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Hepatoxicity of major constituents and extractions of Radix Polygoni Multiflori and Radix Polygoni Multiflori Praeparata

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ABSTRACT

Ethnopharmacological relevance: Radix Polygoni Multiflori (RPM) and Radix Polygoni Multiflori Praeparata (RPMP) were traditionally widely used as Chinese herbal medicine. However, liver adverse reactions caused by RPM or RPMP were frequently reported all around the world recent years. The aim of this study was to study the cytotoxicities of RPM, RPMP and their major constituents on human liver cell L-02 simultaneously.

Materials and methods: Multi-assays, including MTT assay, neutral red uptake (NRU) assay, LDH leakage percentage and liver enzyme secretion (AST, ALT and ALP) were used. Cytotoxicities of major chemical constituents of RPM, 2, 3, 5, 4′-tetrahydroxy-stilbene-2-*O*-*β*-D-glucoside (TSG), physcion and emodin, were tested. The cytotoxicities of water, 50% ethanol and 95% ethanol extractions of RPM and RPMP were tested. HPLC-DAD analysis was carried to reveal the content change of TSG, physcion and emodin after the processing procedure.

Results: The TD_{50} of TSG, physcion and emodin in MTT assay were >10,000 μM, 2853.61 μM and 520.37 μM. In the NRU assay, the TD_{50} of TSG, physcion and emodin were much smaller (1401.53 μM, 1140.00 μM, and 3.80 μM). Emodin induced much severe liver enzyme secretion than TSG and physcion. Cell proliferation and LDH leakage rate showed no difference between RPM and RPMP extractions, but ALP, AST and ALT secretions in RPMP extractions were significant lower than that of PMR groups. Water extractions of RPM and RPMP were less toxic than any other solvent in most of the assays. Positive correlation was found between the TSG/emodin ratio and MTT survival rate. The emodin/physcion ratio also showed positive correlation with the LDH leakage percentage.

Conclusions: In conclusion, Radix *Polygonum multiflorum* and Radix *Polygonum multiflorum* Praeparata were not liver injure inducing in our *in vitro* assays. However, the processing produce of RPM could reduce its effect on both cell proliferation and enzyme secretion of liver cell. Judging from cell proliferation, integrity of cell membrane and enzyme secretion, three major chemical constituents of RPM: TSG, physcion and emodin showed no, moderate and severe cytotoxicity against human liver cell line L-02 respectively. Chemical constituents-cytotoxicity relationship investigation revealed that TSG and physcion probably had attenuating effect to emodin. The attenuating mechanisms were still under investigation.

1. Introduction

Radix Polygoni Multiflori (RPM, Heshouwu in Chinese) and Radix Polygoni Multiflori Praeparata (RPMP, Zhiheshouwu in Chinese) are originated from the root of *Polygonum multiflorum* Thunb. (Polygonaceae) and used in oriental counties for centuries.

Varieties of processing methods were used in the RPM processing procedure. Steaming or steaming with black soybean decoction was the most frequently used one recorded by the Pharmacopoeia of the People's Republic of China, 2010 edition (Commission of Chinese Pharmacopoeia, 2010).

RPM and RPMP appeared different pharmacological effects and were used in the treatments for different diseases. RPM was used for the treatment of constipation. RPMP, thought as herbs that tonify the kidney and liver, was used in the treatment of early graying of hair and hyperlipemia. Both of them were considered as non-toxic medicine since ancient times.

Furthermore, RPM and RPMP were authorized as health food additive by the government of People's Republic of China (Ministry

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransaminase; AST, aspartate aminotransferase; FBS, fetal bovine serum; LDH, lactate dehydrogenase; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; NRU, neutral red uptake assay; RPM, Radix Polygoni Multiflori; RPMI 640, Roswell Park Memorial Institute medium 1640; RPMP, Radix Polygoni Multiflori Praeparata; TD_{50}, median toxic dose; TSG, 2,3,5,4′-tetrahydroxy-stilbene-2-*O*-*β*-D-glucoside.

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of Health of the People's Republic of China, 2002). Both of them were frequently added to variety of lipid-lowering and anti-obesity health foods.

However, hepatic adverse effect was constantly reported since 1990s in China (Niu, 1996; Ye, 1996; Sun, 2002; Zhao, 2007) and other countries (But et al., 1996; Park et al., 2001; Battinelli et al., 2004; Panis et al., 2005; Cárdenas et al., 2006; Yuen et al., 2006; Min et al., 2008; Cho et al., 2009). Rash, fever, abdominal pain, dyspnea, vision problems and palpitations were common adverse effects induced by *Polygonum multiflorum*. Acute toxic hepatitis was most frequently reported hepatic adverse effect (Navarro and Senior, 2006; Murray et al., 2008). Liver enzyme (ALT, AST, bilirubin, gamma glutamyl transpeptidase and LDH) elevation and normal ultrasound examination were presented in most of these patients. Cholestasis and jaundice were sporadically reported. Virus affection could be excluded in all the cases.

Supervisions of usage of *Polygonum multiflorum* were conducted by drug regulatory agencies of Canada (Canadian Adverse Reaction Newsletter, 2003), British (Medicines and Healthcare products Regulatory Agency, 2006a,b) and Australia (Complementary Medicines Evaluation Committee, 2007; Therapeutic Goods Administration, 2008). The MHRA (Medicines and Healthcare products Regulatory Agency, UK) had continuously released press to raise concern about the safety of *Polygonum multiflorum*, owing to 7 liver damage cases collected through the Yellow Card Scheme (an adverse effect reporting system in UK). TGA (Therapeutic Goods Administration, Australia) commanded all the RPM contained preparation to print "Warning: *Polygonum multiflorum* may harm the liver in some people" on its labels (Therapeutic Goods Administration, 2008). The recommended RPM dosage list Pharmacopoeia of the People's Republic of China was reduced from 6–12 g in 2005 edition (Commission of Chinese Pharmacopoeia, 2005) to 3–6 g in 2010 edition (Commission of Chinese Pharmacopoeia, 2010). This amendment was partly due to these continually adverse effect reports.

RPM and RPMP were used as medication for different diseases, but both of them were suspected to be liver damage inducing crude drug. The hepatotoxicity was most likely caused by the direct action of some mutual chemical constituents in RPM and RPMP, or a reactive metabolite of the mutual chemical constituents against hepatocytes. Irrational drug use and drug interaction might also have something to do with the RPM and RPMP induced liver injury. Drug accumulation might also play crucial role in the liver injury, because RPM and RPMP were frequently taken for several months.

Usually, these adverse effects were thought to be induced directly by the anthraquinones contained in RPM and RPMP, however, the relevance of the data relating to the anthraquinone component of *Polygonum multiflorum* is not clear, as traditional preparations of this herb contain very small amount of these compounds. For instance, anthraquinones in "Shen Min" products were below the level of detection in five of the hepatitis cases induced by "Shen Min" (Complementary Medicines Evaluation Committee, 2007). Complementary Medicines Evaluation Committee in Australian (Therapeutic goods administration of Australian, CMEC) had previously considered scientific data on the chemistry, pharmacology, pharmacokinetics and toxicology of laxative anthraquinones. Most previous literature search did not reveal any additional data relating to hepatotoxicity associated with anthraquinones (Complementary Medicines Evaluation Committee, 2007). A research, carried out by Department of Biochemistry at Hong Kong University of Science and Technology, indicated that the anthraquinone component may had liver protection effect, however, higher dose of *Polygonum multiflorum* may over-ride this protective effect and result in hepatotoxicity. Although this group of researchers postulated that their results may indicate a dose-dependant hepatotoxicity of *Polygonum multiflorum*, their subsequent studies unfortunately did not further investigate this matter (Complementary Medicines Evaluation Committee, 2007).

Recent research showed that emodin effectively reversed toxic events induced by acetaminophen (Bhadauria, 2010). However, another research reported emodin was one of the important chemical constituents leading to liver cell damage (Zhang et al., 2010).

Constituents other than anthraquinones, such as impurities and other constituents contained in these crude drugs, might also contribute to the liver injure. In one of these adverse effect cases (Yuen et al., 2006), N-nitrosofenfluramine, a well-known liver toxicant, was adulterated in the RPM and Semen Cassiae contained preparation. In another case, appreciable amount of hydrolysable tannins was detected in leaf of witch hazel (*Hamamelis mollis* Oliver), one of the crude drugs in the preparation (Canadian Adverse Reaction Newsletter, 2003). The hydrolysable tannins were reported to be liver damage constituents (Racela et al., 1967; Reddy et al., 1970). Although the tannins in witch hazel were poorly absorbed following oral administration, hepatic damage might occur if they were gradually absorbed to an appreciable extent. Furthermore, long period of organic solvent and painting contacting working experience might also be one of the causes of liver damage (Canadian Adverse Reaction Newsletter, 2003).

Stilbenes and anthraquinones were the major compounds in RPM and RPMP. TSG, the quality control constituent of RPM and RPMP (Commission of Chinese Pharmacopoeia, 2010), showed great lipid-regulating and antioxidant activity (Lv et al., 2006; Gao et al., 2007). Emodin and physcion were highly-enriched anthraquinones contained in *Polygonum multiflorum* (Dai and Zhao, 2005). They showed great immunoregulation effects (Sun et al., 2006) and myocardial protective effect (Yim et al., 1998), however, they may also contribute to the laxative adverse effect (Qu et al., 2008).

This paper primarily investigated the cytotoxicity of TSG, emodin and physcion and different extractions of RPM and RPMP in order to give some information about the relationship between the liver injure and RPM or RPMP.

2. Materials and methods

2.1. Chemicals

TSG, emodin and physcion were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. The purities of all the standards were not less than 98%. Structures of TSG, emodin and physicon were listed in Fig. 1.

The stock solutions of all tested compounds were achieved by dissolved them in dimethylsulfoxide (DMSO) at a concentration of 10 mmol/ml. Then they were further diluted with 0.2% FBS-RPMI 1640 to graded concentrations from 20 to 300 μM.

2.2. Processing procedure of Radix Polygoni Multiflori

Radix Polygoni Multiflori was collected in Luquan County of Yunnan Province by the authors. The plants were collected in June 2008 and identified as the root of *Polygonum multiflorum* Thunb. by Prof. Rong-hua Zhao, Yunnan University of Traditional Chinese Medicine. Voucher specimens were deposited in the Herbarium of Pharmacognosy, Yunnan University of Traditional Chinese Medicine. RPMP was processed with black soybean decoction according to the procedure recorded in Commission of Chinese Pharmacopoeia (2010).

2.3. Preparation of extraction of RPM and RPMP

100 g RPM or RPMP powder was decocted with water (1000 ml, 800 ml and 600 ml), refluxed with 50% ethanol (800 ml, 600 ml and