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Antitumor activity of bacterial exopolysaccharides from the endophyte *Bacillus amyloliquefaciens sp.* isolated from *Ophiopogon japonicus*

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Abstract. The endophytic bacterium, MD-b1, was isolated from the medicinal plant *Ophiopogon japonicas* and identified as the *Bacillus amyloliquefaciens* sp. with 99% similarity based on the partial sequence analysis of 16S rDNA. Exopolysaccharides were extracted from the endophyte for the evaluation of its antitumor activity against gastric carcinoma cell lines (MC-4 and SGC-7901). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and microscopy were performed to estimate the cell viability and morphological changes of the MC-4 and SGC-7901 cells following treatment with the exopolysaccharides at 14, 22 and 30 μg/μl. The results revealed that the exopolysaccharides displayed concentration-dependent inhibitory effects against the MC-4 and SGC-7901 cells, with an IC_{50} of 19.7 and 26.8 μg/μl, respectively. The exopolysaccharides also induced morphological abnormalities in the cells. These effects indicated the the exopolysaccharides had an antitumoral mechanism of action associated with the mitochondrial dysfunction of the treated cells. This is the first study to investigate the endophytic microorganism isolated from *O. japonicas* and also the first discovery of such antitumoral exopolysaccharides derived from the genus *Bacillus*. This provides a promising and reproducible natural product source with high therapeutic value for anticancer treatment, thereby facilitating the development of new anticancer agents.

Introduction

Despite recent advances in our knowledge of the molecular pathogenesis and targeted therapy of cancer, it remains one of the most malignant diseases threatening human health and quality of life. The World Health Organization has defined cancer as one of the top ten leading causes of mortality worldwide (1). Chemotherapy, combined with radiotherapy and surgery, is the main strategy for anticancer treatment. However, this strategy has limitations, including multidrug resistance and severe side effects in the clinical application, therefore impelling the search for new anticancer drugs with greater therapeutic efficiency or fewer side effects. Recently, natural products have gained increasing attention from a therapeutic point of view and have become the most consistently successful source of potential new drugs (2). Endophytic bacteria are one chief source of natural anticancer products, and are well-known for being producers of vast bioactive anticancer compounds, including anthracyclines, glycopeptides, aureolic acids, anthraquinones, enediynes, antimetabolites, carzinophilin and mitomycins (3-5).

Endophytic bacteria are beneficial microbes that reside in living plant tissues, mainly in the intercellular space and inside vascular tissues, without either doing harm to the host or providing any benefit to other microbial residents (6-8). The bacteria ubiquitously colonize and persist on the inner organs of plants, including the leaves, stems, seeds, tubers, fruits, ovules and, in particular, the roots, during their life-cycles (9-11). Although the interaction between these microorganisms and their respective host-plant is not, as yet, fully understood, progress has been made in the application of such bacteria as their metabolites have diverse biological functions. In total, >129 species representing >54 genera, including the *Bacillus*, *Pseudomonas* and *Agrobacterium* genera, have been isolated from agricultural plants and macrophytes (6,12,13). To date, an increasing number of bacterial endophytes have been idenitified in medicinal herbs commonly used as traditional Chinese medicines (14-16).

The plant *Ophiopogon japonicus* (Thunb) Ker-Gawl, an evergreen perennial medicinal herb, is widely distributed in South-East Asia, particularly in mainland China (Sichuan and Zhejiang provinces) (17). Its tuberous roots (known as

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Mai-dong in China) have been extensively used in traditional Chinese medicine to treat acute and chronic inflammatory diseases, as well as cardiovascular diseases, for thousands of years, as originally recorded by the 'Shennong Materia Medica' (Shen Nong Ben Cao Jing) (18-20), in the Eastern Han Dynasty of China (24-220 AD) and officially listed in the China Pharmacopeia (21). Phytochemical studies have revealed that *O. japonicus* is rich in the polysaccharides, homoisoflavonoids and saponins to which *Mai-dong's* medicinal activities are largely ascribed (22-25). Recently, the majority of studies have focused on these bioactive compounds and their therapeutic functions, while little attention has been paid to the endophytes of *O. japonicas*, with only one study concerned with the isolation of actinomycetes from this herb (26). In the majority of plants, endophytes inhabit roots more easily than the aboveground tissues (27). In this regard, *Mai-dong*, the roots of *O. japonicas*, may be an overlooked promising niche for endophytic bacteria that warrants further investigation.

In order to exploit the untapped microbial resources capable of producing useful mebabolites, we isolated a variety of endophytic fungi and bacteria in our preliminary study (28), and then discovered a Gram-positive bacteria strain with sticky colonies (Fig. 1). Further study confirmed the existence of exopolysaccharides in the colonies which might have resulted in the sticky characteristics. However, the species this strain belongs to and whether its exopolysaccharides possess useful biological activities remains unknown. Therefore, the present study was conducted to identify the endophytic bacteria strain and evaluate the bacterial polysaccharides for their antitumoral activity against gastric tumors.

Materials and methods

Collection and preparation of plant material. The dried tuberous roots of *O. japonicas* (*Mai-dong*) were collected from its trueborn cultivating area in Hangzhou (Zhejiang, China), and then identified and authenticated on the basis of its botanical characteristics. A voucher specimen (No. Md100912) was deposited at the College of Life Science, Zhejiang Chinese Medical University, Zhenjiang, China.

For surface sterilization, the fresh tuberous roots were thoroughly washed in running tap water to remove adhered epiphytes and soil debris, followed by washing them three times in sterile distilled water. Subsequent to being dried in sterile conditions, the root surfaces were sterilized by sequential immersion in 75% (v/v) ethanol for 5 min and 0.1% (v/v) mercury bichloride solution for 5 min. The sterilized roots were rinsed three times with sterile distilled water and excess surface sterilant was evaporated in a hot-air oven. To confirm the success of the surface sterilization, 100 μl aliquots of the last washing solution were plated on Luria-Bertani (LB) media; a lack of bacterial colony growth was consequently observed on the plates.

Isolation of endophytic bacteria. The root samples were sectioned into 4-6-mm slabs using a sterile scalpel and then transferred onto LB plates, followed by incubation at 25±2°C for 7-14 days to allow the growth of endophytic bacteria from the sections. The colonies were isolated and sequentially subcultured onto fresh LB plates for further purification. The purified colonies on the last LB plate were transferred into 5 ml liquid LB medium in conical flasks maintained at 37°C and agitated at 39 x g for 12 h. Subsequently, each suspension was centrifuged at 3,914 x g for 1 min and the deposits were dissolved in 1 ml TE buffer (1 M Tris-HCl, 0.5 M EDTA; pH 8.0). Following further centrifugation at 3,914 x g for 1 min, the deposits were collected as isolated endophytic bacteria and stored at -20°C prior to use.

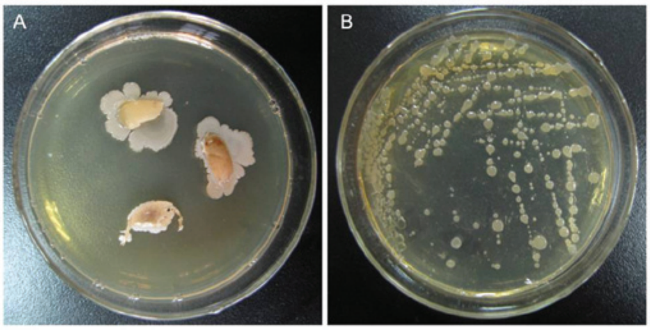

Figure 1. (A) Growth of endophytic bacteria from cut pieces of tuberous roots of *O. japonicas* (*Mai-dong*) on nutrient agar. (B) Sticky colonies of the endophytic bacteria growing throughout the agar medium.

DNA extraction and identification of endophytic bacteria. The isolated endophytic bacteria were resuspended in 200 μl TE buffer with 50 μl lysozyme and then incubated in a water bath at 37°C for 60 min with gentle agitation. A total of 10 μl proteinase K was added to the suspension, followed by incubation at 37°C for 30 min with gentle agitation. The suspension was then supplemented with 40 μl 10% SDS and incubated at 37°C for 30 min with gentle agitation. Subsequent to being mixed with 100 μl 5 M NaCl and 200 μl 3 M sodium acetate solutions, an equal volume of chloroform:isoamylol was added and then the suspension was gently shaken. Following being left to stand at room temperature for 5 min, the mixture was centrifuged at 2,609 x g for 10 min and the supernatant was transferred into precooled isopropanol and maintained for 5 min. Following centrifugation at 3,914 x g for 10 min, the deposit was washed with 1 ml 70% ethanol and dried by evaporation at room temperature. The final deposit was dissolved with 30 μl high-salt TE buffer and collected as extracted DNA for the following analysis.

The extracted DNA was electrophoresed in 1% (w/v) agarose gel, stained with ethidium bromide and UV-visualized. To determine the 16S rDNA gene sequence, PCR was conducted using the universal primers (forward, 5'-AGAGTTTGA TCCTGGCTCAG-3'; reverse, 5'-AAGGAGGTGATCCAG CCGCA-3'). PCR amplification consisted of an initial denaturation at 94°C for 5 min followed by 30 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min) and a final extension at 72°C for 8 min. Amplified DNA was purified using a Takara agarose gel DNA purification kit (Takara Biotechnology Co., Ltd., Dalian, China) and sequenced by Takara Biotechnology Co., Ltd. The 16S rDNA sequence was subjected to BLAST analysis with the NCBI database and aligned by using the multiple sequence alignment program CLUSTAL W (43). A phylogenetic analysis was performed using CLUSTAL X (44) and MEGA 4.0 (45) software, based on the neighbor-joining (46), maximum-likelihood (47) and maximum-parsimony methods (48).

Extraction and quantification of exopolysaccharides from endophytic bacteria. The isolated endophytic bacteria were incubated in liquid LB medium and agitated at 37˚C for 48 h, followed by 10 min of boiling for enzyme inactivation. Following centrifugation at 1,292 x g for 30 min, the supernatant of the bacteria suspension was decolored with active carbon in a water bath at 40˚C for 30 min, and then deproteinized using the Sevag method. Following subsequent centrifugation at 1,292 x g for 30 min, the supernatant was concentrated by evaporation and mixed with a 3-fold volume of 95% ethanol. The mixture was maintained overnight at 4˚C, then centrifuged and its deposit dissolved with double distilled water. Following the final centrifugation, the supernatant was collected as bacteria exopolysaccharides following dialysis against double distilled water for 24 h. The content of the polysaccharides was measured using a phenol-sulfuric acid colorimetric method (49), with glucose as the reference. The concentration of polysaccharides was calculated as the polysaccharide content of extraction (mg) divided by the volume of the last liquid LB medium (3,600 ml).

Tumor cell lines and culture condition. Human gastric carcinoma cell lines (MC-4 and SGC-7901) were provided by the Zhejiang Provincial Center for Disease Control and Prevention (Zhejiang CDC; Hangzhou, China). The MC-4 and SGC-7901 cells were cultured as described in our previous study (38). The cultured cells collected at the stage of logarithmic growth were detached using 0.25% trypsin and their viabilities were shown to be >98%, as revealed using the Trypan blue exclusion test. A suspension of each cell line containing $5x10^4$ cells was pipetted into a 96-well flat-bottomed plate and maintained in a humidified incubator at 37˚C with 5% CO_2 for 24 h.

Antitumor evaluation of the exopolysaccharides. Samples of the extracted bacterial polysaccharides, diluted with distilled water into three concentrations (30, 22 and 14 μg/μl), were added to each well of each cell line respectively, followed by incubation for 18 h at 37˚C with 5% CO_2. The polysaccharide-induced cell damage was morphologically observed under a Leica DMIRE2 inverted fluorescence microscope (Leica Microsystems Corp., Bensheim, Germany). The inhibitory effects of the polysaccharides on MC-4 and SGC-7901 cell proliferation were evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (38). MTT solution (10 μl/well, 5 mg/ml) was added to each well and the plate was incubated for 4 h at 37˚C. A total of 150 μl DMSO was added to replace the supernatant in each well and the plate was gently agitated for 10 min for the dissolution of formazan crystals. The absorbance of each well at 490 nm was measured by an ELISA plate reader (Wellscan MK3; Thermo Labsystems, Helsinki, Finland). The cell viability of each treated group was calculated as the percentage of the untreated control group which was assumed to be 100%. The cytotoxicity of the polysaccharides was expressed as the IC_{50} (sample concentration causing 50% inhibition of cell proliferation) and calculated by Bliss's method. Three replicates were conducted for the experiment.

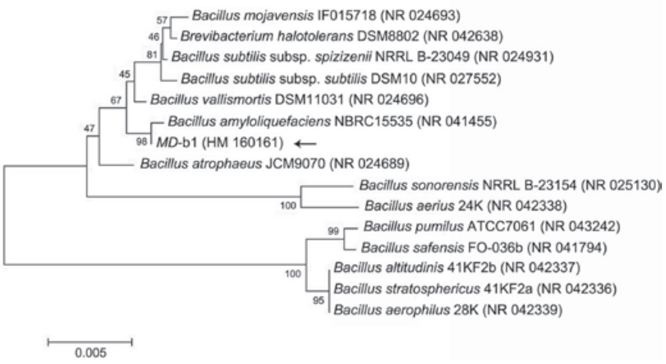

Figure 2. Phylogenetic neighbor-joining tree of the endophytic bacterium (MD-b1) of *Ophiopogon japonicas* tuberous root (*Mai-dong*) based on 16S rDNA gene sequences. The numbers above each branch point are confidence levels (%) generated from 1,000 bootstrap trees. Only the closest associated species with high similarities (≥97%) are indicated. Bar, 0.005 nucleotide substitutions per site.

Statistical analysis. All measurements are expressed as the mean ± standard deviation and were subjected to one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) comparison. $P<0.05$ and $P<0.01$ were considered to indicate statistically significant differences. All analyses were performed using DPS software (Refine Information Tech. Co., Ltd., Hangzhou, China) (50).

Results and Discussion

Isolation and identification of endophytic bacterium from Mai-dong. A Gram-positive bacterium , MD-b1, with sticky colony characteristics was isolated from the inner section of *Mai-dong* and confirmed as an endophyte when all surface microbes on the plant were killed through surface sterilization. A partial sequence of the 16S rDNA gene (1,500 bp) was identified and deposited in GenBank at NCBI (Accession no. HM 160161). BLAST analysis revealed that MD-b1 belonged to the genus *Bacillus* and demonstrated the highest similarity of 99% with the *Bacillus amyloliquefaciens* strain (Accession no. NR 041455). A neighbor-joining dentrogram was constructed for the phylogenetic analysis of this endophyte bacterium, as shown in Fig. 2.

Although numerous endophytes have been isolated from traditional Chinese medicines (29), to the best of our knowledge, the present study is the first with regard to the endophytic microorganism isolated from the *O. japonicas* tuberous root (*Mai-dong*). The finding that endophytes mostly colonize the underground plant tissues, i.e., the root interior, was expected and is in agreement with previous studies (30,31). The root endophytic bacteria from the *Bacillus* genus, including the isolated MD-b1 in the present study, are capable of producing bioactive substances (e.g., mycosubtilin, iturin and surfactin) with antifungal, antibacterial and biosurfactant properties (14,32,33). Besides their application in the areas of biocontrol and agriculture, the medicinal use of metabolites from such endophytes has gained increasing attention from a pharmaceutical perspective. The only reported medicinal effect of the endophytic *Bacillus* strains was antioxidation derived from their production of extracellular enzymes, including amylase, cellulose, pectinase and xylanase (34).

Exopolysaccharide production of MD-b1. As a major class of natural products, metabolites from microorganisms are one of the most reproducible and dependable sources for the development of 'first-in-class' drugs (35). The sticky characteristics of the MD-b1 colonies indicated that the MD-b1 were able to produce metabolites consisting mainly of polysaccharides. Therefore, in the present study, exopolysaccharides were extracted from this endophytic bacterium through fermentation. The content of the polysaccharides in the fermentation broth was 660 mg with a concentration of 0.22 mg/ml. No matter how many of the polysaccharides were biosynthesized by MD-b1, the large-scale production of such metabolites was always achievable using the conventional LB liquid media, indicating the potential scientific and commercial implications for its applications. Polysaccharides are a group of water-soluble bioactive compounds that have attracted considerable interest due to their wide spectrum of bioactivities and their low toxicity. The most common biological functions of polysaccharides are associated with immune system modulation, including antitumoral, antiviral and antioxidant activities. It is noteworthy that the host plants that generate the bioactive products have associated endophytes that are also able to produce the same natural products (36). Considering the antitumoral activity of the *Mai-dong* polysaccharides (37), the MD-b1-produced polysaccharides are thereby expected to have the same activity against tumors.

Antitumoral activity of the polysaccharides derived from MD-b1. Light microscopy was used to observe the morphological changes to the tumor cells (MC-4 and SGC-7901). As shown in Fig. 3A and B, the untreated MC-4 and SGC-7901 cells displayed a normal shape with no apoptosis, indicating the normal condition of these cells. However, following treatment with MD-b1-derived polysaccharides at increasing concentrations (14, 22 and 30 μg/μl), the MC-4 and SGC-7901 cells were found to be damaged or dead with evident cell morphological abnormalities (Fig. 3A1-3 and B1-3), indicating the apoptosis-inducing effect of the polysaccharides against gastric tumor cells. Cell apoptosis may occur with cell shrinkage or collapse, membrane blebbing, boundary splitting or aggregation and nuclei condensation or nucleus fragmentation (38). Additionally, MC-4 and SGC-7901 underwent increasing morphological changes with the increasing polysaccharides concentrations, suggesting that the apoptosis-inducing effect occurred in a dose-dependent manner. The MTT assay also revealed a result consistent with this, where the significant inhibitory effect of the MD-b1-derived polysaccharides against the proliferation of the MC-4 and SGC-7901 cells was identified. As expressed by the tumor cell viability, as well as the IC$_{50}$ estimates in Fig. 4, the polysaccharides exerted a concentration-dependent inhibitory effect against the MC-4 and SGC-7901 cells with an IC$_{50}$ of 19.7 and 26.8 μg/μl, respectively. Compared with the untreated controls, significant inhibition ($P<0.01$) of the MC-4 and SGC-7901 cells was observed at the polysaccharide concentrations of 14-30 μg/μl and 22-30 μg/μl, respectively ($P<0.01$). Together, the antitumoral activity of the MD-b1-derived polysaccharides against human gastric tumor cells *in vitro* was thereby confirmed for the first time in the present study.

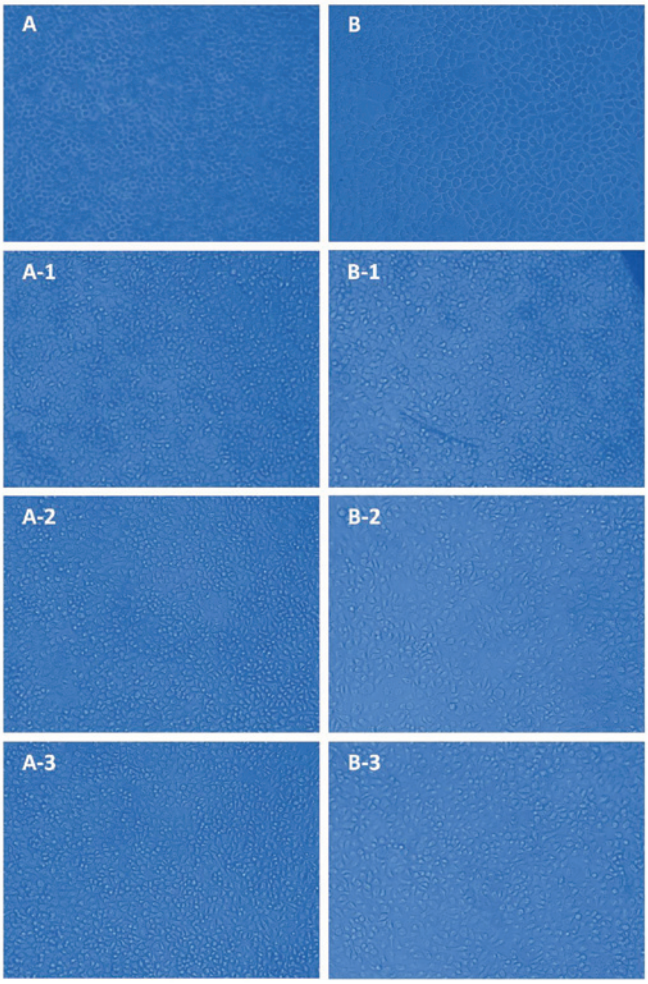

Figure 3. Effect of the endophytic bacterium (MD-b1)-derived exopolysaccharides on the morphology of MC-4 (A series) and SGC-7901 (B series) cells treated for 18 h. (A) Untreated MC-4 and (B) untreated SGC-7901 cells, 14 μg/μl polysaccharides treated MC-4 (A-1) and SGC-7901 (B-1); 22 μg/μl polysaccharides treated MC-4 (A-2) and SGC-7901 (B-2); 30 μg/μl polysaccharides treated MC-4 (A-3) and SGC-7901 (B-3).

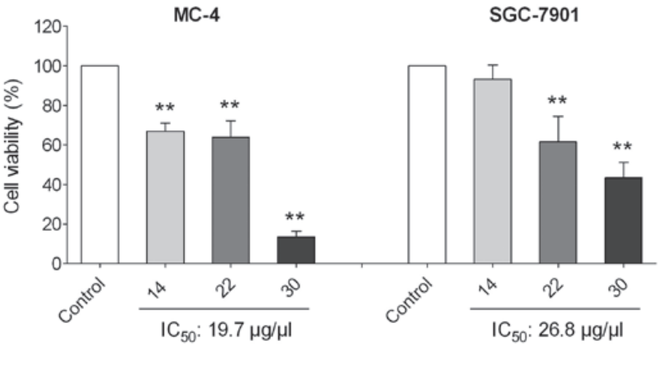

Figure 4. Cytotoxicity of the endophytic bacterium (MD-b1)-derived exopolysaccharides on the viability of the tumor cells (MC-4 and SGC-7901) determined by MTT assay. Results are expressed as a percentage of the untreated control group viability, and the IC$_{50}$ for each cell line was calculated. Values are mean ± SD. $^{*}P<0.05$ and $^{**}P<0.01$ vs. untreated control group.

In recent years, gastric carcinoma has contributed to the high mortality rate of cancer worldwide, attracting increasing attention for the development of specific agents from new sources of natural products. Cytotoxicity-based screening for compounds with antitumoral activities has been previously proven to be successful in the identification of clinically applied natural anticancer products (39). In the present study, we demonstrated the prominent *in vitro* antitumoral activity of MD-b1-derived polysaccharides based on cytotoxicity assays. This demonstrated the promising prospects of such compounds in anticancer applications. The antitumor mechanism may be associated with mitochondrial dysfunction leading to mitochondrial potential loss of the tumor cells (40,41). However, the low oral bioavailability of polysaccharides, caused by their large molecular size and hydrophilic property, has limited their therapeutic applications in the clinic. However, with the co-use of absorption enhancers, such as sodium caprate, this problem may currently be overcome (23). Further chemical analysis is required to determine whether MD-b1-derived polysaccharides contain similar components to *Mai-dong* polysaccharides, since these two differently-sourced polysaccharides exhibit similar antitumoral activities. A generally accepted theory regarding this issue has suggested that the genetic recombination of the endophytes with the host may have occurred in their evolutionary period (42), resulting in the possibility that MD-b1-derived polysaccharides and *Mai-dong* polysaccharides have the same origin. If the endophytes, including MD-b1, produce the same bioactive compounds as their host plants, the fact that the rare and important natural products may be readily available and reproducible via fermentation would be noteworthy, as it may preserve the world's ever-diminishing biodiversity by reducing the requirement for harvesting slow-growing plants. Therefore, the present study provides a promising microbial source of high-value products with significant therapeutic activities against gastric tumors, thereby facilitating the natural product identification process for new anticancer agents and benefiting anticancer therapies in practice.

Acknowledgements

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References

1. GLOBOCAN 2008 (IARC): Section of Cancer Information WHO. http://www.who.int/mediacentre/factsheets/fs297/en/. Accessed July 20, 2012.
2. Gutierrez RM, Gonzalez AM and Ramirez AM: Compounds derived from endophytes: a review of phytochemistry and pharmacology. Curr Med Chem 19: 2992-3030, 2012.
3. Igarashi Y, Trujillo ME, Martínez-Molina E, Yanase S, Miyanaga S, Obata T, Sakurai H, Saiki I, Fujita T and Furumai T: Antitumor anthraquinones from an endophytic actinomycete *Micromonospora lupine* sp, nov.. Bioorg Med Chem Lett 17: 3702-3705, 2007.
4. Taechowisan TC, Lu CH, Shen YM and Lumyong S: Antitumor activity of 4-Arylcoumarins from endophytic *Streptomyces aureofaciens* CMUAc130. J Cancer Res Ther 3: 86-91, 2007.
5. Blunt JW, Copp BR, Hu WP, Munro MH, Northcote PT and Prinsep MR: Marine natural products. Nat Prod Rep 25: 35-94, 2008.
6. Hallmann J, Quadt-Hallmann A, Mahaffee WF and Kloepper JW: Bacterial endophytes in agricultural crops. Can J Microbiol 43: 895-914, 1997.
7. Kobayashi DY and Palumbo JD: Bacterial endophytes and their effects on plants and uses in agriculture. In: Microbial Endophytes. Bacon CW and White JF Jr (eds). Marcel Dekker, NY, New York, pp.199-233, 2000.
8. Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishmaru CA, Arunakumari A, Barletta RG and Vidaver AK: Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl Environ Microbiol 68: 2198-2208, 2002.
9. Azevedo JL, Maccheroni W Jr, Pereira JO and Araújo WL: Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron J Biotechnol 3: e1-e4, 2000.
10. Sturz AV and Nowak J: Endophytic communities of Rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl Soil Ecol 15: 183-190, 2000.
11. Lodewyck C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M and van der Lelie D: Endophytic bacteria and their potential application. Crit Rev Plant Sci 86: 583-606, 2002.
12. Mundt JO and Hinkle NF: Bacteria within ovules and seeds. Appl Environ Microbiol 32: 694-698, 1976.
13. McInroy JA, and Kloepper JW: Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Can J Microbiol 41: 895-901, 1995.
14. Cho KM, Hong SY, Lee SM, Kim YH, Kahng GG, Lim YP, Kim H and Yun HD: Endophytic bacterial communities in ginseng and their antifungal activity against pathogens. Microbial Ecol 54: 341-351, 2007.
15. Tiwari R, Kalra A, Darokar MP, Chandra M, Aggarwal N, Singh AK and Khanuja SP. Endohytic bacteria from *Ocimum sanctum* and their yield enchancing capabilities. Curr Micobiol 60: 167-171, 2010.
16. Vendan RT, Yu YJ, Lee SH and Rhee YH: Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. J Microbiol 48: 559-565, 2010.
17. Yu BY and Xu GJ: Studies on resource utilization of Chinese drug Dwarf lilyturf (*Ophiopogon japonicus*). J Chin Herbs 26: 205-210, 1995.
18. Xiao PG (ed): Modern Chinese Materia Medica. Chemical Indestry Press, Beijing, pp.77, 2002.
19. Kou J, Yu B and Xu Q: Inhibitory effects of ethanol extract from *Radix Ophiopogon japonicus* on venous thrombosis linked with its endothelium-protective and anti-adhesive activities. Vascul Pharmacol 43: 157-163, 2005.
20. Zhou YF, Qi J, Zhu DN and Yu BY: Two new steroidal glycosides from *Ophiopogon japonicus*. Chin Chem Lett 19: 1086-1088, 2008.
21. China Pharmacopoeia Committee: Pharmacopoeia of the People's Republic of China. China Chemical Industry Press, volume I, Beijing, China, p64, 2010.
22. Kou J, Sun Y, Lin Y, Cheng Z, Zheng W, Yu B and Xu Q: Anti-inflammatory activities of aqueous extract from *Radix Ophiopogon japonicus* and its two constituents. Biol Pharm Bull 28: 1234-1238, 2005.
23. Lin X, Xu DS, Feng Y, Li SM, Lu ZL and Shen L: Release-controlling absorption enhancement of enterally administered *Ophiopogon japonicus* polysaccharide by sodium caprate in rats. J Pharm Sci 95: 2534-2542, 2006.
24. Li N, Zhang JY, Zeng KW, Zhang L, Che YY and Tu PF: Anti-inflammatory homoisoflavonoids from the tuberous roots of *Ophiopogon japonicus*. Fitoterapia 83: 1042-1045, 2012.
25. Wang LY, Wang Y, Xu DS, Ruan KF and Wang S: MDG-1, a polysaccharide from *Ophiopogon japonicas* exerts hypoglycemic effects through the PI3K/Akt pathway in a diabetic KKAy mouse model. J Ethnopharmacol 143: 347-354, 2012.
26. Koyama R, Matsumoto A, Inahashi Y, Ōmura S and Takahashi Y: Isolation of actinomycetes from the root of the plant, *Ophiopogon japonicus*, and proposal of two new species, *Actinoallomurus liliacearum* sp. nov. and *Actinoallomurus vinaceus* sp. nov.. J Antibiot (Tokyo) 65: 335-340, 2012.
27. Rosenblueth M and Martinez-Romero E: *Rhizobium etli* maize populations and their competitiveness for root colonization. Arch Microbiol 181: 337-344, 2004.
28. Chen YT, Ding LX, Cheng DQ, Ding ZS, Lin MA and Pan PL: Isolation and identification of endofungi from *Liriope spicata*. J Laiyang Agri Col (Nat Sci) 23: 13-16, 2006 (In Chinese).
29. Miller KI, Qing C, Sze DM and Neilan BA: Investigation of the biosynthetic potential of endophytes in traditional Chinese anticancer herbs. PLoS One 7: e35953, 2012.
30. Rosenblueth M and Martínez-Romero E: Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact 19: 827-837, 2006.

31. Compant S, Mitter B, Colli-Mull JG, Gangl H and Sessitsch A: Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microbial Ecol 62: 188-197, 2011.
32. Bonmatin J, Laprévote O and Peypoux F: Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Comb Chem High Throughput Screen 6: 541-556, 2003.
33. Snook ME, Mitchell T, Hinton DM and Bacon CW: Isolation and characterization of leu7-surfactin from the endophytic bacterium *Bacillus mojavensis* RRC 101, a biocontrol agent for *Fusarium verticillioides*. J Agric Food Chem 57: 4287-4292, 2009.
34. Krishnan P, Bhat R, Kush A and Ravikumar P: Isolation and functional characterization of bacterial endophytes from *Carica papaya* fruits. J Appl Microbiol 113: 308-317, 2012.
35. Newman DJ and Cragg GM: Natural products as sources of new drugs over the last 25 years. J Nat Prod 70: 461-477, 2007.
36. Strobel G, Daisy B, Castillo U and Harper J: Natural products from endophytic microorganisms. J Nat Prod 67: 257-268, 2004.
37. Yang MP, Wu H, Yin L, Zhang X and Duan JA: Advances in research of saponins and polysaccharides in *Ophiopogonis japonicus*. Chin Arch Trad Chin Med 10: 2169-2171, 2008.
38. Chen YT, Lu QY, Lin MA, Cheng DQ, Ding ZS and Shan LT: A PVP-extract fungal protein of *Omphalia lapideacens* and its antitumor activity on human gastric tumors and normal cells. Oncol Rep 26: 1519-1526, 2011.
39. Xia M, Huang R, Witt KL, Southall N, Fostel J, Cho MH, Jadhav A, Smith CS, Inglese J, Portier CJ, Tice RR and Austin CP: Compound cytotoxicity profiling using quantitative high-throughput screening. Environ Health Perspect 116: 284-291, 2008.
40. Chiu TH, Lai WW, Hsia TC, Yang JS, Lai TY, Wu PP, Ma CY, Yeh CC, Ho CC, Lu HF, Wood WG and Chung JG: Aloe-emodin induces cell death through S-phase arrest and caspase-dependent pathways in human tongue squamous cancer SCC-4 cells. Anticancer Res 29: 4503-4511, 2009.
41. Zheng JY, Tao LY, Liang YJ, Chen LM, Mi YJ, Zheng LS, Wang F, She ZG, Lin YC, To KK and Fu LW: Anthracenedione derivatives as anticancer agents isolated from secondary metabolites of the mangrove endophytic fungi. Mar Drugs 8: 1469-1481, 2010.
42. Tan RX and Zou WX: Endophytes: a rich source of functional metabolites. Nat Prod Rep 18: 448-459, 2001.
43. Thompson JD, Higgins DG and Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680, 1994.
44. Thompson JD, Gibson TJ, PleWniak F, Jeanmougin F and Higgins DG: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882, 1997.
45. Tamura K, Dudley J, Nei M and Kumar S: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599, 2007.
46. Saitou N and Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425, 1987.
47. Felsenstein J: Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368-376, 1981.
48. Fitch WM: Toward defining the course of evolution: minimum change for a specific tree topology. Syst Zool 20: 406-416, 1971.
49. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S and Lee YC: Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339: 69-72, 2005.
50. Tang QY and Feng MG (eds): DPS Data Processing System: Experimental Design, Statistical Analysis, and Data Mining. Science Press, Beijing, pp.146-164, 2007.