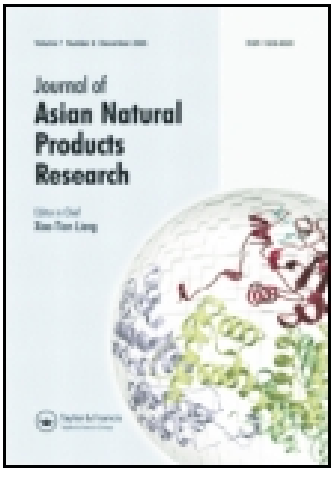

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A new kaempferol trioside from *Silphium perfoliatum*

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A new apiose-containing kaempferol trioside, kaempferol-3-*O*-α-L-rhamnosyl-(1‴ → 6″)-*O*-β-D-galactopyranosyl-7-*O*-β-D-apiofuranoside, along with 16 known compounds, were isolated from 50% acetone extract of *Silphium perfoliatum* L. Their structures were elucidated by acid hydrolysis and spectroscopic techniques including UV, IR, MS, ^{1}H, ^{13}C, and 2D-NMR. In addition, the pharmacological activity of compound **1** was tested with HepG2 and Balb/c mice (splenic lymphocytes and thymic lymphocytes) *in vitro*, and it exhibited inhibitory effect on the proliferation of HepG2 cells and showed the immunosuppressive activity.

Keywords: Asteraceae; *Silphium perfoliatum*; kaempferol trioside

1. Introduction

Silphium perfoliatum L. tribe Heliantheae (Asteraceae) is a perennial herb native to North America, also known as cup plant or Indian cup. Various North American India tribes applied *S. perfoliatum* for medical purposes. The herb is used as a tonic, diuretic, and diaphoretic agent, whereas its roots are used as a poultice for bleeding, wounds, and backache. It was brought to Europe from North America in the eighteenth century because of its ornamental traits. Beijing Botanical Garden has brought *S. perfoliatum* to China from Korea Central Botanical Garden in 1979. *S. perfoliatum* has already been cultivated in every province of China. Earlier, chemical screening of *S. perfoliatum* detected mono-, di-, tri-, and sesqui-terpenoids, carbohydrates, simple flavonoids, ascorbic acid, coumarins, protein complexes, and phenolic acids. Terpenoids from the leaves were found to have hypocholesterolemic and hypotriglyceridemic effects [1,2]. Kaempferol trioside from the aerial parts of *S. perfoliatum* displayed antiproliferative activity against MCF7 cells. However, it was mainly used as fodder in our country, and few of the phytochemical studies have been reported previously. In this paper, we report here the isolation and characterization of a new compound (Figure 1), kaempferol-3-*O*-α-L-rhamnosyl-(1‴ → 6″)-*O*-β-D-galactopyranosyl-7-*O*-β-D-apiofuranoside, together with 16 known compounds, oleanolic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranosyl ester (chikusetsusaponin-IVa) (**2**) [3], 3-*O*-β-D-glucuronopyranosyl echinocystic acid 28-*O*-β-D-glucopyranoside (acanthopanaxoside E) (**3**) [4], 3-*O*-[β-D-glucuronic acid pyranosyl]-29-hydroxyoleanolic acid-28-*O*-[β-D-glucopyranosyl] ester (zygophyloside K) (**4**) [5], erythro-1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(*E*)-3-hydroxy-1-propenyl]-2-methoxyphenoxy}-1,3-propanediol (**5**) [6], *p*-hydroxyphenol(**6**) [7], gallic acid (**7**) [8], pyrogallic acid (**8**) [9], gentisic acid (**9**) [10], hydroferulic acid (**10**) [11], 3-(4-hydroxyphenyl) propionic acid (**11**) [12], vanillin (**12**) [13], protocatechuic acid (**13**) [10], salicylic acid (**14**) [14], *p*-hydro-

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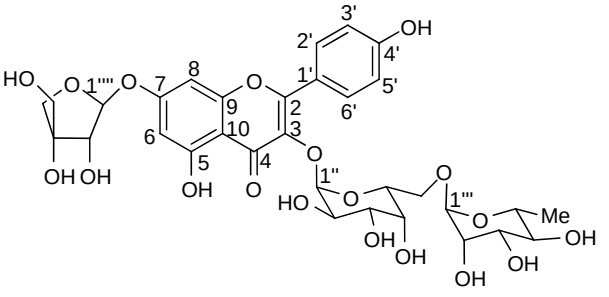

Figure 1. The structure of compound **1**.

xycinnamic acid (**15**) [13], kaempferol-3-*O*-β-D-glucopyranoside (**16**) [15], and kaempferol (**17**) [15], were isolated from 50% acetone extract of *S. perfoliatum*. Ten of these known compounds (**3**–**12**) were isolated from this plant for the first time. In addition to the detailed chemical investigations, this paper describes the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) proliferation activity of compound **1** against HepG2 cell lines, the splenic lymphocytes, and thymic lymphocytes.

2. Results and discussion

Compound **1** was obtained as a yellow amorphous powder, with the molecular formula of $C_{32}H_{38}O_{19}$, as deduced from the $[M + Na]^+$ peak at *m/z* 749.1934 by ESI-Q-TOF-MS. The IR spectrum exhibited the absorption of hydroxyl (3415 cm^{-1}), carbonyl (1658 cm^{-1}), and aromatic rings (1598 and 1491 cm^{-1}). The UV spectrum showed absorption maxima at 265 and 346 nm. Compound **1** afforded kaempferol, galactose, rhamnose, and apiose, which was identified by comparison of its NMR spectral data with those in literature, and confirmed by acid hydrolysis experiment [1,16]. The ^{1}H NMR spectrum for compound **1** (Table 1) exhibited six aromatic proton signals, four of which resonated at δ 8.10 (2H, d, $J = 8.8$ Hz, H-2′, 6′), 6.85 (2H, d, $J = 8.8$ Hz, H-3′, 5′), being ascribed to a

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data of compound **1** (DMSO, δ ppm).

No.	δ_H (J, Hz)	δ_c
2		157.1
3		133.5
4		177.6
5		160.9
6	6.38 (1H, d, $J = 1.5$)	99.2
7		162.5
8	6.72 (1H, d, $J = 1.5$)	94.4
9		156.0
10		105.4
1′		120.3
2′, 6′	8.10 (2H, d, $J = 8.8$)	131.1
3′, 5′	6.85 (2H, d, $J = 8.8$)	115.2
4′		160.6
Gal″		
1″	5.34 (1H, d, $J = 7.7$)	101.9
2″	3.56 (1H, m)	71.1
3″	3.37 (1H, m)	72.9
4″	3.60 (1H, m)	68.0
5″	3.54 (1H, m)	73.6
6″	3.57 (1H, dd, $J = 11.4, 3.5$), 3.25 (1H, m)	65.3
Rha‴		
1‴	4.38 (1H, d, $J = 1.2$)	100.1
2‴	3.28 (1H, dd, $J = 9.4, 3.2$)	70.6
3‴	3.34 (1H, m)	70.4
4‴	3.07 (1H, t, $J = 9.3$)	71.9
5‴	3.34 (1H, m)	68.2
6‴	1.05 (3H, d, $J = 6.2$)	17.9
Api⁗		
1⁗	5.62 (1H, d, $J = 3.2$)	107.1
2⁗	4.17 (1H, d, $J = 3.2$)	76.0
3⁗		78.7
4⁗	4.07 (1H, d, $J = 9.5$), 3.77 (1H, d, $J = 9.5$)	74.6
5⁗	3.35 (1H, m)	61.9

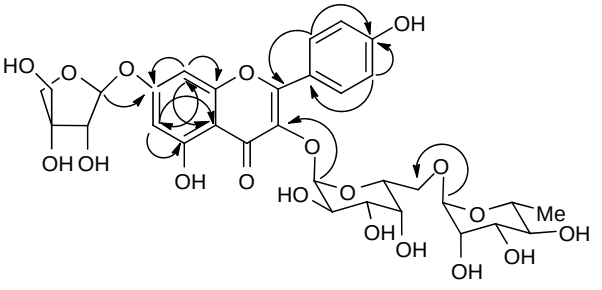

Figure 2. The HMBC correlations of compound **1**.

set of AA′BB′ system of flavone B-ring. Two deshielded one-proton doublets at δ 6.38 (1H, d, $J = 1.5$ Hz) and 6.72 (1H, d, $J = 1.5$ Hz) were assigned to aromatic ring A *meta*-coupled H-6 and H-8, respectively. In addition, three anomeric proton signals were found at δ 5.62 ($J = 3.2$ Hz), 5.34 ($J = 7.7$ Hz), and 4.38 ($J = 1.2$ Hz). Finally, 18 proton signals were found in the range of δ 3.06–4.17 and one methyl at δ 1.05, which suggested the existence of three sugar moieties. The ^{13}C NMR spectrum clearly indicated that **1** is a *C*-methylated flavonoid glycoside including 32 carbons and also indicated the presence of three sugar moieties, together with a C15 aglycone unit. These spectral data of **1** were almost identical with those of kaempferol trioside. According to the HSQC spectrum, three anomeric protons at δ 5.62 (1H, d, $J = 3.2$ Hz, H-1‴′), 5.34 (1H, d, $J = 7.7$ Hz, H-1″), and 4.38 (1H, d, $J = 1.2$ Hz, H-1‴), which were correlated with three anomeric carbons at δ 107.1, 101.9, and 100.1, respectively. In addition, from the ^{1}H NMR and ^{13}C NMR spectral data, **1** was supposed to be a kaempferol glycoside with apiofuranose, galactose, and rhamnose moieties. The *J* value (3.2 Hz) of the anomeric proton signal (δ 5.62, apiofuranosyl H-1‴′) in the ^{1}H NMR spectrum of **1** confirmed a β-apiofuranose structure, The *J* value (7.7 Hz) of the anomeric proton signal (δ 5.34, galactosyl H-1″) in the ^{1}H NMR spectrum of **1** confirmed a β-galactose structure. The *J* value (1.2 Hz) of the anomeric proton signal (δ 4.38, rhamnosyl H-1‴) in the ^{1}H NMR spectrum of **1** confirmed a L-rhamnose structure. The absolute configurations of monosaccharide units mentioned above were successively determined as D, D, and L, with the help of GC analysis of their respective derivatives. In the HMBC spectrum, the correlations of the anomeric proton at δ 5.62 (1H, d, $J = 3.2$ Hz, apiofuranosyl H-1‴′) with C-7 at δ 162.5 revealed that the apiofuranose was linked to C-7; the correlations of the anomeric proton at δ 5.34 (1H, d, $J = 7.7$ Hz, galactosyl H-1″) with C-3 at δ 133.5 revealed that the galactose was linked to C-3; the correlations of the anomeric proton at δ 4.38 (1H, d, $J = 1.2$ Hz, rhamnosyl H-1‴) with C-6″ at δ 65.3 revealed that the rhamnose was linked to C-6″ (Figure 2). The data indicated that **1** was kaempferol 3-*O*-α-L-rhamnosyl-(1‴ → 6″)-*O*-β-D-galactopyranosyl-7-*O*-β-D-apiofuranoside (Figure 1).

Growth assay analysis of **1** from *S. perfoliatum* was performed in our lab. First, the cell viability of **1** was tested on HepG2 cells by the MTT method. As shown in Figure 3, compound **1** exhibited significant inhibitory effect on HepG2 cells proliferation. Second, the cell viability of **1** was tested on Balb/c mice splenic lymphocytes and thymic lymphocytes with ConA by the MTT method *in vitro*. As shown in Figure 4, compound **1** displayed antiproliferative activity against the splenic lymphocytes and thymic lymphocytes.

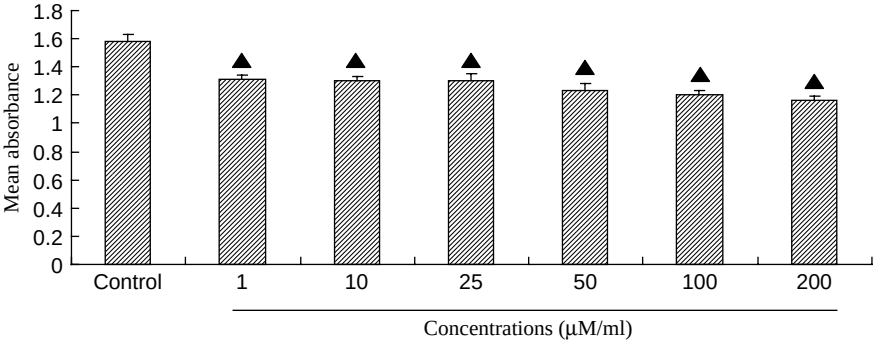

Figure 3. Effects of compound **1** on HepG2 cells proliferation by MTT. HepG2 cells were treated with control and compound **1** at 1, 10, 25, 50, 100, and 200 μM/ml for 96 h (▲, $p < 0.01$, compared with control).

3. Experimental

3.1 General experimental procedures

Optical rotations were obtained using a PerkinElmer 341 polarimeter (PerkinElmer Corporation, Waltham, MA, USA). UV spectra were obtained on a Shimadzu UV-VIS 2201 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). IR spectra were recorded with a Shimadzu FTIR-8201 PC spectrometer

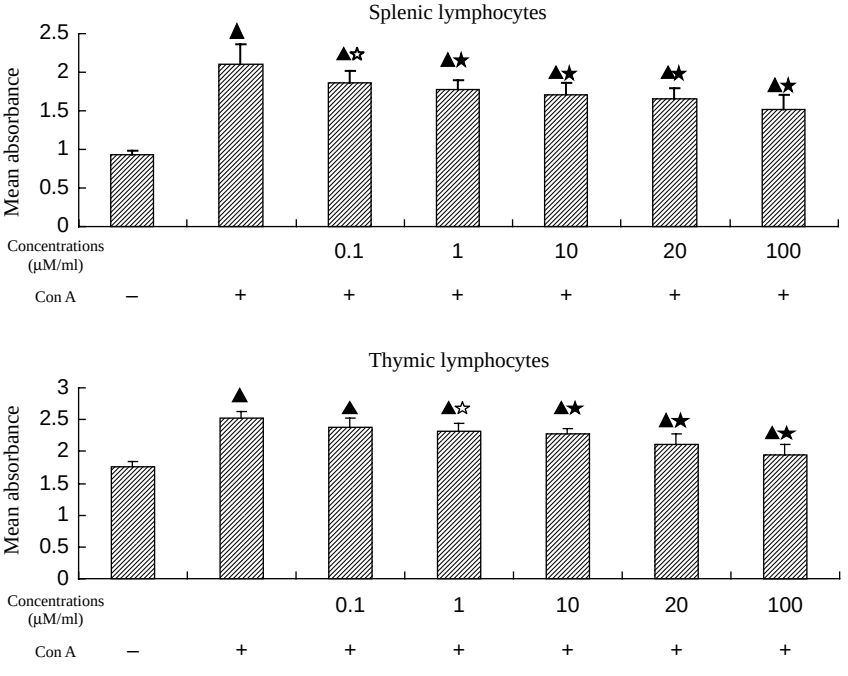

Figure 4. Effects of compound **1** on proliferation of splenic lymphocytes and thymic lymphocytes by MTT assay *in vitro.* Splenic lymphocytes and thymic lymphocytes were treated with vehicle, Con A (5 μg/ml), 0.1, 1, 10, 20, and 100 μM/ml of compound **1**, along with Con A (5 μg/ml) as indicated (▲, $p < 0.01$, compared with the vehicle-treated cells; ☆, $p < 0.05$, compared with the Con A-treated cells; ★, $p < 0.01$, compared with the Con A-treated cells).

(Shimadzu Corporation, Kyoto, Japan). NMR spectra were recorded on a BRUKER AVANCE III-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane as internal reference (Bruker Corporation, Ettlingen, Germany). ESI-Q-TOF-MS were recorded on a micro Mass Q-TOF Micro spectrometer (Waters Corporation, Milford, MA, USA). GC analysis was carried out with an Agilent 7890A instrument (Agilent Technologies Corporation, Santa Clara, CA, USA). Column chromatography was performed over Diaion HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (160–200 mesh, Qingdao Marine Chemical Industry, Qingdao, China), Toyopearl HW-40 (TOSOH Corporation, Tokyo, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), respectively. Thin layer chromatography (TLC) was conducted on self-made silica gel G (Qingdao Marine Chemical Industry, Qingdao, China) plates. The chemical reagents were supplied by Beijing Chemical Plant (Beijing, China) and Tianjin No. 3 Reagent Plant (Tianjin, China). Cell viability was quantitatively obtained by MTT assay with a BIO-RAD Model 680 microplate reader (BIO-RAD Corporation, Hercule, CA, USA).

3.2 Plant material

The air-dried aerial parts of *S. perfoliatum* were collected from Taihang Mountain, Henan Province of China, in June 2011. The plant was identified by Prof. Cheng-Ming Dong. A voucher sample (20110714) was deposited in our laboratory.

3.3 Extraction and isolation

The air-dried aerial plants of *S. perfoliatum* (3.3 kg) were extracted three times with 50% of aqueous acetone at room temperature. The solvent was concentrated *in vacuum* and subjected to a column of Diaion HP-20, which was eluted with H_2O containing an increasing amount of MeOH to afford H_2O eluate 30.7 g (A), 10% MeOH eluate 22.3 g (B), 20% MeOH eluate 15.5 g (C), 30% MeOH eluate 17.4 g (D), and 40% MeOH eluate 10.6 g (E). Fraction A was subjected to Toyopearl HW-40 column (coarse grade) using H_2O–MeOH (50%). The H_2O eluate of A (21.6 g) was rechromatographed on Toyopearl HW-40 (stepwise elution with H_2O and MeOH) to afford two fractions (A-1 and A-2). Fraction A-1 (0.6 g) was purified with Toyopearl HW-40 (elution with 50% aqueous MeOH) to yield compound **6** (11 mg). Fraction A-2 (0.4 g) was purified with Toyopearl HW-40 (50% MeOH) and Sephadex LH-20 (70% MeOH) to give compounds **13** (15 mg) and **14** (11 mg). The 10% MeOH eluate of A (4.1 g) was rechromatographed on Toyopearl HW-40 (70% MeOH) to yield compound **7** (10 mg). The 30% MeOH eluate of A (1.2 g) was purified with Toyopearl HW-40 (70% MeOH) to yield compound **8** (13 mg). Fraction B was subjected to Toyopearl HW-40 column (coarse grade) using H_2O–MeOH (50%). The H_2O eluate of B (14.5 g) was rechromatographed on Toyopearl HW-40 (stepwise elution with H_2O and MeOH) to afford two fractions (B-1 and B-2). Fraction B-1 (0.2 g) was chromatographed on Toyopearl HW-40 (70% MeOH) followed by Sephadex LH-20 with MeOH to give compound **9** (15 mg). Fraction B-2 (0.7 g) was further purified by column chromatography over Toyopearl HW-40 (70% MeOH) and Sephadex LH-20 (MeOH), affording compounds **10** (12 mg) and **11** (11 mg). The 30% MeOH eluate of B (0.9 g) was purified with Toyopearl HW-40 (MeOH) to give compound **15** (9 mg). Fraction C was subjected to Toyopearl HW-40 column (coarse grade) using H_2O–MeOH (50%). The 10% MeOH eluate of C (2.3 g) was purified with Toyopearl HW-40 (70% MeOH) to give compound **12** (9 mg). The 30% MeOH eluate of C (0.8 g) was purified with Toyopearl HW-40 (MeOH) to

give compound **5** (13 mg). Fraction D was subjected to Toyopearl HW-40 column (coarse grade) using H_2O–MeOH (50%). The 10% MeOH eluate of D (3.6 g) was purified with Toyopearl HW-40 (30% MeOH) to give compound **2** (15 mg). The 20% MeOH eluate of D (2.7 g) was purified with Sephadex LH-20 (30% MeOH) to give compound **1** (25 mg). The 30% MeOH eluate of D (2.5 g) was purified with Toyopearl HW-40 (50% MeOH) to give compounds **3** (12 mg) and **4** (9 mg). Fraction E was subjected to Toyopearl HW-40 column (coarse grade) using H_2O–MeOH (50%). The 10% MeOH eluate of E (1.3 g) was purified with Toyopearl HW-40 (70% MeOH) and on silica gel ($CHCl_3$: MeOH, 10:1) to give compounds **16** (13 mg) and **17** (14 mg).

*3.3.1 Kaempferol-3-O-α-L-rhamnosyl-(1‴ → 6″)-O-β-D-galactopyranosyl-7-O-β-D-apiofuranoside (**1**)*

Yellow amorphous powder; $[\alpha]_D^{20}$ − 60 (H_2O, *c* 0.18); UV (H_2O) λ_{max}: 265, 346 nm. IR (KBr) ν_{max}: 3415, 2927, 1658, 1598, 1491, 1347, 1209, 1176, and 811 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) spectral data are shown in Table 1. ESI-Q-TOF-MS: *m/z* 749.1934 $[M + Na]^+$ (calcd for $C_{32}H_{38}O_{19}Na$, 749.1905).

3.4 Acid hydrolysis of compound 1

Compound **1** (4.2 mg) was dissolved in 2 mol/l HCl:dioxane (1:1, 1 ml) and refluxed in a water bath at 90°C for 2 h. After dioxane was removed, the solution was extracted with EtOAc (1 ml × 3). The aqueous layer was neutralized by passing through an Amberlite MB-3 resin column eluted with H_2O, then concentrated and dried to furnish a monosaccharide residue. Then, the sugars were detected by TLC analysis (EtOAc: EtOH:H_2O, 5:2:1, detection solution: anisaldehyde concentrated sulphuric acid) by comparison with authentic samples: galactose (R_f 0.35), apiose (R_f 0.48), and rhamnose (R_f 0.56). Furthermore, the residue was dissolved in pyridine (0.2 ml), and then a pyridine solution (0.3 ml) of L-cysteine methyl ester hydrochloride (5 mg) was added to the solution. The mixture was kept at 60°C for 1.5 h, dried *in vacuo*, and trimethylsilylated with hexamethyldisilazane–trimethylchlorosilane (HMDS–TMCS) (0.1 ml) at 60°C for 1 h. After being partitioned between *n*-hexane (0.5 ml) and H_2O (0.5 ml), the *n*-hexane extract was concentrated and analyzed by GC under the following conditions: HP-5 capillary column (30 m × 0.25 mm × 0.25 μm), column temperature at 230°C, injection temperature at 250°C, N_2 as carrier gas. Ultimately, the D-configurations of galactose, apiose, and the L-configuration of rhamnose were confirmed by comparing the retention times with those of standard samples, respectively. t_R: D-galactose (25.34 and 26.55 min), D-apiose (11.59 and 12.82 min), and L-rhamnose (18.02 and 19.27 min).

3.5 Bioassy

The cytotoxic activity of compound **1** isolated from *S. perfoliatum* on cell proliferation in HepG2 cells was evaluated in accordance with a reported protocol [17]. The experiment was conducted using Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with the cells being grown 24 h prior to treatment. MTT assay was carried out after 96 h cultivation. The absorbencies were measured at 570 nm.

Growth assay analysis of compound **1** in Balb/c mice primary splenic lymphocytes and thymic lymphocytes was evaluated in accordance with a reported protocol [18]. First, the splenic lymphocytes and thymic lymphocytes were separated from the Balb/c mice in sterile conditions. Then, the lymphocytes were suspended by treatment with trypsin (0.25%) and were maintained in 1640 medium. After that, the cells were plated

into 96-well (30,000 and 40,000 cells/well, respectively) tissue confluence plates. The medium was replaced by new medium containing compound **1** (0.1, 1, 10, 20, and 100 μM/ml, respectively) after the cells reached confluence. To observe the cell proliferation, MTT assay was carried out after 24 and 48 h cultivation, respectively. The absorbencies were measured at 570 nm.

Acknowledgements

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