

Pharmacological Investigation of “Meridian Tropism” in Three “Shen” Chinese Herbs

Pou-Kuan Leong, Hoi-Yan Leung, Wing-Man Chan, Kam-Ming Ko*

Division of Life Science, Hong Kong University of Science & Technology, Clear Water Bay, Hong Kong SAR, China

Email: *bcrko@ust.hk

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Abstract

“Meridian tropism” refers to the organ-specific biological action(s) produced by a Chinese herb following its oral administration, which is analogous to the concept of “bioavailability” in Western medicine. In this study, we compared the *in vitro* and *ex vivo* pharmacological actions of three herbs [namely, Dangshen (DS, *Codonopsis Radix*), Ranshen (RS, *Ginseng Radix*) and Xiyangshen (XYS, *Panaxis quinquefolii Radix*)] to validate their meridian tropism. We compared the *in vitro* and *ex vivo* pharmacological actions [*i.e.* the ability to increase splenocyte proliferation and adenosine triphosphate-generation capacity (ATP-GC)] of the ethanolic extracts of DS, RS and YYS to validate their meridian tropism. Results showed that DS, RS and YYS (at 30 - 300 µg/mL) can both stimulate the proliferation of primary mouse splenocytes *in vitro* and increase adenosine triphosphate-generation capacity (ATP-GC) in cultured Caco 2 colon epithelial cells *in vitro*. Interestingly, oral administration of DS and RS (but not YYS, at 3 and 6 g/kg/day × 3 consecutive days) was found to stimulate the proliferation of splenocytes *ex vivo* at 24 h post-treatment in mice. Similarly, DS and RS (but not YYS) increased the ATP-GC of mitochondrial fractions isolated from a small segment of mouse intestine at 48 h post-treatment. This observation is consistent with the meridian tropism of the pharmacological action of “Shen”, *i.e.*, the accessibility of DS and RS (but not YYS) to the “Spleen” meridian. The comparison between the results obtained from *in vitro* and *in vivo/ex vivo* bioassays may offer a potential method for assessing meridian tropism in Chinese herbs.

Keywords

Codonopsis Radix, *Ginseng Radix*, *Panaxis quinquefolii Radix*, ATP-Generation Capacity, Splenocyte Proliferation

1. Introduction

Traditional Chinese medicine theory posits that various visceral organs are func-

tionally connected by a network of channels and collaterals (or meridians) which determines physiological functions in a holistic manner [1]. With this concept in mind, meridian tropism refers to a selective biological action produced by a Chinese herb on one or more target visceral organs following its oral administration [1]. The meridian tropism theory is therefore analogous to the pharmacokinetic concept of “bioavailability” of an herb to a target organ(s) in Western medicine. In this regard, the investigation of the mechanism underlying the organ-specific pharmacological action(s) of Chinese medicinal herbs is crucial for establishing the scientific basis (in relation to Western medicine) for the meridian tropism theory.

In TCM theory, “Qi” refers to the vital energy required for the growth and development of the human body as well as performing physiological functions through visceral organs [2]. Dangshen (DS, *Codonopsis Radix*), Ranshen (RS, *Ginseng Radix*) and Xiyangshen (XYS, *Panaxisquinquifolii Radix*) are Qi-invigorating herbs, which are commonly called “Shen” in Chinese and commonly used for safeguarding health in TCM. As such, Qi-invigorating herbs have been found to reduce the expression of senescent proteins and promote cell proliferation in cultured senescent 2BS fibroblasts [3]. In addition, a recent study has shown that Qi-invigorating herbs can increase the energy charge in cultured rat myocytes as well as in rat skeletal muscle [4]. Consistent with this, a previous study in our laboratory has found that the three Qi-invigorating herbs (DS, RS and YYS) can increase adenosine triphosphate (ATP)-generation capacity (ATP-GC) in cultured H9c2 cardiomyocytes [5]. Interestingly, DS, RS and YYS differ in the nature of their meridian tropism, in which DS and RS but not YYS can act through the “Spleen” meridian.

In the present study, we compared the *in vitro* and *ex vivo* pharmacological actions of the three Qi-invigorating herbs (“Shen”) in an effort to validate their meridian tropism. The effects of ethanolic extracts of DS, RS and YYS on splenocyte proliferation *in vitro* and *ex vivo* were first examined. As the function of “Spleen” in TCM also relates to intestinal digestion and absorption, we also compared the effect of the three “Shen” ethanolic extracts on ATP-GC in culture Caco-2 intestinal epithelial cells *in vitro* and in mitochondrial fractions isolated from mouse intestine *ex vivo*. The chemical constituents in the “Shen” ethanolic extracts were also analyzed by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) in an effort to identify the chemical component(s) that might be responsible for the organ-specific Qi-invigorating action of the “Shen” ethanolic extracts.

2. Materials and Methods

2.1. Chemical and Reagents

Eagle’s Minimum Essential Medium (EMEM) and fetal bovine serum were purchased from Gibco BRL Life Technologies (Grand Island, NY, US). RPMI1640 medium, dimethyl sulfoxide (DMSO), concanavalin A (Con A), adenosine di-

phosphate (ADP), adenosine triphosphate (ATP), sodium pyruvate and sodium malate were bought from Sigma Chemical Co. (St. Louis, MO, USA). Evans Blue was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). All other chemicals were of analytical grade.

2.2. Preparation of Herbal Extracts

DS, RS and YYS were purchased from a local herb dealer (Lee HoongKee Limited, HKSAR, China). The herbs were authenticated by the supplier and voucher specimens were deposited in the Division of Life Science, Hong Kong University of Science and Technology (HKUST). Each herb was extracted with 95% ethanol under reflux for 2 h, as previously described [6]. For the HPLC-MS analysis of “Shen” extracts, chromatographic separation was performed on a Cortecs UPLC C18 column (100 × 2.1 mm, 1.6 µm) using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA). A Thermo LTQ Velos Pro (Thermo Fisher Scientific) multistage MS was used for detection.

2.3. Animal Care

Adult Imprinting Control Region (ICR) female mice (8 - 10 weeks old, 20 - 25 g) were maintained under a 12-h dark/light cycle at about 22°C and allowed food and water ad libitum in the Animal and Plant Care Facility at the Hong Kong University of Science and Technology (HKUST). All experimental protocols were approved by the University Committee on Research Practice at HKUST with the protocol number being 2,013,050.

2.4. Isolation of Splenocytes from Mouse Spleen

Adult female ICR mice were sacrificed by cardiac excision under ketamine chloride-induced anesthesia, using a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine in sterile saline. Splenocyte suspensions were obtained from mice as described [7]. Cell suspensions were re-suspended in RPMI-1640 medium supplemented with 10% FBS at a concentration of 6×10^6 viable cells/mL. The viability of isolated splenocytes was determined by the Trypan Blue exclusion assay.

2.5. Investigation of the Effects of “Shen” Ethanolic Extracts on Splenocytes *in vitro*

Isolated splenocytes were incubated with concanavalin A (Con A, at 4 µg/mL), Dangshen (DS, 30, 100 and 300 µg/mL), Renshen (RS, 30, 100 and 300 µg/mL) or Xiyangshen (YYS, µg/mL) for 48 h prior to conducting the cell proliferation assay. Following a 28 h incubation with Con A, DS, RS and YYS, bromodeoxyuridine (BrdU, 10 µM) was co-incubated with the splenocytes for 20 h prior to conducting the cell proliferation assay.

2.6. Investigation on the Effects of “Shen” Ethanolic Extracts on Splenocytes *ex vivo*

Adult female IRC mice were intragastrically administered by gavage DS, RS or

XYs ethanolic extract at doses of 3 or 6 g/kg for three consecutive days. Twenty-four hours after the last dosing, mice were sacrificed by cardiac excision under ketamine chloride anesthesia. Isolated splenocytes were cultured for 28 h and then incubated with BrdU for 20 h prior to conducting the cell proliferation assay.

2.7. Cell Proliferation Assay

The cell proliferation of splenocytes was measured using a BrdU cell proliferation assay kit (Cell Signaling Technology Inc, MA, USA) according to the manufacturer's instructions. The extent of splenocyte proliferation was determined by measuring absorbance at 450 nm using a Victor V3 Multi-label Counter (Perkin Elmer, Turku, Finland).

2.8. Caco-2 Cell Culture

Caco-2 cells, which are a subclone of the original clonal cell line derived from human colon epithelial cells, were purchased from the American Tissue Culture Centre (ATCC). The cells were cultured as mono-layers in EMEM, supplemented with 10% (v/v) FBS, 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 1.5 g/L of NaHCO₃. All cells were grown under an atmosphere of 5% (v/v) CO₂ in air at 37°C.

2.9. Measurement of ATP-GC in Situ

Caco-2 colon epithelial cells were seeded at a density of 6.5×10^4 cells/well into 24-well microtiter plates. Following attachment, cells were incubated with “Shen” ethanolic extracts for 4 h at 37°C. The control group was given the vehicle (DMSO) only. After the incubation, the ATP-GC assay was performed as previously described [6]. The ATP content generated from cells was measured by the luciferase assay (ATPlite, PerkinElmer Inc., MA). The ATP-GC of “Shen”-incubated cells (and the vehicle control group) was calculated by a two-step processing as described [8]. Data of “Shen” ethanolic extract-treated groups were expressed as percent of control.

2.10. Small Intestinal Motility Following Treatment with “Shen” Ethanolic Extracts in Mice

Small intestinal motility after “Shen” ethanol extraction was measured by monitoring the migration of Evans blue from the pylorus to a region 30 cm distant, as described [9]. In brief, Evans blue [at 50 mg/mL in 0.9% NaCl (w/v), 0.01 mL/g for each mouse] was intragastrically co-administered by gavage with various “Shen” ethanolic extracts (at a dose of 6 g/kg). Non-Evans Blue control animals received “Shen” ethanolic extracts (at a dose of 6 g/kg) only. At 30 min and 48 h post-treatment with Evans Blue, mice were sacrificed by cardiac excision under ketamine chloride anesthesia. The Evans blue in intestinal section was extracted and the absorbance at 600 nm was measured and quantified as area under the

curve (AUC), as described [9]. Non-Evans Blue-treated mice were used as controls (*i.e.* blank). The clearance of Evans Blue at 48 h post-treatment was estimated as follows: $[(\text{Evans Blue AUC}_{30 \text{ min}} - \text{blank AUC}_{30 \text{ min}}) - (\text{Evans Blue AUC}_{48 \text{ h}} - \text{blank AUC}_{48 \text{ h}})] / (\text{Evans Blue AUC}_{30 \text{ min}} - \text{blank AUC}_{30 \text{ min}}) \times 100\%$.

2.11. Effects of “Shen” Ethanolic Extracts on ATP-GC in Mitochondrial Fractions Isolated from a Small Segment of Mouse Intestine

Female ICR mice were randomly divided into 7 groups, with 5 - 7 animals in each. To investigate the effect of “Shen” ethanolic extracts on mitochondrial ATP-GC in a small segment of intestine, mice were administered “Shen” ethanolic extracts at doses of 3 or 6 g/kg, while control animals were given vehicle (water) only. Mice were sacrificed by cardiac excision under ketamine chloride anesthesia 48 h following dosing with the “Shen” ethanolic extracts. The mitochondrial fractions of mouse intestinal epithelium were prepared as described [10]. The mitochondrial ATP-GC of each sample was measured as described previously [8].

2.12. Protein Assay

Protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as standard.

2.13. Statistical Analysis

All data were expressed as mean \pm standard derivation (SD) unless otherwise specified. The homogeneity of variance among various groups was analyzed by Levene’s test. Depending on the results of the Levene test, data were analyzed by one-way analysis of variance (one-way ANOVA) or Welch analysis of variance (Welch ANOVA). Inter-group differences were assessed by Tukey or Games-Howell analysis respectively with $p < 0.05$.

3. Results

3.1. Differing Effects (*in vitro* and *ex vivo*) of “Shen” Ethanolic Extracts on Cell Proliferation in Isolated Mouse Splenocytes

Con A (4 $\mu\text{g/mL}$) stimulated the proliferation of cultured primary mouse splenocyte cells, with the extent of increase being 139% (**Figure 1(a)**). Incubations with DS (100 and 300 $\mu\text{g/mL}$), RS (100 and 300 $\mu\text{g/mL}$) and YYS (30 $\mu\text{g/mL}$) were found to increase cell proliferation in cultured primary mouse splenocytes, with a similar degree of stimulation (43% - 59%) (**Figure 1(a)**).

The effects of orally administered “Shen” ethanolic extracts on splenocyte proliferation were also examined *ex vivo*. The results showed that treatments with DS and RS (6 g/kg), but not YYS, increased the proliferation of splenocytes, with the extent of increase being 24% and 52%, respectively (**Figure 1(b)-(d)**).

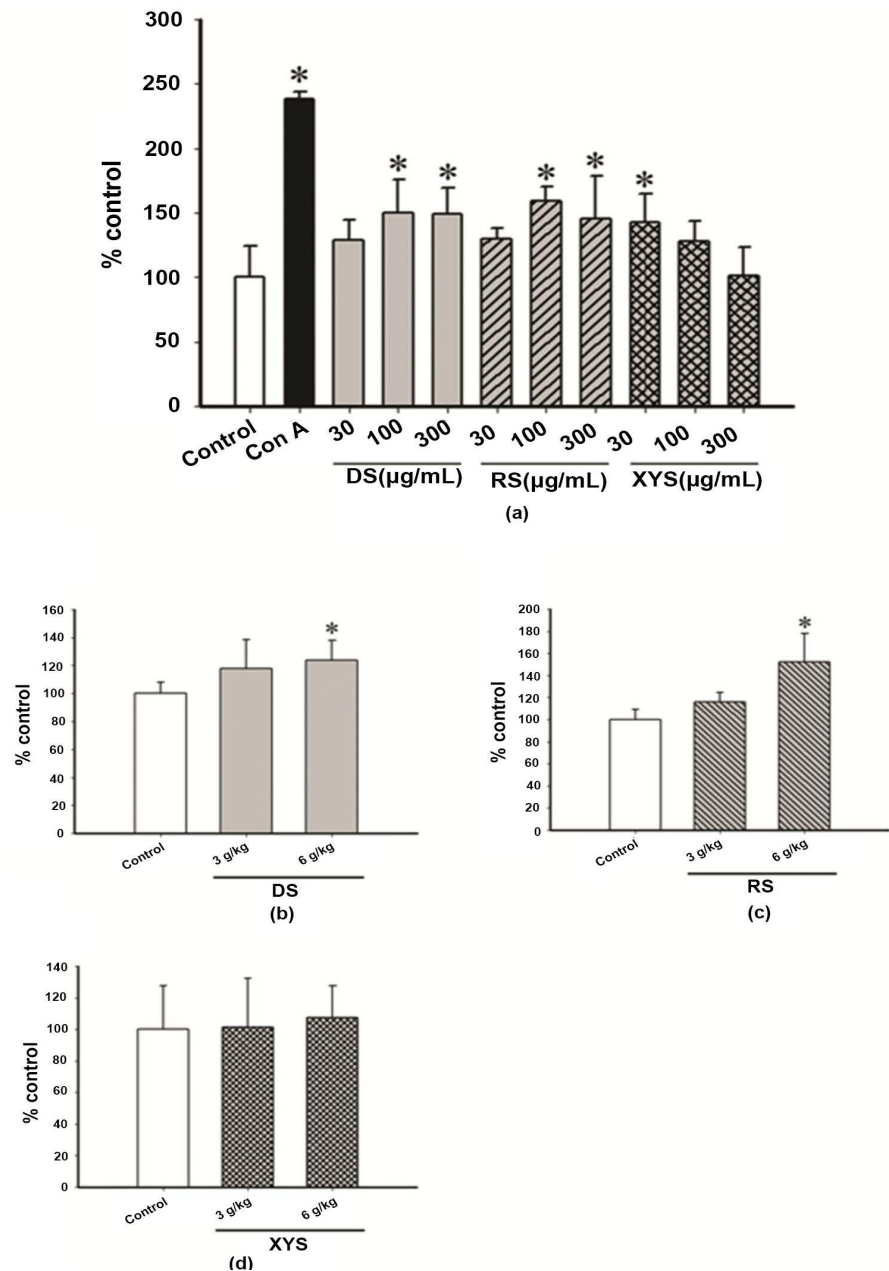


Figure 1. Differing effects (*in vitro* and *ex vivo*) of “Shen” ethanolic extracts on cell proliferation in isolated mouse splenocytes. Splenocytes were isolated from the spleens of female ICR mice as described in Materials and Methods. Isolated splenocytes were incubated with Dangshen (DS), Ranshen (RS) or Xiyangshen (XYS) ethanolic extracts [30, 100 and 300 µg/mL] or concanavalin A (Con A, 4 µg/mL) for 48 h. Cell proliferation was measured using a BrdU cell proliferation assay kit (panel A). Data are expressed in % control (mean absorbance at 450 nm of control = 0.310 ± 0.075). Female ICR mice were intragastrically administered DS (panel B), RS (panel C) or XYS (panel D) ethanolic extracts (3 and 6 g/kg/d \times 3 d), as described in Materials and Methods. Splenocytes were isolated from the spleens of “Shen” ethanolic extract-treated mice. Isolated splenocytes were cultured for 48 h prior to the cell proliferation assay. Data are expressed in % control (mean absorbance at 450 nm of control for DS, RS and XYS were 0.221 ± 0.018 , 0.323 ± 0.029 and 0.389 ± 0.106 , respectively). Values given are means \pm SD, with $n = 4 - 5$. * Significantly different from the control group.

3.2. Differing Effects (*in vitro* and *ex vivo*) of “Shen” Ethanolic Extracts on ATP-GC in Cultured Caco-2 Cells and Mitochondrial Fractions Isolated from Mouse Small Intestine

Incubation with DS, RS or XYS ethanolic extracts increased ATP-GC in cultured Caco-2 intestinal epithelial cells, with the extent of stimulation being 14% and 32% (DS, at 100 and 300 µg/mL), 26% and 15% (RS, at 100 and 300 µg/mL) as well as 22%, 15% and 25% (XYS, at 30, 100, 300 µg/mL), respectively (**Figures 2(a)-(c)**). Small intestinal motility following oral treatment with the “Shen” extracts was also measured. Results indicated that 48 h post-treatment was sufficient to completely expel the ingested “Shen” ethanolic extract from the small intestine, as indicated by the complete clearance of the Evans blue dye (**Table 1**). ATP-GC of mitochondrial fractions isolated from the small intestinal mucosa in “Shen” ethanolic extract-treated mice was also measured at 48 h post-treatment. Treatments with DS and RS (6 g/kg), but not XYS, increased mitochondrial ATP-GC in the small intestinal mucosa of mice *ex vivo*, with the degree of stimulation being 81% and 132%, respectively (**Figure 2(d)**).

3.3. Qualitative Analysis of Chemical Constituents in “Shen” Ethanolic Extracts

While HPLC-MS analysis of RS and XYS ethanolic extracts showed a similar chemical profile in ion chromatograms, the DS extract exhibited an ion chromatogram pattern which was different from those of RS and XYS (**Figure 3(a)-(c)**).

4. Discussion

According to TCM theory, the three “Shen” herbs (DS, RS and XYS) are traditionally used for Qi-invigoration, which can be viewed in terms of an up-regulation of energy status as well as the stimulation of growth and development of the human body [2]. A previous study in our laboratory has demonstrated that the “Shen” herbs under investigation are able to increase ATP-GC in cultured H9c2 cardiomyocytes [5]. Results obtained in the present study showed that the three “Shen” ethanolic extracts stimulated the proliferation of primary splenocytes *in vitro* and increased ATP-GC in cultured Caco 2 colon epithelial cells *in vitro*. Based on the “meridian tropism” theory, DS and RS but not XYS can preferentially invigorate the Qi in the “Spleen”, which, in the context of Chinese medicine, refers not only to the physiological function of the spleen but also the processes of intestinal digestion and absorption. In the present study, oral administration of DS and RS (but not XYS) was found to stimulate the proliferation of splenocytes *ex vivo* 24 h after the last dosing. Similarly, DS and RS (but not XYS) increased ATP-GC in mitochondrial fractions isolated at 48 h post-treatment from a small segment of mouse intestine. Since complete clearance of Evan Blue dye, co-administered with the “Shen” extracts, was observed at 48 h post-oral treatment, it seems unlikely that the DS- and RS-induced enhancement

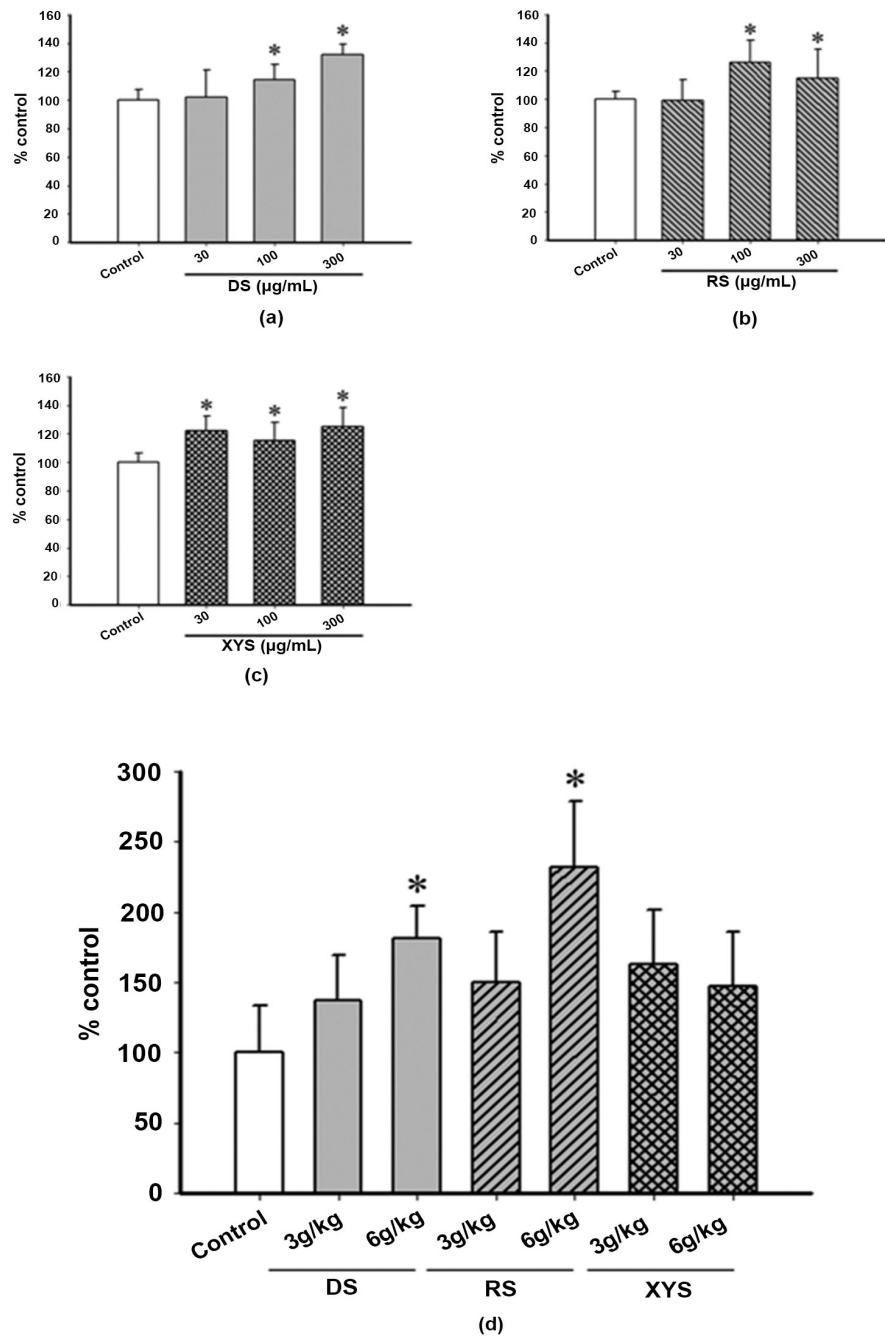


Figure 2. Differing effects (in vitro and ex vivo) of “Shen” ethanolic extracts on ATP-GC in cultured Caco-2 cells and mitochondrial fractions isolated from mouse small intestine. Cells were pre-incubated with DS (panel a), RS (panel b) or YYS (panel c) ethanolic extracts at concentrations of 30 to 300 µg/mL for 4 h. Mitochondrial ATP-GC was measured as described. Data are expressed in percent control with respect to the untreated control (mean of control values of DS, RS and YYS were = 500 ± 38 , 500 ± 29 and 500 ± 33 , respectively). Female ICR mice were intragastrically administered with DS, RS or YYS ethanolic extract (3 and 6 g/kg), as described in Materials and Methods (panel d). Mice were sacrificed at 48 h post-treatment and mitochondrial fractions were isolated from the mucosal layer of the small intestine. Mitochondrial ATP-GC was measured. Data are expressed in percent control (mean of control value = 700 ± 233). Values given are means \pm SD, with $n \geq 3$. *Significantly different from the control group.

Table 1. The extent of clearance of Evans Blue at 48 h post-treatment. The clearance of Evans Blue at 48 h post-treatment (as compared to that of 30 min post-treatment) was estimated as described in Materials and methods.

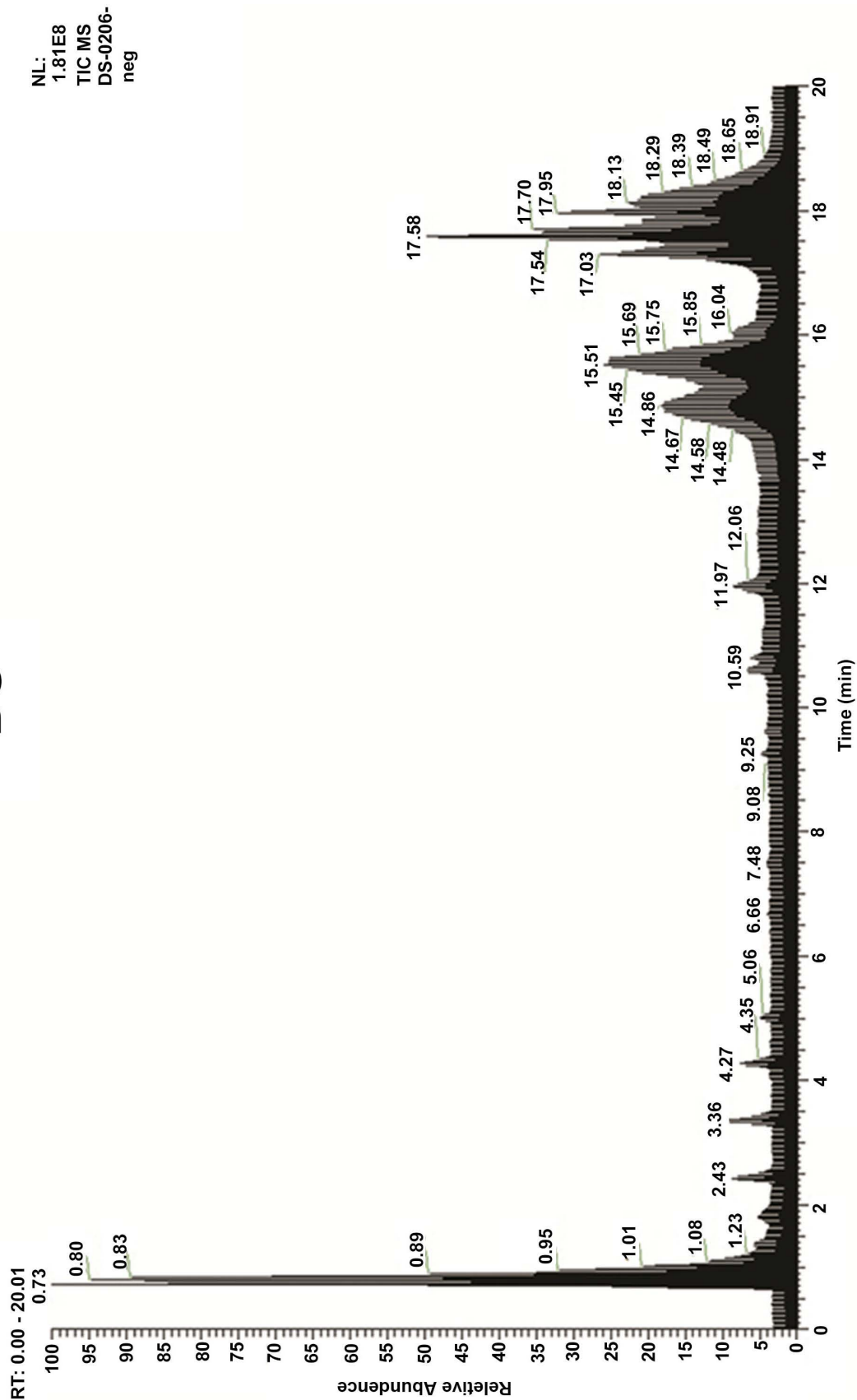
Absorbance at 600 nm	30 min post-treatment		48 h post-treatment		Clearance (%)
	Blank	Evans Blue	Blank	Evans Blue	
Dangshen	0.010	1.40 ± 0.41	0.032	0.030 ± 0.002	100
Renshen	0.005	1.90 ± 0.36	0.031	0.024 ± 0.003	100
Xiyangshen	0.013	1.60 ± 0.20	0.026	0.023 ± 0.002	100

of mitochondrial ATP-GC is caused by a direct stimulatory action on intestinal epithelial cells prior to the isolation of mitochondrial fractions. As for YYS, it stimulated mitochondrial ATP-GC at 24 h post-treatment when the YYS ethanolic extract remained in the intestine. The inability of orally administered YYS to stimulate splenocyte proliferation *ex vivo* or increase mitochondrial ATP-GC in mouse intestinal epithelial cells seems to be in accordance with the meridian tropism of YYS, which describes its accessibility to the meridians of “Heart”, “Lung” and “Kidney”. Differences in the pharmacological effects produced by the three “Shen” ethanolic extracts in cell-based and animal-based assay systems can, therefore, be readily explained by the meridian tropism theory.

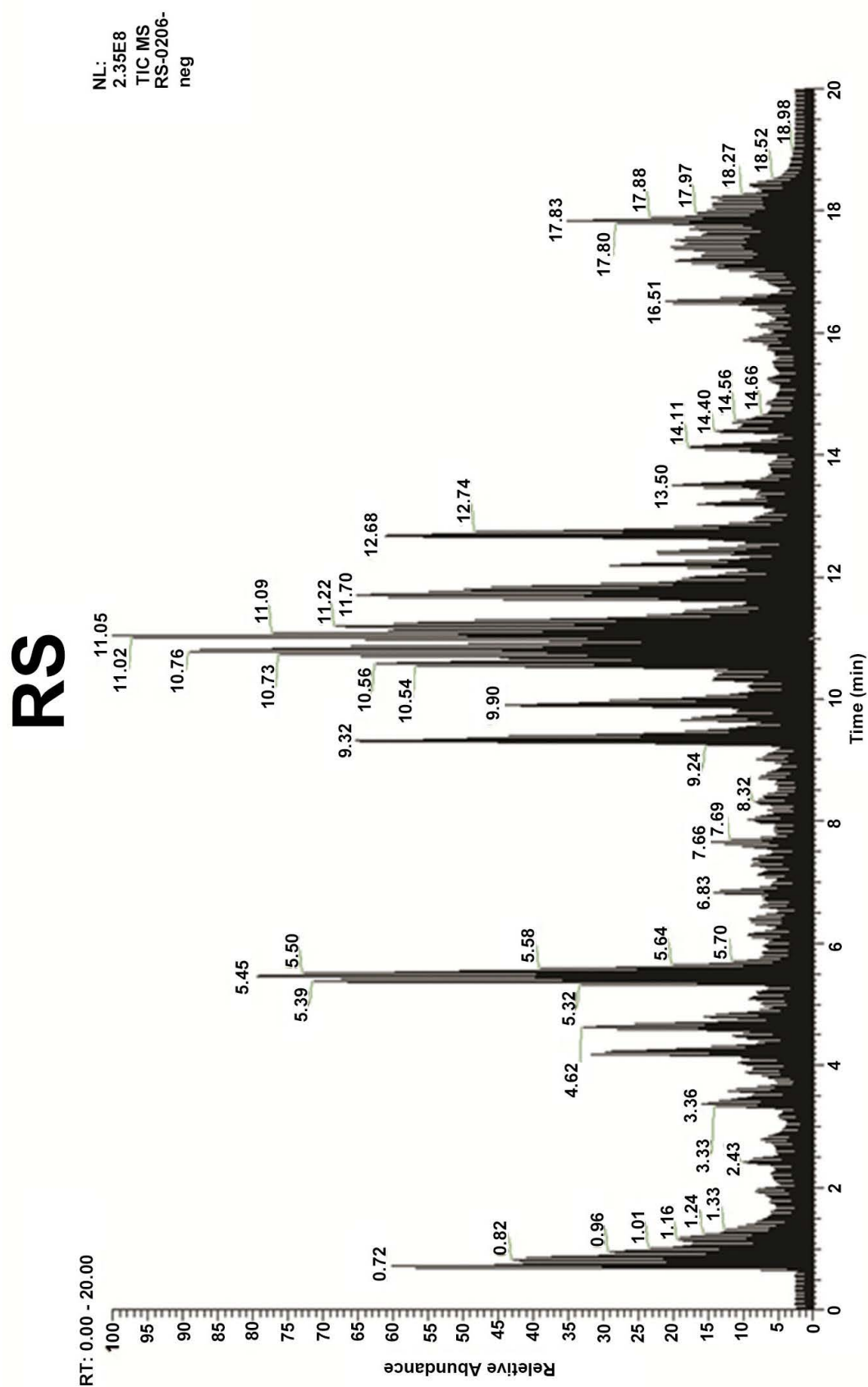
Ginsenosides, which belong to a group of triterpene saponins, have been shown to be active components of RS and YYS [11]. The protection against apoptosis in splenocytes of rats with d-galactose-induced aging [12] and the improvement in energy metabolism in skeletal muscle in rats with postoperative fatigue syndrome [13], both of which are related to the enhancement of “Spleen” function in Chinese medicine theory, suggest a “Spleen-Qi” invigorating action of ginsenosides. While HPLC-MS analysis performed in the present study did not show any differences in chemical profiles of RS and YYS ethanolic extracts, a recent study by Kim *et al.* have revealed notable differences between RS and YYS in terms of their chemical components [11]. RS possesses Rb1, Rg1 and Rb2 as major ginsenosides, a low ratio of protopanaxadiol (PPD)-groups to protopanaxatriol (PPT)-groups and a low ratio of Rb1 to Rg1, whereas YYS possesses Rb1, Re and Rd as major ginsenosides, a high ratio of PPD-groups to PPT-groups and a high ratio of Rb1 to Rg1. Intriguingly, various studies have demonstrated that the biotransformation of ginsenosides to deglycosylated ginsenosides (namely, ginsenoside compound K) is essential for producing their pharmacological actions in whole body assay conditions [14]. For instance, ginsenosides can be metabolized by intestinal flora into ginsenoside compound K, which is more readily absorbed in the intestine [15] [16]. Whether or not the difference in chemical composition and/or in the formation of ginsenoside compound K is a crucial determinant for producing the organ-specific pharmacological actions of RS and YYS ethanol extracts requires further investigation.

Both RS and DS ethanolic extracts exhibited pharmacological effects on the “Spleen” *ex vivo* irrespective of their difference in chemical composition. Active

DS



(a)



(b)

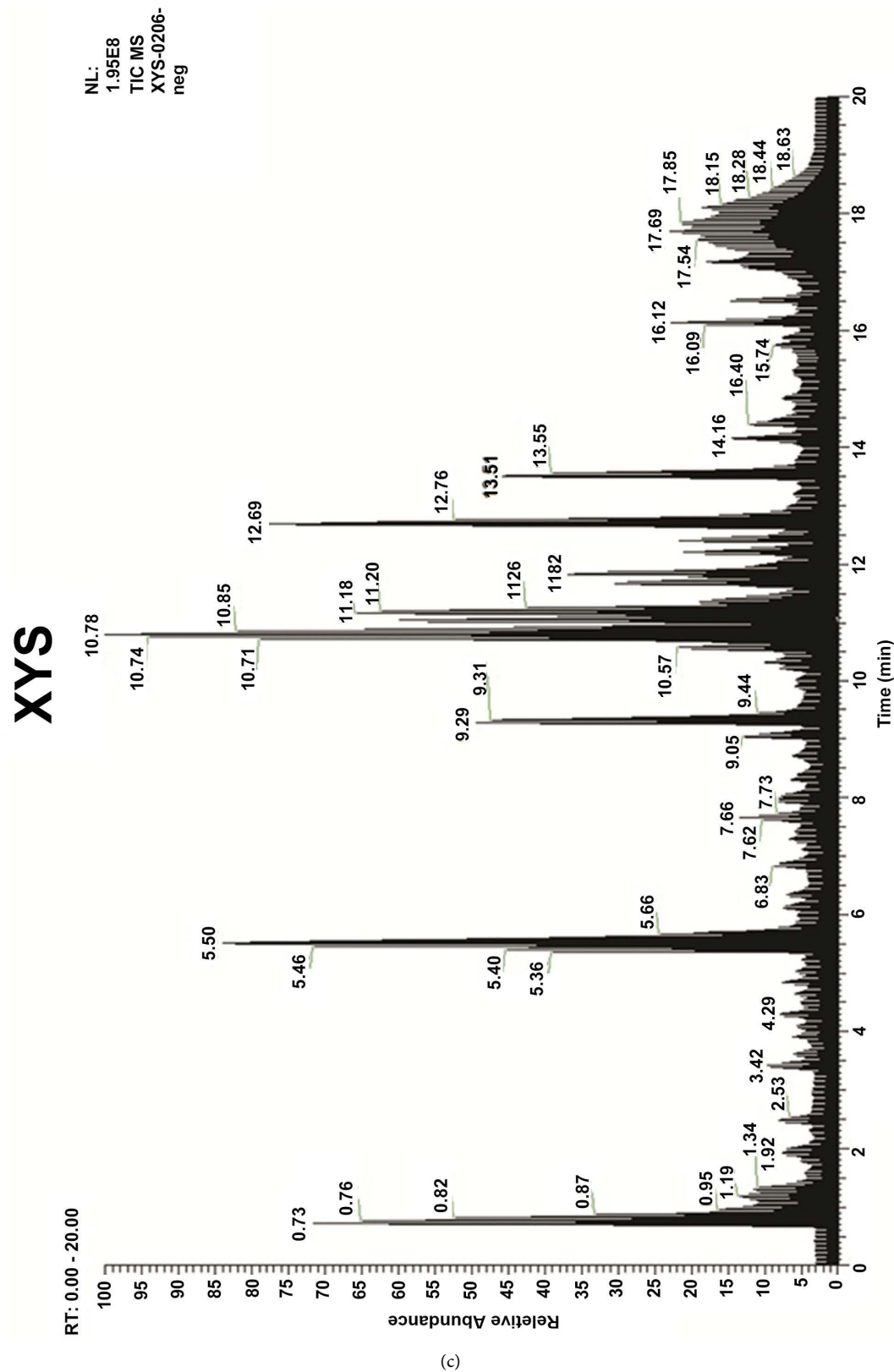


Figure 3. (a) HPLC-MS total ion chromatograms of DS extracts in negative-ion mode. (b) HPLC-MS total ion chromatograms of RS extracts in negative-ion mode. (c). HPLC-MS total ion chromatograms of XYs extracts in negative-ion mode.

components of DS, such as salvianolic acids, tanshinones and dihydrotanshinone, have been shown to produce immunomodulatory actions [17] [18] [19], which may be related to an enhancement of “Spleen” function. Given that no common chemical constituents have been identified in RS and DS [20] [21], the active component(s) in DS responsible for eliciting the organ-specific response is yet to be determined.

In conclusion, our findings indicate that the three “Shen” ethanolic extracts (DS, RS and YYS) can stimulate spleen proliferation *in vitro* and increase ATP-GC in Caco-2 cells *in vitro*. DS and RS (but not YYS) can stimulate splenocyte proliferation *ex vivo* and increase mitochondrial ATP-GC in mouse intestinal epithelial cells *ex vivo*. Despite the fact that the active components responsible for producing the organ-specific action have not as yet been identified, the results obtained from the present study are consistent with the “meridian tropism” of DS, RS and YYS. While the pharmacokinetic study of the organ distribution of chemical components in Chinese herbs has been commonly adopted for investigating the phenomenon of “meridian tropism” [22], the comparison between the results obtained from *in vitro* and *in vivo/ex vivo* bioassays, as shown in the present study, may offer an alternative method for validating “meridian tropism” in Chinese herbs generally.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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