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Response surface methodology-based optimization of ultrasound-assisted extraction of β-sitosterol and lupeol from *Astragalus atropilosus* (roots) and validation by HPTLC method

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ABSTRACT

Objective: To optimize the ultrasonication method for efficient extraction of β-sitosterol and lupeol from the roots of *Astragalus atropilosus* using Box-Behnken design of response surface methodology (RSM), and its validation by high performance thin layer chromatography (HPTLC) method.

Methods: Ultrasonication method was used to extract β-sitosterol and lupeol from *Astragalus atropilosus* (roots). RSM was used to optimize the different extraction parameters *viz.* liquid to solid ratio (10–14 mL/g), temperature (60-80 ℃) and time (40–60 min) to maximize the yield of β-sitosterol and lupeol. The quantitative estimation of β-sitosterol and lupeol was done in chloroform extract of *Astragalus atropilosus* by validated HPTLC method on 10 cm × 20 cm glass-backed silica gel $60F_{254}$ plate using hexane and ethyl acetate (8:2, v/v) as mobile phase.

Results: A quadratic polynomial model was found to be most appropriate with regard to R_1 (yield of total extraction; R^2/% CV = 0.994 8/0.28), R_2 (β-sitosterol yield; R^2/% CV = 0.992 3/0.39) and R_3 (lupeol yield; R^2/% CV = 0.994 2/0.97). The values of adjusted R^2/predicted R^2/signal to noise ratio for R_1, R_2, and R_3 were 0.978 2/0.955 1/48.77, 0.990 4/0.911 0/31.33, and 0.992 7/0.940 1/36.08, respectively, indicating a high degree of correlation and adequate signal. The linear correlation plot between the predicted and experimental values for R_1, R_2, and R_3 showed high values of R^2 ranging from 0.990 5-0.997 3. β-sitosterol and lupeol in chloroform extract of *Astragalus atropilosus* were detected at R_f values of 0.22 and 0.34, respectively, at λ max = 518 nm. The optimized ultrasonic extraction produced 8.462% w/w of R_1, 0.451% w/w of R_2 and 0.172% w/w of R_3 at 13.5 mL/g liquid to solid ratio, 78 ℃ of temperature and 60 min of time.

Conclusions: The experimental findings of RSM optimized extraction and HPTLC analysis can be further applied for the efficient extraction of β-sitosterol and lupeol in other species of *Astragalus*.

KEYWORDS: β-sitosterol; Lupeol; Box-Behnken design; *Astragalus*; High performance thin layer chromatography

1. Introduction

β-sitosterol, a plant steroid has been extensively studied and shows anti-HIV (by immunomodulatory mechanism), antiviral (against tobacco mosaic virus), anti-hepatotoxic, anti-cardiotoxic, and anti-oxidative activities[1]. The triterpenoids have also been found to possess a wide spectrum of biological activities such as anti-inflammatory, hypocholesterolemic, insulin-regulating potential,

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antiviral (particularly lupeol for hepatitis), anti-herpes simplex virus, anti-microbial, and anti-proliferative acitivities[2]. β-sitosterol and lupeol have been analyzed using HPTLC in different plants such as *Tephrosia purpurea* (L.) Pers. (β-sitosterol and lupeol ranged from 0.043% to 0.125%, 0.023% to 0.045% w/w, respectively), *Hibiscus* species (aerial parts; 1.18% and 0.75% w/w for β-sitosterol and lupeol, respectively), *Sisymbrium irio* L (0.21% w/w for β-sitosterol). Some compounds have been analyzed using HPLC in different species of *Astragalus* like astragalosides and isoflavonoids[3-6], but till now, no report has been published on the quantification of these biologically important phytoconstituents in *Astragalus atropilosus* (*A. atropilosus*) using HPTLC method.

Ultrasonic-assisted extraction is an economical and easily operated extraction technique in comparison to the other techniques such as supercritical fluid extraction and microwave-assisted extraction. The enhanced extraction by ultrasonic treatment is mainly attributed to its mechanical effects, which largely expedite the mass transfer between immiscible phases at low frequency by super agitation[7,8].

Response surface methodology (RSM) is a more economical, convenient, diversified, logical and time-saving statistical technique than the conventional single parameter optimization, and has been used to simultaneously optimize different variables involved in the process. The various extraction parameters such as extraction time, extraction temperature, liquid to solid ratio, solvent ratio, *etc*. have been optimized for several phytoconstituents *viz.* betulinic acid from *Tecomella undulata*, triterpenoids from *Jatropha curcas*, embelin from *Embelia ribes* and phytosterol from *Saccharum officinarum* L[9-13]. Among various response surface designs available in RSM, Box-Behnken design (BBD) is more labor efficient (requiring the minimum number of experimental runs), and quite suitable for fitting second-order polynomial equations of three or more experimental factors[14-17]. BBD is known to be more competent than central composite and three-level full factorial RSM designs, as it allows estimation of quadratic model parameters, sequential design building and lack of fit determination for the proposed model. In this experiment, only 17 runs were needed for a three-factorial (3^3) study. BBD can also help in analyzing the quadratic response surface and generating a second-order polynomial model. The HPTLC is a widely used chromatographic technique in the quantitative analysis of herbal extracts, herbal drugs, and its supplements because it is rapid, less expensive, highly sensitive, precise, and has the potential to measure a large number of samples efficiently[18-22].

In the present experiment, authors planned to optimize various extraction parameters such as liquid to solid ratio, extraction temperature and extraction time for the maximum yield of β-sitosterol and lupeol in chloroform extract of *A. atropilosus* (AACE) by applying Box-Behnken design of RSM along with the quantitative estimation of β-sitosterol and lupeol in AACE for the first time by a validated, simple and efficient HPTLC method.

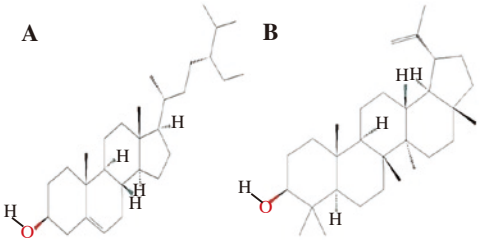

Figure 1. Chemical structure of β-sitosterol (A) and lupeol (B).

2. Materials and methods

2.1. Plant material and chemicals

Roots of *A. atropilosus* (voucher no. 14471) were collected from the Tamniah area of Saudi Arabia. The plant material was authenticated by Dr. Mohamed Yousef, a taxonomist at Pharmacognosy Department, College of Pharmacy, King Saud University, and the voucher specimens were deposited in the herbarium, Department of Pharmacognosy. The roots were washed and dried at room temperature. After drying, roots were broken into small pieces, powdered and stored for further processing. The standards β-sitosterol and lupeol (Figure 1) were procured from Sigma Aldrich. The chemicals hexane, ethyl acetate, and chloroform were purchased from BDH chemicals.

2.2. Ultrasonic extraction and determination of β-sitosterol and lupeol

The extraction of the powdered root of *A. atropilosus* was carried out by ultrasonic vibrations (ultrasound-assisted extraction) using Sonics Vibra cell (Model VCX-750; Sonics, USA). The effect of single factors on extraction procedures was determined as follows:

(1) Effect of liquid to solid ratio on the extraction:

Root powder (1.0 g) was put into a 50 mL conical flask and extracted with various volumes of chloroform (4, 6, 8, 10, 12, 16, 20 mL) to get different liquid-to-solid ratios keeping the extraction time (40 min) and extraction temperature (40 ℃) constant throughout the experiment. Each experiment was repeated 5 times (n=5) and the obtained extracts were merged, filtered and dried at low pressure with rotavapour to get the final extractive yield.

(2) The effect of temperature on extraction:

A total of 10 mL of chloroform was added to 1 g of powdered root in a 50 mL flask for each experiment, and the extraction was performed for different extraction temperatures (30, 40, 50, 60, 70, 80 ℃) at constant extraction time (40 min). Each experiment was repeated 5 times (n=5) and the obtained extracts were merged, filtered and dried at low pressure with rotavapour to get the final extractive yield.

(3) The influence of time on the extraction:

A total of 10 mL of chloroform was added to 1 g of powdered root in a 50 mL flask for each experiment and the extraction was executed for different time variables (10, 20, 30, 40, 50, 60 min) at constant extraction temperature (40 ℃). Each experiment was repeated 5 times (n=5) and the obtained extracts were merged, filtered and dried at low pressure with rotavapour to get the final extractive yield.

On the basis of the above finding of the influence of single factor on extraction yield, the ultrasound-assisted extraction procedure for RSM was set as below: root powder was taken into a conical flask (50 mL) and chloroform was added with different liquid-to-solid ratios (10–14 mL/g), temperature (60–80 ℃) and time (40–60 min).

2.3. Experimental design of RSM

A 3^3 factorial BBD (Design-Expert Software, Trial version 12, Stat-Ease Inc., Minneapolis, MN, USA) of RSM (17 runs) was applied to optimize the extraction variables *viz*. liquid to solid ratio (P_1: 10, 12, 14 mL/g), temperature (P_2: 60, 70, 80 ℃) and time (P_3: 40, 50, 60 min) to get the maximum yield (% w/w) of total extractive matter (R_1), β-sitosterol (R_2) and lupeol (R_3). The appropriate range of different variables was determined according to single-factor experiments. The preparation and analysis of all the samples were carried out in triplicate. A nonlinear quadratic model equation generated by this experimental design is shown below:

$$R_1 = k_0 + q_1P_1 + q_2P_2 + q_3P_3 - q_{12}P_1P_2 + q_{13}P_1P_3 + q_{23}P_2P_3 - q_{11}P_1^2 + q_{22}P_2^2 + q_{33}P_3^2;$$

$$R_2 = k_0 + q_1P_1 + q_2P_2 + q_3P_3 + q_{12}P_1P_2 + q_{13}P_1P_3 + q_{23}P_2P_3 + q_{11}P_1^2 + q_{22}P_2^2 + q_{33}P_3^2;$$

$$R_3 = k_0 + q_1P_1 + q_2P_2 + q_3P_3 - q_{12}P_1P_2 - q_{13}P_1P_3 - q_{23}P_2P_3 + q_{11}P_1^2 + q_{22}P_2^2 + q_{33}P_3^2,$$

Where, R is the response related to each factor level combinations; k_0 is intercept; q_1, q_2, q_3 are linear coefficients; q_{12}, q_{13}, and q_{23} are the interaction coefficients while q_{11}, q_{22}, and q_{33} are the quadratic coefficients. The independent variables were P_1, P_2, and P_3 while R_1, R_2 and R_3 were the dependent variables. The results of various initial trials were used to choose the range of independent variables. Here all the variables including solvent to solid ratio, temperature and sonication time were studied at three levels, low (–1), medium (0) and high (+1). The obtained extracts were filtered using filter papers and used to determine the content of β-sitosterol and lupeol by using validated HPTLC method.

2.4. HPTLC analyses of β–sitosterol and lupeol

All the 17 BBD runs of the AACE (2 mg/mL) were applied as spots (10 μL) on a 10 cm×20 cm glass-backed silica gel $60F_{254}$ HPTLC plate (Merck, Germany) with a band size of 6 mm using Automatic sampler-4 (CAMAG, Switzerland). Before the application, the extract solutions were filtered using a 0.22 μm filter fitted with a microliter syringe (CAMAG). Then the post-application of the plate was developed in a twin trough glass chamber (Automatic Development Chamber-2, CAMAG) saturated with the mobile phase [mixture of hexane and ethyl acetate in the ratio of 8:2 (v/v)] for 20 min at controlled temperature [(25 ± 2) ℃] and controlled humidity [(60 ± 5)%] and the chromatogram was developed up to a height of 8.0 cm. The post-development of the TLC plate was air-dried (30 min), derivatized with p-anisaldehyde reagent and dried again at 110 ℃ for 10 min (in a hot air oven) to furnish the clear and compact spots of all the phytoconstituents present in the sample along with the markers (β-sitosterol and lupeol). The plate was scanned by using TLC scanner-3 (CAMAG) in absorbance mode at λ max = 518 nm and the concentrations of β-sitosterol and lupeol in all the seventeen runs were quantified by using regression equation obtained from the calibration curve of a β-sitosterol and lupeol standards.

2.4.1. Calibration curve preparation

A stock solution (1 mg/mL) of standards β-sitosterol and lupeol in chloroform was prepared. The stock solution was further diluted with chloroform in order to get seven different dilutions *viz*. 10, 20, 40, 60, 80, 100 and 120 μg/mL. All the seven dilutions of β-sitosterol and lupeol (10 μL, each) were applied in triplicate on the HPTLC plate to furnish concentrations of 100, 200, 400, 600, 800, 1 000 and 1 200 ng/band. Furthermore, the linear least-squares regression was used to treat the data of peak area *versus* the concentration of biomarkers.

2.4.2. Validation

The developed HPTLC method was validated for accuracy, precision, robustness, the limit of detection (LOD) and limit of quantification (LOQ) as per the International Conference on Harmonization guidelines[23]. The recovery as accuracy studies of β-sitosterol and lupeol was accomplished by the standard addition method. Additionally, the analyte was spiked with various concentrations (50%, 100%, and 150%) of β-sitosterol and lupeol and reanalyzed by using the proposed HPTLC method in triplicate. The % relative standard deviation (RSD) and recovery were calculated, and the intra and inter-day precisions of three replicates for β-sitosterol and lupeol determination were executed at three concentrations (400, 600 and 800 ng/band). The % RSD of peak areas were calculated. However, for the robustness study of the developed HPTLC method, a small intentional modification was applied to the composition of the solvent system, the mobile phase volume (18, 20, 22 mL) and the saturation time (10, 20, 30 min). Moreover, the effects on the result were calculated as SD and % RSD. The LOD and LOQ for the β-sitosterol and lupeol were calculated by using equations (1) and (2), respectively:

$$LOD = (3.3\times SD)/\alpha \quad (1)$$

$LOQ = (10 \times SD)/\alpha$ (2)

Where SD is the least standard deviation and α is the slope of the curve.

The specificity of the developed HPTLC method was confirmed by analyzing β-sitosterol and lupeol standards and its presence in AACE. Furthermore, the spots for β-sitosterol and lupeol in AACE were established by comparing the R_f value, color and the peak of the spot in the samples with those of the standard.

2.5. RSM model and validity testing

To analyze the experimental results, BBD of RSM (Design-Expert™ software, version 12) was used, and *P*-value < 0.05 were considered to be significant. Additionally, independent variables of the extraction process such as P_1, P_2, and P_3 were concurrently optimized by using BBD. The ultrasonication extraction of the crude drug was executed by using the optimized conditions in triplicate and the yield of R_1, R_2 and R_3 was compared with predicted values for the model validation.

3. Results

3.1. HPTLC analysis of β-sitosterol and lupeol

Out of these solvents, hexane and ethyl acetate in the ratio of 8:2, v/v was found to be quite selective. The developed HPTLC method provided a sharp, compact and well-defined peaks of β-sitosterol and lupeol at the R_f values of 0.22 and 0.34, respectively (Figure 2A). Figure 2B shows that the selected solvent system had a very good resolution for the separation of β-sitosterol and lupeol from other constituents of AACE. The identities of the bands were confirmed by overlaying the spectra of all the extracts with the spectra of β-sitosterol and lupeol (Figure 2C). Furthermore, the linear regression data obtained for the calibration curves (*n*=6) showed a good linear relationship over a wide range of concentrations (100–1 200 ng/band) with respect to peak area (Supplementary Table 1). The linear equation/correlation coefficients (r^2) for β-sitosterol and lupeol were found as Y= 10.363X + 522.03/0.997 2 and Y= 11.442X + 790.77/0.994 1, respectively. The LOD/LOQ (ng) for β-sitosterol and lupeol were found as 10.32/31.28 and 21.17/64.16,

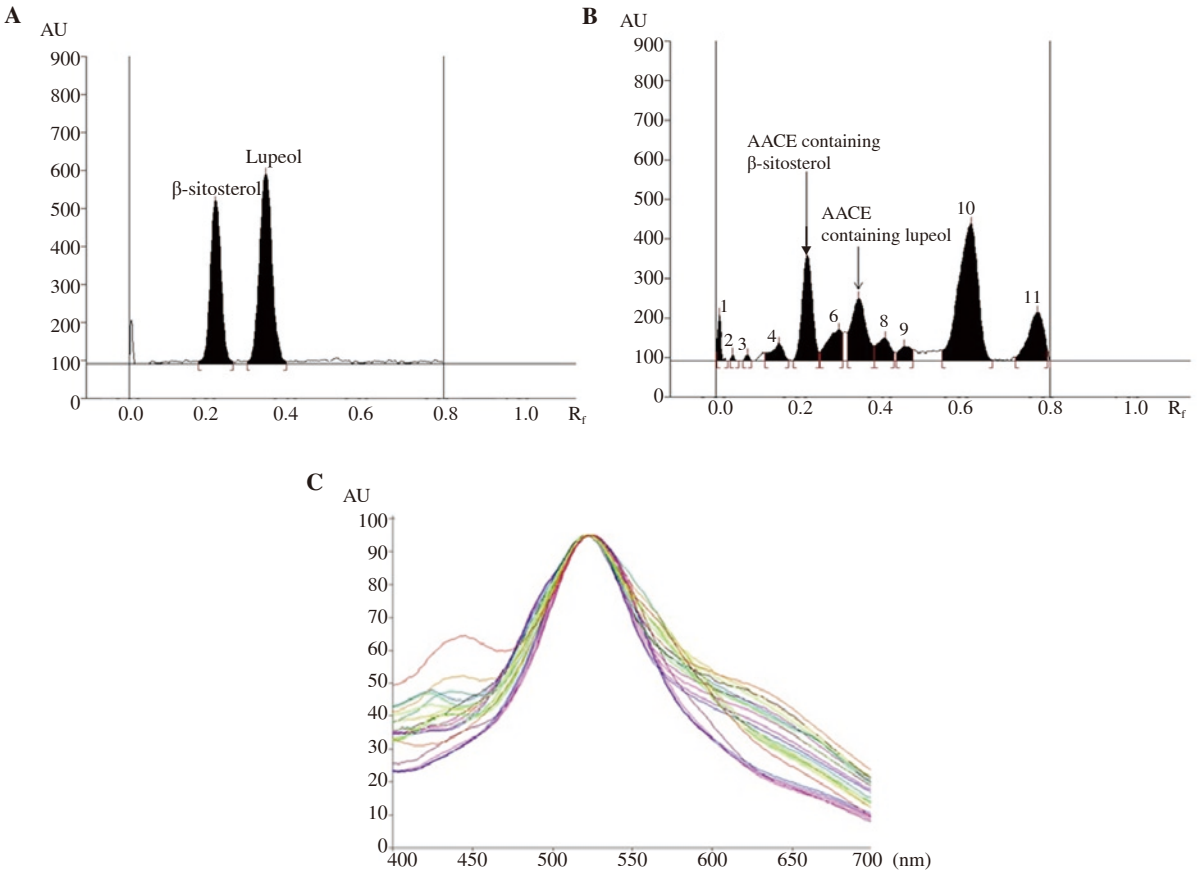

Figure 2. High performance thin layer chromatography (HPTLC) chromatogram and spectral comparison. A: HPTLC chromatogram of standards β-sitosterol and lupeol; B: HPTLC chromatogram of β-sitosterol and lupeol estimation in chloroform extract of *Astragalus atropilosus* (β-sitosterol, spot 5, R_f = 0.22; lupeol, spot 7, R_f = 0.34) using mobile phase: hexane: ethyl acetate (8:2, v/v) at λ max= 518 nm; C: Spectral comparison of all tracks. AACE: chloroform extract of *Astragalus atropilosus*.

respectively.

On one hand, the % RSD for intra-day/inter-day precision of β-sitosterol and lupeol were found as 1.209-1.337/1.087-1.287 and 1.231-1.522/1.219-1.447, respectively (Supplementary Table 2). The accuracy was calculated by recovery analysis which afforded recovery of 98.26%-99.59% and 98.15%-99.43%, respectively for β-sitosterol and lupeol (Supplementary Table 3). On the other hand, the % RSD of β-sitosterol and lupeol were found as 0.978-1.423 and 0.999-1.315, respectively for the accuracy of the proposed method. The low values of % RSD of β-sitosterol and lupeol in robustness studies are recorded in Supplementary Table 4.

3.2. Effect of single-factor tests with ultrasonic extraction of AACE

3.2.1. Effect of extraction time (P_3) on the yield of AACE

The Supplementary Figure 1A shows that the extraction yield (% w/w) of AACE was affected by variation in P_3 (10, 20, 30, 40, 50 and 60 min) [where the other two factors P_1 (liquid to solid ratio) and P_2 (extraction temperature) were fixed at 10 mL/g and 40 ℃, respectively]. In addition, the (%) extraction yields of the AACE increased significantly from 4.10% to 5.71% when P_3 increased from 10 to 40 min. However, no change was observed with further increase in P_3 from 40-60 min, which indicated that 40 min was the time limit to get the maximum AACE yield.

3.2.2. Effect of extraction temperature (P_2) on the yield of AACE

The selected extraction temperatures (P_2) were 30, 40, 50, 60, 70 and 80 ℃, respectively, to study the impact of extraction temperature on the AACE extraction yield (%), keeping the other two factors P_1 (10 mL/g) and P_3 (40 min) constant (Supplementary Figure 1B). The result demonstrated that the AACE extraction yield was increased with an increase in P_2, reaching the maximum at 60 ℃. However, no significant difference was observed by further increasing P_2 from 60 ℃-80 ℃.

3.2.3. Effect of liquid to solid ratio (P_1) on the yield of AACE

The impact of P_1 on the extraction yield of AACE is shown in Supplementary Figure 1C. The AACE extraction yield was significantly increased from 35.1 to 53.3 mg/g as the P_1 increased within the range of 4 to 12 mL/g, due to the increase of the driving force for the mass transfer. However, as the P_1 continued to increase, the extraction yields did not differ significantly any longer.

3.3. Model fitting

The (% w/w) quantity of β-sitosterol (R_2) and lupeol (R_3) of each experimental BBD run was estimated by validated HPTLC method, and the results are shown in Table 1 along with the total extraction yield (R_1). A quadratic model was found to be the best fit model and the comparative results of regression analysis for model and response regression equation for the final proposed model are listed in Supplementary Table 5. The values of adjusted R^2/predicted R^2 for R_1, R_2, and R_3 were found as 0.978 2/0.955 1, 0.990 4/0.911 0 and 0.992 7/0.940 1, respectively which were close to 1. This indicated a high degree of correlation between the observed and predicted values. Similarly, the difference between the adjusted R^2 and predicted R^2 is less than 2, which is required to fit the model. "Adequate Precision" measures the signal to noise ratio which should be greater than 4 to fit the model. In this experiment, the signal to noise ratios were found as 48.77, 31.33 and 36.08 for R_1, R_2, and R_3, respectively. Table 2 showed the analysis of variance (ANOVA) for the fitted quadratic polynomial of R_1, R_2, and R_3 from *A. atropilosus*. The "lack of fit *F*-value" was found as 1.38, 3.01, and 2.05 for R_1, R_2, and R_3 which showed that the "lack of fit" was not significant and showed the validity of RSM results. Furthermore, in this experiment, the model *F*-value for R_1, R_2, and R_3 was found as 149.82/100.33/133.09 which suggests that the model was significant.

3.4. Effect of extraction parameters (P_1, P_2, P_3) on R_1, R_2 and R_3, and RSM analysis

The contributions of each independent variable are shown in Table 3. The linear variables (P_1, P_2, P_3), the interaction variables (P_1P_2) and the quadratic variables (P_1^2, P_3^2) were found significant (P<0.05), and affected the R_1 whereas other variables (P_2P_3, P_1P_3 and P_2^2) were found insignificant (P>0.05). All the linear and the quadratic variables along with the interaction variables (P_1P_2, P_2P_3) were found significant (P<0.05) and affected the R_2 except the interaction variable P_1P_3 (P>0.05). In the case of R_3, all the variables (the linear, quadratic and interaction variables) were found significant (P<0.05) and affected it. Furthermore, the R^2/coefficient of variation (% CV) of the model for R_1, R_2 and R_3 were found as 0.994 8/0.28, 0.992 3/0.39 and 0.994 2/0.97, which indicated a good precision and reliability of the experimental values. Moreover, three-dimensional (3D) plots were constructed to visualize the relationship between independent variables and R_1, R_2 and R_3 according to the generated quadratic polynomial model equation of the coded factors:

R_1 (%) = +7.95 + 0.042 5 P_1 + 0.236 3 P_2 + 0.078 7 P_3 + 0.145 P_1P_2 –-0.02 P_1P_3 + 0.017 5 P_2P_3 – 0.043 3 P_1^2 + 0.004 2 P_2^2 + 0.109 3 P_3^2

R_2 (%) = + 0.396 6 + 0.004 1 P_1 + 0.010 4 P_2 + 0.003 2 P_3 + 0.005 8 P_1P_2 + 0.002 5 P_1P_3 + 0.006 5 P_2P_3 + 0.004 6 P_1^2 + 0.007 1 P_2^2 + 0.009 8 P_3^2

R_3 (%) = + 0.133 6 + 0.005 P_1 + 0.008 P_2 + 0.011 P_3 – 0.001 P_1P_2 – 0.001 P_1P_3 – 0.001 P_2P_3 + 0.005 2 P_1^2 + 0.005 2 P_2^2 + 0.008 2 P_3^2

A positive value of the variables' coefficients indicated that it is in favor of optimization. However, a negative value indicated a reverse

Table 1. Response surface central composite design (uncoded) and results for R_1, R_2, and R_3.

Run	Coded variables			Actual variables			Total extraction yield (R_1) (% w/w)			β-sitosterol yield (R_2) (% w/w)			Lupeol yield (R_3) (% w/w)		
	P_1 (mL/g)	P_2 (℃)	P_3 (min)	P_1 (mL/g)	P_2 (℃)	P_3 (min)	Experimental	Predicted	Residue	Experimental	Predicted	Residue	Experimental	Predicted	Residue
1	-1	1	0	10	80	50	7.951 ± 0.341	7.914	0.037	0.408 ± 0.017	0.411	-0.003	0.148 ± 0.004	0.156	-0.001
2	-1	0	-1	10	70	40	7.892 ± 0.366	7.814	0.078	0.406 ± 0.016	0.408	-0.002	0.131 ± 0.002	0.130	0.001
3	-1	-1	0	10	60	50	7.790 ± 0.305	7.932	-0.113	0.401 ± 0.012	0.399	0.002	0.129 ± 0.003	0.131	-0.002
4	0	-1	-1	12	60	40	7.754 ± 0.448	7.711	0.043	0.405 ± 0.014	0.406	-0.001	0.130 ± 0.004	0.127	0.003
5	1	0	1	14	70	60	8.113 ± 0.503	8.127	-0.014	0.423 ± 0.018	0.421	0.002	0.161 ± 0.006	0.162	-0.001
6	0	1	1	12	80	60	8.425 ± 0.251	8.283	0.142	0.435 ± 0.021	0.434	0.002	0.169 ± 0.007	0.165	0.004
7	-1	0	1	10	70	60	8.071 ± 0.309	8.119	-0.047	0.407 ± 0.008	0.409	-0.002	0.156 ± 0.008	0.154	0.002
8	1	-1	0	14	60	50	7.592 ± 0.269	7.485	0.107	0.398 ± 0.011	0.396	0.002	0.144 ± 0.005	0.142	0.002
9	0	-1	1	12	60	60	7.891 ± 0.409	7.256	0.064	0.399 ± 0.013	0.401	-0.002	0.153 ± 0.004	0.151	0.002
10	1	0	-1	14	70	40	8.052 ± 0.376	8.025	0.027	0.411 ± 0.015	0.409	0.002	0.141 ± 0.004	0.142	-0.001
11	1	1	0	14	80	50	8.272 ± 0.221	8.365	-0.093	0.427 ± 0.018	0.429	-0.002	0.157 ± 0.007	0.156	0.001
12	0	0	0	12	60	50	7.951 ± 0.236	7.909	0.043	0.399 ± 0.011	0.397	0.002	0.135 ±0.005	0.134	0.001
13	0	0	0	12	60	50	7.982 ± 0.262	7.923	0.059	0.399 ± 0.009	0.397	0.001	0.132 ±0.006	0.134	-0.002
14	0	0	0	12	60	50	7.942 ± 0.331	7.984	-0.042	0.399 ± 0.014	0.398	0.001	0.133 ± 0.004	0.134	-0.001
15	0	0	0	12	60	50	7.933 ± 0.414	7.869	0.063	0.397 ± 0.016	0.399	-0.002	0.134 ± 0.003	0.134	0.001
16	0	0	0	12	60	50	7.973 ± 0.153	7.997	-0.024	0.398 ± 0.018	0.399	-0.002	0.134 ± 0.006	0.134	0.001
17	0	1	-1	12	80	40	8.214 ± 0.317	8.160	0.054	0.416 ± 0.016	0.414	0.002	0.147 ±0.007	0.145	0.002

Table 2. ANOVA for the fitted quadratic polynomial model of R_1, R_2 and R_3.

Dependent variables	Source	Sum of square	Degree of freedom	Mean square	*F*-value	*P* value
R_1	Model	0.654	9	0.0727	149.82	< 0.001
	Residual	0.034	7	0.0050	-	-
	Lack of fit	0.017	3	0.0060	1.38	0.390
	Pure error	0.002	4	0.0004	-	-
R_2	Model	0.022	9	0.0020	100.33	< 0.001
	Residual	0.029	7	0.0023	-	-
	Lack of fit	0.031	3	0.0039	3.01	0.257
	Pure error	0.005	4	0.0001	-	-
R_3	Model	0.002	9	0.0003	133.09	< 0.001
	Residual	0.003	7	0.0002	-	-
	Lack of fit	0.008	3	0.0026	2.05	0.249
	Pure error	0.005	4	0.0001	-	-

Table 3. Significance of each response variable effect showed by using the *F* ratio and *P*-value in the nonlinear second-order model.

Dependent variables	Independent variables	SS[a]	DF[b]	MS[c]	*F*-value	*P*-value[d]
R_1	Linear effects					
	P_1	0.0150	1	0.0150	29.79	0.001
	P_2	0.4470	1	0.4470	920.64	< 0.001
	P_3	0.0490	1	0.0490	102.29	< 0.001
	Quadratic effects					
	P_1^2	0.0080	1	0.0080	16.24	0.005
	P_2^2	0.0001	1	0.0001	0.16	0.704[ns]
	P_3^2	0.0500	1	0.0500	103.62	< 0.001
	Interaction effects					
	P_1P_2	0.0840	1	0.0840	173.40	< 0.001
	P_1P_3	0.0020	1	0.0020	3.30	0.112[ns]
	P_2P_3	0.0010	1	0.0010	2.53	0.156[ns]
R_2	Linear effects					
	P_1	0.0010	1	0.0010	56.22	<0.001
	P_2	0.0090	1	0.0090	355.63	0.001
	P_3	0.0010	1	0.0010	34.90	<0.001
	Quadratic effects					
	P_1^2	0.0001	1	0.0001	36.40	<0.001
	P_2^2	0.0002	1	0.0002	87.04	<0.001
	P_3^2	0.0004	1	0.0004	167.85	<0.001
	Interaction effects					
	P_1P_2	0.0001	1	0.0001	54.62	<0.001
	P_1P_3	0.0003	1	0.0003	10.32	0.015
	P_2P_3	0.0002	1	0.0002	69.79	<0.001
R_3	Linear effects					
	P_1	0.0002	1	0.0002	106.06	<0.001
	P_2	0.0005	1	0.0005	271.52	<0.001
	P_3	0.0010	1	0.0010	513.33	<0.001
	Quadratic effects					
	P_1^2	0.0001	1	0.0001	60.38	<0.001
	P_2^2	0.0001	1	0.0001	60.38	<0.001
	P_3^2	0.0003	1	0.0003	150.14	<0.001
	Interaction effects					
	P_1P_2	0.0004	1	0.0004	51.75	<0.001
	P_1P_3	0.0006	1	0.0006	112.18	<0.001
	P_2P_3	0.0009	1	0.0009	198.35	<0.001

[a]Sum of squares; [b]Degree of freedom; [c]Mean sum of squares; [d]*P*-values < 0.05 were considered to be significant; ns: insignificant.

relationship between the independent variables and the response (R_1, R_2 and R_3). Therefore, it is evident from the equation 1, 2 and 3 that the variables such as P_1, P_2 and P_3 had a positive effect on R_1, R_2 and R_3, respectively. It also revealed that the relationship between the response and the variables was not constantly linear. When more than one variable is changed simultaneously, the variables can show various degrees of response.

The combination ratio of all the variables (P_1, P_2 and P_3) for the extraction was selected based on the results of R_1, R_2 and R_3 using three-dimensional response surface plots. As shown in Figures 3A&C, 4A&C and 5A&C, the R_1, R_2 and R_3 were increased positively with the increase in P_2 up to 78℃ when P_1 and P_3 were fixed at 12 mL/g and 50 min, respectively. Figures 3B, 4B and 5B showed an increase in R_1, R_2 and R_3 at longer P_3 and lower P_1 when P_2 was fixed at 60℃.

3.5. RSM validation

For the R_1, R_2 and R_3 checkpoints, the yield evaluation result was found to be within the limits. For the validation of the RSM results of R_1, R_2 and R_3, the experimental values of the responses were compared with the anticipated values and the percentage prediction errors were found to be 1.40%, 1.60%, and 1.05%, respectively. This helps in establishing the validity of the generated equation and describing the domain of applicability of the RSM model. The linear correlation plot between predicted and experimental values for R_1, R_2 and R_3 showed a high value of R^2 (ranging from 0.990 5-0.997 3), indicating excellent goodness of fit (P<0.001) (Supplementary Figure 2).

3.6. Optimization and verification of the model for the extraction parameters

The optimum extraction process parameters were determined by maximizing the responses R_1, R_2 and R_3. During the optimization stage, the desirability function of the Design-Expert™ (version 12) statistical software was applied to obtain the best compromise of response. The predicted optimal condition for the extraction process was found at 13.0 mL/g (P_1), 76℃ (P_2), and 56.5 min (P_3) which resulted in the extraction of 8.496%, 0.445% and 0.169% w/w of R_1, R_2 and R_3, respectively. The extraction process once more repeated by modifying the optimum extraction conditions *viz*. 13.5

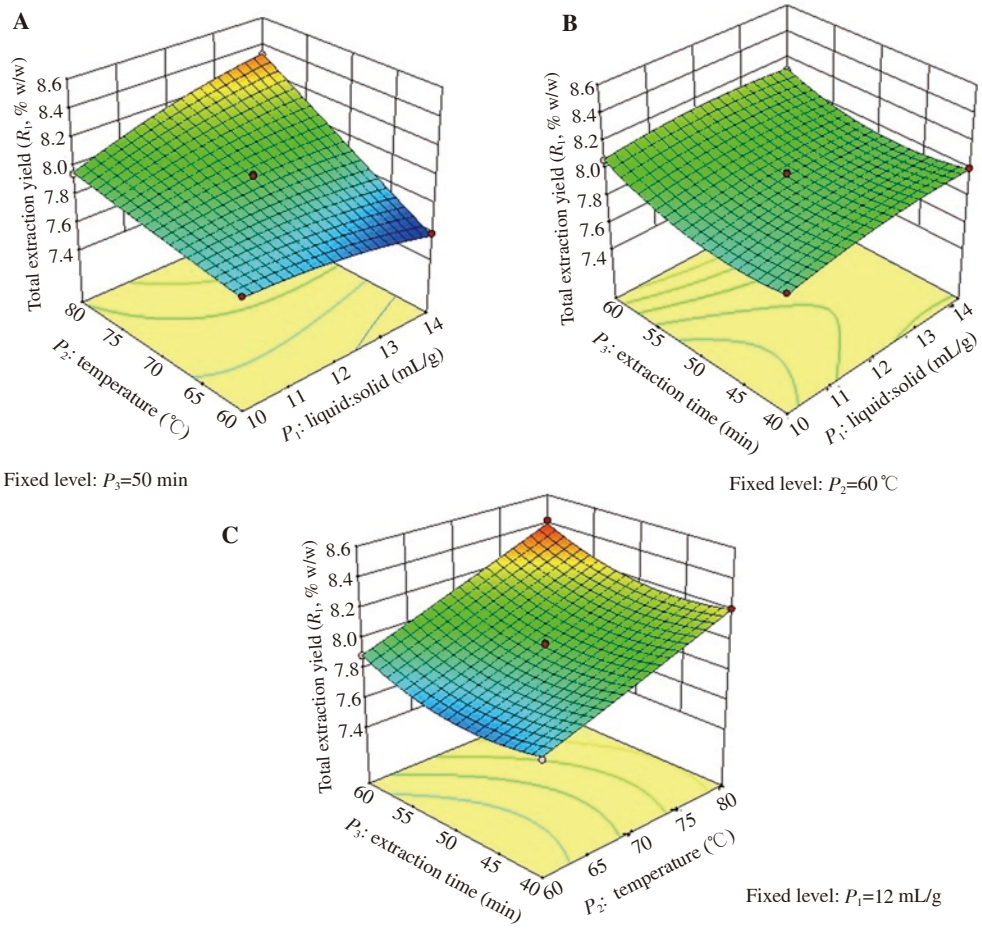

Figure 3. Response surface model 3D plots showing the effects of P_1, P_2 and P_3 on R_1. (A) the effect of P_1 and P_2 on R_1; (B) the effect of P_1 and P_3 on R_1; (C) the effect of P_2 and P_3 on R_1.

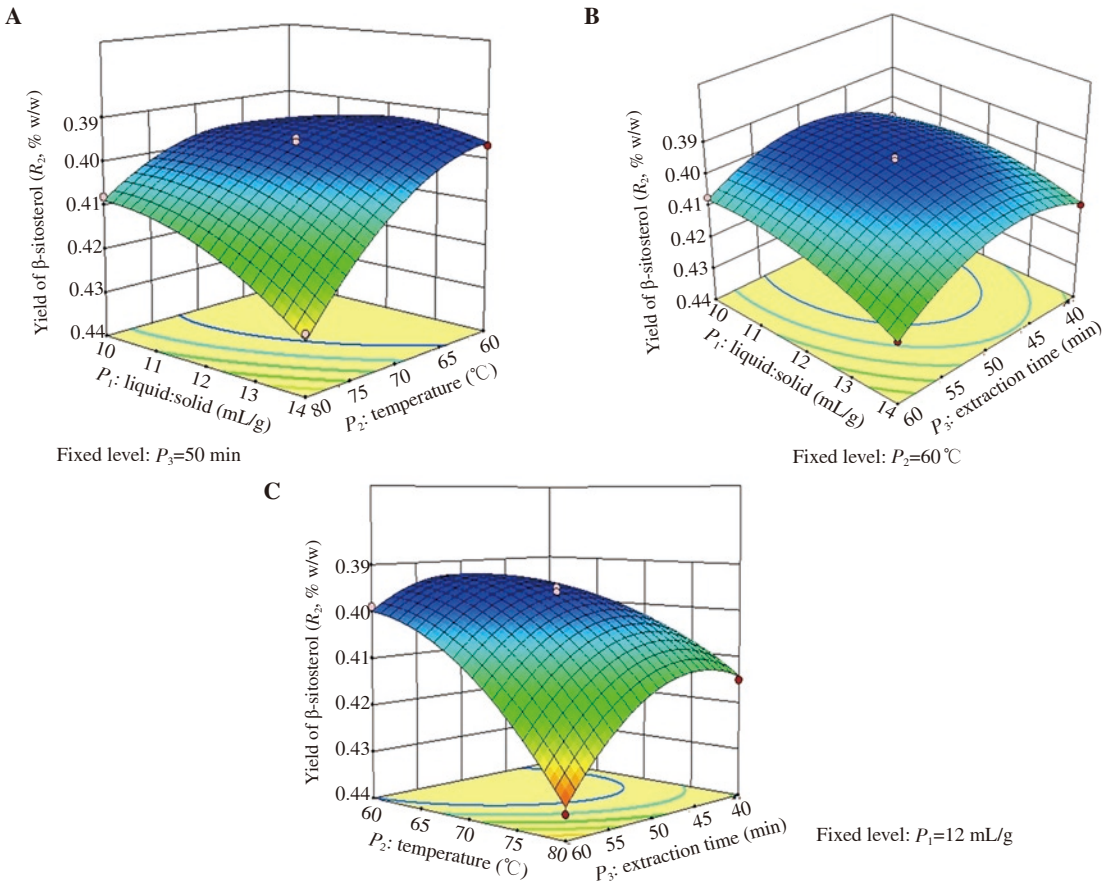

Figure 4. Response surface model 3D plots showing the effects of P_1, P_2 and P_3 on R_2. (A) the effect of P_1 and P_2 on R_2; (B) the effect of P_1 and P_3 on R_2; (C) the effect of P_2 and P_3 on R_2.

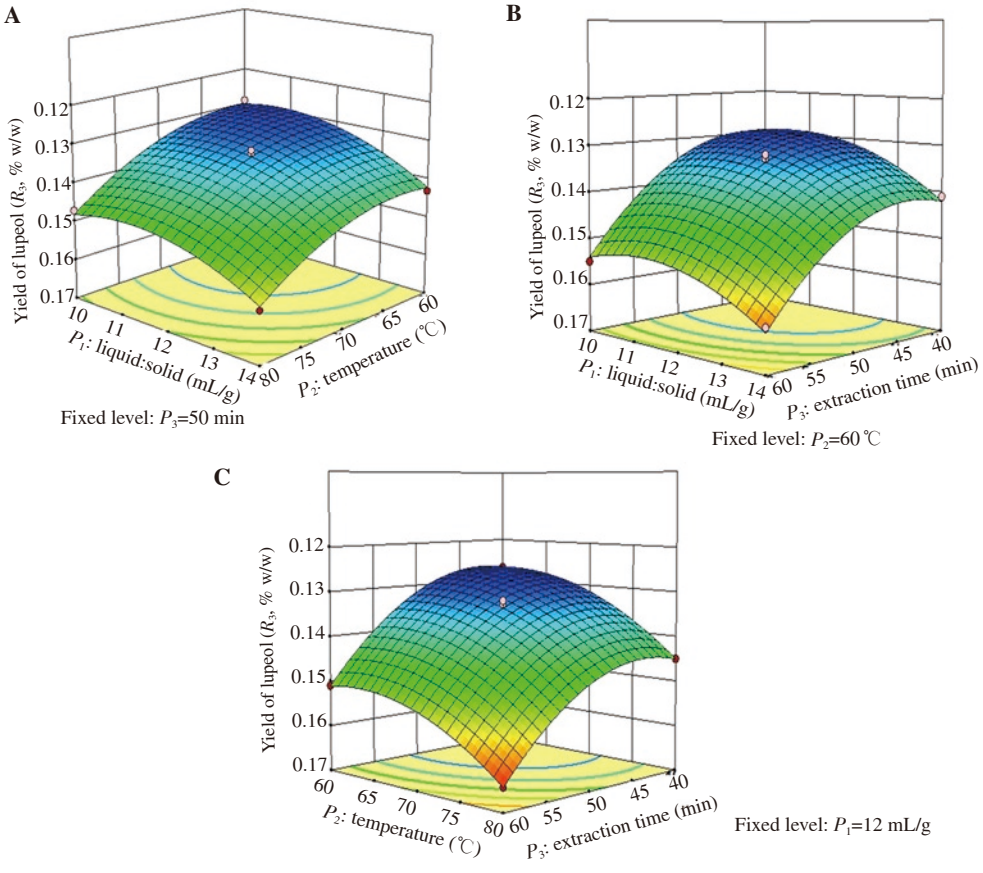

Figure 5. Response surface model 3D plots showing the effects of P_1, P_2 and P_3 on R_3. (A) the effect of P_1 and P_2 on R_3; (B) the effect of P_1 and P_3 on R_3; (C) the effect of P_2 and P_3 on R_3.

mL/g (P_1), 78 ℃ (P_2) and 60 min (P_3) and the total extraction yield (R_1), β-sitosterol yield (R_2) and lupeol yield (R_3) were found as (8.462±0.440)% w/w, (0.451±0.020)% w/w and (0.172± 0.010)% w/w, respectively. There was no significant difference (P>0.05) between the predicted and obtained values. Therefore, this model may be applied for the optimization of the extraction process of β-sitosterol and lupeol from the roots of *A. atropilosus*.

4. Discussion

The simultaneous quantification of β-sitosterol and lupeol in all the fractions of AACE collected during BBD runs was carried out by validated HPTLC method using hexane and ethyl acetate as suitable mobile phase, which showed a good resolution and a separation of β-sitosterol and lupeol along with the other phytoconstituents available in all the fractions of AACE. The developed method was validated as per the guideline of WHO. The low values of % RSD of β-sitosterol and lupeol indicated an excellent precision for intra-day/inter-day study and the highest accuracy of the proposed method. Furthermore, the low values of % RSD for β-sitosterol and lupeol obtained by deliberately changing the mobile phase composition and the time and temperature of the saturation clearly indicate that the mobile phase is robust.

The effect of extraction time as a single factor for the ultrasonic extraction of AACE was tested. The result showed that the (%) extraction yields of the AACE increased significantly with the increase in time from 10 to 40 min. However, no significant increase was seen after that. Consequently, the time affects the liquid circulation and turbulence produced by the cavitation, which causes an increase in the extraction efficiency by increasing the contact surface area between the solvent and the targeted compounds[24]. The increase in the extraction time may lead to the degradation of the triterpenoidal compounds. Similarly, in the case of the extraction, the temperature affects the yield increased with temperature till 60 ℃. However, no significant increase was observed after that. The increase in the extraction yield with increasing temperature is because of the higher mass transfer rate, which leads to higher molecular diffusion[25]. The effect of liquid to solid ratio on the extraction yield of AACE was also studied. It was observed that yield increased with the increase in liquid to solid ratio, which may be due to the increase of the driving force for the mass transfer. Therefore, it is consistent with the fact that higher liquid to solid ratios increases the contact surface between the plant material and the solvent, which enhances the mass transfer of soluble compounds from material to solvent[26,27]. Based on these observations, the ranges of the three independent variables for the optimization of the ultrasonic extraction method by BBD of RSM were selected as liquid to solid ratio:10-14 mL/g, the extraction temperature: 60-80 ℃ and the extraction time: 40-60 min.

The seventeen runs of BBD were carried out and analyzed with the help of validated HPTLC method to find out the quantity of β-sitosterol and lupeol. Consequently, a quadratic model was found to be the best fit model for the BBD analysis. The values of the adjusted R^2/predicted R^2 for R_1, R_2 and R_3 were found to be close to 1, which indicated a high degree of correlation between the observed and predicted values. Furthermore, the difference between the adjusted R^2 and the predicted R^2 is less than 2, which is required to fit the model. The "Adequate Precision" measuring the signal to noise ratio was found more than 4, which indicated an adequate signal and can be used to navigate the design space. In addition, the low "lack of fit F-value" was found for R_1, R_2 and R_3, which indicated that the "lack of fit" is not significant and showed the validity of RSM results. The "lack of fit F-value" test for the model explains the deviation in the data around the fitted model. If it is significant, it means that the model does not fit the data well, hence the insignificant lack of fit is good to fit the model. In this experiment, the model F-value for R_1, R_2 and R_3 was found to be high, which suggests that the model was significant.

The significance of each extraction variables (P_1, P_2, P_3) effects on R_1, R_2 and R_3 and the RSM analysis was evaluated. The interactions of P_1 and P_3 and the square root of P_1 produced negative effects on R_1 which indicated that if P_1 were to be doubled then R_1 will robustly decrease. Moreover, the interactions of P_1 and P_2, P_1 and P_3, and P_2 and P_3 along with the square roots of P_1, P_2 and P_3 produced positive effects on the R_2, which suggested that the increase in any variable will increase the R_2. The interactions of P_1 and P_2, P_1 and P_3, and P_2 and P_3 produced negative effects on R_3 while the square root of P_1, P_2 and P_3 produced the positive effects, which indicated that if the square root of variables P_1, P_2 and P_3 were to be doubled then R_3 will greatly increase.

The 3D plots were constructed to visualize the relationship between the independent variables (P_1, P_2, P_3) and R_1, R_2 and R_3. It was clear from the 3D plot that P_2 (extraction temperature) had a more significant effect on the R_1, R_2 and R_3. The maximum yield of R_1, R_2 and R_3 were obtained at an optimum temperature of 78 ℃. This proves that a higher temperature is helpful in enhancing the compound yield as it increases the diffusion coefficient and solubility, although it may also cause compound degradation[28]. For a high yield of R_1, R_2 and R_3, the optimum extraction temperature, the extraction time and the liquid to solid ratio were found as 78 ℃, 60 min and 13.5 mL/g, respectively.

To validate the RSM results, the experimental values of the responses were compared with the anticipated values. In addition, the percentage prediction errors were evaluated which established the validity of the generated equation and the applicability of the RSM model. The low magnitudes of error, as well as the significant values of R^2 in the present experiment, prove the high prognostic ability of the RSM.

In summary, the experimental findings indicated that BBD for RSM and a validated HPTLC method may be highly efficient and promising techniques for optimizing the extraction conditions and the quantitative

analysis of β-sitosterol and lupeol from *A. atropilosus* roots. All the selected variables, their interactions, and quadratic terms had a significant impact on the yield of the total extraction (R_1), β-sitosterol (R_2) and lupeol (R_3). The model prediction can be used to optimize the yield of R_1, R_2 and R_3 from *A. atropilosus* (roots) within the limits of the experimental variables. The modified optimal extraction conditions for R_1, R_2 and R_3 in the *A. atropilosus* root were found as P_1 (liquid to solid ratio) of 13.5 mL/g, P_2 (extraction temperature) of 78 ℃ and P_3 (extraction time) of 60 min. Under these optimal extraction conditions, the experimental yield of R_1, R_2 and R_3 was found as (8.462±0.440)% w/w, (0.451±0.020)% w/w and (0.172±0.010)% w/w, respectively, which agreed closely with the predicted yield value. The quadratic polynomial model was most appropriate with regard to R_1 (R^2/% CV= 0.994 8/0.28), R_2 (R^2/% CV= 0.992 3/0.39) and R_3 (R^2/% CV= 0.994 2/0.97). The values of adjusted R^2/predicted R^2 for R_1, R_2 and R_3 were found as 0.978 2/0.955 1, 0.990 4/0.911 0 and 0.992 7/0.940 1, respectively (close to 1) and its difference was less than 2. This indicated a high degree of correlation and good model fitting. The signal to noise ratio were 48.77, 31.33 and 36.08 for R_1, R_2 and R_3, respectively, which indicated an adequate signal and can be used to navigate the design space. Furthermore, the linear correlation plot between the predicted and experimental values for R_1, R_2 and R_3 showed a high value of R^2 (ranging from 0.990 5-0.997 3), indicating the prognostic ability of the RSM design. In this study, the solvent system developed for the HPTLC analysis of β-sitosterol and lupeol was found to be excellent in resolving their peaks efficiently and the low values of LOD and LOQ showed the great sensitivity of the developed method.

In the future, the extraction of β-sitosterol and lupeol from the *A. atropilosus* (roots) using the ultrasonic extraction can be used as an alternative natural source of β-sitosterol and lupeol for the pharmaceutical industries. The findings of the RSM analysis can be applied in the future for the maximum extraction of the β-sitosterol and lupeol in other species of genus *Astragalus*. The obtained statistical data supports the applicability of the developed HPTLC method for the quality control of herbal preparations containing β-sitosterol and lupeol.

Conflict of interest statement

We declare that there is no conflict of interest.

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Authors' contributions

PA: Planning, execution, manuscript writing and correspondence; NAS: HPTLC analysis of different exttracts; ASA: BBD analysis; AH: literature survey; OAB: ultrasonic extraction of crude drug; SIA: manuscript writing; AAM: collection, drying and storage of crude drug; MUK: data analysis.

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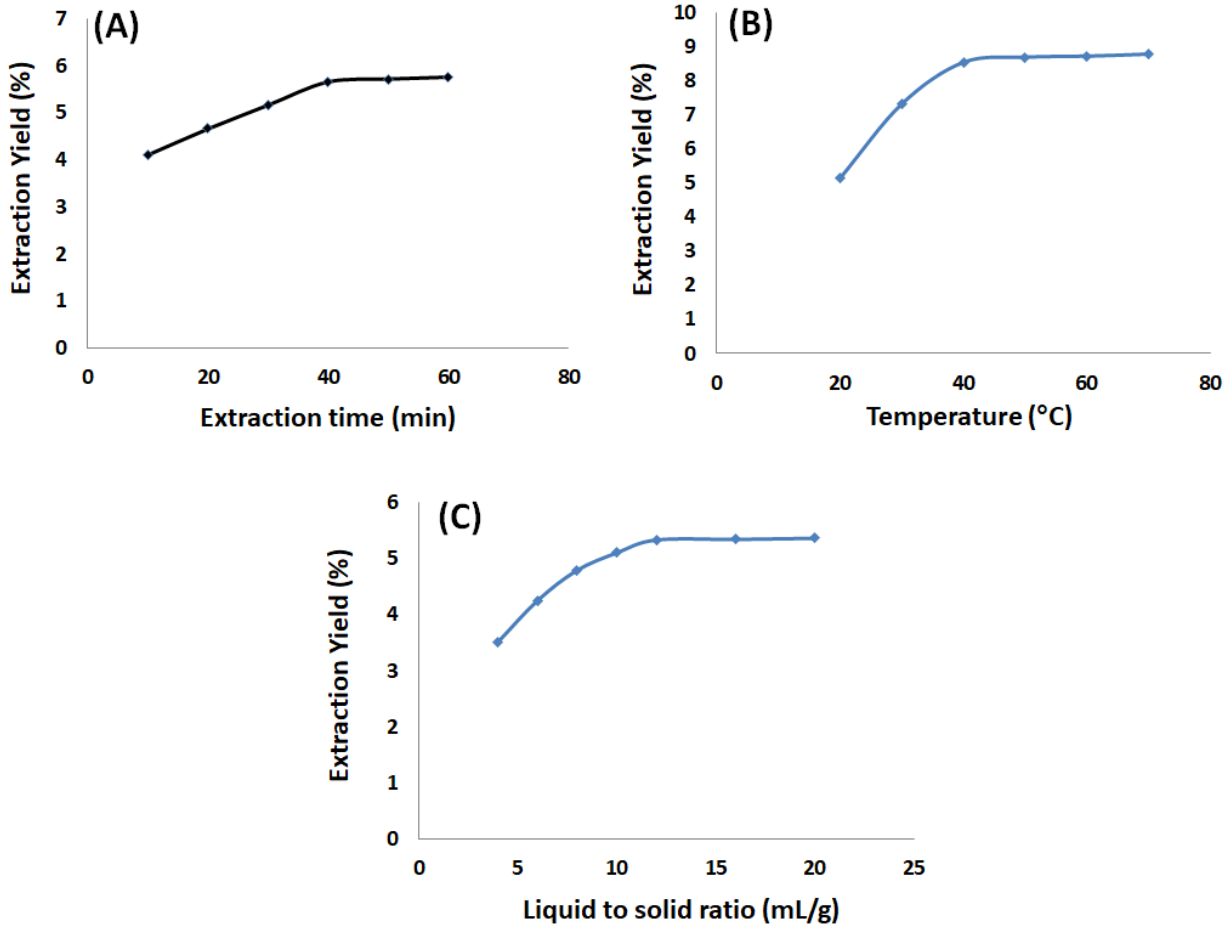
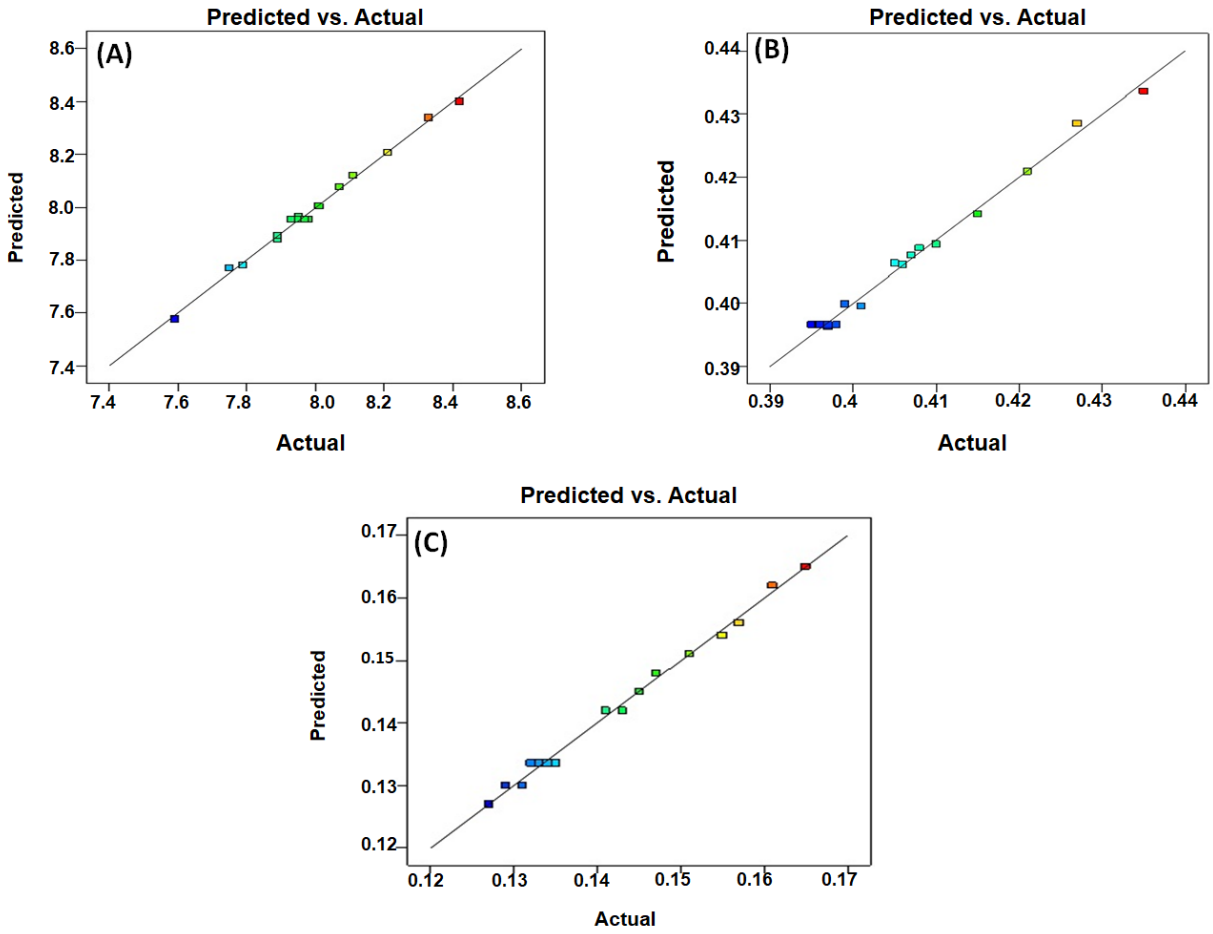


Supplementary Figure S1: The effects of extraction variables on yield of AACE. (A) Effect of P_3 on AACE yield (P_1 = 10 mL/g, P_2 = 40ºC); (B) Effect of P_2 AACE yield (P_1 = 10 mL/g, P_3 = 40 min); (C) Effect of P_1 on AACE yield (P_2 = 40ºC, P_3 = 40 min). Each value represents a mean ± SD (n = 5).



Supplementary Figure S2: Linear correlation plot between actual and predicted values for R_1 (A), R_2 (B) and R_3 (C).

Table S1: R_f, Linear regression data for the calibration curve of β-sitosterol and lupeol (n=6)

Parameters	β-sitosterol	Lupeol
Linearity range (ng/band)	100-1200	100-1200
Regression equation	Y= 10.363X + 522.03	Y= 11.442X + 790.77
Correlation coefficient (r^2)	0.9972	0.9941
Slope ± SD	10.363 ± 0.032	11.442 ± 0.073
Intercept ± SD	522.03 ± 24.026	790.77 ± 16.92
Standard error of slope	0.013	0.029
Standard error of intercept	9.806	6.906
R_f	0.22 ± 0.001	0.34 ± 0.002
LOD (ng)	10.32	21.17
LOQ (ng)	31.28	64.16

Table S2. Precision of the proposed HPTLC Method (n=6)

Conc. of standard added (ng/band)	β-sitosterol				Lupeol			
	Intra-day Precision		Inter-day Precision		Intra-day Precision		Inter-day Precision	
	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD
400	395.56 ± 5.29	1.337	393.92 ± 5.07	1.287	394.84 ± 6.01	1.522	391.61 ± 5.67	1.447
600	593.84 ± 7.38	1.242	590.94 ± 6.87	1.162	592.60 ± 7.83	1.321	589.92 ± 7.26	1.230
800	794.50 ± 9.61	1.209	789.67 ± 8.59	1.087	791.88 ± 9.75	1.231	784.89 ± 7.57	1.219

Table S3. Recovery as accuracy studies of the proposed HPTLC Method (n=6)

Percent (%) of β-sitosterol and lupeol added to analyte	Theoretical concentration of β-sitosterol and lupeol (ng/band)	Concentration found (ng/band) ± SD		%RSD		% Recovery	
		β-sitosterol	Lupeol	β-sitosterol	Lupeol	β-sitosterol	Lupeol
0	400	398.39 ± 5.67	394.39 ± 5.19	1.423	1.315	99.59	98.59
50	600	595.88 ± 7.08	588.94 ± 7.23	1.188	1.227	99.31	98.15
100	800	786.09 ± 8.57	795.46 ± 9.01	1.0901	1.133	98.26	99.43
150	1000	993.94 ± 9.73	984.75 ± 9.84	0.978	0.999	99.39	98.47

Table S4. Robustness of the proposed HPTLC Method (n=6)

Optimization condition	β-sitosterol (400 ng/band)		Lupeol (400 ng/band)	
	SD	%RSD	SD	%RSD
Mobile phase composition; (hexane: ethyl acetate)				
(8:2)	5.67	1.42	6.31	1.60
(7.8:2.2)	5.49	1.37	6.27	1.59
(8.2:1.8)	5.56	1.40	6.24	1.57
Mobile phase volume (for saturation)				
(18 mL)	5.27	1.32	6.41	1.62
(20 mL)	5.18	1.30	6.35	1.60
(22 mL)	5.21	1.31	6.32	1.59
Duration of saturation				
(10 min)	5.73	1.44	6.13	1.54
(20 min)	5.71	1.43	6.09	1.51
(30 min)	5.78	1.46	6.03	1.53

Table S5. Result of regression analysis for model and response regression equation for the final proposed model

Dependent variables	Model F Value	R^2	Adjusted R^2	Predicted R^2	SD
R_1	Linear	0.7767	0.7252	0.5431	0.1063
	2F1	0.9089	0.8543	0.5760	0.0774
	Cubic	0.9914	0.9598	-	0.0207
	Quadratic	0.9948	0.9782	0.9551	0.022
R_2	Linear	0.4909	0.3734	0.1601	0.0093
	2F1	0.6390	0.4224	0.0836	0.0089
	Cubic	0.9916	0.9901	-	0.0011
	Quadratic	0.9923	0.9904	0.9110	0.0016
R_3	Linear	0.7395	0.6794	0.6195	0.0067
	2F1	0.7448	0.5916	0.3737	0.0076
	Cubic	0.9919	0.9908	-	0.0011
	Quadratic	0.9942	0.9927	0.9401	0.0014