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ORIGINAL ARTICLE

Computer-Aided Box–Behnken Outlook Towards Optimization of Extraction of Lawsone from Mehendi Leaves

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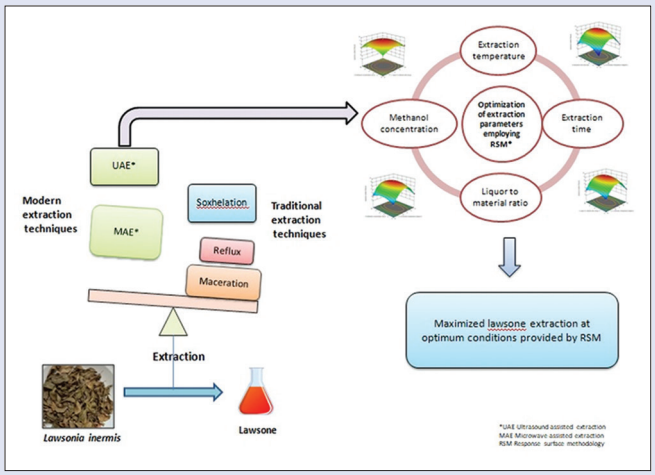
ABSTRACT

Background: Since ages lawsone, an orange-colored dye from leaves of *Lawsonia inermis* is being used by women for self-adornment and is also used in textile industries for dyeing reasons. **Objective:** Comparing traditional and modern extraction modes for extraction of lawsone from *L. inermis* leaves and simultaneous use of response surface methodology for optimizing its extraction procedure. **Materials and Methods:** Design-Expert software was exploited for optimization task. Quantification of lawsone in different extracts was done by high-performance liquid chromatography using aqueous acetic acid and methanol in the mobile phase. **Results:** Ultrasound-assisted extraction stood out to be a supreme technique among all. Moreover, of all solvents (ethylene glycol, dimethylformamide, and methanol) examined methanol stood out to be the most effective solvent for lawsone extraction. By the inverse matrix of the regression model and point prediction, optimal conditions for lawsone extraction were laid down as - extraction temperature - 50.24°C, extraction time - 15.70 min, liquor to material ratio - 24.16 mL/g, and methanol concentration - 75.15%v/v which yielded 17.129 g of lawsone. Under similar conditions (extraction temperature - 50°C, extraction time - 16 min, liquor to material ratio - 24 mL/g, and methanol concentration - 75%v/v), 16.98 g of lawsone was yielded which was close to predicted value. **Conclusion:** We conclude that a non-thermal method proved to a supreme technique for lawsone extraction which has an additional benefit which of avoiding thermal degradation of compound. Furthermore, by model fitting and analysis of regression coefficients, it was confirmed that all the factors studied significantly affected lawsone yield.

Key words: Box–Behnken design, lawsone, *Lawsonia inermis,* naphthoquinone, response surface methodology, ultrasonication

SUMMARY

- *Lawsonia inermis*, commonly known as mehendi is an herb that has been used since ancient times by people for dyeing reasons as well as curing certain ailments. It is a treasurer of a wide range of phytochemical profile. One such phytocompound found in major portion is lawsone, which is an orange dye. Since lawsone is pharmacologically active compound optimization of its extraction process becomes a crucial task so that the maximum amount of the compound can be isolated in a single go. Box–Behnken design (response surface methodology) was opted for optimization to study the effect of four variables – extraction temperature, extraction time, liquor to material ratio, and solvent concentration on lawsone extraction. Of all the extraction modes, ultrasound-assisted extraction stood out to be a supreme technique among all. Moreover, among all solvents (ethylene glycol, dimethylformamide, and methanol), examined methanol stood out to be the most effective solvent for lawsone extraction. By the inverse matrix of the regression model and point prediction, optimal conditions for lawsone extraction were laid down as - extraction temperature - 50.24°C, extraction time - 15.70 min, liquor to material ratio - 24.16 mL/g, and methanol concentration - 75.15%v/v which yielded 17.129 g of lawsone. Under similar conditions (extraction temperature - 50°C, extraction time - 16 min, liquor to material ratio - 24 mL/g, and methanol concentration - 75%v/v), 16.98 g of lawsone was yielded which was close to predicted value. Thereby, it was seen that a non-thermal modern extraction mode provided better yields of lawsone.



Abbreviations used: %v/v: Percent volume by volume; °C: Degree Celsius; 3D: Three dimensional; ANN: Artificial neural network; ANOVA: Analysis of variance; BBD: Box–Behnken design; G: Gram; HPLC: High-performance liquid chromatography; mL: Milli liter; RSM: Response surface methodology; UAE: Ultrasound-assisted extraction.

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INTRODUCTION

Quinones are a class of organic compounds that are not aromatic but are conjugated cyclic diketones. Harbourne classifies this class of phytocompounds into four categories, namely benzoquinones, naphthoquinones, anthraquinones, and isoprenoid, quinones.[1] The benzoquinone moiety in quinones is responsible for imparting color to them. Over 200 species are reported to produce naphthoquinones.

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Of 65 orders, only 16 reports their occurrence, which suggests that phylogenetically more advanced orders namely Caryophyllales, Ericales, and Lamiales have a higher percentage of naphthoquinones producing families.[2] However, they make little contribution to color in higher plants.[1] In lower plants such as bacteria and fungi they contribute to color like their presence in basidiomycetes.[2] Although anthraquinones have been widely studied because of their purgative actions, naphthoquinones are also treasurer of many other pharmacological activities. In plants, they are generated through acetate malonate, the mevalonic and chorismic acids and the p-hydroxybenzoic acid pathways[3,4] and are usually colored and so attract pollinators.[5] Their colors may vary between yellow, orange, and brown and is attributed to the double bond.[5]

Lawsone is a napthhoquinone well known for its coloring properties. Chemically lawsone is 2-hydroxy-1,4 naphthoquinone and is also referred to as hennotannic acid [Figure 1] In major portions lawsone is found in leaves of *Lawsonia inermis* (Lythraceae), commonly called "Henna." Its occurrence has also been reported in *Eichhornia crassipes* (Pontederiaceae).[6] Lawsone has also been produced through hairy root cultures.[7] Henna leaves paste is very popularly used for coloring hair because of the natural burgundy color it provides. It is also used for body art where designs are made on hand and feet from henna paste mostly to beautify the bride to be. Apart from this, lawsone is also used in perfume industry as well as the textile industry for staining fabrics as well as leather.[8] Besides coloring properties, this phytocompound is valued because of a wide spectrum of biological activity it serves. Naphthoquinones including lawsone acts through two mechanisms. First through redox cycling thereby generating reactive oxyen species and second by acting as electrophiles, i.e., accepting an electron pair.[3,9] This class of compounds has proven their actions against parasites such as *Leishmania* spp., *Schistosoma* spp. *Tripanosoma cruzi*, apicomplexans parasites, as *Plasmodium falciparum, Babesia equi, Toxoplasma gondii*, and *Theileria* spp.[3]

The portion in which any compound constitutes a plant depends on various factors such as the type of soil, topography of the geographical area, rainfall, manure used, and many more factors. The extraction of a phytocompound simply means separating it from plant matrix and certain factors such as extraction process, polarity of solvent, extraction temperature, and others play a very crucial role in the extraction process. Whenever any phytocompound is possessed with a wide range of pharmacological activities, its extraction becomes a priority so as to gain maximum benefits from it. Optimization of the extraction process comes into play in such cases. Originally, optimization was done by "one– factor-at– a-time" method where one factor was studied at a time. However, this method fails to study the interactions of different variables being studied. Some computer-aided techniques such as artificial neural network (ANN) and response surface methodology (RSM) are available which helps to provide the interactive effects of the input variables. Both ANN and RSM are mathematical-statistical technique that is void of negative aspects of traditional optimization methods. ANN is a logic programming technique which imitates the human brain features such as learning, remembering, deciding and inference, without receiving any aid. RSM, on the other hand, is statistical method developed by Box and Wilson in 1951 for modeling and analyzing a process in which the response of interest is affected by various variables.[10,11]

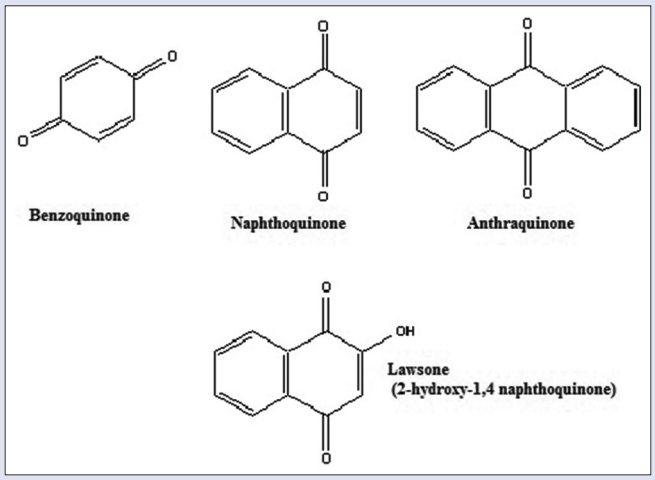

Figure 1: Chemical structure for different classes of quinones

RSM can be successfully used where different combinations of input variables (such as extraction temperature, extraction time, and pH) are given and its response (quantity of phytocompound) is studied. Besides providing interactive effects of the variables, RSM is time-saving and economical.[12] This approach has been opted by various researchers for optimizing biotechnological processes. Hafshejani *et al.* optimized decolorization and mineralization of triazo dye Direct Blue 71 by *Pseudomonas aeruginosa* using RSM.[13] A comparison of RSM and ANN to enhance the release of reducing sugars from nonedible seed cake by autoclave assisted HCl hydrolysis was done by Shet *et al.*[14] Similarly, optimization of extracellular fungal-mediated nanosilver green synthesis was done using RSM by Othman *et al.*[15] Phytocompound extraction field has also exploited this multivariate technique to a large extent [Table 1].

In the present study, we have utilized RSM to optimize the extraction parameters of lawsone from leaves of *L. inermis* and concomitant use of high-performance liquid chromatography (HPLC) for its quantification. The optimization of extraction of total phenolics from leaves of *L. inermis* has been reported by Uma *et al.* in 2010,[34] but the optimization of extraction of lawsone through RSM has not been reported yet.

MATERIALS AND METHODS

Plant material and chemicals

Leaves of *L. inermis* were procured from Herbal garden, Jamia Hamdard University, New Delhi, India. After authentication from a taxonomist, a specimen was preserved in School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India (Voucher number-PPLI/2015/20). HPLC grade solvents-methanol, water, and other analytical grade solvents were purchased from S.D. Fine Chemicals, India. Standard lawsone was bought from Sigma-Aldrich, St. Louis, Missouri, USA.

Statistical technique

Design-Expert Software (version 11, Stat-Ease, Minneapolis, USA) was used for optimization needs.

Precursory experiments

Initial examinations were conducted to determine the best mode and most effective solvent for lawsone extraction. Dried leaves were powdered in a grinder (Sujata Supermix, 900W) and subjected to extraction through maceration, hot solvent extraction through reflux and soxhlet technique, microwave-assisted extraction, and ultrasound-assisted extraction (UAE) in solvents of different polarity.

Quantification of lawsone by high-performance liquid chromatography

Preparation of standard lawsone solution

Stock solution in a concentration of 1 mg/mL of lawsone was prepared in HPLC grade methanol. Aliquots were prepared from stock solution

Table 1: The use of response surface methodology in phytocompound extraction field

Phytocompound extracted	Plant used	Extraction method
Luteolin[16]	*Vitex negundo*	Hot solvent extraction by reflux technique
Embelin[17]	*Embelia ribes*	UAE
Betulinic acid[18]	*Tecomella undulata*	Hot solvent extraction by soxhelation
Anthocyanin pigments[19]	*Melastoma malabathricum*	Hot maceration
Phenolic and anti-oxidant compounds[20]	*Rheum moorcroftianum*	UAE
Puerarin and Daidzein[21]	*Radix Puerariaethomsonii*	MAE
Swertiamarin[22]	*Enicostema littorale*	UAE
Carotenoids[23]	*Diospyros kaki*	SFE
Stigmasterol[24]	*Tecomella undulata*	UAE
Gymnemic acids[25]	*Gymnema sylvestre*	UAE
Anthraquinones[26]	*Rheum emodi*	MAE
Polyphenols[27]	*Punica granatum*	UAE
Rosmarinic acid[28]	*Melissa officinalis*	UAE
Shikmic acid[29]	*Illicium verum*	UAE
Polyphenol[30]	*Prunus virginiana*	MAE
Iridoids[31]	*Gentiana rigescens*	UAE
Polysaccharide[32]	*Angelica sinensis*	UAE
Pentacyclic triterpenoids[33]	*Swertia chirata*	Solid-liquid reflux

UAE: Ultrasound- assisted extraction; MAE: Microwave-assisted extraction

ranging from 50 μg/ml to 0.039 μg/ml. The solutions were filtered through 0.2 μm membrane filter (Axiva) and then stored at −20°C till further use. Before subjecting the solutions to HPLC analysis, they were bought to room temperature. Peak area versus concentration graph was plotted for the same using Microsoft excel 2007 and the linear equation obtained was used to determine the concentration of lawsone in sample solutions.

Preparation of test solution

Accurately weighed 10 mg of each extract was dissolved in HPLC grade methanol, filtered through 0.2 μm membrane filter (Axiva) and then subjected to HPLC analysis.

Chromatographic conditions

HPLC Quaternary System (Shimadzu, Japan) with LC10AT VP pumps (Shimadzu, Japan), single wavelength programmable ultraviolet-visible detector and a system controller was used for HPLC analysis. Lichrosphere C_{18} reverse-phase column (Merck, Germany) with 25 mm × 4.6 mm, particle size 5 μm was used for separation. The sample injection was done using rheodyne injector fitted with a 20 μL fixed loop. 0.1% mol L^{-1} acetic acid: Methanol in ratio of 33:67%v/v at a flow rate of 1 mL min^{-1} and 42°C was used as mobile phase.[35]

Single factorial experiments

Ranging a particular variable over a range while keeping other variables constant helps to analyze the effect of the variable. The variables studied here included extraction temperature, extraction time, liquor to material ratio, and percentage of solvent. The extracts were quantified by HPLC as discussed above.

Statistical optimization

Box–Behnken Design (BBD) was opted since it is devoid of any embedded factorial design.[12] The experimental design consisted of 29 runs, 24 factorial experiments, and 5 replicates of the center points. Coding of the independent variables was done as per below given equation where x_i is coded value of an independent variable, X_i is actual value of independent variable, X_o is actual value of independent variable at center point and ΔX is step-change value of independent variable.

$$x_i = \frac{(X_i - X_o)}{\Delta X}$$

Table 2: Levels of variables for Box-Behnken design

Variable	Levels		
	-1	0	+1
Extraction time (min) A	10	15	20
Extraction temperature (°C) B	45	50	55
Liquor to material ratio (mL/g) C	22	24	26
Methanol concentration (%) D	75	85	95

The three variables were designated as A, B, C, and D and were ordered into three levels coded as + 1, 0, and −1 for high, intermediate, and low levels, respectively. Tables 2 and 3 give the coded and actual values of variables and the BBD runs, respectively. All extracts were quantified by HPLC as discussed above.

RESULTS AND DISCUSSION

Precursory experiments

HPLC analysis showed a retention time of 6.2 min for standard lawsone [Figure 2a and b]. Initial experiments were conducted to determine the best mode and most effective solvent for extraction of lawsone. Among the three solvents investigated, namely, methanol, ethylene glycol and dimethylformamide, methanol extracted maximum amount of lawsone. Simultaneously UAE stood out to be the best mode of extraction [Figure 2c].

Six dilutions were taken to prepare the calibration plot of standard lawsone (peak area versus concentration) which gave a good correlation coefficient (R^2) of 0.994 [Figure 2d]. The corresponding linear equation (y = mx + c) obtained was used to calculate the content of lawsone in each extract.

Single factorial experiments

These trials guided to select ranges of factors for RSM, BBD. The leveling for BBD was done based on the results of these trials. Results are given in Figure 3.

Optimization by response surface methodology

BBD provided 29 runs with different combinations of four variables. By multiple regression analysis, second-order polynomial models were established for the variables and the relationship between

Table 3: Design of experiments by Box-Behnken design

Run	Factor 1 A: Extraction temperature (°C)	Factor 2 B: Extraction time (min)	Factor 3 C: Liquor to material ratio (mL/g)	Factor 4 D: Methanol concentration (% v/v)	Response Lawsone content (% w/w)	
					Experimental value	Predicted value
1	0	+1	0	-1	5.09	4.79
2	0	0	0	0	9.08	8.88
3	0	0	-1	+1	4.65	4.73
4	0	0	+1	-1	5.68	5.15
5	0	0	0	0	9.08	8.88
6	0	+1	-1	0	4.78	4.62
7	+1	+1	0	0	0.78	1.79
8	0	0	0	0	9.09	8.88
9	0	-1	0	-1	3.33	3.74
10	+1	0	+1	0	1.97	2.20
11	+1	0	0	+1	1.89	1.89
12	+1	0	0	-1	1.94	1.73
13	-1	0	-1	0	0.45	0.4475
14	+1	-1	0	0	0.85	0.5767
15	0	-1	0	+1	3.03	3.56
16	0	-1	+1	0	3.43	3.81
17	0	0	-1	-1	4.12	4.53
18	0	0	0	0	7.97	8.88
19	0	+1	0	+1	5.55	5.38
20	0	0	0	0	9.06	8.88
21	-1	0	0	-1	0.34	0.5625
22	-1	0	0	+1	0.37	0.8058
23	0	+1	+1	0	5.12	5.44
24	-1	+1	0	0	1.07	0.8833
25	0	-1	-1	0	3.47	3.38
26	-1	0	+1	0	0.33	0.8108
27	-1	-1	0	0	0.39	0.7700
28	+1	0	-1	0	1.56	1.31
29	0	0	+1	+1	7.23	5.36

lawsone content and the variables was given as per the equation given below:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_0 A_1 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2$$

Where Y is the predicted response, i.e., lawsone content, β_0 is model constant, β_1, β_2, β_3, and β_4 linear coefficients, A, B, C, and D are independent variables, $\beta_{12,}$ $\beta_{13,}$ $\beta_{14,}$ $\beta_{23,}$ β_{24}, and β_{34} are cross-product coefficients, $\beta_{11,}$ $\beta_{22,}$ β_{33}, and β_{44} are the quadratic coefficients.

Model fitting, analysis, and response surface curves

The analysis of the results was done through the coefficient of regression (R^2), analysis of variance (ANOVA), and response surfaces. R^2 value of 0.9860 implies the closeness of the data to the fitted regression. The values of Adjusted-R^2 (0.9611) and Predicted-R^2 (0.9018) were close enough signifying excellent fit of the model. Meantime, the coefficient of variance (%CV) of 15.27% which is small value advocated a good dependency on the experimental model. "Adequate Precision" (signal to noise ratio) which is desired to be more than 4 was 22.9270 further showing goodness of the model. The lack of Fit test gives the variation of the data around the fitted model. The *F*-value and *P* value for the lack-of-fit were 1.96 and 0.2703, respectively, implying it to be non-significant which was good for the model. By multiple regression analysis, the following second-order polynomial equation was obtained:

$$\text{Lawsone content} = 8.88 + 0.5633\,A + 0.7167\,B + 0.3117\,C + 0.1017\,D - 0.1100\,AB + 0.1300\,AC - 0.200\,AD + 0.0950\,BC + 0.1900\,BD + 0.0050\,CD - 5.69\,A^2 - 2.57\,B^2 - 02.00\,C^2 - 1.94\,D^2$$

The results for ANOVA have been tabulated in Table 4. F and *P* values give the significance of coefficient terms. The larger the *F*-value and the smaller the *P* value, more significant is the model.[36] Here, A, B, A^2, B^2, C^2, and D^2 had $P < 0.0500$ proposing that the model could be used to predict these responses.

Three dimensional response surface plots help to understand the interactive effects of variables more clearly. Figure 4a helps to understand the interactions between A and B. As the extraction temperature is increased from 45°C to 52°C, an increase in lawsone content is noted. However, further increment in temperature brings a drop in lawsone content. Similarly, increasing the extraction time from 10 min to 17 min increases the lawsone content, beyond which a dip is observed. As shown in Figure 4b, as the liquor to material ratio is varied from 22 mL/g to 24.9 mL/g lawsone extracted also increases after which a decrement in seen till 26 mL/g. Extraction temperature had the same effect as seen with extraction time, i.e., increment in lawsone content till 52°C. Figure 4c shows the interactive effects of A and D which shows that as methanol concentration rises from 70%v/v to 76.8%v/v lawsone content also increases. Further increasing the methanol concentration do not further increase lawsone content. Furthermore, extraction temperature till 52.5°C shows an uphill trend toward lawsone but beyond that a downhill trend is observed. Figure 4d shows that maximum lawsone content can be achieved at extraction time of 19 min and liquor to the material ratio of 25.8 mL/g. Interactive effects of B and D from Figure 4e shows lawsone content to increase as the extraction time increases till 19.41 min and methanol concentration till 79.41%v/v after which both factors shows a dip in lawsone concentration. In case of C and D [Figure 4f], both the factors shows maximum lawsone extracted at 25.6 mL/g liquor to material ratio and 79.28%v/v of methanol concentration. Further enhancement in both factors decreased the lawsone content in the extracts.

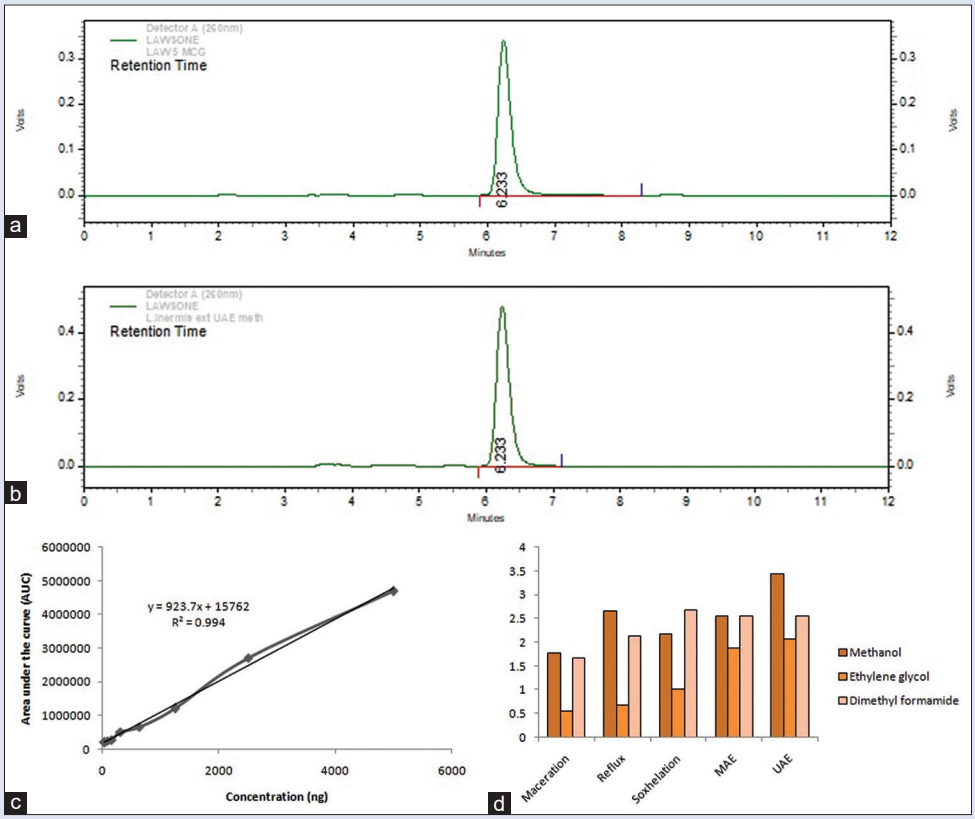

Figure 2: (a) High performance liquid chromatography chromatogram of pure lawsone (2.5 µg). (b) High performance liquid chromatography chromatogram of *Lawsonia inermis* ultrasound-assisted extraction methanolic extract. (c) Calibration curve of pure lawsone with respect to peak area. (d) Comparative chart of different methods and solvents for extraction of Lawsone

Table 4: Analysis of variance for quadratic model

Source	Sum of squares	Mean square	*F*	*P*
Model	241.60	17.26	50.43	<0.0001 (significant)
A-extraction temperature	3.81	3.81	11.13	0.0049
B-extraction time	6.16	6.16	18.01	0.0008
C-liquor to material ratio	1.17	1.17	3.41	0.0862
D-methanol concentration	0.1240	0.1240	0.3624	0.5568
AB	0.0484	0.0484	0.1414	0.7125
AC	0.0676	0.0676	0.1975	0.6635
AD	0.0016	0.0016	0.0047	0.9465
BC	0.0361	0.0361	0.1055	0.7501
BD	0.1444	0.1444	0.4220	0.5265
CD	0.0001	0.0001	0.0003	0.9866
A^2	209.86	209.86	613.23	<0.0001
B^2	42.78	42.78	125.00	<0.0001
C^2	25.83	25.83	75.48	<0.0001
D^2	24.43	24.43	71.37	<0.0001
Residual	4.79	0.3422		
Lack of fit	3.98	0.3979	1.96	0.2703 (not significant)
Pure error	0.8125	0.2031		
Cor total	246.40			

Validation of the model

Through point prediction analysis and inverse matrix of regression polynomial equation, the optimal levels of variables (A - 50.24°C, B -15.70 min, C - 24.16 mL/g, and D - 75.16%v/v) as well as predicted value of lawsone content (17.12 g/25 g raw material) were obtained. Under similar conditions (A - 50°C, B - 16 min, C - 24 mL/g, and D - 75%v/v) experiments in triplicate (n = 3) were conducted as recheck run. Lawsone content in these experiments was found to be 16.98 g/25 g of raw material which was slightly lower than predicted value [Figure 5]. However, no significant difference was observed between the predicted yield and experimental one when the Student's *t*-test was conducted, indicating that the model was satisfactory and adequate for reflecting the expected optimization.

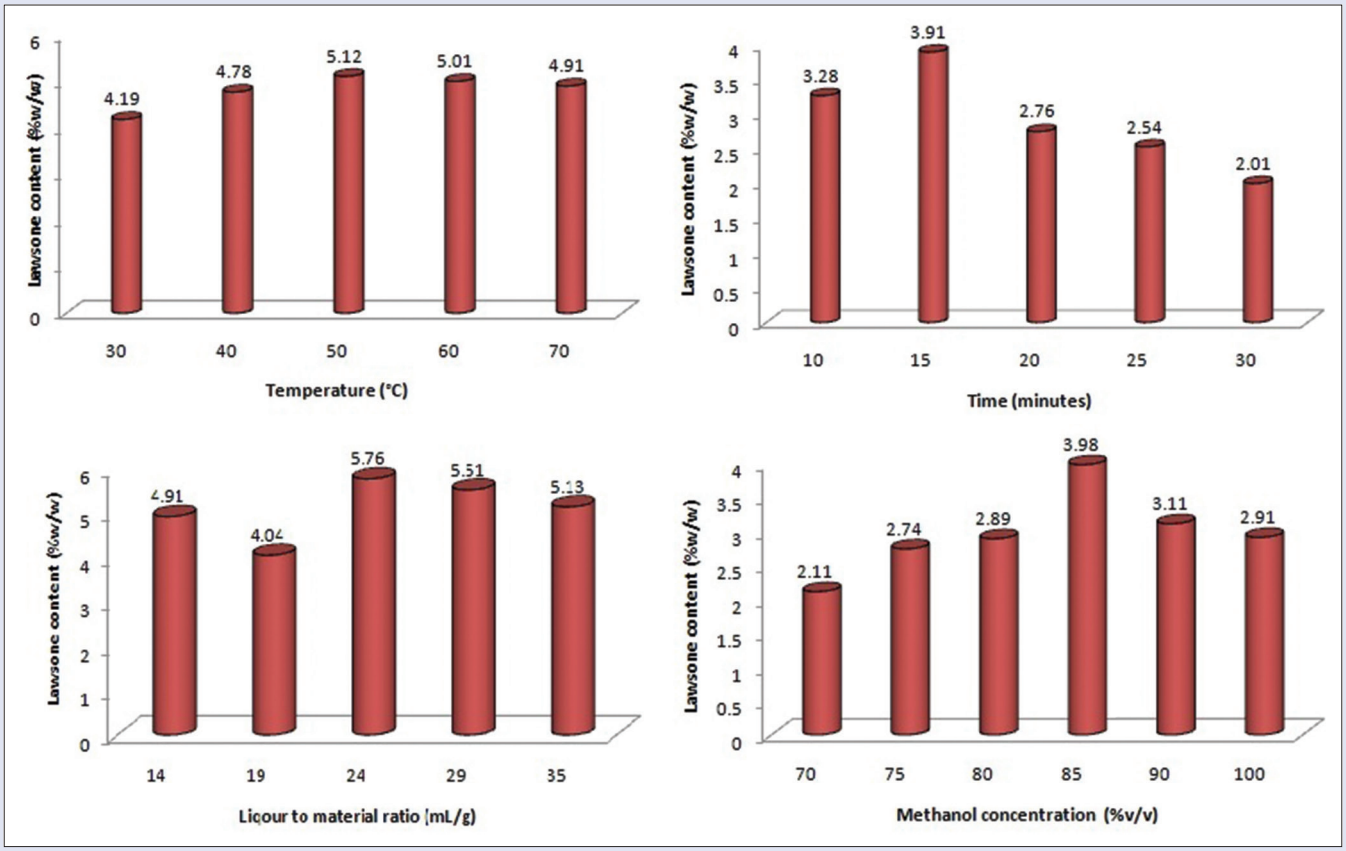

Figure 3: Results of single factorial experiments

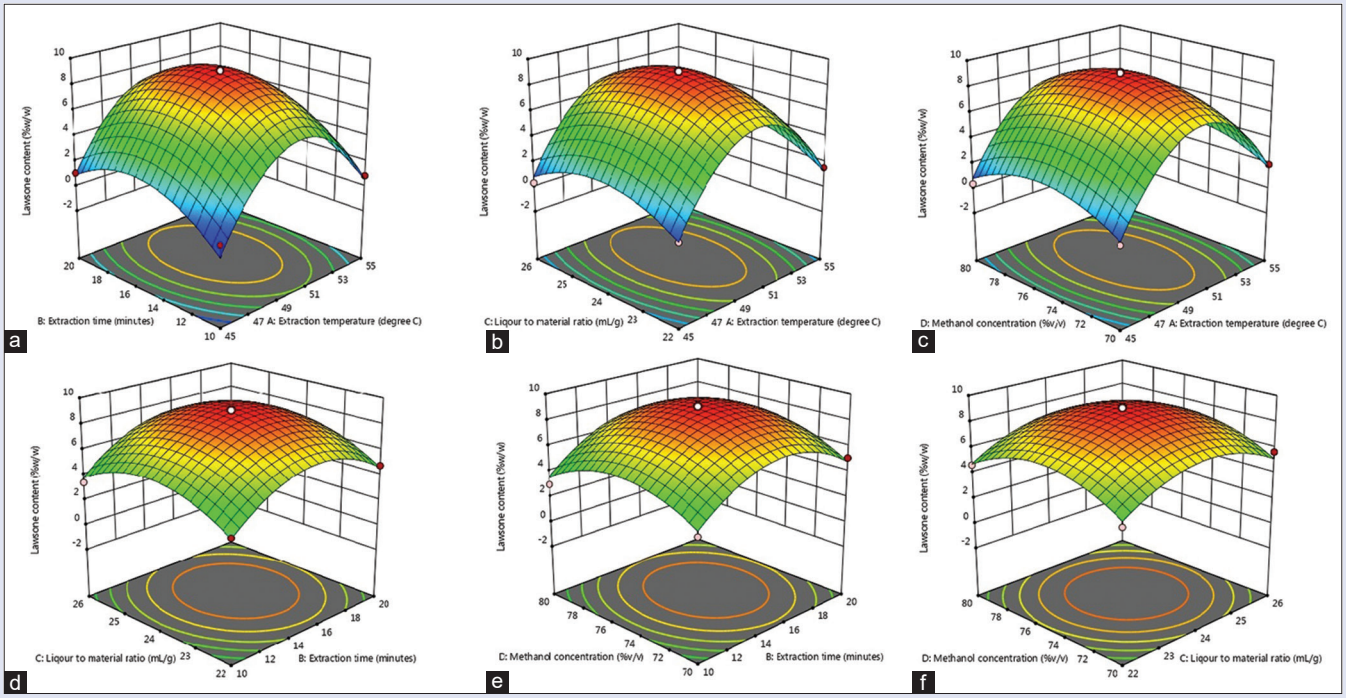

Figure 4: (a) 3D surface plot for A (extraction temperature) and B (extraction time). (b) 3D surface plot for A (extraction temperature) and C (liquor to material ratio). (c) 3D surface plot for A (extraction temperature) and D (methanol concentration).(d)- 3D surface plot for B (extraction time) and C (liquor to material ratio). (e) 3D surface plot for B (extraction time) and D (methanol concentration). (f) 3D surface plot for C (liquor to material ratio) and D (methanol concentration)

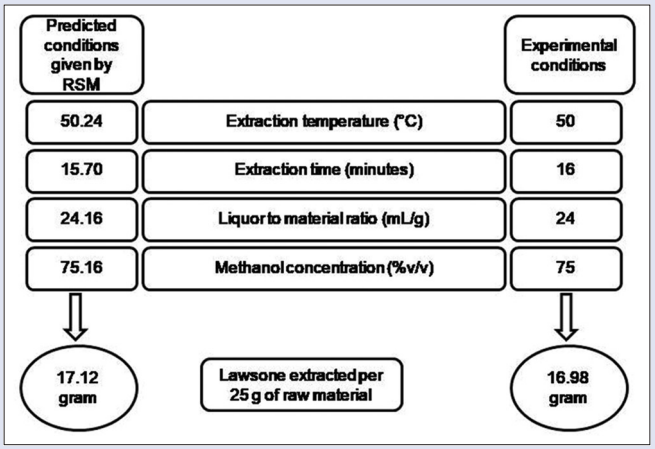

Figure 5: The illustration of predicted and experimental conditions for lawsone extraction

CONCLUSION

Extraction and isolation of natural dyes is an indispensible act for textile industries as well as pharmaceutical firms, especially where the dye is credited with biological activities too. Stereotypically extraction was done by traditional means which includes solid–liquid extraction which are still most commonly used methods for extraction because of ease of use, efficiency, and wide-ranging applicability.[36] However, modern extraction techniques provide a upper hand over conventional methods of being ecofriendly. Our study explored both ways and found out that UAE which is a modern and a greener approach of extraction provides better yields of lawsone. The solvent also plays a very vital role in extraction. A solvent apart from being according to the polarity of the compound being extracted should also be able to withstand the interfering substances. In this piece of work, we optimized that extraction process of lawsone, which is natural dye from henna leaves through UAE. RSM, a multivariate statistical approach was utilized in this regard to reduce the number of experimental trials and simultaneously studying the interactive effects of various independent variables being studied. The extraction optimization of dye from *Butea monosperma* by RSM has been done earlier by Sinha *et al.* (2012)[37] but for lawsone has not been reported yet. The optimized conditions for lawsone extraction were found to be as extraction temperature - 50.24°C, extraction time - 15.70 min, liquor to material ratio - 24.16 mL/g, and methanol concentration - 75.16%v/v. Under such conditions 16.98 g/25 g of raw material lawsone was yielded which was slightly lower than the value predicted by BBD.

The outcome of our research will help the other researchers to take advantage of the conditions given by RSM, BBD to isolate maximum amount of lawsone from *L. inermis* leaves by UAE. UAE which is also a non-thermal method provides additional benefit of avoiding thermal degradation of the phytocompound as well as being environmentally friendly.

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Conflicts of interest

There are no conflicts of interest.

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