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Optimum conditions for *Radix Rehmanniae* polysaccharides by RSM and its antioxidant and immunity activity in UVB mice

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ABSTRACT

Optimization of *Radix Rehmanniae* polysaccharides (RRPs) was done using response surface methodology (RSM). Optimum extraction conditions for maximizing the determination of *Radix Rehmanniae* polysaccharides were: extraction temperature 100 °C, extraction time 2 h and ratio of liquid to solid 6. The model had a satisfactory coefficient of R^2 (=0.9815) and was verified experimentally. The results suggested that the conditions were mild and useful for extraction yield of *Radix Rehmanniae* polysaccharides. Pharmacological experiment showed that *Radix Rehmanniae* polysaccharides could enhance serum interleukin-2 (IL-2), interleukin-4 (IL-4) and interleukin-10 (IL-10) levels, skin glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities, and decrease skin malondialdehyde (MDA) level in ultraviolet B (UVB) ray treated mice, this study suggests that *Radix Rehmanniae* polysaccharides extract may prove to be a useful therapeutic option in the reversal of skin diseases.

1. Introduction

Radix Rehmanniae (RR) has a long history of usage in traditional Chinese medicine (TCM). The first record appeared in the Canon on Medicinal Herbs by the Divine Ploughman (Ca. 200 BC) Shen-nong Bencao Jing. It was classified as high-grade (very safe) medicine. RR is derived from the root of *Rehmannia glutinosa Libosch.* (family Scrophulariaceae) and its unprocessed form is called "Sheng Dihuang". RR has been applied in many TCM applications for wound healing. According to the classical TCM interpretation, RR can reduce heat in blood, nourish yin and promote the production of body fluid (Yen, 1997; Yi, Feng, & Yu, 1988; Zhang et al., 2011). Traditionally, raw *Radix Rehmanniae* has been known to "reduce heat in blood, nourish yin and promote the production of body fluids", and used for treating maculation, nosebleeds, rash, and skin eruptions, while processed *Radix Rehmanniae* can "nourish yin and replenish blood, reinforcing essence and marrow", and has been used for treating anemia, diabetes, dizziness, tinnitus, nocturnal emission and palpitation (Chinese Pharmacopeia Commission, 2005).

UVB radiation is known to affect many biological processes, and is largely detrimental to individual organisms (Davies & Truscott, 2001; Longstreth et al., 1998; Ravanat, Douki, & Cadet, 2001). It has been reported that UVB radiation induces an increase in lipid peroxide (LPO) (Girotti, 1990, 2001), changes of antioxidant enzyme activities (Dreher & Maibach, 2001; Ichihashi et al., 2000; Shindo, Witt, & Packer, 1993), apoptosis (Aragane et al., 1998; Kulms et al., 1999) and sunburn cell formation in various animal skin or cultured cells (Aov et al., 1991; Bayerl, Taake, Moll, & Jung, 1995; Cope, Fabacher, Lieske, & Miller, 2001; Hofer & Mokri, 2000; Parrish, Anderson, Urbach, & Pitts, 1978; Teifke & Lohr, 1996). UVB radiation genemicees reactive oxygen species (ROS) and by this also induces cellular oxidative stress. Superoxide dismutase (SOD) and catalase (CAT) are among the most active scavengers of ROS, providing defence against cellular oxidative stress.

In the present study, we investigated optimal extraction parameters of *Radix Rehmanniae* polysaccharides using RSM. Then, we examined the antioxidant and immunity activities in UVB mice.

2. Materials and methods

2.1. Experimental design

A number of factors such as extraction temperature, extraction time and ratio of liquid to solid significantly affect the extraction efficiency. Therefore, a standard RSM design (Box–Behnken design) was used to identify the relationship between the response function (total amount of polysaccharides extracted) and the process variables (extraction temperature, extraction time and ratio of liquid to solid). The experimental range of the selected process

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Table 1
Design and results of central composition design.

Run	A	B	C	R
1	−1.00	−1.00	0.00	27.8
2	1.00	−1.00	0.00	34.05
3	−1.00	1.00	0.00	35.11
4	1.00	1.00	0.00	40.71
5	−1.00	0.00	−1.00	32.72
6	1.00	0.00	−1.00	40.31
7	−1.00	0.00	1.00	38.17
8	1.00	0.00	1.00	36.71
9	0.00	−1.00	−1.00	30.6
10	0.00	1.00	−1.00	42.75
11	0.00	−1.00	1.00	39.99
12	0.00	1.00	1.00	36.16
13	0.00	0.00	0.00	44.57
14	0.00	0.00	0.00	45.01
15	0.00	0.00	0.00	45.12
16	0.00	0.00	0.00	44.83
17	0.00	0.00	0.00	44.18

variables with their units and notation is given in Table 1. The response variable, polysaccharides (total amount of polysaccharides extracted) can be expressed as a function of the independent process variables according to the following response surface quadratic model.

A total of 17 experiments were performed in duplicate according to the Box–Behnken design matrix in Table 1 and the average values were used in data analysis. The experimental data were analyzed by the software, Design Expert Version 7.1.6 (Stat-Ease, USA). The adequacy of the developed model and statistical significance of the regression coefficients were tested using the analysis of variance (ANOVA). The interaction among the different independent variables and their corresponding effect on the response was studied by analyzing the response surface contour plots.

2.2. Animals and treatment

Six weeks old mice were purchased from the labomiceory animal center and animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed individually in a well-ventilated room maintained at 22 ± 2 °C, and 12 h light–dark cycle. The animals were divided into the normal control (n = 10) and the test group (n = 50). The test group was further divided into UVB group (n = 10) and four UVB + RRP groups (n = 40). Four UVB + RRP groups were orally fed with RRP (400 mg/kg b.w.) for 2 weeks, 4 weeks, 6 weeks and 8 weeks, respectively. RRP was diluted in the saline. The normal and UVB groups were orally fed with equal volume of saline. Then, the backs of the animals were exposed to UVB (180 mJ/cm^2) radiation for 24 h. Animals were sacrificed at the end-point of this experiment, skin tissues were isolated immediately and stored at −30 to −80 °C for further analysis.

2.3. Biochemical parameters assaying

IL-2, IL-4, IL-10 and GSH contents in skin were determined with enzymatic method kits.

Tissues lipid peroxidation was assessed by the complex formed between malondialdehyde (MDA) and thiobarbituric acid (TBA) (Ohkawa, Ohishi, & Yagi, 1979). Briefly, skin tissue (0.5 g) was homogenized with 4.5 mL of KCl (1.15%). The homogenate (100 μL) was mixed with 0.1 mL sodium dodecylsulfate (SDS) (8.1%), 750 μL acetic acid (20%) and 750 μL TBA reagent (0.8%). The reaction mixture was heated at 95 °C for 60 min. After heating, the tubes were cooled and 2.5 mL of n-butanol:pyridine (15:1) was added. After mixing and centrifugation at 4000 × g for 10 min, the upper phase was taken for measurement at 532 nm.

The activity of superoxide dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 mL reaction mixture contained 100 mM Tris/HCl (pH 7.8), 75 mM NBT, 2 μM riboflavin, 6 mM EDTA, and 200 μL of supernatant. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the micee of NBT reduction by 50% as described by Winterbourn, Hawkins, Brian, and Carrell (1975).

The enzyme catalase converts H_2O_2 into water. In brief, 0.25 g of tissue was homogenated in 1 mL of 50 mM Tris–HCl and centrifuged at 2000 × g for 15 min. Then 10 μL of supernatant was added to a quartz cuvette containing 980 μL of distilled water, and 10 μL of 0.066 M H_2O_2 (dissolved in sodium phosphate buffer) was added to start the reaction. The testicular CAT activity was measured spectrophotometrically at 240 nm by calculating the micee of degradation of H_2O_2, the substmicee of the enzyme (Xu, Yuan, & Lang, 1997). Activity of CAT is expressed as units/mg protein.

Glutathione peroxidase (GSH-Px) activity was analyzed by a spectrophotometric assay. A reaction mixture consisting of 1 mL of 0.4 M phosphate buffer (pH 7.0) containing 0.4 mM EDTA, 1 mL of 5 mM NaN_3, 1 mL of 4 mM GSH, and 0.2 mL of supernatant was preincubated at 37 °C for 5 min. Then 1 mL of 4 mM H_2O_2 was added and incubated at 37 °C for further 5 min. The excess amount of GSH was quantified by the DTNB method as described by Sharma and Gupta (2002). One unit of GSH-Px is defined.

2.4. Statistical analysis

All values in the figures and text were expressed as means ± SD. The results were analyzed by one-way ANOVA. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A p-value less than 0.05 was considered significant.

3. Results and discussion

According to the single-factor investigation results, the coded factor levels and real values of three factors (extraction temperature, extraction time and ratio of liquid to solid) are presented in Table 1. The 17 runs and the results of the response (polysaccharides production) in the factorial design are presented in Table 1.

With the Design Expert software, the ANOVA for response of variable polysaccharides production for the factorial design is shown in Table 1. The first-order model fitting the experiment results well was formulated as follows:

$$R_1 = +44.74+2.25 \times A+2.79 \times B + 0.58 \times C - 0.16 \times A \times B - 2.26 \times A \times C - 400 \times B \times C - 5.36 \times A^2 - 4.96 \times B^2 - 2.40 \times C^2 \quad (1)$$

The ANOVA of the linear regression model demonstmiceed that the model was highly significant from the evidence of the low p-value ($p > F$, <0.0001). The high determination coefficient (R^2 = 0.9815) proved the goodness fit of the model, suggesting that the sample variation of 98.15% for RRP was attributed to the variable factors. As can be seen from Table 2, the p-values of extraction temperature 0.0007 and extraction time 0.0002 were both significant, suggesting that extraction temperature and extraction time were the factors that greatly influenced polysaccharides production, while the ratio of liquid to solid was not greatly influenced factors because of the high p-values (>0.05). The value of the adjusted determination coefficient (adjusted R^2 = 0.9577) was also high, which advocated a high significance of the model (Table 3). Fig. 1(A)–(C) presents the plot of actual values and predicted values of polysaccharides production. From the above, it could be concluded that the model could

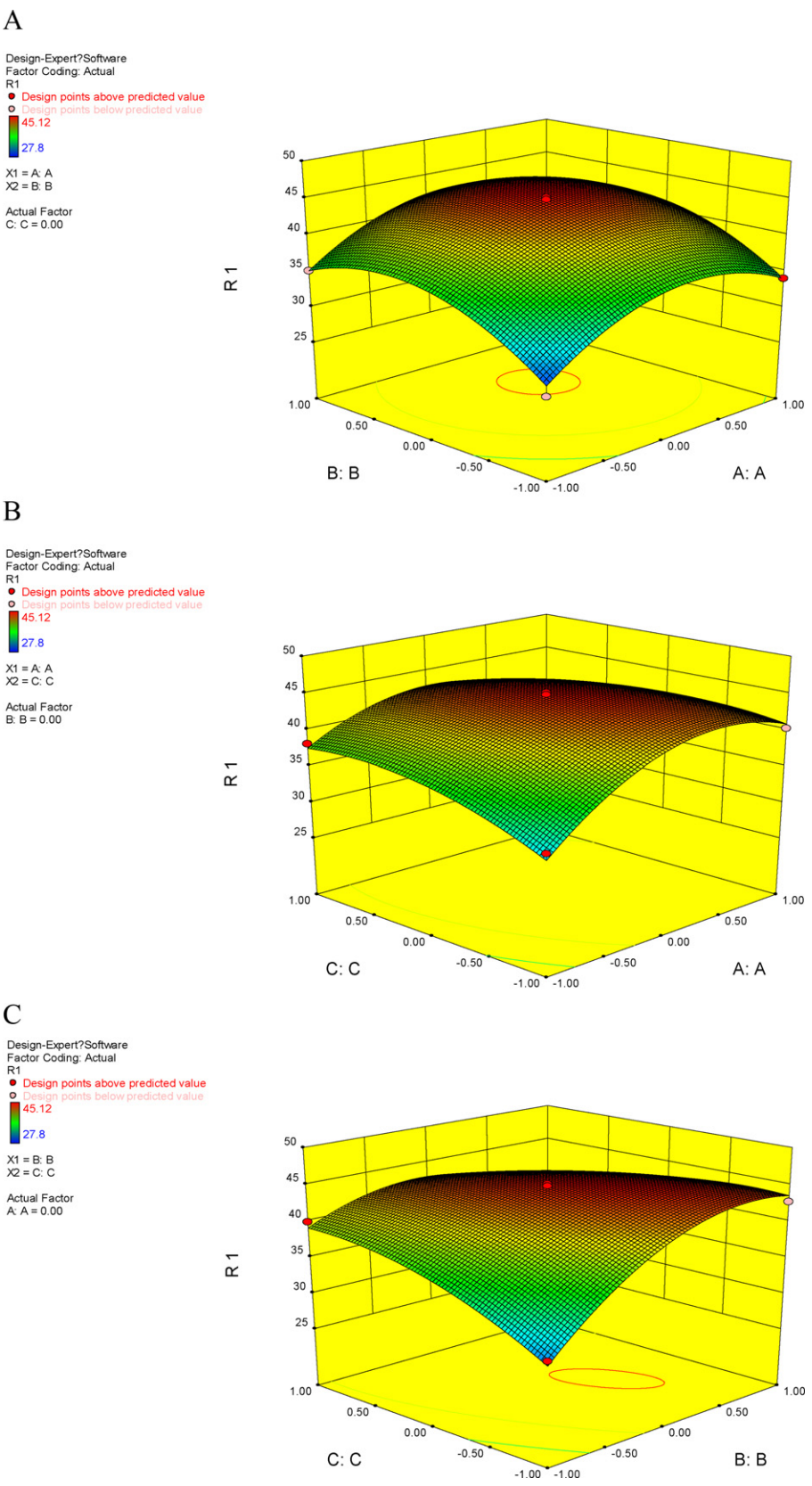

Fig. 1. Response surface (a) and contour plots (b) of the polysaccharides production with the extraction temperature and extraction time.

Table 2
ANOVA for response surface quadratic model analysis of variance.

Source	Sum of squares	*df*	Mean square	*F* value	*p*-Value prob > *F*	
Model	464.37	9	51.60	41.23	<0.0001	Significant
A-A	40.41	1	40.41	32.29	0.0007	
B-B	62.11	1	62.11	49.62	0.0002	
C-C	2.70	1	2.70	2.16	0.1851	
AB	0.11	1	0.11	0.084	0.7798	
AC	20.48	1	20.48	16.36	0.0049	
BC	63.84	1	63.84	51.01	0.0002	
A^2	121.01	1	121.01	96.69	<0.0001	
B^2	103.73	1	103.73	82.88	<0.0001	
C^2	24.32	1	24.32	19.43	0.0031	
Residual	8.76	7	1.25			
Lack of fit	8.19	3	2.73	19.24	0.0077	Significant
Pure error	0.57	4	0.14			
Cor total	473.13	16				

successfully predict the polysaccharides production and the obtained coefficients could be the direction miceio of the steepest ascent method.

IL-2 is a growth and activating factor for Th1 and Th2 cells (Robbins, Klassen, Rasmussen, Clayton, & Russ, 1986; Upham, McMenamin, Schon-Hegrad, Robinson, & Holt, 1994). The relationship and balance between IL-2 and IL-10 play a crucial role in operating cellular complex network in asthma and remain to be elucidated. Interleukin 4 (IL-4), a multifunctional cytokine secreted by Thelper 2 (Th2)-type lymphocytes, mast cells, basophils, and eosinophils, is implicated in the pathogenesis of allergic disease states (MacGlashan et al., 1994; Moqbel et al., 1995). This cytokine has several important immunomodulatory functions, such as the regulation of IgE synthesis, the development of Th2-type lymphocytes (Le Gros, Ben-Sasson, Seder, Finkelman, & Paul, 1990; Swain, Weinberg, English, & Huston, 1990) and enhancement of B-cell surface antigens such as the low-affinity receptor for IgE (Fc RII/CD23) and class II major histocompatibility complex molecules (Defrance et al., 1987). IL-10 is a potent regulatory cytokine that decreases inflammatory responses and protects airway from developing inflammatory responses to inhaled allergens (Moore, O'Garra, de Waal Malefyt, Vieira, & Mosmann, 1993; Umetsu & Dekruyff, 1999). It is now appreciated that human IL-10 is produced by Th0, Th1 and Th2 lymphocytes, although monocytes and tissue macrophages are important major sources of IL-10 (de Waal Malefyt, Abrams, Bennet, Figdor, & de Vries, 1991; Del Prete et al., 1993; Yssel et al., 1992). It inhibits the production of proinflammatory cytokines and chemokines by monocytes, T cells, neutrophils, and eosinophils (de Waal Malefyt et al., 1991; de Waal Malefyt, Yssel, & de Vries, 1993; Del Prete et al., 1993; Kasama, Strieter, Lukacs, Burdick, & Kunkel, 1994; Taga, Mostowski, & Giovanna, 1993; Takanashi et al., 1994).

In this study, the levels of plasma IL-2, IL-4 and IL-10 of control and experiment mice are presented in Table 4. A significant decrease in plasma IL-2, IL-4 and IL-10 levels was observed in UVB rats compared to normal control mice. A significant enhancement in the levels of plasma IL-2, IL-4 and IL-10 was observed in UVB + RRP groups animals compared to control animals. Moreover, plasma IL-2, IL-4 and IL-10 levels increased with increasing time of RRP pretreatment.

As the outermost organ of the body, the skin is frequently and directly exposed to a prooxidative environment, including ultraviolet radiation, drugs, and air pollutants. Besides external inducers of oxidative attack, the skin has to cope with endogenous generation of reactive oxygen species (ROS) and other free radicals, which are continuously produced during physiological cellular metabolism. To counteract the harmful effects of ROS, the various compartments of the skin (stratum corneum/skin barrier, epidermis, dermis, subcutis) are equipped with layerspecific antioxidant systems, which help to maintain an equilibrium between ROS and antioxidants and thus prevent oxidative stress. Previous report (Hayashi, Kayasuga, Nagao, & Miwa, 2000) showed that the application of UV-A and UV-B was caused by the production of free-radicals in the nucleus.

To cope with the increasing oxidative stress, skin has developed two groups of antioxidant systems. The first group is composed of several enzymes including superoxide dismutase, catalase, peroxidase, and glutathione reductase. The second group is composed of many low-molecular-weight antioxidants (LMWAs) such as GSH, NADH, carnosine, uric acid, carotene, polyphenols, and lipoic acid (Kohen & Gati, 2000; Rieger & Pains, 1993; Schalbreuter & Wood, 1989; Shindo et al., 1993). They can scavenge ROS by donating electrons. SOD is an enzyme that primarily contributes to cellular defenses against oxidative stress, and plays a role in the conversion of superoxide anion to hydrogen peroxide. CAT is an enzyme that protects the body from active oxygen-induced oxidative damage by converting endogenous H_2O_2 to H_2O. It is thought that the increase in CAT activity was induced in order to degrade hydrogen peroxide produced by SOD activity. Human skin has an inherent antioxidant capacity to reduce the potential damage caused by free radicals. This inherent capacity can be significantly depleted by moderate UV light exposure (Shindo, Witt, Han, & Packer, 1994; Thiele, Dreher, & Packer, 2000). The loss of this natural epidermal and dermal antioxidant capacity may be counteracted by topical and systemic administration of antioxidants to the skin (Aust, Stahl, Sies, Tronnier, & Heinrich, 2005; Mathews-Roth et al., 1972; Pillai, 2005).

Table 3
ANOVA results for the quadratic equation of Design-Expert 6.0 for the response.

Std. dev	1.12	R^2	0.9815
Mean	38.75	Adj R^2	0.9577
C.V. %	2.89	Pred R^2	0.7211
Press	131.97	Adeq precision	18.089

Table 4
Serum IL-2, IL-4 and IL-10 levels in different groups.

Group	IL-2 (ng/mL)	IL-4 (ng/mL)	IL-10 (ng/mL)
NC	7.87 ± 0.49	25.74 ± 1.85	165.39 ± 10.63
UVB	3.88 ± 0.22[b]	13.96 ± 1.25[b]	88.48 ± 5.49[b]
UV + RRP (2 weeks)	4.93 ± 0.42[c]	16.09 ± 1.37[c]	101.42 ± 6.95[d]
UV + RRP (4 weeks)	6.02 ± 0.53[d]	19.11 ± 1.72[d]	118.75 ± 9.61[d]
UV + RRP (6 weeks)	6.91 ± 0.56[d]	20.64 ± 1.99[d]	128.41 ± 11.25[d]
UV + RRP (8 weeks)	7.42 ± 0.71[d]	21.09 ± 2.05[d]	139.64 ± 12.63[d]

[b] $p < 0.01$, compared with group NC group.
[c] $p < 0.05$, compared with UVB group.
[d] $p < 0.01$, compared with UVB group.

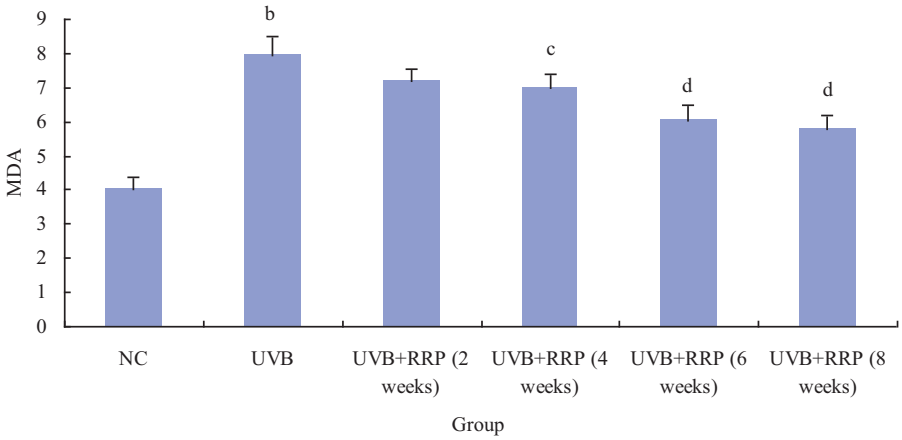

Fig. 2. Skin MDA level in different groups: $^{b}p < 0.01$, compared with group NC group; $^{c}p < 0.05$, $^{d}p < 0.01$, compared with UVB group.

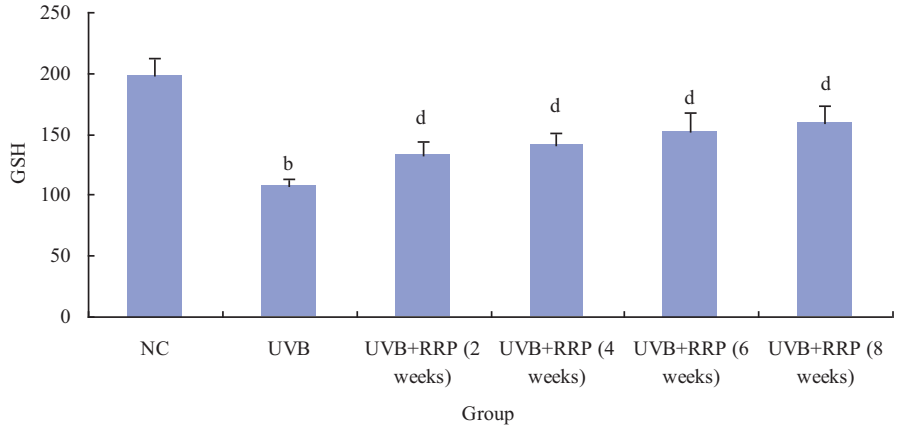

Fig. 3. Skin GSH level in different groups: $^{b}p < 0.01$, compared with group NC group; $^{d}p < 0.01$, compared with UVB group.

The levels of skin MDA of control and experiment rats are presented in Fig. 2. A significant increase in skin MDA levels was observed in UVB rats compared to normal control rats. A significant decrease in the levels of skin MDA was observed in UVB + RRP groups animals compared to control animals. Moreover, skin MDA levels decreased with increasing time of RRP pretreatment.

Skin glutathione (GSH) level of UVB group rats was significantly decreased ($p < 0.01$) than normal control group. UVB + RRP-pretreated groups showed significant restoration of GSH level. With increasing RRP-pretreatment time, skin GSH level continuously increased in UVB + RRP groups (Fig. 3).

Skin SOD, CAT and GSH-Px activities were significantly decreased in the UVB group compared to the NC group as shown in Fig. 4. There was a significant increase in skin SOD, CAT and GSH-Px activities by pre-administration of the 400 mg/kg RRP in UVB + RRP groups compared to UVB group.

Therefore, it is considered that the protective effect of RRP against UVB-induced skin injury may be associated with the scavenging of free-radicals.

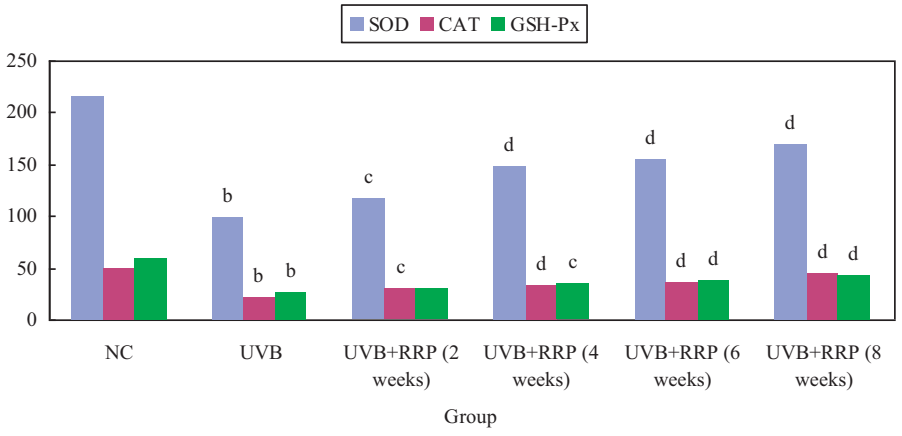

Fig. 4. Skin SOD, CAT and GSH-Px activities in different groups: $^{b}p < 0.01$, compared with group NC group; $^{c}p < 0.05$, $^{d}p < 0.01$, compared with UVB group.

4. Conclusion

Optimum extraction conditions for maximizing the determination of *Radix Rehmanniae* polysaccharides were: extraction temperature 100 °C, extraction time 2 h and ratio of liquid to solid 6. *Radix Rehmanniae* polysaccharides extract may prove to be a useful therapeutic option in the reversal of skin diseases.

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