Isolation and identification of antiproliferative compounds from the roots of *Tetrastigma hemsleyanum* against MDA-MB-435S cell lines

Zhucan Lin[1†], Liyun Chen[1†], Qi Qiu[2] and Suhua Guo[1,2*]
[1]State Key Laboratory of Chinese Pharmacies, Pharmacy College, Fujian University of Traditional Chinese Medicine, Fuzhou, China
[2]Fujian Health College, Fuzhou, China

Abstract: This present study aimed to elucidate antiproliferative activity of four extracts ($CHCl_3$, EtOAc, *n*-BuOH and H_2O) and chemical constituents isolated from the most potent extract of *Tetrastigma hemsleyanum* Diels et. Gilg (TDG) against MDA-MB-435S cell lines using the MTT assay at various concentrations *in vitro*. Ten compounds were isolated and identified as (1) β-sitosterol, (2) palmitic acid, (3) protocatechuic acid, (4) salicylic acid, (5) *p*-hydroxybenzoic acid, (6) resveratrol, (7) *trans*-4-hydroxycinnamic acid, (8) kaempferol, (9) quercetin, and (10) isoquercitrin. Compounds 3, 5-7, 10 were the first report of isolation from this plant. Moreover, antiproliferative activity displayed that the $CHCl_3$, H_2O extracts and compounds 6, 8 exhibited obvious inhibitory effects on MDA-MB-435S cell lines with IC_{50} values 100.28± 2.64, 127.48±3.45, 92.39±1.68 and 120.30±1.97μg/mL, respectively. Thus the obtained results indicate antiproliferative activity of TDG against MDA-MB-435S cell lines is ascribable to the most potent $CHCl_3$ extract along with active compounds 6 and 8, which could be considered as a potential chemotherapeutic agent in breast cancer.

Keywords: Antiproliferative activity; MDA-MB-435S; Phytochemical; *Tetrastigma hemsleyanum*

INTRODUCTION

Tetrastigma hemsleyanum Diels et. Gilg (TDG), a kind of herbaceous vine, belongs to Vitaceae, which mainly distributed in southern China, such as Jiangsu, Zhejiang, Fujian, Jiangxi, and Guangdong provinces. As an edible and medicinal plant, its leaves, fruits and roots are extensively used to treat infantile hyperpyretic convulsion, rheumatism, asthma, menstrual disorders, and numerous inflammatory diseases including viral meningitis, pharyngitis, bronchitis, hepatitis and pneumonia on account of their antipyretic, analgesic and anti-inflammatory properties (Shao *et al*., 2011; Sun *et al*., 2013) and its roots possess the best efficacies.

Now, TDG is a unique rare plant in China, which has antiviral, hepatoprotective, immuneregulatory and anticancer functions (Xu *et al*., 2008; Sun *et al*., 2013; Feng *et al*., 2014). Several types of compounds, such as flavonoids (quercetin, kaempferol, kaempferol-3-O-neohesperidoside, kaempferol-7-O-β-L-rhamnopyranceyl-3-O-β-D-glucopyranoside, apigenin-6-C-α-L-arabinopy-ranosyl-(1-4)-α-L-rhamnopyranoside, apigenin-8-C-α-L-arabinopyranosyl-(1-4)-α-L-rhamnopyranoside and apigenin-6,8-di-C-β-D-glucopyranoside), phenolic acids (salicylic acid, 3-caffeoylquinic acid, 5-caffeoylquinic acid, 1-caffeoylquinic acid, 5-*p*-coumaroylquinic acid, 1-*p*-coumaroylquinic acid, isoorientin, orientin, isoorientin-2″-O-rhamnoside, orientin-2″-O-rhamnoside, isovitexin, vitexin, vitexin-2″-O-rhamnoside and isovitexin-2″-O-rhamnoside), and steroids (β-sitosterol, ergosterol, daucosterol and 6'-O-benzoyl-daucosterol), have been isolated and identified from this plant (Chen and Guo., 2012; Cai *et al*., 2014; Sun *et al*., 2013). Recent extensive research have focused on its anticancer activity, and modern pharmacological studies also indicated that TDG and its flavones could not only inhibit various kinds of tumor cells growth, such as SGC-7901 (human gastric cancer), A549 (human lung cancer), SMMC-7721 (Human hepatoma), A375 (human melanoma), HT-29 (human colon cancer), K562 and HL-60 (human leukemia) cell lines *in vitro*, but also induce their apoptosis (Feng *et al*., 2006; Cheng and Lu., 2007; Wang *et al*., 2012; Zhang *et al*., 2010; Lu *et al*., 2011; Liu and Xia., 2010; Xu *et al*., 2010, 2011). However, there is too little information on the antiproliferative activity of its active extracts and compounds isolated from TDG against MDA-MB-435S (human breast carcinoma) cells. Therefore, in this present study, the extracts and bio-assayed guided isolated chemical constituents of TDG have been preliminary screened for their antiproliferative activity by cell-based model. It is the first report regarding the bioassay-guided isolation of antiproliferative constituents from TDG against MDA-MB-435S cell lines.

MATERIALS AND METHODS

Apparatus and reagents

MS, ^1H and ^{13}C-NMR spectra were measured on ZQ-2000 UPLC/MS spectrometer (Waters, USA), Bruker AV-400 and 100 spectrometer (Bruker, Swiss), respectively. Preparative HPLC was performed by a reversed phase column (Chromatorex C_{18} column, 10μm, 20mm × 250 mm) at 15.0mL/min. Sephadex LH-20 (GE Healthcare, USA) and Silica gel (Qingdao Marine Chemical Factory, 200-300 mesh) were applied to chromatographic

**Corresponding author:* e-mail: guosuhua2005@126.com;

separation. Human breast carcinoma MDA-MB-435S was purchased from Beijing Dingguo Changsheng Biotechnology Co., LTD (Beijing, China). RPMI 1640 (SH30809.01B, USA) and Methyl thiazolyl tetrazolium (MTT, St Louis, MO63103, USA) were obtained from HyClone and Sigma, respectively. Ethanol, chloroform, ethyl acetate, and *n*-butyl alcohol were analytical grade obtained from Shanghai Sinopharm Chemical Reagent Co., LTD.

Plant material

Roots of *Tetrastigma hemsleyanum* Diels et. Gilg were provided by Zhejiang Lishui Green Valley Institute of Rare Plants (Zhejiang, China) and identified by Professor Surong Che at Pharmacy College of Fujian University of TCM (Voucher specimen no. PH 2012071).

Extraction, fractionation and isolation

The dried roots of TDG (5.0kg) were powdered and then extracted with 70% ethanol (30L × 2, 2.0h each time). The combined extract was concentrated in vacuum to yield a brown crude extract (216.0g). The crude extract was suspended in H_2O and then partitioned with $CHCl_3$, EtOAc and *n*-BuOH to render dried $CHCl_3$ (36.2g), EtOAc (15.4g), *n*-BuOH (89.0g) extracts and H_2O residue (60.8g), respectively. Each of these extracts was tested for its antiproliferative activity against the MDA-MB-435s cell lines and the $CHCl_3$ extract exhibited the most activity.

The $CHCl_3$ extract was further separated by chromatography on silica gel (200-300 mesh, 10×120cm, 500g), eluted with CH_2Cl_2-MeOH (100:0-0:100) as solvent gradient system, to give 5 fractions (Fr1-Fr5). Fraction 2 (4.2g) was repeatedly purified over silica gel column (200-300 mesh, 2×60cm, 50g), using PE-EtOAc (50:1-0:100) as the eluent, led to compounds 1 (450mg) and 2 (23mg). Fraction 3 (3.1g) was separated by preparative HPLC (Chromatorex C_{18} column, eluted with MeOH-H_2O from 1:9 to 5:5) to produce 2 fractions (Fr 3.1-Fr 3.2). Further fractionation of Fr 3.1 with preparative HPLC (MeOH-H_2O, 2:8) yielded compound 3 (6.7mg), 4 (5.1mg), and 5 (8.2mg), while Fr 3.2 (MeOH-H_2O, 3:7) afforded compound 6 (6.4mg). Fraction 4 (2.2g) was further subjected to preparative HPLC (MeOH-H_2O, 5:5) to obtain compound 7 (4.6mg), 8 (5.0 mg) and 9 (6.8mg). Compound 10 (10.2mg) was isolated from fraction 5 (0.4g) by Sephadex LH-20 (MeOH) and preparative HPLC (MeOH-H_2O, 4:6). The purities of isolated compounds were more than 95% based on the HPLC method.

Antiproliferative activity

Cell culture

The MDA-MB-435S cells were cultured into RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100U/mL) and streptomycin (100μg/mL) at incubator (37□, 5% CO_2-humidified air).

MTT assay

Evaluation of antiproliferative activity of above four extracts and compounds isolated from the roots of TDG was undertaken by MTT assay (Huang *et al*., 2013; Samarghandian *et al*., 2014). Cells were seeded (100μL, 1×10^5 cells/mL) in a 96-well plate. After 24 h of incubation in the appropriate medium, cells were treated with various concentrations of four extracts (20-500 μg/mL) and pure compounds (15-240μg/mL) from TDG for another 24h of culture. For MTT assay, cells were labeled with10μL of MTT solution (final, 0.5mg/mL) and additionally incubated for 4 h (37□, 5% CO_2). Then, the resulting formozan was solubilized in 100μL dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm by an ELX 800 microplate spectrophotometer. The IC_{50} values were calculated as the concentration of drug yielding 50% cell survival.

RESULTS

Antiproliferative activity

Results for antiproliferative preliminary screening of the partitioned extracts ($CHCl_3$, EtOAc, *n*-BuOH and H_2O) and 10 pure compounds isolated from the roots of TDG are shown in table 1. The $CHCl_3$, EtOAc and H_2O extracts revealed notable antiproliferative activity against MDA-MB-435S cell lines with IC_{50} values 100.28±2.64, 446.94 ±5.32 and 127.48±3.45μg/mL, respectively, indicating that TDG had a strong inhibition on MDA-MB-435S cells growth. Among these extracts, $CHCl_3$ extract displayed the most potent effect.

As presented in table 1, only compounds 6 and 8 showed obvious antiproliferative activity against MDA-MB-435S cells (IC_{50} 92.39±1.68 and 120.30±1.97μg/mL, respectively). However, the activity was obviously inferior to positive control, taxol (2.06±0.11μg/mL). Meanwhile, the other compounds possessed none or weak activity.

Therefore, the $CHCl_3$, H_2O extracts along with compounds 6 and 8 have the potential of chemotherapy in breast cancer since they have an ability to inhibit MDA-MB-435S cells growth.

Identification of isolated compounds

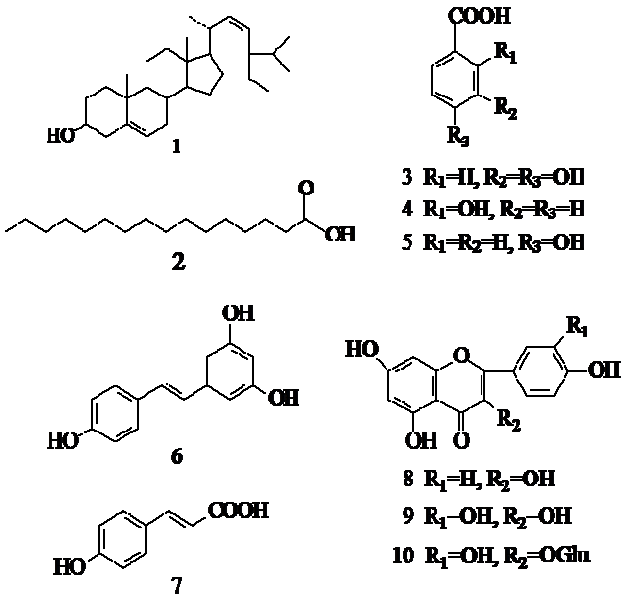
In order to identify the active chemical constituents, the most potent extract, $CHCl_3$ extract was further purified by various chromatography. In summary, 10 known compounds (fig. 1) were identified including five phenols and three flavones consist of β-sitosterol (1), palmitic acid (2), protocatechuic acid (3), salicylic acid (4), *p*-hydroxybenzoic acid (5), resveratrol (6), *trans*-4-hydroxycinnamic acid (7), kaempferol (8), quercetin (9), and isoquercitrin (10). Their spectral and chromatographic data were displayed in table 2.

Table 1: IC_{50} values of different extracts and 10 compounds isolated from TDG on MDA-MB-435S cell lines (n = 3)

Sample	Taxol	$CHCl_3$ extract	EtOAc extract	*n*-BuOH extract	H_2O extract	Compound 1	2	3	4	5	6	7	8	9	10
IC_{50} (μg/mL)	2.06 ±0.11	100.28 ±2.64	446.94 ±5.32	ND	127.48 ±3.45	ND	ND	ND	ND	ND	92.39 ±1.68	ND	120.30 ±1.97	ND	ND

Table 2: The spectral and chromatographic data of 10 compounds isolated from the $CHCl_3$ extract of TDG

No.	Compounds	ESI-MS (m/z)	^{1}H-NMR (400 MHz, $(CD_3)_2CO$)	Chromatographic data	Referees
1	β-sitosterol	413.64 $[M-H]^-$		equal R_f to the standard of β-sitosterol based on TLC	
2	palmitic acid	256.24 $[M]^+$	*δ:* 2.28 (2H, t, *J*=7.4 Hz, H-2), 1.61 (2H, m, H-3), 1.29 (24H, brs, -CH_2), 0.88 (3H, t, *J*=6.8 Hz, H-16)		Yun *et al*., 2014
3	Protocatechuic acid	155.16 $[M+H]^+$	*δ*: 7.53 (1H, d, *J*=2.0 Hz, H-2), 7.48 (1H, dd, *J*=8.0, 2.0 Hz, H-6), 6.90 (1H, d, *J*=8.0 Hz, H-5)		Lin *et al*., 2014
4	salicylic acid	139.15 $[M+H]^+$	*δ*: 11.37 (2H, brs, 7-COOH, 2-OH), 7.90 (1H, dd, *J*=6.7, 1.6 Hz, H-6), 7.54 (1H, m, H-4), 6.96 (1H, d, *J*=7.8 Hz, H-3), 6.93 (1H, m, H-5)		Wang *et al*., 2011
5	*p*-hydroxybenz-oic acid	139.16 $[M+H]^+$	*δ*: 7.92 (2H, d, *J*=8.2 Hz, H-2, 6), 6.92 (2H, d, *J*=8.2 Hz, H-3, 5)		Lin *et al*., 2014
6	resveratrol	229.14 $[M+H]^+$	*δ*: 7.42 (2H, d, *J*=8.6 Hz, H-2′, 6′), 7.02 (1H, d, *J*=16.3 Hz, H-8), 6.89 (1H, d, *J*=16.3 Hz, H-7), 6.84 (2H, d, *J*=8.6 Hz, H-3′, 5′), 6.54 (2H, d, *J*=2.1 Hz, H-2, 6), 6.27 (1H, t, *J*=2.1 Hz, H-4)		Wang *et al*., 2013
7	*trans*-4-hydrox-ycinnamic acid	165.15 $[M+H]^+$	*δ*: 7.61 (1H, d, *J*=15.9 Hz, H-7), 7.56 (2H, d, *J*=8.6 Hz, H-2, 6), 6.90 (2H, d, *J*=8.6 Hz, H-3, 5), 6.34 (1H, d, *J*=15.9 Hz, H-8)		Miyakea *et al*., 2012
8	kaempferol	285.2 $[M-H]^-$		equal R_f and R_t to the standard of kaempferol based on TLC and HPLC	
9	quercetin	301.1 $[M-H]^-$		equal R_f and R_t to the standard of quercetin based on TLC and HPLC	
10	isoquercitrin	465.19 $[M+H]^+$	*δ*: 12.39 (1H, s, 5-OH), 8.04 (1H, d, *J* =2.0 Hz, H-2′), 7.60 (1H, dd, *J*=8.4, 2.0 Hz, H-2′), 6.97 (1H, d, *J*=8.4 Hz, H-5′), 6.53 (1H, d, *J*=1.8 Hz, H-8), 6.30 (1H, d, *J*=1.8 Hz, H-6), 5.27 (1H, d, *J*=7.2 Hz, H-1″), 3.80~3.30 (6H, m, H-2″~6″)		Zhao *et al*., 2013



1. β-sitosterol, 2. palmitic acid, 3. protocatechuic acid, 4. salicylic acid, 5. *p*-hydroxybenzoic acid, 6. resveratrol, 7. *trans*-4-hydroxycinnamic acid, 8. kaempferol, 9. quercetin, 10. isoquercitrin

Fig. 1: Chemical structure of the compounds 1-10 isolated from TDG roots

DISCUSSION

Many studies have demonstrated that a number of phytochemicals or extracts present in medicinal herbs have anticancer potential *in vitro* and *in vivo*. They might be good candidate for the development of anticancer drugs (Lee and Houghton., 2005). It is an attractive strategy of searching for potential agents from medicinal plants with promising features of cytotoxicity and apoptosis induction in cancer cells.

TDG is a well-known traditional medicinal herb, which possesses anti-inflammatory, analgesic, and antipyretic properties. It was recently reported that EtOAc, H_2O extracts of TDG and its flavones significantly inhibited the growth of various cancer cells and induced their apoptosis *in vitro* (Feng *et al*., 2006; Cheng and Lu., 2007; Xu *et al*., 2008; Zhang *et al*., 2010; Lu *et al*., 2011; Liu and Xia., 2010; Xu *et al*., 2010, 2011; Wang *et al*., 2012; Ding *et al*., 2012; Sun *et al*., 2013). It is suggested that TDG and its extracts could be used for the treatment of cancer as a folk remedy. However, there has been no report on the active extracts and chemical constituents responsible for antiproliferative activity against MDA-MB-435S cell lines from TDG so far. In this study, we report, for the first time, that the $CHCl_3$, H_2O extracts along with compounds 6 and 8 isolated from TDG indicated cytotoxicity in MDA-MB-435S cells.

The preliminary screening of extracts and isolated compounds for antiproliferative activity was evaluated by the MTT assay (table 1). According to results, the $CHCl_3$, EtOAc and H_2O extracts showed cytotoxicity in MDA-MB-435S cells, while the $CHCl_3$ extract exhibited the most potent effect. Further isolation and identification of bioactive constituents from the most active extract were carried out and ten compounds including five phenols and three flavones were obtained. Among these compounds, compounds 3, 5-7, 10 were for the first time isolated from this plant. Then, results of the MTT assay showed that only compounds 6 (resveratrol) and 8 (kaempferol) exhibited obvious inhibitory effects on MDA-MB-435S cells, while resveratrol showed a more potent effect than that of kaempferol. Therefore, it is possible that resveratrol and kaempferol in $CHCl_3$ extract of TDG may play a major role in the inhibition of MDA-MB-435S cells growth. Considering the potential of $CHCl_3$ extract, compounds 6 (resveratrol) and 8 (kaempferol) as anticancer agents, further studies on the molecular mechanism of cell death are needed to provide more convincing evidences. In addition, further studies will also be needed to fully understand the compositions and their mechanism of the EtOAc, H_2O extracts of TDG for cancer treatment.

CONCLUSION

The results in this study clearly indicated that the roots of TDG contained a variety of phenols and flavones. Moreover, the antiproliferative activity of TDG against MDA-MB-435S cell lines is ascribable to the most active $CHCl_3$ extract along with active compounds 6 (resveratrol) and 8 (kaempferol). The findings suggest that TDG provides a source of natural antiproliferative agents and can be considered as a potential preventive and chemotherapeutic agent in breast cancer.

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