Food Chemistry 119 (2010) 306–310

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Bioactive dammarane-type triterpenoids derived from the acid hydrolysate of *Gynostemma pentaphyllum* saponins

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ARTICLE INFO

*Article history:*
Received 30 January 2009
Received in revised form 5 May 2009
Accepted 16 June 2009

*Keywords:*
*Gynostemma pentaphyllum*
Cucurbitaceae
Hydrolysate
Dammarane triterpenoid
Cytotoxicity
Porcine pancreatic lipase inhibitor
(23*S*)-3*β*-Hydroxydammar-20,24-dien-21-oic acid 21,23-lactone

ABSTRACT

From an acid hydrolysate of the crude saponins of *Gynostemma pentaphyllum*, three triterpenes, including a new compound (23*S*)-3*β*-hydroxydammar-20,24-dien-21-oic acid 21,23-lactone (**1**) and two known aglycons (20*S*, 23*R*)-3*β*,20*β*-dihydroxydamma-24-dien-21-oic acid 21,23-lactone (**2**) and (20*S*, 24*S*)-20,24-epoxydammarane-3*β*,12*β*,25-triol (**3**), were isolated. Their structures were established on the basis of extensive spectral evidence (HR-ESI-MS, IR, 1D and 2D-NMR experiments). In bioactive assays *in vitro*, compound **1** was found to have potent cytotoxicity against the human breast cancer cells MDA-MB-435, whereas compounds **2** and **3** exhibited modest inhibitory activity toward porcine pancreatic lipase. The results indicated that acid treatment of *G. pentaphyllum* extract could produce diverse structures with interesting bioactivity.



## 1. Introduction

The creeping plant *Gynostemma pentaphyllum* (Thunb.) Makino (Jiaogulan, in Chinese), belonging to the Cucurbitaceae family, is a perennial herb growing wild in the mountainous regions of Japan, Korea, China, and Southeast Asian countries. The application of *G. pentaphyllum* has a history of about 500 years in China. The book 'Herbs for Famine' published in the Ming Dynasty (1368–1644 A.D.) describes the herb's usage as a vegetable, suitable as a food, or a dietary supplement during famine rather than as a medicinal herb (Razmovski et al., 2005). In addition, it was used as a sweetener in Japan and can be taken either as tea or in alcohol in China. It has been used in Chinese and Japanese folk medicine to alleviate various diseases and symptoms including hypertension, cough, migraine, insomnia and diabetes mellitus (Takimoto, Arihara, Nakajima, & Okuhira, 1983).

In traditional Chinese medicine, recent reports have shown that *G. pentaphyllum* exhibits a variety of biological effects, such as cholesterol-lowering, immunopotentiating, as well as antitumor, anti-inflammatory, antioxidant, cardiovascular, anti-hyperlipidemic and hypoglycemic effects (Aktan, Henness, Roufogalis, & Ammit, 2003; Circosta, Pasquale, & Occhinto, 2005; Han, Liu, & Gao, 1995; Huang et al., 2006; Lin, Lin, Chiu, Yang, & Lee, 1993; Megalli, Aktan, Davies, & Roufogalis, 2005; Megalli, Davis, & Roufogalis, 2006; Razmovski et al., 2005; Yeo et al., 2008). The dammarane glycosides isolated from *G. pentaphyllum*, also known as gypenosides or gynosaponins (Kuwahara, Kawanishi, Komiya, & Oshio, 1989; Razmovski et al., 2005; Takimoto et al., 1983), are believed to be the bioactive components responsible for its diverse pharmacological properties and reported clinical effects (Rao & Gurfinkel, 2000; Zhang & Huang, 1993). Notably, these saponins are structurally similar to the ginseng saponins (Razmovski et al., 2005). Since ginsenosides are the well known biologically active principles in *Panax ginseng* C.A. Meyer (Korean ginseng), *G. pentapbyllum* has received much attention. Because of this structural similarity, it is claimed that drinking a tea made of *G. pentaphyllum* leaves could regularly promote good health and lessen the severity of many disorders, including ulcers of the stomach (Lv et al., 2009; Rujjanawate, Kanjanapothi, & Amornlerdpison, 2004). Recently a novel insulin-releasing substance, phanoside has also been isolated from this herb (Norberg et al., 2004). Some gypenosides were reported to be effective in the treatment of tumours (Chen, Chung, & Chen, 1999; Lu, Chen et al., 2008;

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doi:10.1016/j.foodchem.2009.06.033

Lu, Tsai et al., 2008; Razmovski et al., 2005), such as human hepatoma cell lines (Hep3B and HA22T) (Chen et al., 1999) and human lung cancer A-549 cells (Lu, Chen et al., 2008).

The molecular diversity is one of the most important aspects in drug discovery. Thus, the structures of natural-products should be as diverse as possible. One approach for creating such new natural-product derivatives is combinatorial chemistry. Another approach is to chemically modify the plant extracts, such as by reduction, oxidation, and hydrolytic reactions to afford a whole new range of natural or synthetic 'metabolites' (Teng, Li, Wang, & Yang, 2004). In order to search for diversity of new bioactive metabolites from this plant species, in connection with our study, we hydrolysed the crude saponins of *G. pentaphyllum* under acidic conditions (HCl/EtOH, 1:10). Preliminary biological test showed that the resultant hydrolysate exhibited moderate cytotoxic activity, further chemical investigations of the hydrolysate led to the isolation of a new dammarane-type triterpene derivative **1**, together with two known analogues **2** and **3**. In this paper, herein we deal with the isolation and structural elucidation of the new compound **1**, as well as the cytotoxicity and lipase enzyme inhibitory activities of the isolated triterpenes.

## 2. Materials and methods

### 2.1. Plant saponins

The crude saponins of *G. pentaphyllum* were purchased from Acetar Bio-Tech Inc., Shaanxi province, China.

### 2.2. General procedure

The melting point was determined on an XRC-1 micro-melting point apparatus and is uncorrected. Optical rotations were measured on a Perkin–Elmer Model 341 polarmeter. IR spectra were obtained with a Nexus 870 FT-IR with $CHCl_3$ film. NMR spectra were recorded on a Bruker DRX-500 spectrometer in $CDCl_3$, with TMS as an internal standard, $\delta$ in ppm, $J$ in Hz. Positive ESI-MS was recorded with an API QSTAR Pulsar 1 spectrometer.

Column chromatography (CC) was carried out on silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, PR China) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). Fractions were monitored by TLC and spots were visualised by heating silica gel plates sprayed with 10% $H_2SO_4$ in ethanol.

### 2.3. Acid hydrolysis and isolation of compounds

The crude saponins (350 g) of *G. pentaphyllum* dissolved in EtOH (5 L) were treated with 10% HCl (500 mL). The resulting solution was heated at 60 °C for 8 h. The alcoholic solvent was evaporated under reduced pressure to give crude hydrolysates, which were passed through Diaion HP-20 column, washed with $H_2O$, and then eluted with aq. 80% EtOH to furnish a brown residue (100 g). The residue was submitted to silica gel CC ($CHCl_3$/MeOH/$H_2O$ 8:2:0.2) to afford five fractions. Fraction 2 was separated by silica gel CC ($CHCl_3$/MeOH/$H_2O$ 9:1:0.1) to give four subfractions Fr.2.1–Fr.2.4. Fr.2.2 was fractionated over silica gel with petroleum ether/acetone (90:10, 80:20, 70:3) to afford another three fractions. Fr.2.2.2 was purified by CC (RP-18; MeOH/$H_2O$ 70:30–90:10) followed by Sephadex LH-20 column ($CHCl_3$/MeOH 1:1) to yield compound **1** (120 mg). Fr.2.2.3 (60 mg) was further purified by repeated CC over silica gel (petroleum ether/acetone 85:15, 80:20), followed by Sephadex LH-20 column ($CHCl_3$/MeOH 1:1) to afford compound **2** (18 mg). Fr.2.3 was subjected to CC over silica gel ($CHCl_3$/MeOH/$H_2O$ 85:15:0.1) and to CC over silica gel (petroleum ether/acetone 8:2) and purified by Sephadex LH-20 column (methanol) to yield compound **3** (10 mg).

### 2.4. Spectrometric identification of isolated compounds

(23*S*)-3$\beta$-Hydroxydammar-20,24-dien-21-oic acid 21,23-lactone (**1**): amorphous powder, mp 190–191 °C; $[\alpha]_D^{25}$ + 87.5° (*c* 0.25, $CHCl_3$); IR (KBr) $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 3474, 2940, 2854, 1751, 1085, 1030; positive HRESIMS: *m/z* 477.3328 $[M+Na]^+$ (calc. 477.3324); $^1H$ NMR and $^{13}C$ NMR data, see Table 1.

(20*S*, 23*R*)-3$\beta$,20$\beta$-Dihydroxydamma- 24-dien-21-oic acid 21,23-lactone (**2**): amorphous powder; positive ESI-MS, *m/z* 473 $[M+H]^+$; $^1H$ and $^{13}C$ NMR ($CDCl_3$): see Table 1.

(20*S*, 24*S*)-20,24-Epoxydammarane-3$\beta$,12$\beta$,25-triol (**3**): amorphous powder; positive ESI-MS, *m/z* 477 $[M+H]^+$; The $^1H$ and $^{13}C$ NMR ($CDCl_3$) data agreed well with those of literature data (Liu et al., 2004).

### 2.5. In vitro cytotoxicity assay

The antitumor activity was assayed by the MTT method (Skehan et al., 1990). The human mammary gland carcinoma cell lines (MDA-MB-435) were used for the screening. All cell lines were seeded into 96 well plates at a concentration of about 50,000 cells/mL and were incubated in 5% $CO_2$ atmosphere at 37 °C for 24 h. Then 20 μl of the sample (organoantimony complex) were added and further incubation was carried out at 37 °C for 48 h. Fifty microlitre of 0.1% MTT was added to each well. After 4 h, the culture medium was removed, and 150 μl of isopropanol was added to dissolve the insoluble blue formazan precipitates produced by MTT reduction. The plate was shaken for 20 min on a plate shaker to ensure complete dissolution. The optical density of each well was measured at 570 nm (MTT). The antitumor activ-

**Table 1**
$^1H$ (500 MHz) and $^{13}C$ NMR (125 MHz) data of compounds **1** and **2** ($CDCl_3$, $\delta$ in ppm, $J$ in Hz)[a].

| No. | **1** | | **2** | |
|---|---|---|---|---|
| | $\delta_C$ | $\delta_H$; mult; $J$ (Hz) | $\delta_C$ | $\delta_H$; mult; $J$ (Hz) |
| 1 | 39.0 t | 1.62 (m), 0.97(m) | 38.7 t | 1.57 (m), 0.97(m) |
| 2 | 27.3 t | 2.10 (m), 1.72(m) | 27.4 t | 2.17 (m), 1.80(m) |
| 3 | 78.8 d | 3.23 (dd, *J* = 11.2, 4.8) | 78.9 d | 3.25 (dd, *J* = 11.4, 4.9) |
| 4 | 37.5 s | – | 38.2 s | – |
| 5 | 55.7 d | 0.76 (d, 10.9) | 55.3 d | 0.78 (d, *J* = 10.7) |
| 6 | 18.2 t | 1.52 (m), 1.30 (m) | 18.5 t | 1.53 (m), 1.32 (m) |
| 7 | 35.3 t | 1.52 (m), 1.21 (m) | 33.9 t | 1.56 (m), 1.29 (m) |
| 8 | 40.8 s | – | 40.1 s | – |
| 9 | 50.6 d | 1.30 (m) | 50.2 d | 1.32 (m) |
| 10 | 37.1 s | – | 35.9 s | – |
| 11 | 21.2 t | 1.54 (m),1.30 (m) | 20.7 t | 1.49 (m), 1.28 (m) |
| 12 | 24.7 t | 2.11 (m),1.69 (m) | 23.5 t | 2.10 (m), 1.71 (m) |
| 13 | 46.7 d | 2.52 (m) | 45.7 d | 2.52 (m) |
| 14 | 49.8 s | – | 50.1 s | – |
| 15 | 31.2 t | 1.63 (m), 1.17 (m) | 30.8 t | 1.62 (m), 1.15 (m) |
| 16 | 28.7 t | 2.53 (m), 1.37 (m) | 28.3 t | 2.49 (d, *J* = 11.4), 1.37 (m) |
| 17 | 37.5 d | 2.53 (m) | 45.0 d | 2.69 (m) |
| 18 | 15.5 q | 0.91 (s) | 14.9 q | 0.98 (s) |
| 19 | 15.3 q | 0.78 (s) | 16.7 q | 0.80 (s) |
| 20 | 140.3 s | – | 81.3 s | – |
| 21 | 173.9 s | – | 178.3 s | – |
| 22 | 145.7 d | 6.82 (d, *J* = 8.7) | 39.2 t | 2.78 (dd, *J* = 13.1, 7.3), 2.35 (dd, *J* = 13.1, 5.6) |
| 23 | 78.0 d | 4.90 (m) | 75.8 d | 5.54 (m) |
| 24 | 121.9 d | 5.59 (d, J = 8.8) | 120.6 d | 5.39 (m) |
| 25 | 137.8 s | – | 137.9 s | – |
| 26 | 25.7 q | 1.80 (s) | 25.7 q | 1.78 (s) |
| 27 | 18.2 q | 1.78 (s) | 18.0 q | 1.78 (s) |
| 28 | 27.9 q | 0.99 (s) | 27.5 q | 1.05 (s) |
| 29 | 15.6 q | 0.98 (s) | 15.4 q | 1.08 (s) |
| 30 | 16.1 q | 0.84 (s) | 16.1 q | 0.92 (s) |

[a] Assigned by COSY, HSQC, NOESY, and HMBC experiments. $^{13}C$ multiplicities were determined by DEPT experiments.

ity was determined three times in independent experiments, using three replicate wells pertoxicant concentration (10, 1, 0.1 l g/mL) and obtained the mean optical densities for drug-treated cells at each concentration as a percentage of that of untreated cells.

### 2.6. Pancreatic lipase inhibition assay

Lipase activity was measured using 2,4-dinitrophenyl butyrate (DNPB) as a substrate (Won et al., 2007). DNPB was synthesised using Mosmuller's method (Mosmuller, Van Heemst, Van Delden, Franssen, & Engbersen, 1992). Porcine pancreatic lipase (PPL, type II, St. Louis, MO, USA) stock solutions (1 mg/mL) were prepared in 0.1 mM potassium phosphate buffer (pH 6.0), and the solutions were stored at −20 °C to reserve. For determining lipase inhibitory activity, the compounds (25 μg/mL) were pre-incubated with the enzyme for 1 h in the potassium phosphate buffer (0.1 mM, pH 7.2, added with 0.6 mL Tween 80 per 100 mL) at 30 °C before assaying the enzyme activity (Gargouri, Ransac, & Verger, 1997). The reaction was then started by adding 0.1 mL of 25 mM DNPB, all in a final volume of 5.0 mL. After incubation at 30 °C for 5 min, the amount of 2,4-dinitrophenol released in the reaction was measured at 360 nm using the Evolution 300 UV–Visible spectrophotometer (Thermo, England). The activity of the negative controls was also checked with and without inhibitor. The inhibitory activity (I) was calculated according to the following formula (Sharma, Sharma, & Seo, 2005):

$$I\% = \left(1 - \frac{B-b}{A-a}\right) \times 100$$

where, *A* is the activity of enzyme without inhibitor, *a is* the negative control without inhibitor, whilst, *B is* the activity of enzyme with inhibitor and *b* is the negative control with inhibitor.

## 3. Results and discussion

Acid hydrolysis of the crude saponins of *G. pentaphyllum* with an alcoholic solution of HCl provided crude hydrolysates. The hydrolysates were passed through a Diaion HP-20 column, and then subjected to silica gel, Sephadex LH-20 and reversed-phase (RP-18) column chromatography to afford three dammarane-type triterpenes **1**–**3**, including a new compound **1**, as shown in Fig. 1. The structures of two known aglycons **2** and **3** were characterised by comparison of their NMR and MS data with those reported in the literature (Liu et al., 2004; Yin & Hu, 2005). They are (20*S*, 23*R*)-3*β*,20*β*-dihydroxydamma-24-dien-21-oic acid 21,23-lactone (**2**) and (20*S*, 24*S*)-20,24-epoxydammarane-3*β*,12*β*,25-triol (**3**).

Compound **1** was obtained as an amorphous white solid. The molecular formula was determined as $C_{30}H_{46}O_3$, with 8 degrees of unsaturation, on the basis of its pseudomolecular ion peak at *m/z* 477.3328 ($[M+Na]^+$) in positive ion high-resolution ESI-MS as well as $^{13}C$ NMR data and. The IR spectrum indicated absorptions of a hydroxyl group at 3474 $cm^{-1}$ and a $\gamma$ lactone group at 1751 $cm^{-1}$. By comparison of its $^1H$ and $^{13}C$ NMR spectral data with compound **2**, compound **1** has the same nucleus skeleton as **2** but a different side-chain. The structure of **1** was deduced from detailed analyses of $^1H$ and $^{13}C$ NMR data (Table 1), as well as by 2D-NMR experiments (HMBC, HMQC, H–H COSY and ROESY) and comparison with those of known model compounds (Liu et al., 2004; Piacente, Pizza, De Tommasi, & De Simone, 1995; Yin & Hu, 2005).

The $^{13}C$ NMR and DEPT spectra (Table 1) of **1** showed the presence of 30 carbon signals, which were recognised as seven methyls, eight methylenes, eight methines and seven quaternary carbon atoms, including four olefinic carbons [$\delta$ 145.7(CH), 140.3(C), 137.8(C), and 118.9(C)], one axially oriented carbinolic methine group [$\delta$ 78.8(CH)], and one lactone carbonyl carbon ($\delta$ 173.9). The $^1H$ NMR spectrum (Table 1) of **1** showed seven singlets at $\delta$ 0.78–1.80 assigned to tertiary methyls, two of which were diagnostic for methyl groups at $\delta$ 1.80 and 1.78 connected to an olefinic carbon (C-25). In addition, the $^1H$ NMR spectrum of **1** displayed two olefinic protons at $\delta$ 5.59 (d, $J$ = 8.8 Hz) and 6.82 (d, $J$ = 8.7 Hz), and an oxymethine proton at $\delta$ 4.90 (m). A 3β-OH substitution was evident from the chemical shift and the $J$ values of the proton attributable to C-3 at $\delta$ 3.23 (dd, $J$ = 11.2, 4.8 Hz) (Piacente et al., 1995; Yin & Hu, 2005).

The distinct difference in $^{13}C$ NMR between **1** and **2** was that the carbon signals at $\delta$ 81.3 (C-20) and $\delta$ 39.2 (C-22) in **2** were replaced by two olefinic carbons at $\delta$ 140.3 and $\delta$ 145.7 in **1**, suggesting that one double bond at C-20 was formed due to loss of one $H_2O$ from its precursor reported in the literature (Yin & Hu, 2005). The side-chain moiety of **1** was confirmed by selected HMBC (Fig. 2) correlations: from H-17 to C-20, H-22 to C-21, and H-23 to C-22, C-24 and C-25, as further confirmed by key $^1H$–$^1H$ COSY correlations observed between H-22 and H-23, H-23 and H-24 (Fig. 2). The stereochemistry at C-23 of **1** was deduced from ROSEY interactions and NMR analysis. The ROSEY correlations (Fig. 2) of H-23/H-22, H22/H-17α, H-22/H-24, H-24/Me-26 and H-23/Me-27 were observed, revealing that H-23 should be located on the $\beta$-side and the 2-methypropenyl group on the $\alpha$-side (Piacente et al., 1995; Yin & Hu, 2005). Thus, the configuration at C-23 was established as *S* (Yin & Hu, 2005). Accordingly, the structure of **1** was assigned as (23*S*)-3$\beta$-hydroxydammar-20, 24-dien-21-oic acid 21,23-lactone.

Compounds **1–3** were tested for their activities against the human breast cancer cells (MDA-MB-435). Compound **1** was found to have significant cytotoxic activity ($IC_{50}$ = 3.9 μg/mL), whilst compounds **2** and **3** showed no activity. This result suggests that the double bond between C-20 and C-22 of the 21,23-lactone moiety in the molecule is relatively essential for the cytotoxic activity.

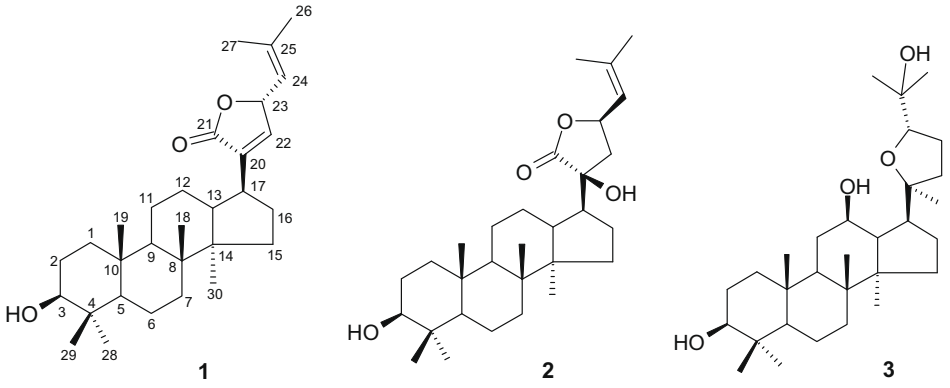


**Fig. 1.** Structures of compounds **1**–**3**.

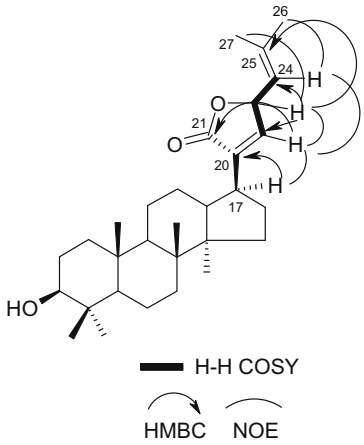


**Fig. 2.** Key HMBC, NOE and COSY correlations in compound **1**.

Moreover, compounds **1–3** were also assayed for their porcine pancreatic lipase (PPL) inhibitory activity. The results (Table 2) showed that **1** displayed no inhibitory function on PPL, whilst **2** and **3** presented a mild inhibitory activity. Pancreatic lipase, the main lipid-digesting enzyme, removes fatty acids from the $\alpha$ and $\alpha'$ position of dietary triglycerides. The inhibition to pancreatic lipase is an attractive targeted approach for the treatment of obesity (Thomson, De Pover, Keelan, Jarocka-Cyrta, & Clandinin, 1997).

These results suggest that the lipase inhibitory activity is partly involved in hypoglycemic and antihyperglycemia effects of an ethanol extract of *G. pentaphyllum* in the type 2 diabetic animal model (Megalli et al., 2005; Megalli et al., 2006; Norberg et al., 2004; Yeo et al., 2008). *In vivo* studies with the hydrolysate should be studied further.

Due to various interesting activities and its non-toxic advantage (Attawish et al., 2004), *G. pentaphyllum* leaf has high potential as raw material for nutraceutical and functional foods. It also has been widely used as beverages and food additives, such as mineral water, cola, wine, beer, chewing gum, milk, biscuits and noodles (Gao & Yu, 1993), for improving resistance and immunity, reducing blood lipid levels, preventing cancer and diabetes due to the presence of important nutritional dammarane-type triterpenes. Therefore, we can assert that this hydrolysate of *G. pentaphyllum* saponins can be considered to be used as a beneficial food with chemopreventive potential.

In conclusion, in this study, a new artificial dammarane-type triterpenoid **1** and two relatives **2** and **3** were obtained from the hydrolysate of the saponins of *G. pentaphyllum*. In *in vitro* bioactive assays, only compound **1** with a $\alpha,\beta$-unsaturated 21,23-lactone skeleton exerted potent cytotoxic activity against the human breast cancer cells MDA-MB-435, whereas two aglycons **2** and **3** inhibited the enzyme activity of porcine pancreatic lipase. The hydrolysate of *G. pentaphyllum* saponins may be effective in the prevention of diabetes and cancers. This present study showed that this acid hydrolytic modification of crude saponins may present a vital alterative for discovery of new structurally simplified bioactive 'metabolites' as functional food additives.

**Table 2**
Inhibitory effects of compounds **1**–**3** on enzyme activity of PPL.[a]

| Compound | Concentration (μg/mL) | | |
|---|---|---|---|
| | 25 | 50 | 100 |
| **1** | −5.8 ± 0.4 | −1.8 ± 0.2 | 2.4 ± 0.2 |
| **2** | 26.1 ± 1.3 | 40.2 ± 1.6 | 65.9 ± 1.4 |
| **3** | 19.9 ± 1.0 | 35.5 ± 0.9 | 51.2 ± 1.0 |
| Orlistat | 86.3 ± 2.5 | 90.3 ± 2.9 | 91.6 ± 3.3 |

[a] The inhibition is expressed in percentage (%). All tests were conducted in triplicate and the means are used. Orlistat was used as a positive control.

## Acknowledgments

This work was financially supported by the Programs for New Century Excellent Talents in University (NCET-05-0852) and for Changjiang Scholars and Innovative Research Team in University (IRT0749).

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