5 Alpha-Reductase and Aromatase Inhibitory Constituents from *Brassica rapa* L. Pollen

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In the screening of biologically active constituents from *Brassica rapa* pollen, the supercritical CO_2 fluid extract (SFE-CO_2) showed potent 5α-reductase and aromatase inhibiting activity. The SFE-CO_2 extract was separated by various chromatographic methods to give two new phytosterol derivatives, 24-methylenecholesterol linolenate (1) and cycloeucalenol linolenate (2), as well as eight known compounds, 24-methylenecholesterol palmitate (3), cycloeucalenol (4), pollinastanol (5), 24-methylenecholesterol (6), linolenic acid (7), palmitic acid (8), monolinolein (9) and monopalmitin (10), compounds 7 and 9 showed potent 5α-reductase inhibitory activity; compounds 1—6 and 10 showed potent aromatase inhibitory activity.

Key words *Brassica rapa* L.; phytosterol; fatty acid; 5α-reductase inhibitory activity; aromatase inhibitory activity

BPH (benign prostatic hyperplasia) is a prevalent syndrome in old man, about 40% men over 50 years suffered from BPH, but no definitive treatment. Pollens extracts have been reported having remarkable anti-BPH activity[1,2] and pollens have similar constitutes, including alkanes, alkenes, fatty acids, triterpene esters triglycerides, flavones, and so on.[3,4] However the anti-BPH active components of pollen remain unknown. In our screening for anti-BPH pollen, *Brassica rapa* pollen exhibited a notable activity for reducing the volume of prostate in rat model with BPH ($p<0.01$, compared with model). In order to clarify the active principles of *Brassica rapa* pollen, water, 95% (v/v) ethanol, acetone and SFE-CO_2 (supercritical CO_2 fluid extract), four extracts were screened on anti-BPH activity. The SFE-CO_2 extract exhibited the remarkable bioactivity than other solvent extracts. In the following target screening the SFE-CO_2 extract showed potent 5α-reductase and aromatase inhibiting activity. Under bioactive guidance this extract was chromatographied by gel column and afforded two new phytosterol derivatives (**1**, **2**) and eight known compounds (**3**—**10**) (Fig. 1). The structure of new compounds was determined using chemical and spectral methods. This report describes the isolation of the constitutes of *Brassica rapa* pollen and determination of their structure. We also report their 5α-reductase and aromatase inhibition bioactivities.

Results and Discussion

Air-dried pollen of *Brassica rapa* L. was extracted with SFE-CO_2 two times. The concentrated extract was fractionated by SiO_2 c.c. (silica gel column chromatography) to afford fractions A—D. Fraction B showed potent 5α-reductase inhibition with inhibitory rate as 48.88±4.6%, fractions A and B showed potent aromatase inhibition with inhibitory rate as 79.6±6.9% and 84.0±0.1%, respectively. So the fractions A and B were successively separated by means of SiO_2 c.c. and HPLC using a reverse phase column to give two new compounds **1** and **2** along with eight known compounds **3**—**10**. The compounds of 24-methylenecholesterol palmitate (**3**), cycloeucalenol (**4**), pollinastanol (**5**), 24-methylenecholesterol (**6**), linolenic acid (**7**), palmitic acid (**8**), monolinolein (**9**) and monopalmitin (**10**) were identified by comparing their ^{1}H-NMR and ^{13}C-NMR and MS data with those reported in literature.[5—9]

Compound **1** was obtained as a colorless oil, and its molecular formula $C_{46}H_{76}O_2$ was established from HR-ESI-MS, which gave a pseudomolecular ion peak at *m*/*z* 681.5585 $[M+Na]^+$. The ^{1}H-NMR, ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectrum (in $CDCl_3$, Table 1) showed the presence of a 24-methylenecholesterol moiety compared with compound **6** and a unsatu-

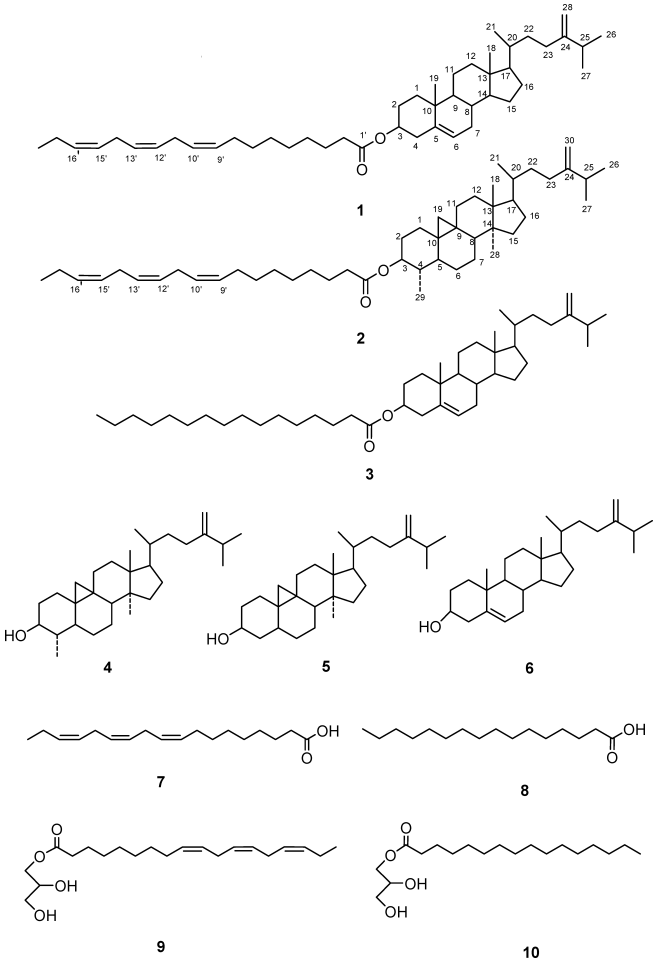

Fig. 1. Chemical Structures of Compounds **1**—**10**

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1** and **2** in $CDCl_3$ (400 MHz and 100 MHz)

Position	**1**		**2**	
	δ_C	δ_H (mult., Hz)	δ_C	δ_H (mult., Hz)
1	36.9 (t)	1.82, m; 1.15, m	31.0 (t)	2.15, m; 1.82, m
2	31.1 (t)	2.10, m; 1.86, m	35.4 (t)	2.58, m; 1.84, m
3	73.2 (d)	4.72 (overlapped with H-28)	78.5 (d)	4.52, m
4	39.5 (t)	2.01, m	43.4 (d)	1.38, m
5	139.5 (s)		41.6 (d)	1.46, m
6	117.4 (d)	5.15, s	24.7 (t)	1.68, m; 1.33, m
7	32.3 (t)	1.26—1.32, m	28.1 (t)	1.80, 1.30, m
8	33.8 (d)	1.34, m	46.9 (d)	1.51, m
9	49.2 (d)	1.65—1.70, m	23.5 (s)	
10	36.5 (s)		29.5 (s)	
11	21.5 (t)	1.55, m; 1.43—1.50, m	25.1 (t)	1.72, m
12	39.5 (t)	2.04, m; 1.18—1.28, m	35.4 (t)	1.43, m
13	42.3 (s)		45.2 (s)	
14	55.0 (d)	1.20—1.30, m	48.7 (s)	
15	25.5 (t)	1.61, m	32.9 (t)	1.61, m
16	27.9 (t)	1.80, m; 1.42—1.50, m	27.1 (t)	1.92, m; 1.34, m
17	56.0 (d)	1.82, m	52.2 (d)	1.72, m
18	12.0 (q)	0.53, s	17.8 (q)	0.90, s
19	13.3 (q)	0.82, s	27.0 (t)	0.40, d (3.8); 0.14, d (3.8)
20	36.2 (d)	1.40, m	36.2 (d)	1.40, m
21	18.4 (q)	0.95, d (7.5)	18.4 (q)	0.90, d (6.2)
22	34.7 (t)	1.54—1.63, m; 1.12—1.21, m	35.0 (t)	1.44—1.53, m; 1.12—1.21, m
23	31.3 (t)	2.05—2.14, m; 1.85—1.94, m	31.3 (t)	2.05—2.14, m; 1.85—1.94, m
24	156.9 (s)		156.9 (s)	
25	33.8 (d)	1.72, m; 1.32, m	33.9 (d)	1.72, m; 1.32, m
26	21.9 (q)	1.03, d (6.8)	21.9 (q)	1.03, d (6.8)
27	22.0 (q)	1.02, d (6.8)	22.0 (q)	1.02, d (6.8)
28	106.0 (t)	4.72, s; 4.66, s	19.2 (q)	0.89, s
29	—	—	14.4 (q)	0.84, d (6.1)
30	—	—	105.9 (t)	4.72, s; 4.66, s
1′	173.6 (s)		173.6 (s)	
2′	34.8 (t)	2.28, t (7.2)	34.8 (t)	2.28, t (7.2)
3′	25.6 (t)	1.57, m	25.6 $(t)_2$	1.57, m
4′	29.1 (t)	1.26, m	29.1 (t)	1.26, m
5′	29.1 (t)	1.27, m	29.1 (t)	1.27, m
6′	29.2 (t)	1.30, m	29.2 (t)	1.30, m
7′	29.6 (t)	1.35, m	29.6 (t)	1.35, m
8′	27.2 (t)	2.04, m	27.2 (t)	2.04, m
9′	132.0 (d)	5.28—5.42, m	132.0 (d)	5.28—5.42, m
10′	128.2 (d)	5.28—5.42, m	128.2 (d)	5.28—5.42, m
11′	25.6 (t)	2.81, m	25.6 (t)	2.81, m
12′	127.8 (d)	5.28—5.42, m	127.8 (d)	5.28—5.42, m
13′	127.2 (d)	5.28—5.42, m	127.2 (d)	5.28—5.42, m
14′	25.5 (t)	2.81, m	25.5 (t)	2.81, m
15′	128.2 (d)	5.28—5.42, m	128.2 (d)	5.28—5.42, m
16′	128.3 (d)	5.28—5.42, m	128.3 (d)	5.28—5.42, m
17′	20.4 (t)	2.04, m	20.4 (t)	2.04, m
18′	14.3 (q)	0.98, t (7.5)	14.3 (q)	0.98, t (7.5)

rated fat acid moiety.[10] The ^{1}H- and ^{13}C-NMR spectral data were almost consistent with those of compound **6** except that the chemical shift of C-3 of **6** was shifted downfield by 1.25 ppm in ^{1}H-NMR and 2.5 ppm in ^{13}C-NMR, respectively. Therefore it was concluded that compound **1** is a 24-methylenecholesterol derivative possessing a fatty acid ester unit at the C-3 position and the C-3 position is the only site which can be esterificated. The HMBC spectrum shown in Fig. 2 also indicates that fatty acid esterificated at C-3. The fatty acid moiety of compound **1** contains 18 carbons and three double bonds, which can be inferred from NMR and ESI-MS data, and was confirmed by the fragment at *m*/*z* 381 $[M-C_{18}H_{29}O_2$ (fatty acid)$]^+$ (base peak, 100%) in its EI-MS. According to published reports[10] on the ^{13}C-NMR chemical shifts of allylic methylene carbons (*Z* alkenes, δ_C <27 ppm; *E* alkenes, δ_C >30 ppm) of alkenes, all the three double bonds of fatty acid part of compound **1** should be in the *Z* configuration (δ 27.2, C-8′; δ 25.6, C-11′; δ 25.5, C-14′; δ 20.4, C-17′). In order to identify the position of three double bonds (C-9′, 10′, 12′, 13′, 15′, 16′), the fatty acid part was analyzed by GC-MS after saponification and derivatization.[11] Compound **1** gave a main peak at 18.6 min, the retention time and mass fragments were both in agreement with that of methyl linolenate. So the fatty acid moiety of compound **1** was a linolenic acid. Therefore, the compound **1** was identified as 24-methylenechlolesterol linolenate.

Compound **2** was obtained as colorless oil, and the molecular was determined to be $C_{48}H_{78}O_2$ from its HR-ESI-MS.

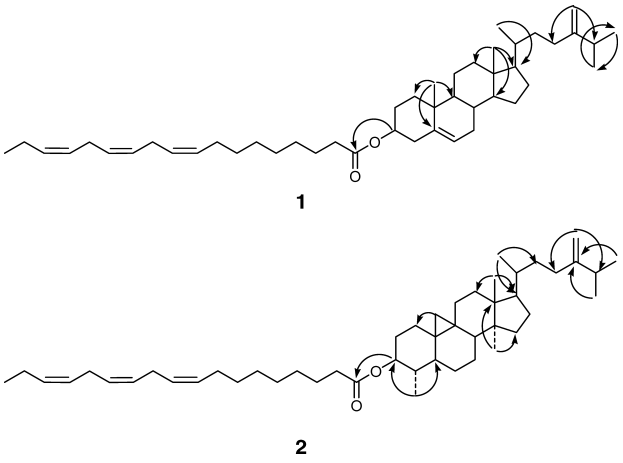

Fig. 2. Key HMBC Correlations from H to C for Compounds **1** and **2**

The ^{1}H-NMR, ^{13}C-NMR and DEPT spectrum (in $CDCl_3$, Table 1) showed the presence of a cycloeucalenol moiety compared with compound **4** and a unsaturated fat acid moiety.[10] The ^{1}H- and ^{13}C-NMR spectral data were similar with those of compound **4** except that the chemical shift of C-3 of **4** was shifted downfield by 1.31 ppm in ^{1}H-NMR and 1.9 ppm in ^{13}C-NMR, respectively. Therefore it was concluded that compound **2** is a cycloeucalenol derivative with a fatty acid ester unit at the C-3 position and the C-3 position is the only site which can be esterificated. The HMBC spectrum shown in Fig. 2 also indicates that fatty acid esterificated at C-3. The fatty acid moiety of compound **2** was deduced as linolenic acid by the same method of compound **1**. So the compound **2** was determined as cycloeucalenol linolenate.

All the compounds isolated from *Brassica rapa* pollen were evaluated for their *in vitro* 5α-reductase and aromatase inhibitory activities. Firstly, the compounds were screened at a concentration of 100 μg/ml. And then the candidate compounds with an inhibitory rate over 50% were examined at concentrate of 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml to calculate their IC_{50}. The results show that compounds **7** and **9** are effective for 5α-reductase inhibition with inhibitory rate as 86.9±0.80% and 64.3±0.48% at 100 μg/ml, compounds **1**—**6** and **10** exhibited potent activity for aromatase inhibition with inhibitory rate as 58.5±12.3%, 61.6±5.3%, 45.9±4.6%, 56.3±0.20%, 67.6±5.7%, 64.1±0.7% and 73.1±1.1% at 100 μg/ml, respectively; the following test show that compounds **7** and **9** have an IC_{50} for 5α-reductase inhibition with values of 0.07 mM and 0.18 mM, and compounds **1**—**6** and **10** have an IC_{50} for anti-aromatase with values of 0.26 mM, 0.22 mM, 0.13 mM, 0.45 mM, 0.03 mM and 0.10 mM, respectively (Tables 2, 3). We can easily come to the conclusion that linolenic acid derivatives (compounds **7**, **9**) have more potent 5α-reductase inhibitory activity, whereas phytosterol derivatives (compounds **1**—**6**) have more potent aromatase inhibitory activity. The two new compounds exhibited similar aromatase inhibitory activity with other phytosterols, but as phytosterol unsaturated fatty esters the new compounds have more double bonds and are liquid at room temperature. They have different solubility and chemical polarity from phytosterol, that is essential factors for the absorption of phytosterol.[12] So compounds **1** and **2** are interesting compounds in term of their chemical structure and biological activity.

Table 2. 5α-Reductase Inhibitory Activity of Compounds **1**—**10**

Compound	Inhibition rate at 100 μg/ml	IC_{50} *a)*
1	6.9±5.1%	—
2	11.5±4.1%	—
3	−71.0±10.8%	—
4	7.3±2.9%	—
5	−10.4±0.8%	—
6	−10.9±3.7%	—
7	86.9±0.80%	0.07 mM
8	25.4±0.80%	—
9	64.3±0.48%	0.18 mM
10	−3.5±0.30%	—
Linolenic acid	86.9±0.80%	0.07 mM

a) IC_{50} values were determined by regression analysis and expressed as the mean of three replicates.

Table 3. Aromatase Inhibitory Activity of Compounds **1**—**10**

Compound	Inhibition rate at 100 μg/ml	IC_{50} *a)*
1	58.5±12.3%	0.26 mM
2	61.6±5.3%	0.22 mM
3	45.9±4.6%	0.13 mM
4	56.3±0.20%	0.45 mM
5	67.6±5.7%	0.03 mM
6	64.1±0.7%	0.0327 mmol/l
7	24.4±4.4%	—
8	28.6±2.1%	—
9	34.2±1.5%	—
10	73.1±1.1%	0.10 mM
Anlumitepian	92.8±2.58%	2.53 μM

a) IC_{50} values were determined by regression analysis and expressed as the mean of three replicates.

Experimental

General Experimental Procedures Optical rotations were determined with a JASCO P-1020 polarimeter. UV spectra were measured on a Shimadzu UV-2500PC UV–Vis spectrometer and ATR-FTIR spectra were recorded on a Nicolet/Nexus 670 FTIR spectrometer with a Miracle single bounce ATR accessory. ^{1}H- and ^{13}C-NMR, spectra were recorded in $CDCl_3$ on a Bruker DRX400 NMR spectrometer. HR-ESI-MS was obtained with a YA019 Q-Tof micro mass spectrometer, ESI-MS on ZQ4000 MAA249 and EI-MS on Autospec Premier P708 mass spectrometer. Column chromatography was carried out on silica gel (Qingdao Haiyang, 300—400 mesh), octadecyl silica (ODS) (Fuji Silesia 40 μm), HPLC column (Agilent 9.4×30 mm). [1β-^{3}H] Androstenedione (23.5 Ci/mM) was purchased from Perkin-Elmer Life and Analytical Science Company.

Plant Material The pollen of *Brassica rapa* L. was collected in September 2005 in Jilin, China. This plant material was identified by Prof. Pu Shu (Institute of botany, Jiangsu province, China) and a voucher specimen (PN 05-009) was preserved in our laboratory.

Extraction and Isolation Dried pollens (2000 g) of *Brassica rapa* L. was extracted two times by SFE-CO_2 at 40 MPa, 55 °C. The combined extract was evaporated under a reduced pressure and given a yellow gum (105 g). Then the extract was chromatographied over a silica gel column (40×800 mm), eluted with petroleum ether, petroleum ether (60—90 °C)–EtOAc mixture, methanol. Fractions were collected as follows: fraction A, petroleum ether–EtOAc (100 : 1); fraction B, petroleum ether–EtOAc (20 : 1); fraction C, petroleum ether–EtOAc (5 : 1); fraction D, methanol. Fraction A (35.7 g) was re-chromatographed by silica gel column [petroleum ether–$CHCl_3$ (1 : 1—1 : 2)] to obtain compound **3** (15 mg) and fractions A1—A3. Fraction A1 [petroleum ether–$CHCl_3$ (1 : 2)] was purified by an Agilent HPLC column (9.8×30 mm) eluted with 100% ethanol to obtain compound **1** (12 mg) and compound **2** (9 mg). Fraction A2 [petroleum

ether–$CHCl_3$ (1 : 1)] was repurified with crystal to afford compound **8** (40 mg). Fraction A3 was re-chromatographed by silica gel column [$CHCl_3$] and crystallized under −20 °C to afford compound **7** (22 mg). Fraction B (42.4 g) was separated into two fractions (B1, B2) over a silica gel column eluting with $CHCl_3$–MeOH (from 100 : 1 to 100 : 2). Fraction B1 (780 mg) was chromatographed on a ODS column (2.4×60 cm) with EtOH–H_2O (3 : 1) to afford compound **4** (28 mg), compound **5** (19 mg) and compound **6** (25 mg). Fraction B2 (1580 mg) was re-chromatographed on a silica gel column (2.4×60 cm) eluted with $CHCl_3$–MeOH (100 : 2) to afford compound **9** (208 mg), compound **10** (10 mg).

Compound **1**: Colorless oil. HR-ESI-MS *m/z*: 681.5585 $[M+Na]^+$ (Calcd for $C_{46}H_{76}O_2Na$, 681.5587), ESI-MS (positive) *m/z* 681 $[M+Na]^+$, 1339 $[2M+Na]^+$, EI-MS 70 eV *m/z* 658 $[M]^+$ (48), 531 (26), 394 (35), 381 (100), 296 (32), 276 (40), 107 (53), 95 (80). $[\alpha]_D^{25}$ +35.1° (*c*=0.022, $CHCl_3$). UV (hexan) λ_{max} (ε) 195 (39810). IR (ATR) cm^{-1}: 3010, 2930, 2857, 1733, 1463, 1178. ^1H- and ^{13}C-NMR spectroscopic data shown in Table 1.

Compound **2**: Colorless oil. HR-ESI-MS *m/z*: 709.5898 $[M+Na]^+$ (Calcd for $C_{48}H_{78}O_2Na$: 709.5900), ESI-MS (positive) *m/z* 709 $[M+Na]^+$, EI-MS 70 eV *m/z* 686 $[M]^+$ (18), 422 (17), 409 (100), 394 (65), 380 (21), 284 (27), 121 (46), 95 (73). $[\alpha]_D^{25}$ +47.3° (*c*=0.03, $CHCl_3$). UV (hexane) λ_{max} (ε) 195 (33113). IR (ATR) cm^{-1}: 3015, 2930, 2857, 1735, 1175. ^1H- and ^{13}C-NMR spectral data shown in Table 1.

Determination of Fatty Acid of Compounds 1 and 2 by GC-MS The determination of fatty acid was done by modification of previous method.[13] Compounds **1** or **2** (2.0 mg) were treated with 0.5 M methanolic NaOH solution (0.4 ml) at 65 °C, 5 min later 0.5 ml of 20% (w/v) boron trifluoride–methanol solution was added. After 2 min the methylated production was extracted with *n*-hexane (0.2 ml) and analyzed on a Hewlett Packard HP 6890/5973 series GC-MS apparatus combined with a HP-5MS column (30 m×025 mm×0.25 μm). Helium was used as carrier gas at a flow rate of 1.0 ml/min. Samples were analyzed with the column held initially at 150 °C for 5 min after injection, then increased to 300 °C with 5 °C/min heating ramp with 10 min hold time. The injection was performed in split mode (split ratio: 30 : 1). Detector and injector temperatures were 250 °C and 310 °C, respectively. Run time was 45 min. MS scan range was (*m/z*): 20—440 atomic mass units (AMU) under EI (electron impact) ionization (70 eV). Ion source temperature: 250 °C. The fatty acid components of compounds **1** and **2** were determined by comparing their mass fragmentations with those of mass spectra from the NIST database search as well as comparison of the retention times and mass spectrums of authentic samples of linolenic acid methyl ester.

5α-Reductase Inhibitory Activity Test The 5α-reductase assay was done by previous method.[14] Briefly the reaction mixture, in a final volume of 3.0 ml, contained liver microsomes, 150 μM testosterone in 100 μl of ethanol, 167 μM NADPH, and medium A, with or without the indicated amount of a sample in 100 μl of DMSO. The reaction was started by the addition of microsomes to the pre-heated reaction solution in a tube. After 10 min the incubation was terminated by adding 100 μl of 3 M NaOH, and then 100 μl of 1.0 mM cholesterol acetate in *n*-hexane were added as the internal standard for GC-MS. Forty milliliters of diethyl ether were added to extract metabolites, and the tubes were capped and shaken. The organic phase was decanted and evaporated under reduced pressure. Residue was dissolved in 100 μl ethyl acetate for GC-MS. GC-MS analyses were conducted on Agilent 6890 gas chromatograph equipped with a HP-5 (30 m by 0.25 mm; film thickness, 0.25 μm) and coupled to an Agilent 5975 mass spectrometer. The mass spectrometer was operated in the EI mode at 70 eV. Helium was used as the carrier gas with a flow rate of 1.0 ml/min. The first oven temperature was 150 °C for 3 min after injection, and the temperature was then increased to 300 °C at a rate of 30 °C/min. The sample (1 μl) was injected into the GC at an injector temperature of 310 °C. The 5α-reductase activity was measured by analyzing the extent of the conversion of testosterone to dihydrotestosterone. The 5α-reductase inhibitory activity of each sample was calculated with following equation using peak-area ratios (*R*=A/I.S., A represent the area of dihydrotestosterone, I.S. represent the area of internal standard, cholesterol acetate.):

$$5\alpha\text{-reductase inhibitory activity (\%)} = (Ro - Rs)/Ro \times 100$$

Ro and Rs represent the peak-area ratios of dihydrotestosterone in the absence and presence of the sample, respectively. α-Linolenic acid was used as a positive standard. Each experiment was carried out in triplicate.

Aromatase Inhibitory Activity Test The aromatase assay was done by previous method.[15] Briefly the substrate, [1β-^{3}H] androstenedione was dissolved in serum-free cell culture medium. Placental microsomes were prepared as 0.1 mg/ml in a potassium phosphate buffer (67 mM, pH 7.4) containing 20% (w/v) glycerol, 0.5 mM dothiothreitol, and 0.25 M sucrose. The assay reaction mixture (225 μl), containing placental microsomes (2.5 μg), [^{3}H]androstenedione (50 nM), progesterone (10 μM), and bovineserum albumin (0.1%, w/v) in potassium phosphate (67 mM, pH 7.4), with sample solution in DMSO (final DMSO concentration 2.22%, v/v), was introduced in a 3 ml tube and pre-incubated at room temperature for 10 min; then, 25 μl of NADPH (3 mM) were added and the mixture was incubated at 37 °C for 15 min. The reaction was terminated by addition of 50 μl of 20% (w/v) trichloroacetic acid, and 250 μl of the solution were transferred to another well containing the charcoal-dextran pellet. The solution was thoroughly mixed and centrifuged (1000×***g***, 5 min) to remove non-reacted substrate; an aliquot of the supernatant containing $[^3H]H_2O$ as reaction product was counted in a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter. Aromatase inhibition activity was calculated as the percentage of remaining activity from the reaction without sample. Anlumitepian was used as positive control. Analyses were carried out in triplicate.[16]

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