Biotransformation of furannoligularenone by transgenic crown galls of *Panax quinquefolium*

Chunyan Yan[1,2], Jue Wang[3], Guoyan Duan[2], Rongmin Yu[2]

[1]Department of Natural Medicinal Chemistry, College of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, [2]Biotechnology Institute of Chinese Materia Medica, Jinan University, Guangzhou, [3]Center of Forecasting and Analysis, Shenzhen Institute for Drug Control, Shenzhen, China

Submitted: 12-08-2011 Revised: 14-09-2011 Published: 23-05-2012

Access this article online
Website: www.phcog.com
DOI: 10.4103/0973-1296.96555
Quick Response Code:

ABSTRACT

Background: Transgenic plant suspension cultures could be used as an effective tool for the biotransformation of exogenous compounds. **Objective:** To investigate the biotransformation of furannoligularenone (**1**) by transgenic crown galls of *Panax quinquefolium*. **Materials and Methods:** Compound **1** was administered into the crown gall cultures and co-cultured for 6 days. The cultures were dried and extracted with methanol for HPLC analyses. The extract was separated on column chromatography, and biotransformation products' structures were elucidated by the physicochemical properties and the data of NMR and MS. Moreover, three flasks were randomly chosen each day to establish time–course during the period for co-culturing. **Results:** Co-culturing compound **1** with crown galls yielded two compounds, 3-oxo-eremophila-1,7(11)-dien-12,8-olide (**2**) and 3-oxo-8-hydroxy-eremophila-1,7(11)-dien-12,8-olide (**3**), which were obtained by biotransformation using *P. quinquefolium* crown galls for the first time. Time–course investigation revealed that the mole conversion ratio reached the highest level of 45.5% and 33.9% on fourth and fifth day after substrate administration, respectively. Furthermore, a proposal biosynthesis pathway was given from compound **1** to compounds **2** and **3**. **Conclusion:** This was the first example of compound **1** being successfully converted into compounds **2** and **3** by transgenic crown galls of *P. quinquefolium*.

Key words: Biotransformation, furannoligularenone, transgenic crown galls, *Panax quinquefolium*

INTRODUCTION

Plant suspension cultures can serve as tools for the *in vivo* production of secondary metabolites[1] as well as for the biotransformation of exogenous compounds.[2] The ability to biocatalyze foreign substrates region-specifically and stereo-selectively under mild conditions is of great interest since some of the target products are difficult to be prepared by microorganic biotransformation or chemical synthesis manner.[3] There are many kinds of biotransformation patterns in plant cells, such as oxidation, reduction, hydroxylation, esterification, methylation, isomerization, glycosylation, etc.[4,5] Considerable progress on biotransformation of exogenous compounds by plant cultures has been made during the last two decades.

Address for correspondence:
Dr. Rongmin Yu, College of Pharmacy, Jinan University, Guangzhou 510632, People's Republic of China.
E-mail: tyrm@jnu.edu.cn

Crown galls are kind of irregular transgenic tissues induced by *Agrobacterium tumefaciens*, which are capable of introducing and replicating their own DNAs to the plant's nuclear genome.[6] They are free of extra administration of phytohormones. Furthermore, they are superior to callus and cell cultures in the aspects of faster growth and more secondary metabolites. Thus, crown galls can be applied to produce useful secondary metabolites efficiently and effectively.

Recently, scientists pay more and more attention to eremophilane-type sesquiterpenes because these compounds have demonstrated anti-HIV,[7] antibacterial,[8] and cytotoxic activities.[9,10] Furannoligularenone, one of the bioactive eremophilane sesquiterpene component obtained from *Ligularia pleurocaulis*, has demonstrated some bioactivities, such as relieve cough, reduce sputum, and promote blood flow. Previously, we succeeded for the first time in inducing transgenic crown galls from the stems of *Panax quinquefolium* and reported the production of bioactive ginsenosides from this culture.[11,12] To propose biosynthesis pathway of eremophilane-type sesquiterpenes and look for new bioactive compounds by biotransformation methods as well as investigate the ability of transgenic crown galls of *P. quinquefolium* as a new biocatalyst, furannoligularenone was chosen as the exogenous substrate in this study.

MATERIALS AND METHODS

General

Melting points were determined on an X-4 apparatus and corrected. NMR spectra were recorded on a Bruker Advance 400 MHz (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR) with TMS as the internal standard. ESI–mass spectra were measured on a Bruker Esquire 2000 mass spectrometer. HPLC was performed on an Agilent 1200 liquid chromatograph instrument, equipped with a photodiode array detector, a quaternary pump, a vacuum degasser module, Phenomenex C_{18} (5 μm, 4.6 × 250 mm). Silica gel (200–300 mesh) was purchased from the Qingdao Marine Chemical Group, China. Furannoligularenone (compound **1**) was donated by Professor Naili Wang, Graduate School at Shenzhen, Tsinghua University, China, and its purity was above 98% measured by HPLC.

Plant cultures

The crown galls were induced from the direct infection of *P. quinquefolium* sterile stems with *A. tumefaciens* C_{58},[11,12] and then were cultured in the MS liquid medium (3% (w/v) sucrose) without phytohormones. The crown galls were sub-cultured into a fresh medium at 30 day intervals.

The pH value of the medium was adjusted to 5.75 before sterilization. Five grams fresh transgenic crown galls were inoculated into a 250 mL Erlenmeyer flask with 100 mL MS medium, which were cultured at 25 °C on an orbital shaker at 110 rpm in the darkness. All the above were prepared for biotransformation of compound **1** and its time-course establishment. The procedure was carried out in triplicate.

Biotransformation of 1 by crown galls of *P. quinquefolium*

Substrate **1** (30 mg) dissolved in 1.5 ml methanol was administered into three flasks of crown galls suspension cultures,[13] which were pre-cultured for 15 days. The co-cultures proceeded for 6 days. The negative control was crown galls without adding substrate **1**.

Crown galls were harvested and the cultures were separated from the medium by filtration. The crown galls were dried, ground, and extracted with methanol by ultrasonic three times and the extract was concentrated by evaporation *in vacuo*. The methanol extract and the medium were used for HPLC analyses. The negative controls were treated with the same method for HPLC analysis.

Isolation of biotransformation products

Compound **1** (180 mg) was added to the crown galls. The residue of cultures extracted by the above procedure was dissolved in water and partitioned between H_2O and ethyl acetate for five times. The organic phase was concentrated *in vacuo* to dryness. The residue was separated on column chromatography by silica gel (200–300 mesh), eluting with a mixture of petroleum ether–acetone in different ratios. The products were further purified by Sephadex LH-20 eluting with methanol to yield compounds **2** (9.3 mg) and **3** (7.1 mg) as judged by NMR and MS analyses and no products in the negative controls.

HPLC analysis

The conditions for HPLC analysis

The elution system was methanol–water [40: 60, (v/v)]. The flow rate was 1.0 ml/min. Column temperature was 25°C. The analysis was monitored at 280 nm. The inject volume was 10 μl.

Establishment of time course of substrate 1

Fresh crown galls (5.0 g) were inoculated into a 250 mL Erlenmeyer flask with 100 mL MS medium and cultured at 25°C on an orbital shaker at 110 rpm in the darkness. After 15 days pre-culturing, 52.5 mg of substrate **1** in 5.3 ml MeOH was added to 21 flasks of cultures. The cultures period was proposed for 6 days, and three flasks were randomly chosen each day. The culture was filtered and the crown galls were dried, ground and extracted with methanol by ultrasonic for three times. The extract was concentrated *in vacuo* and dissolved in 5 ml of methanol.

All the samples were filtered through a 0.45 μm filter membrane just before use. The solution (10 μl) was injected into the HPLC instrument for analysis. The biotransformation compounds **2** and **3** were quantitatively determined, respectively.

RESULTS

Culture of crown galls

Crown galls of *P. quinquefolium* were cultured on solid culture of the MS medium and the system displayed a sigmoidal growth curve.[11]

Analysis of biotransformation products

The result of the biotransformation metabolites of substrate **1** by crown gall cultures showed two new peaks appeared in HPLC of samples. Retention times of products **2** and **3** were 13.87 min and 11.60 min, respectively.

Structural elucidation of biotransformation products

Two biotransformation compounds were purified from the cultures. Their structures were, respectively, identified as 3-oxo-eremophila-1,7(11)-dien-12,8-olide (**2**) and 3-oxo-8-hydroxy-eremophila-1,7(11)-dien-12,8-olide (**3**), on the basis of physicochemical properties and the data of MS, ^{1}H NMR, and ^{13}C NMR. The spectral data for products **2** and **3** (MS, IR and NMR spectra) proved that they should have the same structure of A ring as substrate **1**. These two furanoeremophilanolides differed in the C-8 substitution: product **2** had a hydrogen atom at C-8 ($[M]^+$ 246 amu; δ_H 4.93 br t; δ_C 81.5 ppm), while **3** had a hydroxyl group ($[M]^+$ 262 amu; ν 3240 cm^{-1}; δ_C 104.7 ppm). The ^{13}C NMR signals for **2** and **3** were nearly the same, except for the shielding of C-8. Consequently, the 8α-H and 8α-OH configuration of these lactones (products **2** and **3**) were also proposed, respectively.[14]

3-Oxo-eremophila-1,7(11)-dien-12,8-olide (2): Colorless needles, $C_{15}H_{18}O_3$, mp: 184–186 °C, ^{1}H NMR (CD_3OD, 400 MHz) δ: 6.67 (dd, J = 10.0, 2.0 Hz, H-1), 6.00 (dd, J = 10.0, 3.2 Hz, H-2), 2.50 (q, J = 6.8 Hz, H-4), 2.34 (d, J = 13.5 Hz, H-6a), 2.82 (d, J = 13.5 Hz, H-6b), 4.93 (m, H-8), 1.47 (dd, J = 12.3, 1.4 Hz, H-9a), 2.58 (dd, J = 13.6, 6.8 Hz, H-9b), 2.91 (m, H-10), 1.79 (t, J = 1.71 Hz, H-13), 0.62 (s, H-14), 1.12 (d, J = 6.8 Hz, H-15). ^{13}C NMR δ: 151.9 (C-1), 130.2 (C-2), 202.5 (C-3), 44.2 (C-4), 45.0 (C-5), 35.2 (C-6), 161.9 (C-7), 81.5 (C-8), 37.9 (C-9), 55.0 (C-10), 123.6 (C-11), 176.6 (C-12), 8.0 (C-13), 11.5 (C-14), 7.6 (C-15).

3-Oxo-8-hydroxy-eremophila-1,7(11)-dien-12,8-olide (3): Colorless needles, $C_{15}H_{18}O_4$, mp: 204–206 °C, ^{1}H NMR (CD_3OD, 400 MHz) δ: 6.83 (dd, J = 10.0, 2.0 Hz, H-1), 6.15 (dd, J = 10.0, 3.3 Hz, H-2), 2.78 (q, J = 6.8 Hz, H-4), 2.52 (br. d, J = 13.2 Hz, H-6a), 2.86 (d, J = 13.1 Hz, H-6b), 1.89 (t, J = 13.4 Hz, H-9a), 2.54 (dd, J = 13.2, 3.4 Hz, H-9b), 3.24 (br. dd, J = 11.5, 1.0 Hz, H-10), 1.95(d, J = 1.56 Hz , H-13), 0.78 (s, H-14), 1.27 (d, J = 6.86 Hz, H-15). ^{13}C NMR δ: 152.1 (C-1), 130.0 (C-2), 202.6 (C-3), 44.6 (C-4), 45.6 (C-5), 36.8 (C-6), 169.2 (C-7), 104.7 (C-8), 39.7 (C-9), 55.1 (C-10), 125.4 (C-11), 173.9 (C-12), 8.0 (C-13), 10.9 (C-14), 7.5 (C-15).[15,16]

Establishment of time course of compound 1

Results of the biotransformation products of substrate **1** by crown galls of *P. quinquefolium* were illustrated in Figure 1. Time course investigation revealed that the metabolites emerged in the culture and medium within 1 day after administration. With the co-culture time increased, the yield of **2** in the culture and the medium was increased until the fourth day and fifth day and the maximum concentrations of **3** in the culture and the medium appeared on the fifth and third day, respectively. Then, the concentrations decreased gradually, indicating the formation of other secondary products. Moreover, the mole conversion ratio of two major products both in the culture and medium reached their highest levels of 45.5% and 33.9% on the fourth and fifth day after substrate administration, respectively. On the fourth day, the biotransformation ratio of compound **1** reached highest (45.76%), the yields of compounds **2** and **3** were 87.6 mg/L and 53.8 mg/L, respectively. However, the excrete ratio of products reached highest (7.89%) for **2** and 26.1% for **3** (by weight)) on the first day. A possible biosynthesis pathway in the cultures was proposed in Figure 2. Obviously, compound **2** was an intermediate in the biosynthesis pathway of compound **3**.

The results of comparing the system of crown galls of *P. quinquefolium* with that of hairy roots of *Polygonum multiflorum*[17] demonstrated that the two plant cultures showed similar biotransformation patterns on compound **1**. Compound **2** was the major metabolite in both systems. Compound **3**, a minor product, was also detected in both cultures [Figures 3 and 4].

DISCUSSION

Recently, *A. tumefaciens*-induced crown galls and *Agrobacterium rhizogenes*-induced hairy roots have broadened the application of *in vitro* plant cultures especially for the production of secondary metabolites.[18, 19] During the course of our studies, we discovered that the biotransformation of substrate **1** by hairy roots of *P. multiflorum*[17] biosynthesized the same metabolites with *P. quinquefolium* crown galls, but its conversion ratio was lower than later.

From the results of the time-course experiments, the biotransformation compounds **2** and **3** were found both in the medium and cultures in either biocatalyst, but existed in the cultures in a yield above 90%, and very few existed in

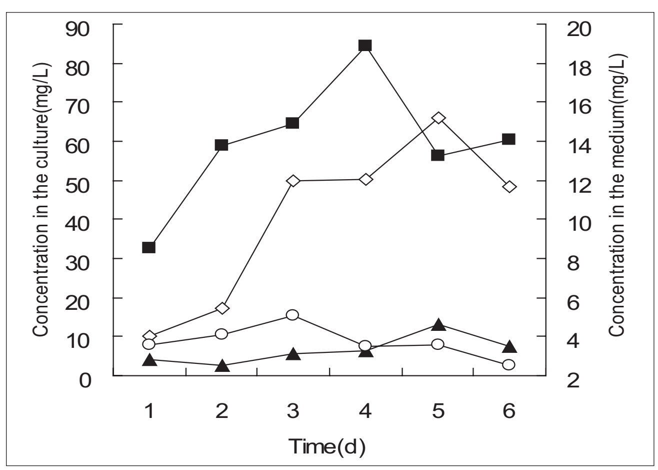
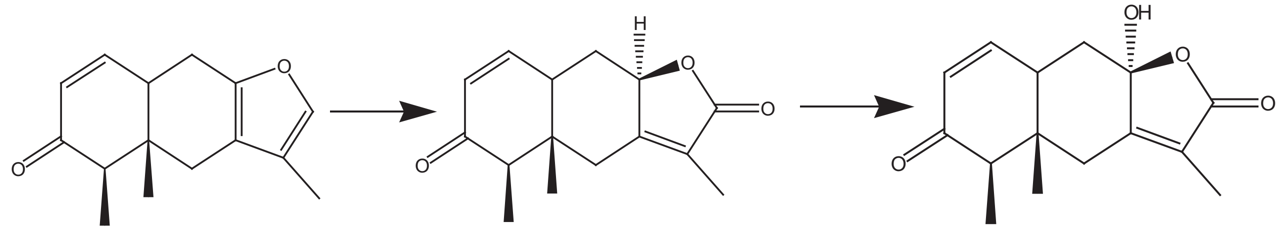


Figure 1: Time–course curve of the biotransformation of 1 in the medium and the transgenic crown galls of *P. quinquefolium*. (—■—)The yield of 2 in the culture; (—◇—) the yield of 3 in the culture; (—▲—) the yield of 2 in the medium; and (—○—) the yield of 3 in the medium



1 2 3

Figure 2: A proposal biosynthesis pathway of furannoligularenone (1)

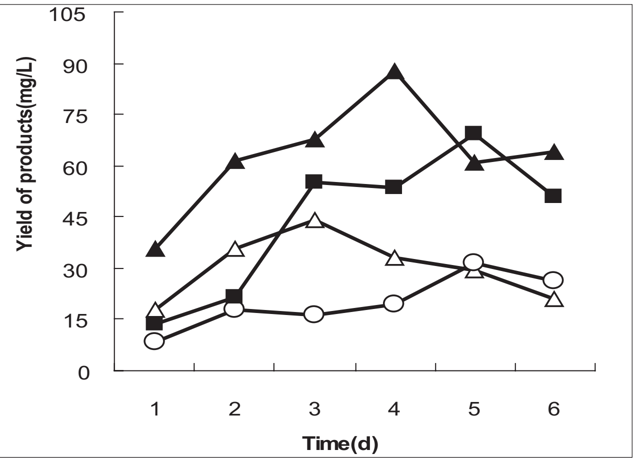


Figure 3: The comparison of time–course curve of compounds 2 and 3 from 1 in crown galls of *P. quinquefolium* and hairy roots of *P. multiflorum*. (—△—) The yield of 2 in hairy roots; (—○—) the yield of 3 in hairy roots; (—▲—) the yield of 2 in crown galls; and (—■—) the yield of 3 in crown galls

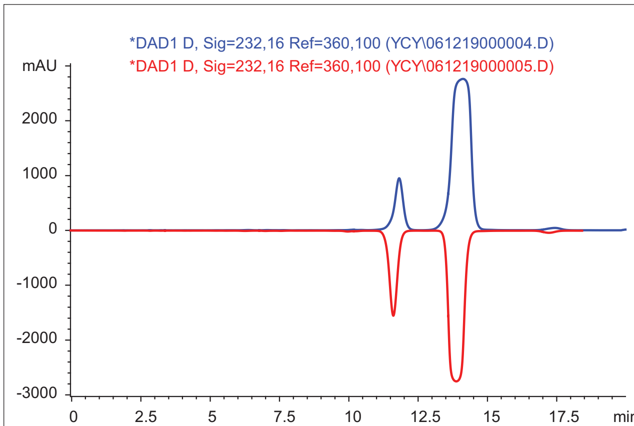


Figure 4: HPLC chromatograms of the biotransformation compound of 1 by crown galls of *P. quinquefolium* and hairy roots of *P. multiflorum*

the medium. Compared with biodegradation of substrate **1** in the two biocatalysts, we found that the content of biotransformation products of *P. quinquefolium* crown galls was relative higher than that of hairy roots of *P. multiflorum*. Thus, crown galls of *P. quinquefolium* might be the optimal biocatalyst for the bioconversion of substrate **1**.

The co-cultured time was different according to the distinct target product during the biotransformation process by crown galls of *P. quinquefolium*. Incubation for 4 days was better to produce compound **2** and 5 days in the case of compound **3** based on their conversion ratio. It was interesting that on the first day of bioconversion the excrete ratio was highest, then with the co-culture time increase it was decreased, this might be due to the feedback of the products in the medium.

In general, exogenous substrates could be toxic for plant cultures. However, biotransformation reactions, such as hydroxylation and glucosylation, were considered to be detoxification reactions. Therefore, hydroxylation offered the best opportunities for the production of more effective drugs with fewer side-effects.[20]

Enzyme-catalyzed reactions did not proceed instantly, but mildly in a stage, so the co-cultured time was the key to raise conversion ratio of metabolites. The enzymes of the cultures varied in different biotransformation system. Characterization of enzymes that catalyze the oxidation and hydroxylation of substrate **1** is now in progress.

CONCLUSION

The transgenic crown gall cultures of *P. quinquefolium* could be used as a potential biocatalyst. This system showed the same potential to produce some useful constituents by its ability of oxidation and hydroxylation of extrinsic organic compounds with *P. multiflorum* hairy roots,[17] and the conversions were region-selective reactions. The two culture systems were found to produce products (**2** and **3**) that are more polar than the parent compound (**1**).

ACKNOWLEDGMENTS

This research work was financially supported by National Natural Sciences Foundation of China (No. 81102779), Guangdong Natural Science Foundation (No. 9451022401003453) and Guangdong Medical Research Foundation of Science and Technology (No.B2011151).

REFERENCES

1. Roberts SC. Production and engineering of terpenoids in plant cell culture. Nat Chem Biol 2007;3:387-95.
2. Shimoda K, Kubota N, Taniuchi K, Sato D, Nakajima N, Hamada H, *et al*. Biotransformation of naringin and naringenin by cultured *Eucalyptus perriniana* cells. Phytochemistry 2010;71:201-5.
3. Liu JH, Yu BY. Biotransformation of bioactive natural products for pharmaceutical lead compounds. Curr Org Chem 2010;14: 1400-6.
4. Thorpe TA. History of plant tissue culture. Mol Biotechnol 2007;37:169-80.
5. Shimoda K, Harada T, Hamada H, Nakajima N, Hamada H. Biotransformation of raspberry ketone and zingerone by cultured cells of *Phytolacca americana*. Phytochemistry 2007;68:487-92.
6. Escobar MA, Dandekar AM. *Agrobacterium tumefaciens* as an agent of disease. Trends Plant Sci 2003;8:380-6.
7. Singh SB, Zink D, Polishook J, Valentino D, Shafiee A, Silverman K, *et al*. Structure and absolute stereochemistry of HIV-1 integrase inhibitor integric acid. A novel eremophilane sesquiterpenoid produced by a *Xylaria* sp. Tetrahedron Lett 1999;40:8775-9.
8. Li XQ, Gao K, Jia ZJ. Eremophilenolides and other constituents from the roots of *Ligularia sagitta*. Planta Med 2003;69:356-60.
9. Beattie KD, Waterman PG, Forster PI, Thompson DR, Leach DN. Chemical composition and cytotoxicity of oils and eremophilanes derived from various parts of *Eremophila mitchellii* Benth. (Myoporaceae). Phytochemistry 2011;72:400-8.
10. Haaften CV, Duke CC, Weerheim AM, Smit NP, Haard PM, Darroudi F, *et al*. Potent cytotoxic effects of *Calomeria amaranthoides* on ovarian cancers. J Exp Clin Cancer Res 2011;30:29-34.
11. Yu RM, Song YB, Zhang H, Ye WC, Zhang YL, Yao XS. Study on the culture of crown gall from *Panax quinquefolium* and the production of its secondary metabolites--ginsenosides Re and Rg_1. Chin J Biotechnol 2003;19:372-5.
12. Yu RM, Jin QX, Sun H, Ye WC, Zhao Y. The growth characteristics and ginsenosides isolation of suspension-cultured crown gall of *Panax quinquefolium.* Chin J Biotechnol 2005;21:754-8.
13. Yan CY, Yu RM, Zhang Z, Kong LY. Biotransformation of 4-hydroxybenzen derivatives by hairy root cultures of *Polygonum multiflorum* Thunb. J Integr Plant Biol 2007;49:207-12.
14. Bohlmann F, Ziesche J. New sesquiterpenes from *Senecio species.* Phytochemistry 1980;19:1851-3.
15. Torres P, Ayala J, Grande C, Anaya J, Grande M. Furanoeremophilane derivatives from *Senecio flavus.* Phytochemistry 1999;52:1507-13.
16. Zhang CF, Zhang M, Qu R, Wang ZT. Studies on sesquiterpenes constituents of *Ligularia pleurocaulis*. Chin J Nat Med 2004;2:341-3.
17. Yan CY, Ma WL, Yan WW, Yu RM. [Biotransformation of furannoligularenone by hairy root cultures of Polygonum multiflorum]. Zhong Yao Cai 2008;31:633-5.
18. Yu RM, Ma N, Yan CY, Zhao Y. Effects of exogenous phytohormones on hairy root growth and biosynthesis of anthraquinones in the hairy root culture of *Polygonum multiflorum*. Chin J Biotechnol 2006;22:619-23.
19. Shi XJ, Zhu JH, Yang L, Yu RM. Chemical constituents from crown gall of transgenic *Panax quinquefolium* (II). Chin Tradit Herbal Drugs 2011;42:870-3.
20. Dai JG, Ye M, Guo HZ, Zhu WH, Zhang DO, Hu Q, *et al*. Regio- and stereo-selective biotransformation of 2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene by *Ginkgo* cell suspension cultures. Tetrahedron 2002;58:5659-68.

Cite this article as: Yan C, Wang J, Duan G, Yu R. Biotransformation of furannoligularenone by transgenic crown galls of *Panax quinquefolium*. Phcog Mag 2012;8:124-8.

Source of Support: Financially supported by National Natural Sciences Foundation of China (No. 81102779), Guangdong Natural Science Foundation (No. 9451022401003453) and Guangdong Medical Research Foundation of Science and Technology (No.B2011151),
Conflict of Interest: None declared.