MTT were purchased from YiFeiXue Biotechnology (Nanjing, China).

The fruit of A. squamosa L. was collected from lincang, Yunnan Province in October 2014 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. 20141017zh) was deposited in the Pharmaceutical College.

2. Isolation and Identification

13 kg dried pericarps of *A. squamosa* L. were powdered. Then the pericarps were extracted by 95% ethanol and concentrated under reduced pressure to obtain a residue (660 g). The extract were subjected to silica gel column with Petroleum ether - ethyl acetate in gradient elution and divided into fifteen fractions, named F1 to F15. The F5 was delivered into silica gel column eluted with Petroleum ether - Acetone 50:1 to 1:1. The crystalline constituent was dissolved and identified compound 1. After the F6 subjected to silica gel column, compound 2, 4 and 5 were purified from this fraction. Compound 3 was purified by silica gel column eluted with methylene chloride -methanol 10:1 to 2:1. All compounds were identified by MS and NMR.

3. Cell culture

The human hepatoma cell lines SMMC-7721 and HepG2 were purchased from KeyGen Biotechnology (Nanjing, China) and cultured in DMEM medium supplemented with 10 % fetal calf serum, 100 μg/mL streptomycin and 100 μg/mL penicillin at 37 °C in a wet atmosphere of 5 % CO₂. Cells were passaged every 2-3 days.

4. Cell viability

To evaluate the viability of SMMC-7721 and HepG2 hepatoma cells, cells were seeded in 96-well flat-bottomed plates both at the density of 8×10³ cell per well and incubated with various concentrations (200 μL) of the compounds 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 GraphPad. Prism 6.0 software. Cell viability assay was repeated 3 times at least.

Mass, ¹H-NMR, ¹³C-NMR, HSQC, HMBC and NOESY spectrums of compound 1 to compound 5
ESI-MS of compound 1

1H NMR of compound 1
$^{13}$C NMR of compound 1

HSQC of compound 1
HMBC of compound 1

NOESY of compound 1
ESI-MS of compound 2

$^{1}H$ NMR of compound 2
$^{13}$C NMR of compound 2

HSQC of compound 2
NOESY of compound 2

MS of compound 3
$^{1}H$ NMR of compound 3

$^{13}C$ NMR of compound 3
HSQC of compound 3

HMBC of compound 3
NOESY of compound 3

MS of compound 4
$^1$H NMR of compound 4

$^{13}$C NMR of compound 4
HSQC of compound 4

HMBC of compound 4
$^1$H NMR of compound 5

$^{13}$C NMR of compound 5
HSQC of compound 5

HMBC of compound 5
NOESY of compound 5