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ORIGINAL ARTICLE

Cost effective quantification of picrosides in *Picrorhiza kurroa* by employing response surface methodology using HPLC-UV

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Abstract This paper reports the optimum conditions for the extraction of picrosides from *Picrorhiza kurroa* using response surface methodology (RSM). A central composite design (CCD) was used to investigate the effects of four independent variables, namely methanol concentration (%), solid-to-liquid ratio (g/mL), incubation time (min) and temperature (°C) on the extraction yield of picroside I (P-I) and picroside II (P-II) quantified by high performance liquid chromatography with ultraviolet detection (HPLC-UV). The cumulative effects of independent variables were studied and the optimal extraction conditions were obtained as methanol concentration (0 %), solid-to-liquid ratio (1:120 g/mL), incubation time (30 min) and temperature (30 °C). The picrosides were extracted under optimum conditions to check the validity of the model. The experimental values agreed with the predicted ones, indicating suitability of the model employed and the success of RSM in optimizing the extraction conditions. The significant achievement of the present work lies in the fact that distilled water rather than methanol, a hazardous and expensive solvent, is an effective and highly selective solvent for the extraction of picrosides from *P. kurroa* which makes this process economical and environment friendly.

Keywords Response surface method · Central composite design · Picrosides · *Picrorhiza kurroa* · HPLC-UV · Extraction

Abbreviations

HPLC-UV	High Performance Liquid Chromatography with Ultraviolet Detection
RSM	Response Surface Methodology
CCD	Central Composite Design
P-I	Picroside-I
P-II	Picroside-II

Introduction

P. kurroa, family Scrophulariaceae, is an endangered plant species which has commodious applications in medicine. The International Union for Conservation of Nature and Natural Resources (IUCN) listed this plant species as 'endangered' due to its voluminous harvesting and lack of organized cultivation (Nayar and Sastri 1990). The active constituents of the herb are picrosides, the terpenoids with an iridane skeleton of monoterpene origin, which have ample usage in medicine due to their pharmacological properties like hepatoprotective and immunomodulatory activity (Dwivedi et al. 1993; Puri et al. 1992; Shukla et al. 1991), antioxidant and anticancerous activity particularly in liver (Joy and Kuttan 1995; Joy et al. 2000) and antiallergic and antiasthmatic activity (Dorsch et al. 1991). Therefore, it is necessary to establish an optimum extraction procedure for picrosides from *P. kurroa*.

Generally, optimization of a process could be achieved by either classical or statistical methods. The classical method, which involves variation of one independent factor at-a-time keeping all other factors constant, is time-consuming and

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incapable of detecting the true optimum conditions, especially due to the interactions among the factors (Liu and Tzeng 1998). Statistical experimental designs have been in use for several decades (Box and Hunter 1957; Plackett and Burman 1946). One of these designs viz. response surface methodology (RSM), first introduced by Box and Wilson (Box and Wilson 1951), has now been established as a convenient method for developing optimum processes with precise conditions along with minimized production cost by efficient screening of process parameters (Vohra and Satyanarayana 2002). The extraction efficacy can be significantly influenced by many factors viz. solvent concentration, extraction time, temperature (Wettasinghe and Shahidi 1999; Xu et al. 2013), and solvent to solid ratio (Cacace and Mazza 2003). As a package of statistical and mathematical techniques, RSM can reduce the number of experiments and provide a mathematical model which can be effectively used to evaluate the effects of multiple factors and their interaction on one or more response variables (Myers and Montgomery 2002). Unfortunately, there is a paucity of literature available on the statistical methods used in the optimization of picroside extraction from *P. kurroa*. Thus, in the present study, central composite design (CCD) and RSM analyses were carried out to optimize the critical factors for realizing the extraction of picrosides from *P. kurroa* through HPLC-UV. To the best of our knowledge, this is the first report on the optimization of picroside extraction from *P. kurroa* using RSM.

Materials and methods

Plant material

P. kurroa rhizome samples were collected from plants grown in nursery (Sairopa, Himachal Pradesh, 4,500 m altitude, 31°38′–31°54′ N and 77°20′–77°45′ E) and a voucher sample has been submitted to HFRI (Himalayan Forest Research Institute, Shimla, HP, INDIA) with Herbarium Acc. No. 0670. Samples were thoroughly washed under running tap water and segregated as shoots, rhizome and roots. The mature rhizome sections were dried and then ground into a fine powder in liquid nitrogen using a pestle mortar.

Extraction of picrosides

Picrosides were extracted from the powdered samples (100 mg of each) using different concentrations of methanol (0–90 %) with a solid–liquid (g/mL) ratio ranging from 1:50 to 1:150. The samples were vortexed for proper mixing and sonicated for a time period ranging from 10 to 50 min with the temperature of the sonicator ranging from 15 to 45 °C and were kept steady by putting ice in it (within±1.0 °C). Samples were then centrifuged at 10,000 × g for 15 min. The supernatants were filtered through 0.22 μm filter (Millipore) and diluted 10-fold with 80 % methanol. An aliquot (20 μL) of the above samples was injected into HPLC for analysis.

HPLC-UV analysis

Picroside-I (P-I) and Picroside-II (P-II) in *P. kurroa* were quantified through HPLC analysis as per the method of Pandit et al. (2012).

Statistical optimization of picroside extraction

To examine the cumulative effect of extraction parameters viz. methanol concentration (%), solid–liquid ratio (g/mL), incubation time (min.) and temperature (°C), response surface methodology was employed using a statistical software package Design Expert® 8.0.7.1, Stat-Ease, Inc. A 2^4 full factorial central composite design (CCD) with 16 trials for factorial design, 8 trials for axial point and 6 replicate trials at the central point, leading to a set of 30 experiments was designed. All the variables were coded as '0' at the central point. The response value from each experiment of CCD was the average of triplicates.

Results and discussion

HPLC-UV analysis

To validate the statistical experimental strategies, experiments were performed in triplicates under selected conditions along with one control sample processed under the same conditions as mentioned by Pandit et al. (2012) with the aim of monitoring the increase in extraction yield as compared to the previously obtained yield. HPLC-UV analysis was performed to identify the two picrosides, P-I and P-II, by comparing their retention times against those of known standards. The extraction yield was calculated and HPLC chromatograms of the standards and samples are shown in Fig. 1.

Analysis of single factor

Effect of methanol concentration

The effect of methanol concentration was determined by reflux extraction of 100 mg of powdered sample with 10 mL of methanol solution at different concentrations ranging from 0–90 % (0, 10, 30, 50, 70 and 90 %). The

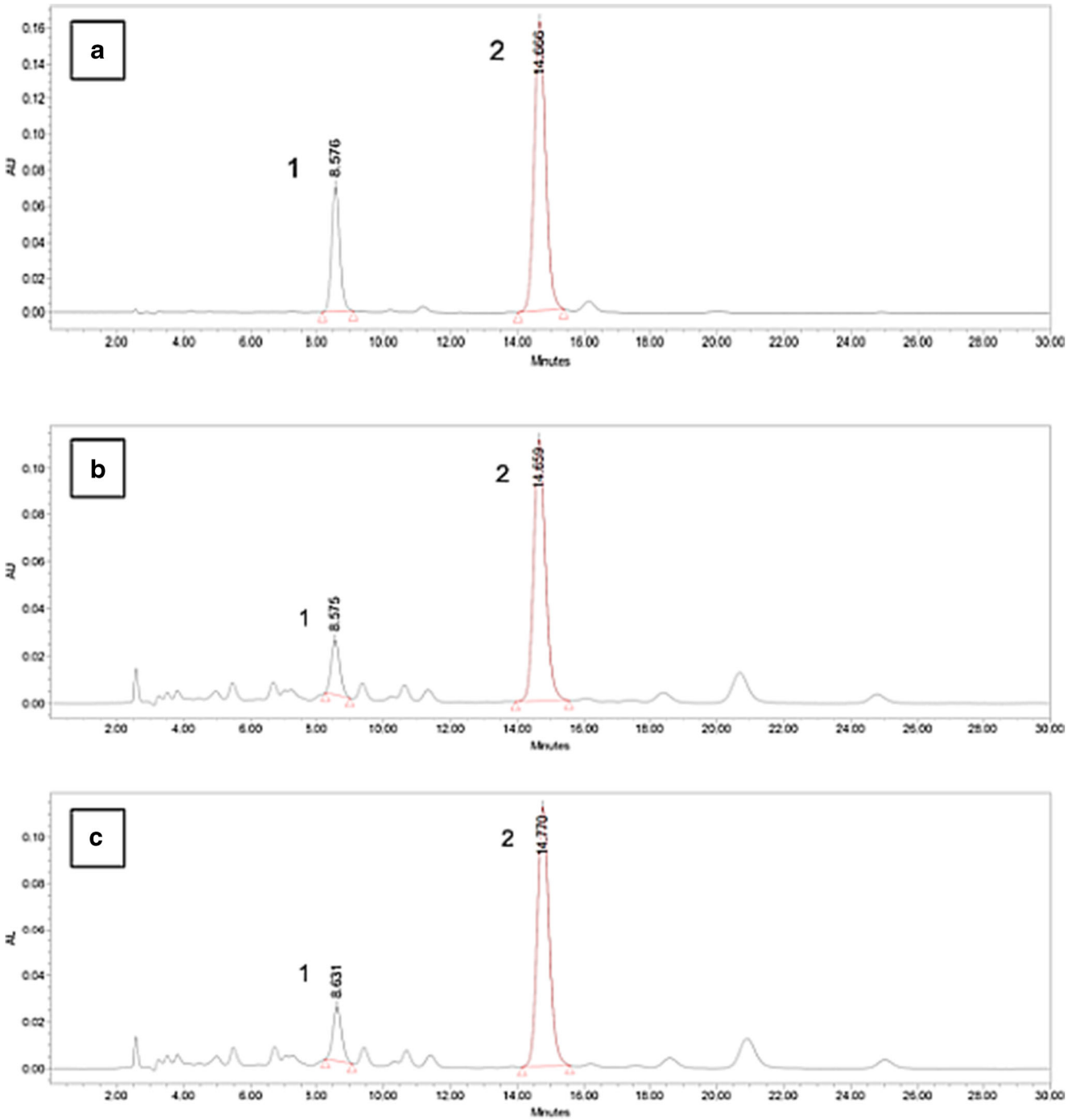


Fig. 1 HPLC chromatograms of the picrosides I and II: (**a**) Standards (1: Picroside II, 2: Picroside I), (**b**) Control (methanol 80 %, solid-to-liquid ratio 10 g/mL, incubation time 30 min and temperature 30 °C) and (**c**) Test (methanol 0 %, solid-to-liquid ratio 10 g/mL, incubation time 30 min and temperature 30 °C)

process was carried out at 25 °C for 30 min in the sonicator. The extracts were filtered and then diluted 10-fold with 80 % methanol. The extract yield was determined according to the methods above and results were shown in Supplementary Figs. 1a and 2a. It was evident from Supplementary Fig. 1a that the extraction yield of P-I decreased with increase in methanol concentration reaching a minimum at 60 % methanol, followed by an increase with further increase in methanol concentration. In contrast to this, Supplementary Fig. 2a showed that the extraction yield of P-II congruently decreased with an increase in methanol concentration. So, considering the cost and the yield, 0 % methanol i.e. pure distilled water should be adopted for picroside extraction.

Effect of solid-to-liquid ratio

The effect of solid-to-liquid ratio on picroside yield was determined by reflux extraction of 100 mg powdered sample with 80 % methanol solution by varying the solid-to-liquid ratio from 1:50 to 1:150 (1:50, 1:70, 1:90, 1:110, 1:130 and 1:150). The extraction and quantification of picrosides was carried out as described above and results are shown in Supplementary Figs. 1b and 2b. The data showed a significant increase in the extraction yield of both P-I and P-II with increasing ratios of liquid-to-solid from 1:50 to 1:110 followed by a decline. Therefore, the optimum solid-to-liquid ratio for the extraction of picrosides was 1:110.

Effect of incubation time

The effect of incubation time was determined by reflux extraction of 100 mg powdered sample with 10 mL of 80 % methanol by performing the extraction in the sonicator at 25 °C for 0, 10, 30, 50 and 70 min. The extracts were filtered and then diluted 10-fold with 80 % methanol. The picroside yield was determined according to the methods described above and results are shown in Supplementary Figs. 1c and 2c. The data revealed a significant increase in the extraction yield of both P-I and P-II with increasing extraction time from 0 to 50 min followed by a decrease. Therefore, the optimum extraction time chosen should be 50 min.

Effect of temperature

The effect of temperature was examined by reflux extraction of 100 mg powdered sample with 10 mL of 80 % methanol by carrying out the extraction at 0 °, 15 °, 25 °, 35 °, 45 ° and 60 °C for 30 min in a sonicator. The extracts were filtered and then diluted 10-fold with 80 % methanol. The extract yield was determined and results are shown in Supplementary Figs. 1d and 2d. It was concluded from the results that extraction yield of both P-I and P-II significantly increased with raising temperature from 0 ° to 45 °C but decreased on further increase in temperature. Therefore, the optimum temperature was taken as 45 °C.

Analysis of the fitted model

The four factors and lower, middle and upper design points for RSM along with the experimental results of extraction yield are shown in Table 1. Multiple regression equations were generated relating response variable to the coded levels of the independent variables. Multiple regression coefficients were determined by employing least squares technique (Myers and Montgomery 2002) to predict the polynomial quadratic equation for the extraction yield of P-I and P-II. The overall polynomial equation for P-I and P-II was

Picroside I (μg/mg) = $+49.95–0.80A+4.40B+1.24C+0.78D – 0.044AB+0.51\ AC+0.23\ AD+0.36BC+0.23BD – 0.46CD+1.47A^2 – 6.10B^2 – 0.47C^2+0.62D^2$

Picroside II (μg/mg) = $+25.41–0.45A+1.66B+0.59C+0.25D – 0.67AB+1.09\ AC+0.35\ AD+0.19BC+0.18BD+0.13CD+0.28A^2 – 3.41B^2 – 0.35C^2+0.15D^2$

Where A is methanol concentration (%), B is solid-to-liquid ratio (g/mL), C is incubation time (min) and D is temperature (°C).

Statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA), which showed that the regression was statistically significant. The model F-value for extraction yield of P-I and P-II was 2.64 and 2.53, respectively as shown by Fisher's test, which indicated that the model was significant. The value of "Prob > F" less than 0.05 was desirable for a significant model. The Prob > F value of the model was <0.05 both in case of P-I and P-II, respectively; therefore, the model terms were also significant (Table 2). There was only a 3.60 and 4.27 % chance in case of P-I and P-II, respectively, that a "Model F-Value" this large could occur due to noise. The "Adeq Precision" measures the signal to noise ratio (a ratio of greater than 4 was desirable) and the ratio of 8.344 and 7.497, of the P-I and P-II model, respectively, indicated an adequate signal. A lower value of coefficient of variation (CV) for P-I (14.87 %) and P-II (15.76 %) showed the experiments conducted were precise and reliable (Du 2003). The coefficient of determination (R^2) explains the level of variability in the model. Le et al. (2010) and Chauhan and Gupta (2004) have emphasised that the R^2 should be >0.75 but in contrast to this, the R^2 of our model was 0.71 (P-I) and 0.70 (P-II). The linear variable X_2 and quadratic variable X_2^2 were statistically very significant at $p<0.05$ whereas the linear variables X_1, X_3, X_4; quadratic variables X_1^1, X_3^3, X_4^4 and the two-variable interaction X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4 had no significant influence ($P>0.1$) on the extraction yield of picrosides.

Analysis of RSM

The three dimensional (3D) and contour plots (2D) were the graphical representations helpful in observing interaction among the variables. The 3D plots are represented in Supplementary Fig. 3 (P-I) and Supplementary Fig. 4 (P-II) while the contour plots are shown in Fig. 2 (P-I) and Fig. 3 (P-II). The cumulative effect of independent variables (methanol concentration, solid-to-liquid ratio, incubation time and temperature) was obtained by keeping two of the variables constant. As shown in Fig. 2a, the cumulative effect of two factors viz. methanol concentration and solid-to-liquid ratio revealed maximum extraction yield of P-I at low and high levels of methanol concentration, and minimum yield at low and high levels of solid-to-liquid ratio. It was clear from the contour plot that maximum yield of P-I was obtained when 12 mL of

Table 1 Factors and levels in the response surface central composite design arrangement along with the experimental and predicted results

N0.	X1	*X*2	X3	X4	Experimental		Predicted	
					P-I (μg/mg)	P-II (μg/mg)	P-I (μg/mg)	P-II (μg/mg)
1	45	10	30	30	49.98	25.54	49.95	25.41
2	90	5	10	45	45.25	23.13	40.14	19.44
3	90	15	10	45	47.98	20.94	48.60	21.40
4	45	10	30	60	49.68	24.42	53.96	26.50
5	90	15	50	45	49.31	24.06	51.91	25.40
6	0	15	50	45	49.44	24.15	52.13	24.74
7	90	15	50	15	47.88	22.68	50.35	23.58
8	90	5	50	15	44.35	23.48	41.38	21.57
9	45	20	30	30	44.15	19.27	34.34	15.06
10	45	10	30	30	49.96	25.39	49.95	25.41
11	0	15	10	45	51.28	25.40	50.86	25.11
12	0	5	10	45	48.25	23.45	42.22	20.49
13	0	5	50	45	46.13	20.63	42.05	19.34
14	45	10	30	30	49.93	25.44	49.95	25.41
15	45	10	70	30	50.27	25.32	50.57	25.17
16	45	10	30	30	49.94	25.36	49.95	25.41
17	90	5	50	45	47.82	25.27	42.00	22.67
18	45	10	30	0	48.21	23.37	50.85	25.51
19	135	10	30	30	50.82	23.26	54.21	25.64
20	45	0	30	30	0	0	16.73	8.43
21	90	5	10	15	43.94	21.50	37.68	18.86
22	45	10	30	30	49.96	23.37	49.95	25.41
23	0	15	50	15	49.76	22.84	51.48	24.33
24	45	10	30	30	49.93	25.36	49.95	25.41
25	0	5	50	15	46.52	22.17	42.33	19.66
26	90	15	10	15	44.52	21.00	45.21	20.06
27	0	5	10	15	46.66	24.86	40.67	21.32
28	−45	10	30	30	53.88	25.60	52.21	26.14
29	0	15	10	15	46.12	24.67	48.37	25.22
30	45	10	−10	30	38.97	18.46	47.03	23.74
Control	80	10	30	30	48.21	24.37	50.21	25.23

X1 Methanol Conc. (%), X2 Solid:Liquid ratio (g/mL), X3 Incubation time (min), X4 Temperature (deg C)

Distilled water was used against 0 and −45 % methanol concentrations

Control sample was processed under unoptimized conditions as mentioned by Pandit et al. (2012)

pure distilled water was used for the extraction. Further, on increasing or decreasing the volume of the solvent, the yield of P-I was decreased. In contrast to this, Fig. 3a showed that the extraction yield of P-II was maximum at low levels of methanol concentration while minimum at low and high levels of solid-to-liquid ratio. However, it was not significant in both P-I and P-II that the increase of methanol concentration affected the extraction yield at certain solid–liquid ratio.

The results shown in Supplementary Fig. 5 indicated that the mutual interactions between methanol concentration and incubation time were not significant as the increase of the methanol concentration did not affect the extraction yield of both P-I and P-II at a certain value of incubation time period and vice versa. In addition, the cumulative effect of methanol concentration and temperature was not significant as either the surface was completely flat (Supplementary Figs. 3 and 4) or showed the complete redness (Supplementary Fig. 6). From Figs. 2b and 3b, it was concluded that when incubation time was at a certain value, the extraction yield of both P-I and P-II increased with the increase of solid–liquid ratio. However, it

Table 2 Analysis of variance (ANOVA) for the quadratic polynomial mode

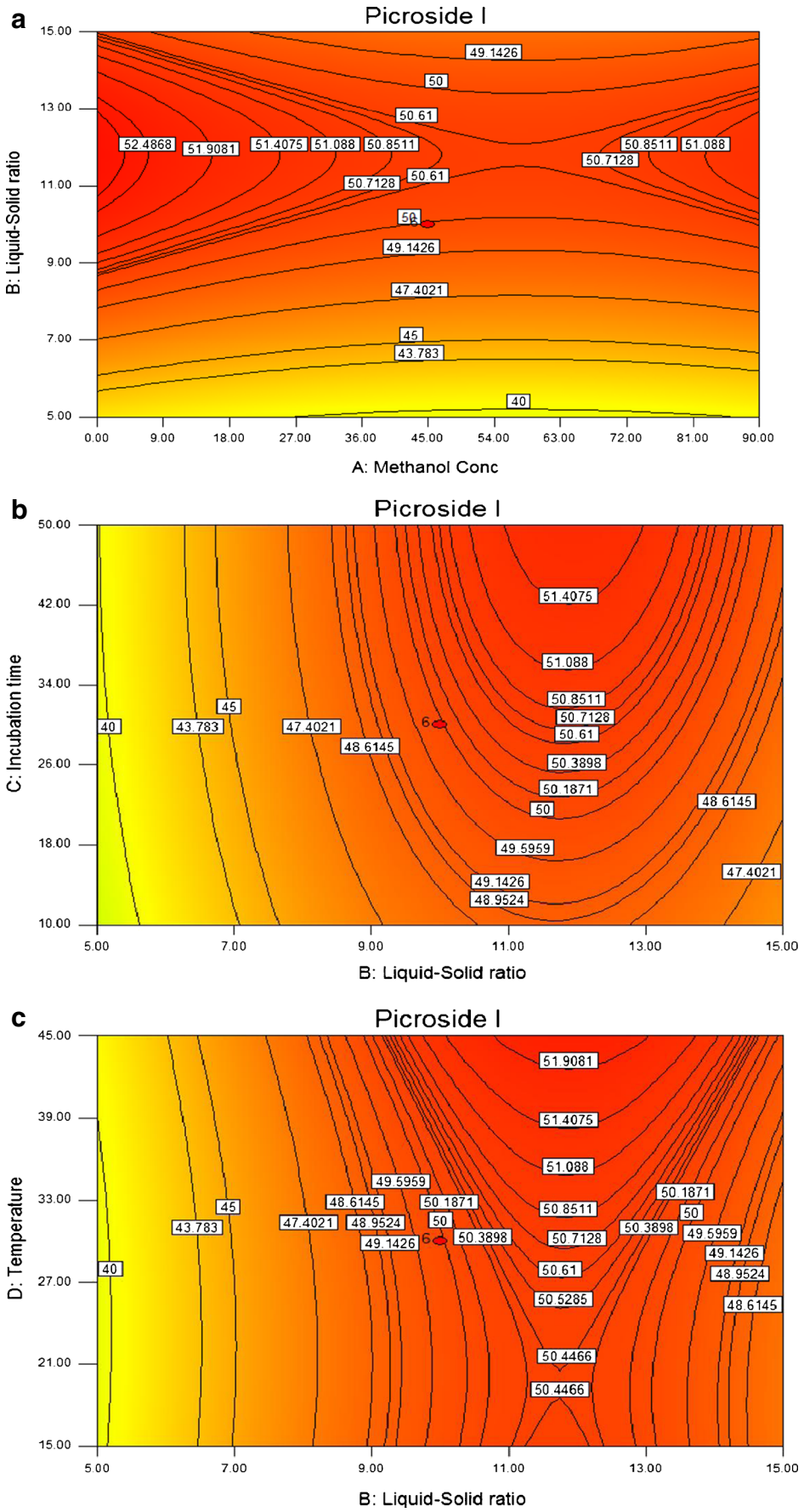
Source	Sum of Squares		df	Mean Square		F Value		p-value Prob > F	
	P-I	P-II		P-I	P-II	P-I	P-II	P-I	P-II
Model	1,758.44	454.95	14	125.60	32.50	2.64	2.53	0.0360*	0.0427*
A-Methanol Conc.	15.41	4.85	1	15.41	4.85	0.32	0.38	0.5776	0.5483
B-Liquid–solid ratio	465.26	65.97	1	465.26	65.97	9.79	5.13	0.0069**	0.0387*
C-Incubation time	37.03	8.23	1	37.03	8.23	0.78	0.64	0.3914	0.4363
D-Temperature	14.49	1.47	1	14.49	1.47	0.30	0.11	0.5890	0.7404
AB	0.032	7.09	1	0.032	7.09	6.627E-004	0.55	0.9798	0.4692
AC	4.13	19.16	1	4.13	19.16	0.087	1.49	0.7722	0.2410
AD	0.82	2.00	1	0.82	2.00	0.017	0.16	0.8970	0.6992
BC	2.08	0.60	1	2.08	0.60	0.044	0.047	0.8371	0.8313
BD	0.88	0.52	1	0.88	0.52	0.018	0.041	0.8937	0.8430
CD	3.36	0.26	1	3.36	0.26	0.071	0.020	0.7940	0.8883
A^2	59.04	2.22	1	59.04	2.22	1.24	0.17	0.2826	0.6837
B^2	1,021.14	319.74	1	1,021.14	319.74	21.48	24.87	0.0003***	0.0002***
C^2	5.94	3.37	1	5.94	3.37	0.12	0.26	0.7287	0.6161
D^2	10.41	0.62	1	10.41	0.62	0.22	0.048	0.6466	0.8287
Residual	713.15	192.87	15	47.54	12.86				
Lack of Fit	713.15	192.84	10	71.31	19.28	1.783E+005	3,887.91	<0.0001 ***	<0.0001 ***
Pure Error	2.000E-003	0.025	5	4.000E-004	4.960E-003				
Cor Total	2,471.59	647.82	29						

*Significant; *** Extremely Significant

was not significant that the increase of the incubation time affected the extraction yield of both P-I and P-II at a certain value of solid-to-liquid ratio. The cumulative effect of solid-to-liquid ratio and temperature shown in Figs. 2c and 3c revealed that at a certain temperature value, the extraction yield of both P-I and P-II increased with an increase in solid-to-liquid ratio while no significant increase was observed upon increasing the temperature at certain value of solid-to-liquid ratio. Also, Supplementary Fig. 7 revealed that incubation time and temperature did not display the quadratic effect on responses as the mutual interactions between them were not significant.

Overall, the response surface data showed that the variation of solid-to-liquid ratio resulted in a noticeable increase in the extraction yield of P-I and P-II whereas methanol concentration, incubation time and temperature had no significant effect on the picroside yield. Using RSM optimized conditions (methanol concentration 0 %, solid: liquid ratio as 1:110 g/mL, incubation time 30 min and temperature 30 °C) for extraction of picrosides from *P. kurroa*, the maximum experimental yield of P-I and P-II was 53.88 and 25.60 μg/mg, respectively which was higher than the corresponding values of 48.21 and 24.37 μg/mg obtained under unoptimized conditions (control). The optimum extraction conditions predicted by the point prediction option in the software were the same except that the solid: liquid ratio was 1:120 g/mL, which would result in maximum yield of picrosides. In contrast to the previous studies (Pandit et al. 2012; Bhandari et al. 2008; Singh et al. 2011), which documented the need of methanol in the picroside extraction, our results came with some important finding of omitting methanol from the extraction procedure. By keeping in view the economy and environmental hazards, this finding meets the commercial needs. In addition, our results showed that solvent concentration was the most important factor contributing to the extraction of picrosides using RSM. This is in congruence with the previous report given by Park et al. (1998) who found that solvent concentration plays a critical role in the extraction of soluble solids from various natural products. The present study also concluded that the picroside extraction did not need specific temperature conditions and extended incubation time; the extraction could easily be carried out at room temperature and less incubation time. This is supported by a number of studies which showed that the picroside extraction was done at room temperature and for an ordinary incubation time period (Singh et al. 2011; Kawoosa et al. 2010).

Fig. 2 **a** Contour plot (2D) showing the effect of methanol concentration and liquid–solid ratio on the yield of Picroside I. **b** Contour plot showing the effect of liquid–solid ratio and incubation time on the yield of Picroside I. **c** Contour plot showing the effect of liquid–solid ratio and temperature on the yield of Picroside I


a
Picroside I
B: Liquid-Solid ratio
A: Methanol Conc
15.00
13.00
11.00
9.00
7.00
5.00
0.00
9.00
18.00
27.00
36.00
45.00
54.00
63.00
72.00
81.00
90.00
49.1426
50
50.61
52.4868
51.9081
51.4075
51.088
50.8511
50.7128
47.4021
45
43.783
40
b
Picroside I
C: Incubation time
B: Liquid-Solid ratio
50.00
42.00
34.00
26.00
18.00
10.00
5.00
7.00
9.00
11.00
13.00
15.00
51.4075
51.088
50.8511
50.7128
50.61
50.3898
50.1871
50
49.5959
49.1426
48.9524
48.6145
47.4021
45
43.783
40
c
Picroside I
D: Temperature
B: Liquid-Solid ratio
45.00
39.00
33.00
27.00
21.00
15.00
5.00
7.00
9.00
11.00
13.00
15.00
51.9081
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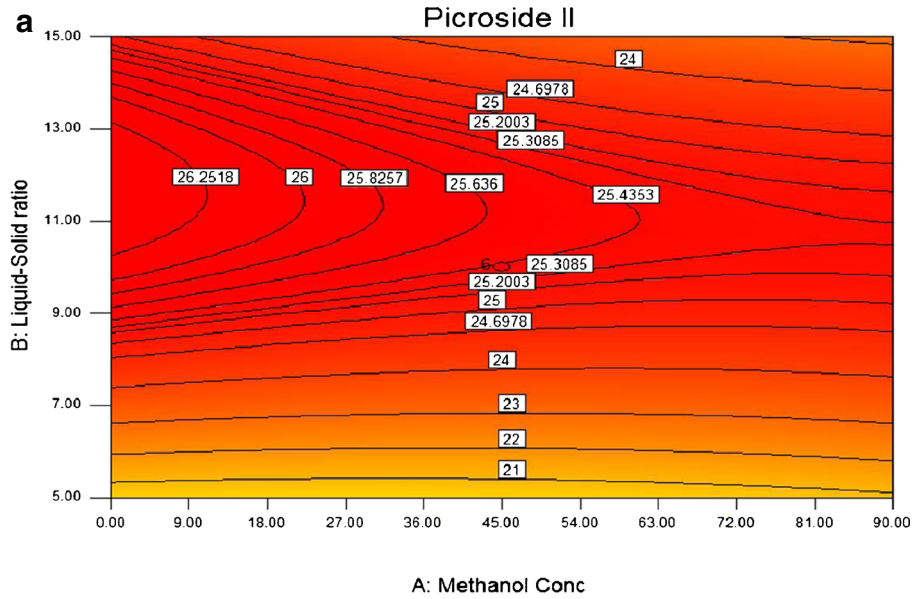
a
Picroside II
B: Liquid-Solid ratio
A: Methanol Conc
15.00
13.00
11.00
9.00
7.00
5.00
0.00
9.00
18.00
27.00
36.00
45.00
54.00
63.00
72.00
81.00
90.00
24
24.6978
25
25.2003
25.3085
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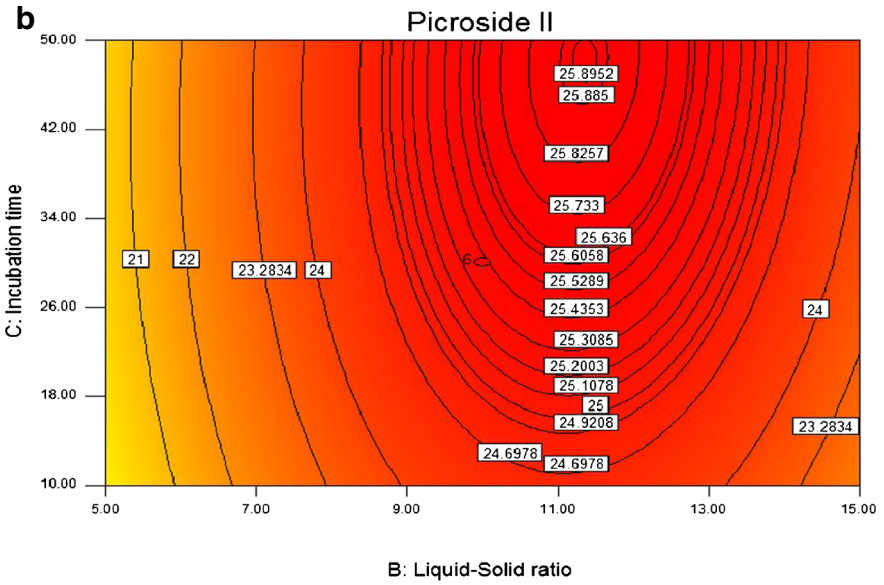
22
21


b
Picroside II
C: Incubation time
B: Liquid-Solid ratio
50.00
42.00
34.00
26.00
18.00
10.00
5.00
7.00
9.00
11.00
13.00
15.00
25.8952
25.885
25.8257
25.733
25.636
25.6058
25.5289
25.4353
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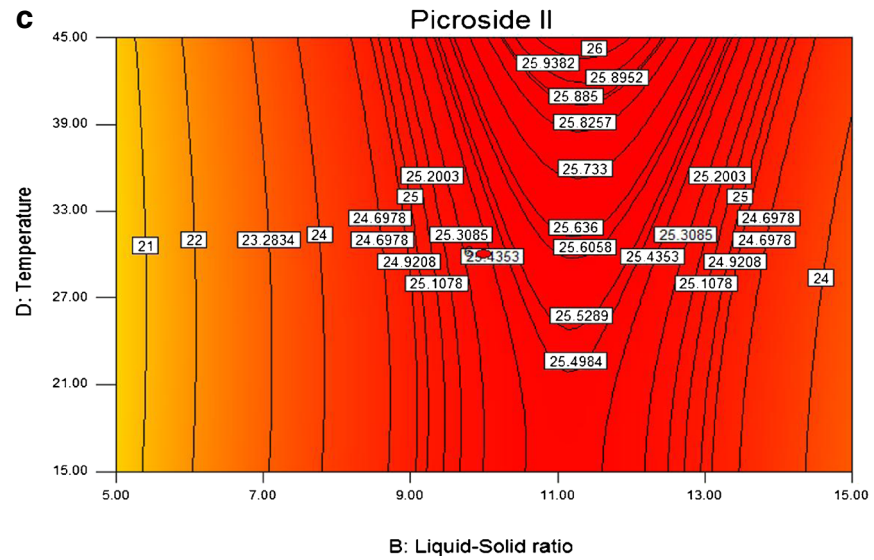
c
Picroside II
D: Temperature
B: Liquid-Solid ratio
45.00
39.00
33.00
27.00
21.00
15.00
5.00
7.00
9.00
11.00
13.00
15.00
26
25.9382
25.8952
25.885
25.8257
25.733
25.2003
25.2003
25
25
24.6978
24.6978
24.6978
24.6978
25.3085
25.3085
25.636
25.6058
25.4353
25.4353
24.9208
24.9208
25.1078
25.1078
25.5289
25.4984
21
22
23.2834
24
24

◀ **Fig. 3** **a** Contour plot (2D) showing the effect of methanol concentration and liquid–solid ratio on the yield of Picroside II. **b** Contour plot showing the effect of liquid–solid ratio and incubation time on the yield of Picroside II. **c** Contour plot showing the effect of liquid–solid ratio and temperature on the yield of Picroside II

This work clearly shows a cost-effective extraction of picrosides from *P. kurroa* which will probably inaugurate commercial interest in utilising picrosides from *P. kurroa* as an inexpensive source of health promoting additives e.g. natural hepatoprotective.

A cost effective method to determine the potent hepatoprotectants, picrosides, in *P. kurroa* has been developed. The method is environmentally safe as it excludes the use of methanol which is hazardous. Response surface methodology was successfully used to determine the optimum extraction conditions for picrosides. The second-order polynomial model gave a satisfactory description of the experimental data. Results showed that pure distilled water was more efficient in extracting picrosides as compared to methanol. The optimal extraction conditions were found to be methanol conc. (0 %), solid-to-liquid ratio (1:120 g/mL), incubation time (30 min) and temperature (30 °C). Under the optimum conditions, the experimental values of P-I and P-II agreed with the predicted ones, indicating suitability of the model employed.

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Conflict of interest The authors declare that they do not have any conflict of interest.

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