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Stachyose: One of the Active Fibroblast-proliferating Components in the Root of Rehmanniae Radix (地黃 dì huáng)

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Abstract

This study aimed to investigate and compare the fibroblast-proliferating activities of different Rehmanniae Radix (RR) samples and its chemical components using human normal fibroblast cells Hs27. Those active components were quantified in differently treated RR samples using UPLC so as to correlate activity with component content. Our results showed that dried RR aqueous extract exhibited the most potent fibroblast-proliferating activity. Stronger effect was observed when ethanol with heating was applied in the extraction process. Stachyose and verbascoside were demonstrated for their first time to exhibit significant stimulatory effects on fibroblast proliferation. However, the proliferating effect of dried RR extract did not correlate with the stachyose content, and verbascoside was not responsible for the fibroblast proliferative effect of RR since it was undetectable in all samples. In conclusion, stachyose only contributed in part to the activity of RR, suggesting that other active components might be present and yet to be found.

Key words: Rehmanniae Radix, *Rehmannia glutinosa*, Stachyose, Verbascoside, Fibroblast proliferation

Introduction

Complications of diabetes mellitus (DM) has been by far the global burden affecting not only individuals but also global economic. There are currently 346 million people worldwide suffering from diabetes (World Health Organization, 2011), of which 15% develop diabetic foot ulcers; and 60% of non-traumatic lower-limb amputations occur in people with diabetes (Macdonald & Geyer, 2010). Diabetic foot ulceration is caused by combination of peripheral neuropathy, ischemia and infection (Kalish & Hamdan, 2010; Levin, 1995), and the risk of limb amputation in diabetic patients is 15 – 46 times higher than in normal person, resulting in much higher rate of hospital admission versus other diabetes complications (Armstrong & Lavery, 1998).

Rehmanniae Radix (地黃 dì huáng; the root of *Rehmannia glutinosa* Libosch) (family Scrophulariaceae), has been widely used in traditional Chinese medicines (TCM) such as 'pills of six drugs with Rehmannia' for DM treatment (Liu & Lauda, 2002). Our recent studies showed that a RR-containing TCM formula could reduce limb amputation rate in diabetic patients by 85%

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(Leung et al., 2008; Wong et al., 2001). Subsequent investigations of the formula showed that RR alone could significantly improve viability of fibroblast cell line CRL-7522 and primary culture of fibroblast from diabetic patients (Lau et al., 2007 and 2009). Fibroblasts are the major cell type found in the granulation of wound tissues and they play an essential role in wound healing including secretion of a series of growth factors that facilitate angiogenesis, proliferation and matrix deposition (Mansbridge et al., 1999; Park et al., 2005). Fibroblast proliferation has been adopted as an indicator for the assessment of wound healing (Adolphe et al., 1984; Bucalo et al., 1993; Park et al., 2005). Besides, *in vivo* investigations on the wound healing effect using animal model with foot ulcer showed that 7 days of RR treatment could significantly reduce wound area when compared with control (Lau et al., 2008).

The form of RR in traditional Chinese medicine can be fresh, dried or steamed with rice wine (State Pharmacopoeia Commission of People's Republic of China, 2005). The chemical profile of RR changes extensively during processing (Kubo et al., 1996; Wang et al., 2009) and thus different commodities of RR can be used in different clinical applications. For example, fresh root can be used for nose bleeding, sore throat, rashes and skin eruptions; dried root is used in the treatment of hematemesis, nose bleeding, rash and diabetes; while steamed root is used for the treatment of anemia, diabetes, dizziness, tinnitus, nocturnal emission and palpitations (Jiangsu Xinyiyuan, 1979). Although different kinds of RR have been widely used and some components in their chemical profiles have been successfully purified and identified as iridoids glycosides (Nishimura et al., 1989), ionone glycosides (Yoshikawa et al., 1986) and furfural derivatives (Lin et al., 2008), the active components for promoting wound healing have not yet been identified.

Five commonly found and commercially available chemical components (Figure 1) in RR were chosen in this study. According to the Chinese Pharmacopoeia (CP) 2005 (State Pharmacopoeia Commission of People's Republic of China, 2005), catalpol and 5-hydroxymethyl-2-furaldehyde (5-HMF) are regarded as the chemical markers in dried and steamed RR, respectively. However, in CP 2010 (State Pharmacopoeia Commission of People's Republic of China, 2010), verbascoside is also chosen as a chemical marker of dried/ steamed RR and the regulatory limit is not less than 0.02% w/w. On the other hand, the major chemical constituents in RR are carbohydrates which

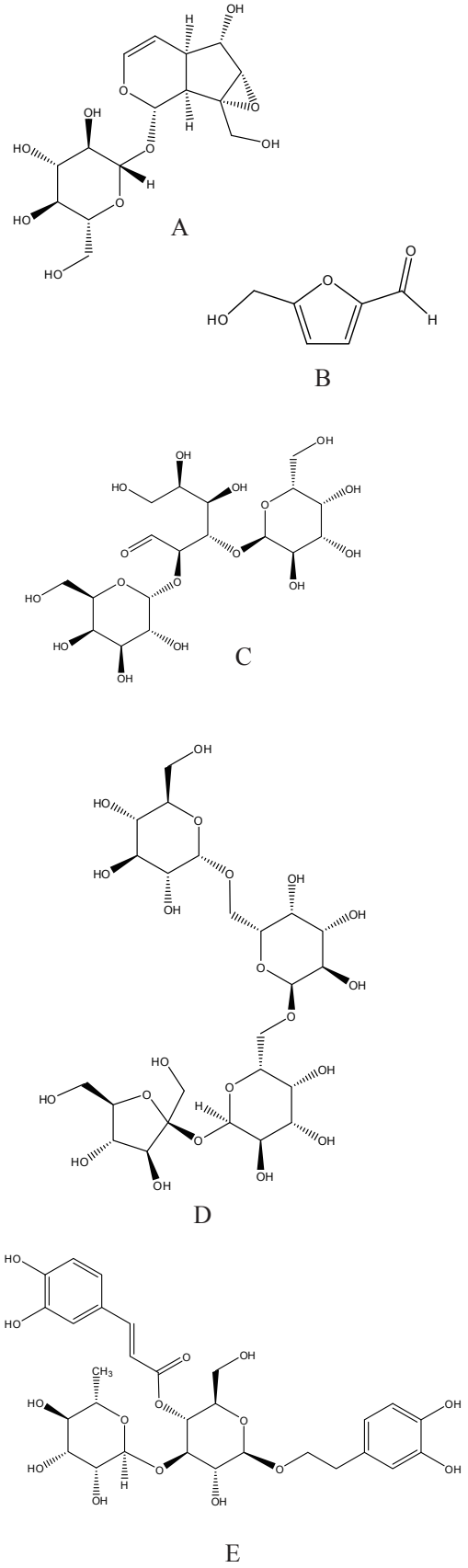

Figure 1. Chemical structures of five known chemical components in RR. A: catalpol; B: 5-hydroxymethyl-2-furaldehyde; C: manninotriose; D: stachyose; E: verbascoside.

constitutes up to 70% w/w of content and are mainly in oligosaccharides form (Tang et al., 2008). In fact, stachyose is the major oligosaccharide in fresh RR and its content (48.3% w/w) is the highest when compared with dried and steamed RR (Hong et al., 2009) whereas manninotriose is the major oligosaccharide found in steamed RR (Guo et al., 2007).

The objectives of this study were: i) to investigate and compare the fibroblast-proliferating activities of three different forms of RR sample (fresh, dried or steamed); ii) to further compare its activity by using four different treatments of dried RR sample (water or ethanol extraction accompanied with or without heating); iii) to evaluate the fibroblast-proliferating activities of five commonly found RR chemical components (catalpol, 5-hydroxymethyl-2-furaldehyde, stachyose, manninotriose, verbascoside); iv) to quantify the amounts of those bioactive components in the four differently treated samples and try to correlate their fibroblast-proliferating effects to the amounts of bioactive components presented. In this study, three different forms of RR (fresh, dried and steamed) aqueous crude extracts were tested for their *in vitro* proliferative effects on Hs27 fibroblast cells. The one exhibited the strongest stimulatory effect would be further investigated on different treatments to see if the proliferative activity would be enhanced. Five commonly found chemical components in RR would be tested for their proliferative effects and those with proliferative activities were chosen for quantitative analysis in four differently treated samples.

Materials & Methods

Authentication of RR Samples

Three different forms of RR sample (fresh RR or 鮮地黃 xiān dì huáng, dried RR or 乾地黃 gān dì huáng and steamed RR or 熟地黃 shóu dì huáng) were purchased from Henan province of China and they were authenticated by thin layer chromatography using chemical markers or reference materials as suggested by Chinese Pharmacopoeia 2005 (State Pharmacopoeia Commission of People's Republic of China, 2005). A certain portion of each sample was deposited in the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong and their voucher specimen numbers were 2008-3179 (fresh RR), 2008-3200 (dried RR) and 2008-3177 (steamed RR).

Preparation of Aqueous Crude Extract (with Heating) of Three Different RR Samples

Two hundred and fifty grams each of the three different forms of RR sample were cut into small pieces. They were immersed in 2.5 liters of water for 1 hour and then heated under reflux for 1 hour twice. The resulting extracts were combined, filtered, concentrated and freeze-dried. The extraction yields of the crude extracts of fresh, dried and steamed RR were 62.8% w/w, 63.2% w/w and 67.2% w/w, respectively.

Preparation of Aqueous (without Heating) and Ethanolic (with or without Heating) Crude Extracts of Dried RR Sample

Aqueous Crude Extract (without Heating)

Ten grams of dried RR herb was cut into small pieces. They were immersed in 100ml of water overnight and then sonicated for 1 hour twice. The resulting extracts were combined, filtered, concentrated and freeze-dried. The extraction yield was 58.0% w/w.

Ethanolic Crude Extract (with Heating)

Ten grams of dried RR herb was cut into small pieces. They were immersed in 100ml of 95% ethanol for 1 hour and then heated under reflux for 1 hour twice. The resulting extracts were combined, filtered and concentrated into dryness. The extraction yield was 7.6% w/w.

Ethanolic Crude Extract (without Heating)

Ten grams of dried RR herb was cut into small pieces. They were immersed in 100ml of 95% ethanol overnight and then sonicated for 1 hour twice. The resulting extracts were combined, filtered and concentrated into dryness. The extraction yield was 2.2% w/w.

Chemicals and Reagents

Acetonitrile (ACN) and methanol (MeOH) (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ethanol (95%, reagent grade) was purchased from Uni-chem (Doha, Qatar). Acetic acid (100%, analytical grade) and triethylamine (TEA) (purity≥ 99.5%, certified grade) were purchased from BDH (London, UK) and Tedia (USA), respectively. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (PS) and phosphate buffered saline (PBS) were purchased from Invitrogen (California, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Catalpol and 5-hydroxymethyl-2-

furaldehyde (5-HMF) were purchased from National Institute for the Control of the Pharmaceutical and Biological Products (Beijing, China). Stachyose (as hydrate, purity≥98%) and verbascoside (purity≥ 98%) were purchased from Sigma-Aldrich (St. Louis, USA) and Topharman Shanghai Co., Ltd. (Shanghai, China), respectively. Manninotriose (purity≥85%) was purchased from Institute of Pharmacognosy, School of Pharmaceutical Sciences of Shandong University, China.

Cell Culture

Human fibroblast cell line Hs27 (ATCC CRL-1634) was obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM with 10% v/v fetal bovine serum (FBS) and 100U/ml of penicillin streptomycin (PS) at 37°C, 5% CO_2 in humidified incubator.

Cell Proliferation Assay

Cell proliferation was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Briefly, cells were seeded onto 96-well plate at density of 3 x 10^3 cells/well in DMEM with 2% v/v FBS and 100U/ml of PS and treated with herbal sample. After 48h of treatment, 30µl of MTT solution (5mg/ml in PBS) was added to each well and further incubated for 3h at 37°C. Subsequently, 150µl of DMSO was added to each well to dissolve the purple formazan crystal. The absorbance of each well at 540nm was measured using microplate spectrophotometer (BioTek, Vermont, USA).

Chemical Analysis

Quantitative analyses of two chemical components in dried RR aqueous or ethanolic crude extracts were performed.

Stachyose

Ultra-performance liquid chromatography-evaporative light scattering detection (UPLC-ELSD) analysis was performed on a liquid chromatography system (Waters ACQUITY UPLC system, Waters Corporation, USA) equipped with binary pump and ACQUITY UPLC ELSD detector. Chromatography was carried out on a Waters ACQUITY UPLC BEH Amide column (2.1 x 100 mm, 1.7 µm) at 40ºC column temperature. The mobile phase consisted of (A) water (0.2% TEA) and (B) acetonitrile (0.2% TEA). The system was run with a gradient program of 75% B to 40% B in 10 min and the flow rate was 0.2ml/min. ELSD parameters were as follows: drift tube temperature – 50.0°C; gas pressure – 50psi; Nebulizer – cooling.

Verbascoside

Ultra-performance liquid chromatography-diode array detection (UPLC-DAD) analysis was performed on a liquid chromatography system (Waters ACQUITY UPLC system, Waters Corporation, USA) equipped with binary pump and ACQUITY UPLC DAD detector. Chromatography was carried out on a Waters ACQUITY UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) at 40ºC column temperature. The mobile phase consisted of water (with 1% acetic acid) and methanol (75: 25). The flow rate was 0.2 ml/min. Verbascoside peak was recorded at 332 nm.

Statistical Analysis

The data are presented as mean ± SD values. Student' s t-test was used for statistical analyses. Differences were considered as statistically significant if $p<0.05$.

Results

Proliferation Effects of the Aqueous Crude Extracts of Three Different Forms of RR

All three RR aqueous crude extracts enhanced Hs27 fibroblast proliferation as measured by MTT assay. A dose-dependent effect of proliferation was observed when the concentration increased from 0.16mg/ml to 1.25 mg/ml (Figure 2). The subsequent proliferation rate decreased as the concentration further increased to 5mg/ml. Dried RR extract exhibited significant stronger stimulatory effect than fresh or steamed RR extracts at the optimum concentration of 1.25 mg/ml ($p<0.05$).

Effects of Dried RR Crude Extracts Prepared from Different Extraction Methods on Fibroblast Proliferation

Dried RR crude extracts were prepared by four different extraction methods: a) extracted with water with heating; b) extracted with water without heating; c) extracted with 95% ethanol with heating; d) extracted with 95% ethanol without heating, and tested for their fibroblast-proliferating effects. As shown in Figure 3, heated ethanolic crude extract, at the concentration range between 0.08 and 0.31mg/ml, was obviously the most potent among the four extracts. At 0.63 mg/ml, it was of similar potency as heated aqueous extract whilst at the optimum concentration of 1.25 mg/ml, it was as potent as the non-heated aqueous extract. Taking the effectiveness at all concentrations tested into consideration, the heated ethanolic crude extract conferred the

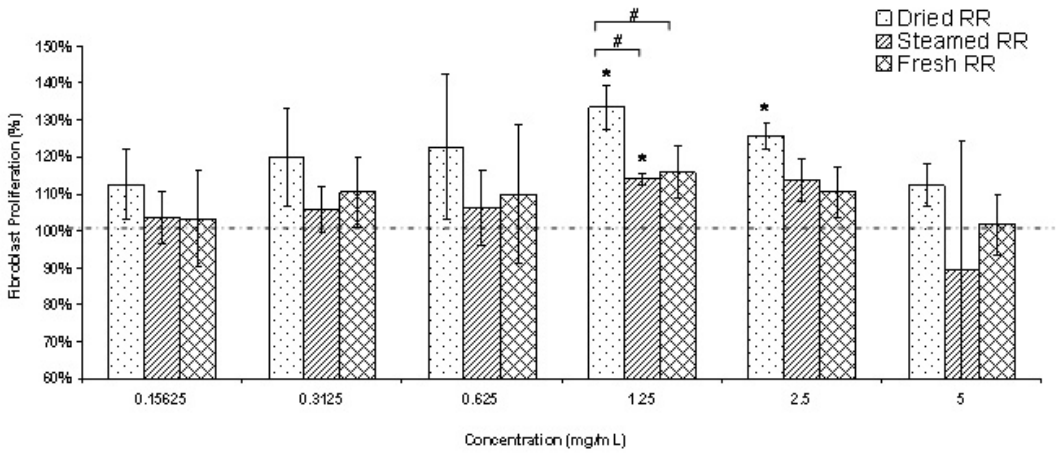

Figure 2. Fibroblast proliferation effect of the aqueous crude extracts of three different forms of RR. * denotes $p<0.05$ when compared with 100% control and # denotes $p<0.05$ when compared with dried RR using Student's t-test.

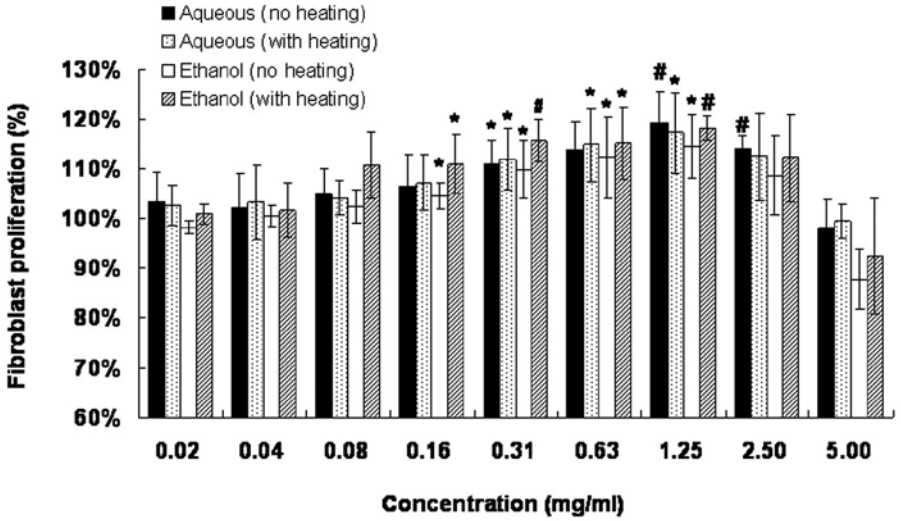

Figure 3. Effect of dried RR crude extracts prepared from different extraction methods on fibroblast proliferation. Data are mean±SD (n=3). * denotes $p<0.05$ and ** denotes $p<0.01$ when compared with 100% control using Student's t-test.

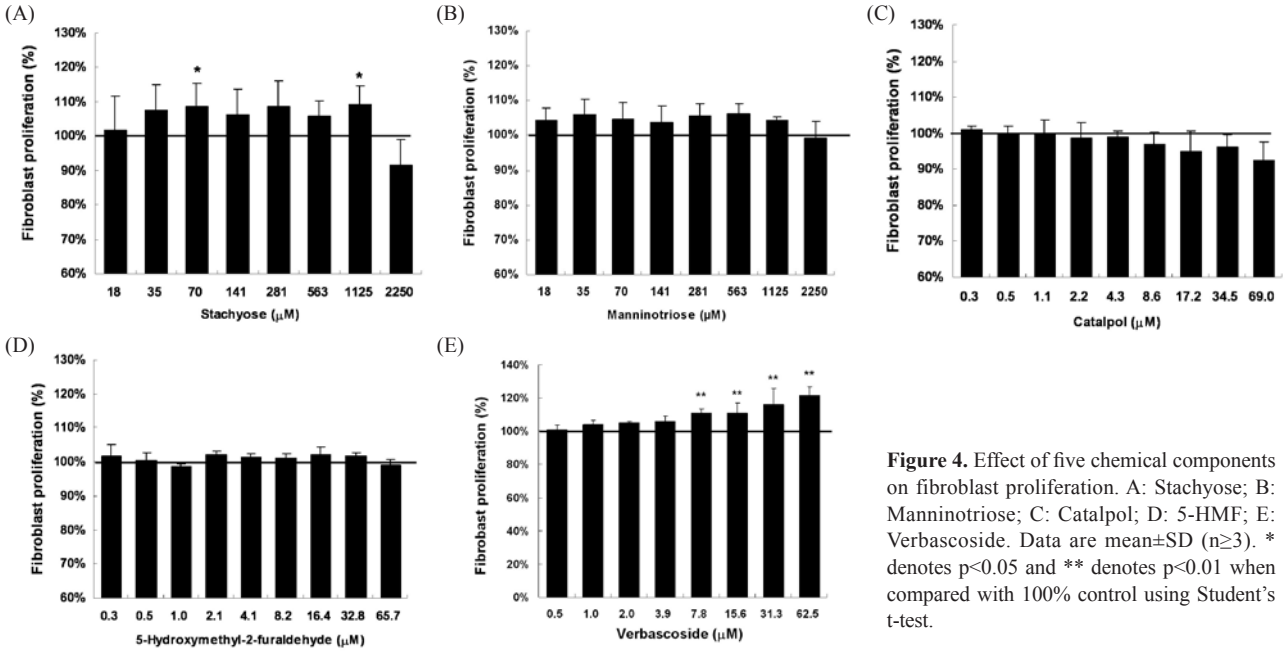

Figure 4. Effect of five chemical components on fibroblast proliferation. A: Stachyose; B: Manninotriose; C: Catalpol; D: 5-HMF; E: Verbascoside. Data are mean±SD (n≥3). * denotes $p<0.05$ and ** denotes $p<0.01$ when compared with 100% control using Student's t-test.

Table 1. Calibration range and linearity of the two chemical components.

Chemical components	Test ranges	r^2
Stachyose	0.125 – 1.0 mg/ml	0.9992
Verbascoside	0.625 – 5.0 μ/ml	0.9996

Key: r^2: correlation coefficient

most promising fibroblast-proliferating activity among the four crude extracts..

Effects of Five Chemical Components on Fibroblast Proliferation

As shown in Figure 4, stachyose and verbascoside exerted certain extent of stimulatory effects on fibroblast proliferation ($p<0.05$ at some particular concentrations), whereas manninotriose, catalpol and 5-HMF had no manifest effect on Hs27 proliferation.

Comparison of Two Bioactive Components among the Dried RR Aqueous and Ethanolic Crude Extracts (with or without Heating)

Calibration curves of the two bioactive components, stachyose and verbascoside, with good linearity ($r^2 \geq 0.992$) were prepared for quantitative analyses of the dried RR crude extracts (Table 1). Their contents in the dried RR extracts were quantified by UPLC-DAD/ELSD and the result was shown in Table 2. Their UPLC profiles were given as supplementary information (Figures S1 and S2). Aqueous extracts were found to have much higher amount of stachyose than ethanolic extracts no matter heating was employed or not. On the other hand, verbascoside was not detected in all dried RR crude extracts with detection limit of 0.0125% (w/w).

Discussion

Fibroblast proliferation is important in granulation formation that leads to wound healing. Rehmanniae Radix was previously found to stimulate proliferation of fibroblast cell line (Lau et al., 2007) and primary fibroblasts cultured from diabetic foot ulcer patients (Lau et al., 2009). In the present study, aqueous crude extracts were prepared from RR samples under different traditional processing methods (fresh, dried or steamed) and tested for their fibroblast proliferation effect. Our results demonstrated that all of them exhibited positive effect and dried RR extract was found to be the most potent (Figure 2). Therefore, it was chosen for further investigation.

In order to further compare the proliferation effect, four different extraction methods were employed for crude extract preparation using different extraction solvent (water or ethanol) and temperature (with or without heating). The ethanol extraction and no heating applied procedures were selected due to higher recovery capacity of organic constituents in ethanol when compared to water, and also heat instability of certain chemical components, such as verbascoside (Bilia et al., 2008). Our results showed that all four dried RR crude extracts possessed positive effect, with heated ethanolic crude extract being the most potent when compared with the other three extracts (Figure 3).

To investigate the active ingredient(s), five commercially available chemical components in RR (Figure 1) were selected to test for their fibroblast-proliferating effects individually. The concentration ranges to be evaluated were based on the previous results of HPLC analyses of the three RR aqueous crude extracts (data not shown). Among the five chemical components tested, only stachyose and verbascoside were found to possess significant positive proliferation effect while no stimulatory effect was found in manninotriose, catalpol and 5-HMF (Figure 4).

Since stachyose and verbascoside exhibited stimulatory effects on fibroblast proliferation (Figure 4A and 4E), it was of great interest to see whether the stimulatory effect of dried RR aqueous or ethanolic extracts were completely attributed to the presence of different amounts of stachyose and verbascoside in them. The contents of two components (stachyose and verbascoside) which possessed positive proliferation effects were determined in these four crude extracts by UPLC. Ethanolic crude extracts were found to have less stachyose than aqueous ones no matter heating was employed or not (Table 2). This was due to higher extractability of stachyose in water (Guo et al., 2007; Hong et al., 2009). On the other hand, verbascoside was not detected in aqueous or ethanolic crude extracts. Taking into account of the findings that ethanolic extract contained less stachyose but exerted stronger fibroblast

Table 2. Stachyose and verbascoside contents in dried RR aqueous/ethanolic crude extracts.

Dried RR crude extracts	Stachyose (% w/w)	Verbascoside (% w/w)
Aqueous (heating)	23.34 ± .26	ND[a]
Aqueous (no heating)	25.89 ± .41	ND
Ethanol (heating)	4.72 ± .17	ND
Ethanol (no heating)	4.05 ± .03	ND

Data are mean ± SD (n=3). [a]: not detected, detection limit is 0.0125% (w/w)

proliferative effect, and verbascoside exhibited significant fibroblast proliferative activity but its amount was too minute to be detectable in all dried RR extracts, it was speculated that verbascoside was not responsible for the fibroblast proliferative effect of the dried RR extracts and stachyose was one of the active components which partially contributed to the activity. Further works for finding the other active component(s) of dried RR aqueous or ethanolic extracts are required.

Conclusion

Among the three RR aqueous crude extracts tested, dried RR was found to possess the most potent effect on fibroblast proliferation which promoted wound healing. Crude extract prepared by heating with ethanol would exhibit stronger fibroblast proliferative effect. Two components of RR (stachyose and verbascoside) were found to stimulate fibroblast proliferation for their first time but their contents in RR aqueous or ethanolic crude extracts could not account for the activity difference among the extracts. Other active component(s) responsible for the fibroblast proliferative effect of RR aqueous or ethanolic crude extracts would need to be further explored and identified.

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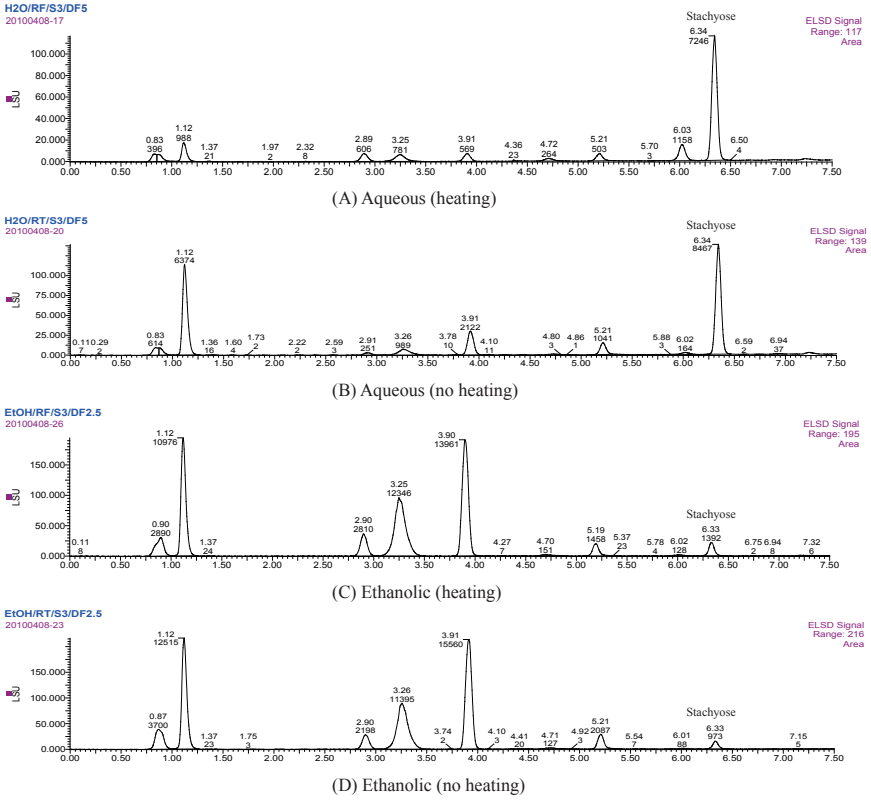

Figure S1. UPLC profiles of stachyose present in four dried RR crude extracts

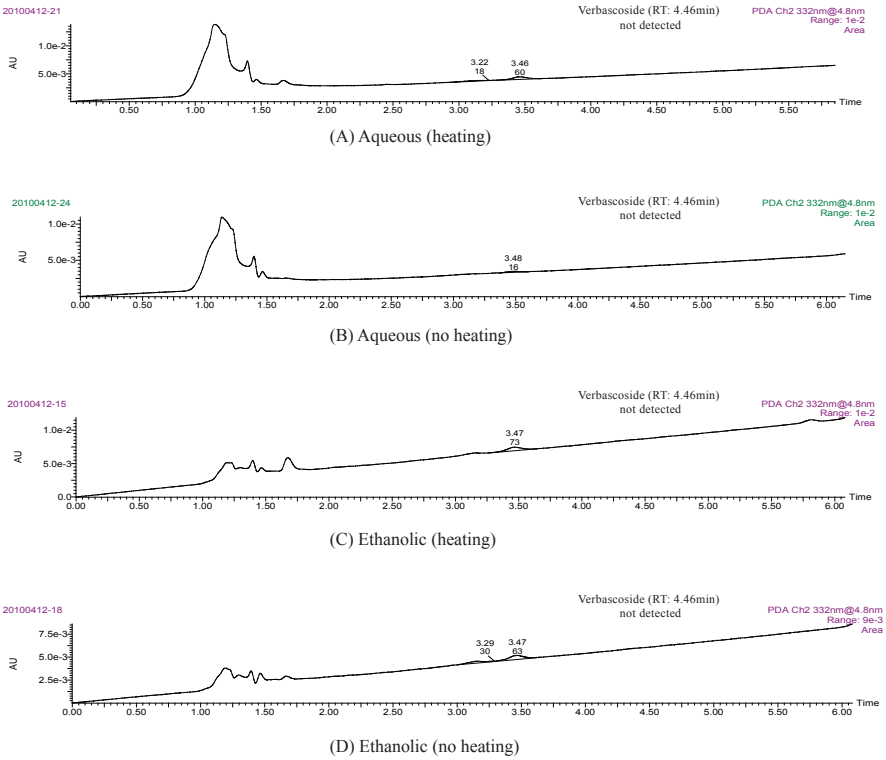

Figure S2. UPLC profiles for the detection of verbascoside in four dried RR crude extracts