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Characterization of chemical components in extracts from *Si-wu* decoction with proliferation-promoting effects on rat mesenchymal stem cells

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Abstract—*Si-wu* decoction (SDE), a classic prescription in Traditional Chinese Medicine, has been used for the treatment of a variety of anaemia in China for centuries. In order to explore the scientific basis of the formula, we investigated the relationship between its chemical components and proliferation-promoting effects on rat marrow-derived mesenchymal stem cells (MSCs). Twenty (F-1–F-20) components were obtained and their proliferation-promoting effects on MSCs were investigated. The results showed that F-4, F-7, F-10, and F-11 stimulated the proliferation of the MSCs. The chemical components with proliferation-promoting effects on the MSCs were further identified by GC–MS, HPLC, LC–MS, and other spectra. Ligustilide (F-4) isolated from SDE showed the best proliferation-promoting effect. Palmitic acid methyl ester and stearic acid ethyl identified from F-7 and F-10 by HPLC were also confirmed to be responsible for stimulating MSC proliferation. A novel compound, 6,7-dihydroxy-3-(-prop-1-enyl)isobenzofuran-1(3H)-one, was found in the SDE for the first time by LC–MS^n, whose structure was similar to ligustilide.

1. Introduction

The proliferative and differentiative effects of organic small molecules have drawn a prevalence attention. Coowar et al. synthesized indole fatty alcohols and reported their effects on the differentiation of neural stem cell.[1] Wu et al. reported that the organic small molecules had proliferative effects on embryonic stem cells.[2] The small organic molecules with promoting proliferation and differentiation of mesenchymal stem cells (MSCs) are potentially important for the development of the tissue engineer and gene therapy.[3]

Traditional Chinese Medicine (TCM) is a unique medical system, which is basically different from western medicines for the treatment of many diseases in Mainland China for centuries. It has attracted great interest that many TCM formulas are able to improve the proliferation and differentiation of stem cells.[4–11] The TCM formulas contain many small organic molecules with bioactivities. For example, in our recent study, a series of 8-hydroxyquinoline derivatives showed the ability to regulate the proliferation of MSCs.[12]

Si-wu decoction (SDE) is one of the famous ancient tonic prescriptions in TCM, which is comprised of four herbs, viz. *Rhizoma chuanxiong*, *Radix angelicae sinensis*, *Radix paeoniae alba*, and *Radix rehmanniae praeparata*. The formula has been used for the treatment of a variety of anaemia.[13] Previous studies indicated that *Si-wu* decoction could improve the RBC immunoadhesive function and stimulate the ability of stem cell multiplication in the irradiated mice .[14] *Si-wu* decoction can also stimulate the proliferation of stem cells in the human bone marrow stromal cells.[15] It attracts our great interests to investigate the chemical components of this

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preparation with the potential proliferation-promoting effects of stem cells.

The major function of MSCs is to assist the microenvironment for substance and differentiation of hematopoietic stem cells with the function of differentiation into multiple cell lines. MSCs differentiate into specialized tissues including cardiomyocytes, neurons, and glial cells, etc.[16–22] MSC transplantation has emerged as a potential restorative therapy for cardiovascular diseases,[17,18] spinal injury[23] and central nerve system disorders.[24] As the growth of MSCs in bone marrow is unable to replace the damaged cells sufficiently, the drug discovery targeted on the MSCs could bring great hope in the treatment of those diseases. One of the important aspects in the drug discovery is to find out the potential chemical substances in TCM formulas which have been clinically demonstrated to be effective.

In this paper, silica gel column chromatography was used to separate the extraction from *Si-wu* decoction. The chromatographic peaks and spectral information obtained by GC–MS and HPLC were compared and analyzed. The effects of the components isolated from *Si-wu* decoction on the proliferation of MSCs were evaluated and the results revealed that fatty acid esters were effective for stimulation of the cell proliferation of MSCs, potentially being used for stem cell replacement therapy.

2. Summary

Si-wu decoction extraction (SDE) was isolated by silica chromatography, and 20 samples were obtained. The experimental results suggest that F-4, F-7, F-10, and F-11 might be the bioactive components with the promoting proliferation effects on MSCs. The chemical components of *Si-wu* decoction with proliferation-promoting effects on rMSCs were investigated by GC–MS, HPLC, LC–MS, and other spectra.

Ligustilide (F-4) showed the best proliferation-promoting effect on MSCs. Palmitic acid methyl ester and stearic acid ethyl ester identified from F-7 and F-10 by HPLC and standard materials were also responsible for stimulating MSC proliferation. 6,7-dihydroxy-3-(-prop-1-enyl) isobenzofuran-1(3*H*)-one, identified for the first time by MS^n, whose structure was similar to ligustilide. We found that 6,7-dihydroxy-3-(-prop-1-enyl)isobenzofuran-1 (3*H*)-one was the main constituent in F-11, whose relative peak area was 84.47% in HPLC.

Nevertheless, we should say that seeking for the chemical basis of herbal medicine is not an easy job. First, analyzing such complex systems containing hundreds of chemical components is indeed a challenge. Second, in contrast to western medicine, the fundamental pharmacological activity of TCM is of synergetic effect based on the multiple target drug system that has different pathways and delivery carriers in a cell on those molecules. Third, still much less is known about the mechanisms of interaction of reactive metabolites with cellular molecules and how it leads to tissue transformation. Therefore, our study can only provide very limited information about the chemical basis of *Si-wu* decoction for the treatment of diseases. It is necessary to further explore the synergic actions of those active components.

3. Results and discussion

3.1. Chemical fractions and proliferation-promoting effects of *Si-wu* decoction extraction (SDE)

Traditional silica column chromatography was used to isolate the chemical constituents from the SDE, which was dissolved in ethyl acetate/chloroform (1:4), gradient-eluted by ethyl acetate and petroleum ether, and 20 samples were obtained, marked with F-1–F-20. Chemical identification shows that F-4 is a single compound isolated from SDE by gradient elution. Others are mixture compounds. Then, GC–MS and HPLC were employed to investigate the components in the fractions. MTT assay was used to evaluate the proliferation-promoting functions of the fractions on rMSCs. The results revealed that F-3, F-4, F-5, F-7, F-9, F-11, F-12, F-13, F-19, and F-20 could stimulate the proliferation of MSCs as shown in Figure 1.

We next compared the proliferation-promoting effects of the fractions extracted from *Si-wu* decoction on MSCs. As shown in Figure 2, there were dose-dependent responses of F3, F4, F-7, and F-9 on MSC proliferation. Among the fractions, F-11 showed the highest optical densities of MSCs. Other fractions had no significant effects on the proliferation of MSCs. The effective doses of SDE were between 0.01 and 3 mg/ml. We used basic fibroblast growth factor (bFGF) as a positive control. SDE at 0.3 mg/ml was equivalent to the effects of bFGF. The results suggest that SDE has the proliferation-promoting effects on MSCs. In addition, we found that F-2 has a dose-dependent inhibitory effect on the proliferation of MSCs.

3.2. Identification of chemical components in F-4, F-11, F-7, and F-10

As F-4, F-11, F-7, and F-10 showed the proliferation-promoting effects on the rMSCs, we specifically selected four samples for further chemical identification. In the fractions, F-4 was identified to be a single pure compound, in mass spectrum and IR spectrum. F-7 and F-10 and F-11 were found to be mixed chemicals which were further analyzed with HPLC–MS.

3.2.1. Chemical identification of F-4 and evaluation of its proliferation-promoting activity. Sample F-4 was obtained by silica gel column chromatography with ethyl acetate/petroleum ether (99:1, v/v) as eluant, and exhibited one spot at R_f of 0.56 on silica gel TLC. The HPLC chromatography showed a single peak at rt of 2.099 min. EI-MS (*m*/*z*)%: 190.03 [M^+], 161.01(100.00), 148.11(95.20), 133.24(24.09), 105.03(65.70), 77.01(25.11), 54.97(36.36). IR (cm^{-1}): 3049.34(=C–H), 1764.66 (lactone), 1671.13 (–C=C–C=C–), 1269.50, 961.53 (–C=C–). The spectral data were consistent with the standard IR spectrum and mass spectrum of ligusti-

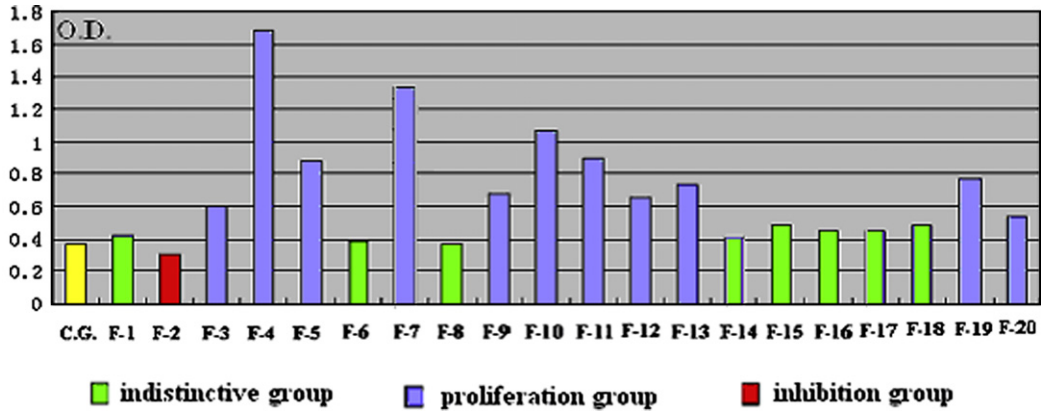


Figure 1. Effects of 20 samples on the proliferation of rMSCs by MTT assay.

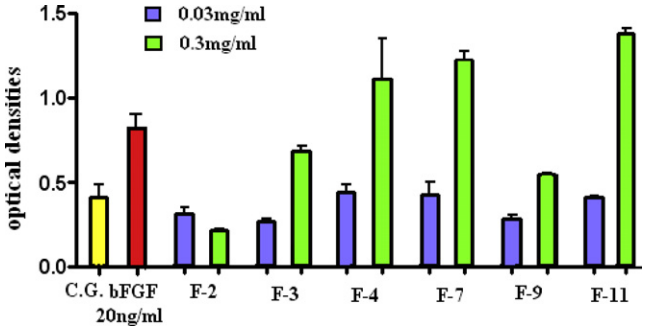


Figure 2. Effect of different doses of SDE on MSC proliferation.

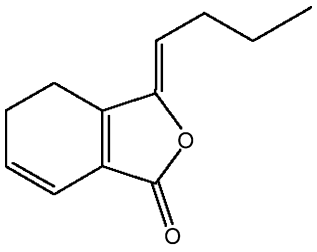


Figure 3. Structure of ligustilide.

lide.[23] Therefore, F-4 was conformed to be ligustilide (as shown in Fig. 3).

To verify the bioactivity of F-4, we conducted cell cycling analysis with flow cytometry. The rates of S-phase population were used as proliferation index (PI). As shown in Figure 4, F-4 treated group had a significantly higher value of PI than the control group, suggesting that F4, identified as ligustilide, has the proliferation-promoting effect on the MSCs.

3.2.2. Chemical identification of F-11. Since F-11 was not a pure compound as identified by HPLC chromatography, we used LC–MS to further compare and analyze the chromatographic peaks and spectral information. The relative peak area at rt of 2.79 min was about 84.47%, referring to the main constituent of F-11. The results of MS^n proved that the chemical was a lactone whose structure is similar to ligustilide (F-4). This compound was identified with MS^n for the first time (Table 1), as shown in Figure 5. Since F-11 is ligustilide derivative, we did not further evaluate its proliferation-promoting effect on the MSCs with flow cytometry.

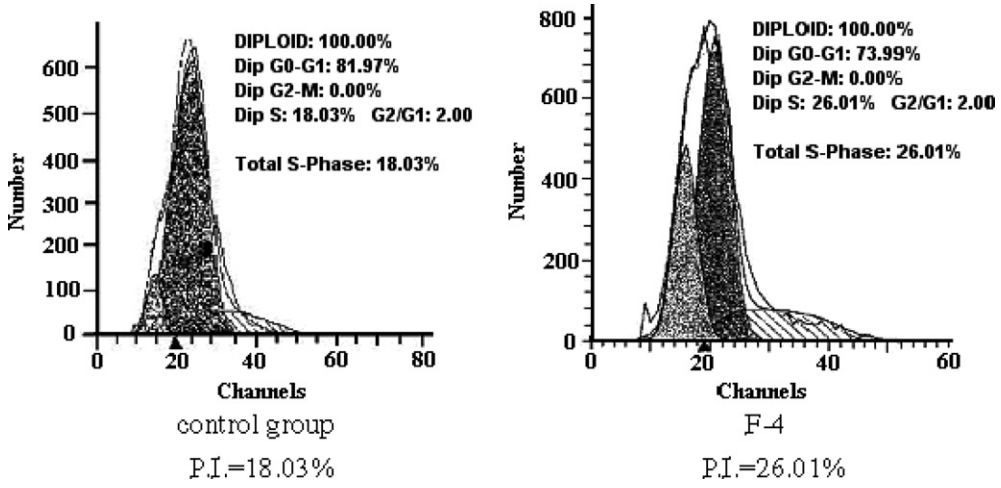


Figure 4. MSCs activity of sample F-4 by flow cytometry.

Table 1. Mass parameter

Sheath gas FR (arb)	Aux gas FR (arb)	I spray voltage (kv)	Capillary temperature (°C)	Capillary voltage (kV)	Tube lens offsets (v)
60	20	2.5	280	4.5	35.0

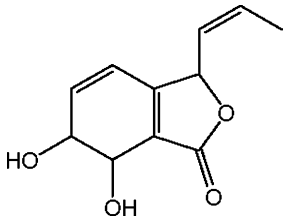


Figure 5. Structure of 6,7-dihydroxy-3-(-prop-1-enyl)isobenzofuran-1(3*H*)-one.

3.2.3. Chemical analysis of F-7 and F-10 and evaluation of their proliferation-promoting activities. By using GC–MS, we analyzed the chromatographic peaks and spectral information and found that four fatty acid esters were presented in F-7 and F-11. We then used four standard fatty acid esters named palmitic methyl ester (S-1), palmitic ethyl ester (S-2), stearic methyl ester (S-3), and stearic ethyl ester (S-4) for HPLC study. The results showed that S-1, S-2, S-3, and S-4 were indeed present in the fractions F-7 and F-10. The percentage of fatty acid esters in F-7 and F-10 is shown in Table 2. The content of stearic ethyl ester (S-4) in F-7 was higher than that of F-10.

In order to verify the proliferation-promoting activities of the fatty acid esters, we investigated the effects of the standard materials on cell cycling in the MSCs with flow cytometry. As shown in Figure 6 and Table 3, treatment of S1, S-2, S-3, and S-4 (90 μg/ml each) significantly increased the population rates of S-phase cells, showing that the P.I. values of S-1 S-2, S-3, and S-4 were 11.69 %, 6.83%, 7.76%, and 11.73%, respectively, whereas that of control groups was 9.32%. Among them, S-4 group (11.73%) had the highest PI values. The results suggest that S-1 and S-4 could stimulate the proliferation of MSCs. It is interesting that S-2 group (6.83%) and S-3 group (7.76%) showed lower PI values than the control group (9.32%), indicating that S-2 and S-3 have proliferation-inhibiting effects. The results suggest that the different chemical components in the SDE might have synergic effects which not only promote the proliferation of mesenchymal stem cells but also prevent the over growth of the MSCs.

4. Experimental

4.1. Materials for chemical analysis

The raw materials of *R. chuanxiong*, *R. angelicae sinensis*, *R. paeoniae alba*, and *R. rehmanniae praeparata* were purchased from Caizhilin pharmaceutical store in Guangzhou, PR China. Ethyl acetate, petroleum ether, and dichloromethane were of analytical grade. Column chromatography was performed with silica gel (200–300 mesh) and TLC was carried out on precoated silica plates. Four standard materials including palmitic acid methyl ester (S-1), palmitic acid ethyl ester (S-2), stearic acid methyl ester (S-3), stearic acid, and ethyl ester (S-4) were purchased from Tokyo, Chemical Industry, Co., Ltd.

Table 2. The percentage of fatty acid esters in F-7 and F-10

	F-7%	F-10%
S-1	3.8	14.46
S-2	4.9	17.47
S-3	—	3.19
S-4	12.0	1.03

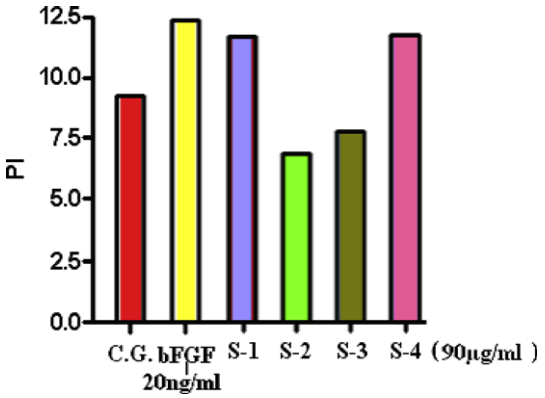


Figure 6. Effects of S-1–S-4 on the PI of rMSCs by cell cycle analysis.

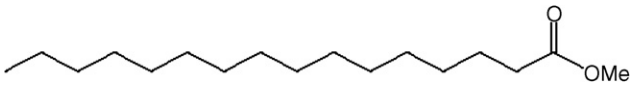
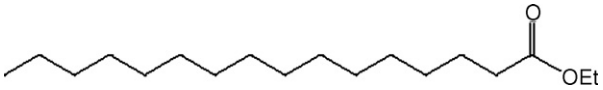
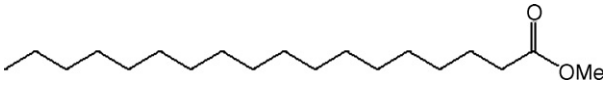
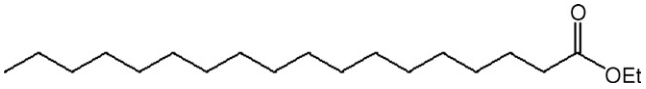
4.2. Separation by column chromatography

The ointment extracted from *Si-wu* decoction was dissolved in ethyl acetate/chloroform(1:4), gradient eluted by ethyl acetate and petroleum ether, and 20 samples marked with F-1–F-20 were obtained. The samples were used for the bioactivity assay and further identification of the chemical components by GC–MS and HPLC.

4.3. GC–MS system

GC–MS analysis was performed on a Finnigan Trace gas chromatograph equipped with a mass-selective detector. A DB-5 MS fused-silica capillary column (30 m × 0.25 mm id) was employed for separation. Electron impact mass spectra were recorded at 70 eV. Ultra-high purity (99.999%) helium was used as the carrier gas at a flow-rate of 1 ml/min. The injector and detector temperatures were set at 280 °C. The injection volume was 10 μl and all the injections were performed in a split ratio of 1:10. The column temperature was 60 °C for 0 min increasing to 280 °C in increments of 8 °C/min and remaining steady at 280 °C for 20 min. Data analy-

Table 3. Statistical data of four standard materials by flow cytometry

	G0/G1(%)	S-phase	Name	Structure
Control group	90.68	9.32	/	/
bFGF (20 ng/ml)	87.56	12.44	/	/
S-1	88.31	11.69	Palmitic acid methyl ester	
S-2	93.17	6.83	Ethyl palmitate	
S-3	92. 24	7.76	Methyl stearate	
S-4	88.27	11.73	Stearic acid ethyl ester	

ses were performed and controlled by an Xcalibur software in a personal computer.

4.4. HPLC system

The HPLC was performed on a Supelco RP C-18 (15 cm × 4.6 mm) reverse-phase analytical column with a mixture of acetonitrile/water (v/v = 80:20 at 0 min, then gradient change to 100% acetonitrile in 2 min) as the mobile phase at a flow-rate of 1.0 ml/min, and a UV detector set at 210 nm.

4.5. LC–MS^n system

LC–MS was performed on a Finnigen LCQ deca XP Max Surveyor.

The liquid chromatography analysis was performed on a Supelco RP C-18 (15 cm × 4.6 mm) analytical column with methanol and water as the mobile phase (v/v = 73:27) at the rate of 1.0 ml/min, and a PDA detector at 275 nm.

Ion source: ESI (cation mode).

4.6. Materials for biological studies

Sprague–Dawley rats at 4 weeks old were obtained from the animal centre of Guangzhou University of Traditional Chinese Medicine. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA); CD44 was purchased from Wuhan Boster Biological Technology Co., Ltd (Wuhan, China); other chemicals were obtained from Sigma (St. Louis, MO, USA).

4.7. Culture of MSCs

Bone marrow was obtained from the femur and tibia of rat. The marrow samples were diluted with DMEM (LG: low glucose) containing 10% FBS. MSCs were prepared by gradient centrifugation at 900*g* for 30 min on Percoll of a density of 1.073 g/ml. The cells were washed, counted, and plated at 1×10^6/cm^2 on Petri dishes in DMEM–LG supplemented with 10% FBS. Medium was replaced and the unattached cells were removed every 3 days. MSCs formed as confluent layers were detached by treatment with 0.25% of trypsin and transferred into cultural flasks at 1×10^4/cm^2. MSCs at passage 3 were evaluated for cultured cell homogeneity using detection of CD44 by flow cytometry and were used for the described experiments. This method has been described previously.[22,25]

4.8. MTT assay

Twenty samples isolated by silica column chromatography from SDE were dissolved in DMSO. The experiment groups included blank control group (culture medium and cells), solvent group (DMSO and cells), sample group (samples dissolved in DMSO and cells), and the group without cells. The effects on the proliferation of rMSCs were measured by MTT assay as previously described.[4,25] NADP related dehydrogenase existing in mitochondria could reduce yellow MTT to insoluble formazan. Formazan was dissolved in DMSO, and the levels of absorbance were then detected by using Bio-kinetics Reader. The rMSC subculture for five generations was cultured with complete culture medium in 96-well culture plate at the density 1×10^8/L. After 72 h of incubation, MTT (5 mg/ml Sigma) was added and incubated at 37 °C for 4 h. The supernatant was then eliminated carefully before adding 150 μl of dimethylsulfoxide and vibrated for 10 min. The values of absorbance (*A*) at 490 nm were detected by Bio-kinetics Reader (PE-1420, USA).

4.9. Flow cytometry

Cell cycling analysis was conducted with flow cytometry. MSCs were sealed in six-well plates followed by stimulation in DMEM supplemented with 10% FBS for 1 day. After being washed twice with PBS, trypsinized, and fixed in methanol, the cells were precipitated (5 min of centrifugation at 500*g*) and re-suspended in 1 ml PBS containing 40 μg of RNase A per ml and 40 μg of propidium iodide (PI) per ml. After incubation for 30 min at 37 °C, flow cytometric analysis was performed with an

EPICS XLMCL flow cytometry (Coulter); then the proliferation index (PI) was detected with the previous method.[26,27]

$$PI = (S + G2/m)/(G0/G1 + S + G2/m) \times 100\%.$$

4.10. Statistical analysis

The results were presented as $\overline{X} \pm SD$ and analyzed statistically using one-way analysis of variance as well as Student's *t*-test. The statistical analyses were performed with SPSS software.

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