Indian Journal of Natural Products and Resources
Vol. 16(3), September 2025, pp. 410-416
DOI: 10.56042/ijnpr.v16i3.17727

Optimization of extraction conditions for *Cupressus torulosa* essential oil using response surface methodology and chemical characterization by GC-MS

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Received 07 March 2025; revised received 09 June 2025; accepted 26 June 2025

This study aims to optimize *Cupressus torulosa* essential oil (CTEO) extraction conditions using Response Surface Methodology (RSM) and analyze its chemical composition. A three-factor, three-level Box-Behnken Design (BBD) was employed to evaluate the effects of extraction time, material-to-liquid ratio (MLR), and drying period on essential oil yield. The optimized conditions—5.355 hours of extraction, a 16:1 MLR, and a 5.285-day drying period—resulted in a predicted yield of 1.121%. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified 31 compounds, comprising 91% of the total oil, with *β*-Caryophyllene (10.91%), *Δ*-3-carene (10.21%), Terpinen-4-ol (9.81%), and Caryophyllene oxide (9.15%) as major constituents. Statistical validation using ANOVA confirmed the significance of the model, with extraction time having the most notable effect on yield. These findings highlight the importance of optimizing extraction parameters to maximize yield and maintain essential oil quality.

Keywords: *Cupressus torulosa*, Essential oil, GC-MS, Optimization, Response surface methodology

IPC code; Int. cl. (2021.01)− A61K 36/00, A61K 127/00

Introduction

Cupressus torulosa, more commonly known as the Himalayan cypress, is an evergreen species native to the northwestern Himalayas including regions of India, Nepal, and Tibet[1]. In Uttarakhand, India, this tree is widely distributed and locally called 'Surai'[2]. Traditionally, the needles of *C. torulosa* are recognized for their notable medicinal applications, such as anti-inflammatory, antimicrobial, and wound-healing properties[3]. Scientific studies back these traditional uses, demonstrating that essential oil derived from the needles (CTEO) possesses significant antioxidant, antimicrobial and anti-inflammatory effects, supported by both *in vitro* and *in vivo* research. CTEO has demonstrated effective inhibition against various bacterial pathogens like *Bacillus subtilis, Micrococcus luteus*, *Pseudomonas alcaligenes*, and *Bacillus cereus*. Regarding antifungal properties, CTEO shows strong bioactivity against plant and human pathogenic fungi such as *Alternaria alternata*, *Curvularia lunata*, *Bipolaris spicifera*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*[3,4]. We confirmed its significant antioxidant potential in a separate study through *in vitro* DPPH, ABTS free radical scavenging assays, and the FRAP assay. Additionally, CTEO demonstrated strong anti-inflammatory activity in both *in vitro* and *in vivo* models[5]. In our previous work, we established the chemical composition of CTEO from 14 different locations in Uttarakhand and Himachal Pradesh. We identified five chemotypes in the Uttarakhand region: terpinen-4-ol/limonene, terpinen-4-ol/sabinene, terpinen-4-ol, terpinen-4-ol/umbellulone, and terpinen-4-ol/totarol[6]. Moreover, Lohani *et al.* reported *α*-pinene-rich CTEO from the Kalsi, Joshimath, and Jeharikhal regions of Uttarakhand, indicating the presence of *α*-pinene chemotype[7]. Lohani *et al.* also documented seasonal and physiological variations, reporting that trees in the Chakrata region yielded the highest essential oil content (1.38%) during the monsoon season in small-sized trees, while the lowest yield (0.36%) was recorded in medium-sized trees during summer. During the monsoon, terpinen-4-ol was dominant across all tree sizes; in the post-monsoon period,

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limonene was more prevalent. In winter, *α*-pinene dominated in small and medium trees, while sabinene was the major constituent in large trees. In summer, terpinen-4-ol remained dominant in small trees, whereas *α*-pinene was prominent in medium and large trees[8]. Such variations in chemical composition due to geographical, seasonal, and physiological factors lead to differing applications. Bhalla *et al.* recommended the terpinen-4-ol chemotype for formulating natural anti-inflammatory products due to its significant biological activity. They reported that the terpinen-4-ol/umbellulone chemotype, characterized by high levels of umbellulone, a known headache-inducing agent, may be less suitable for the fragrance industry but may show potential as a mosquito repellent, given the reported effectiveness of umbellulone against *Aedes aegypti* mosquitoes[6]. Identifying such chemotype-specific applications marks a step forward in the commercialization of *C. torulosa*.

However, the realization of these commercial applications largely depends on the availability and quality of raw material. The efficiency and quality of essential oil production are strongly influenced by the extraction method[9,10]. Various techniques, including traditional hydrodistillation and steam distillation, as well as advanced methods like microwave-assisted distillation and supercritical fluid extraction, significantly affect the yield and chemical profile of essential oils, ultimately impacting their biological activity[11]. Our recent comparative study of hydrodistillation and steam-water distillation for CTEO extraction revealed that hydrodistillation offers higher oil yield while better preserving volatile compounds[2]. Although the extraction methods, biological activities, and chemical variations of CTEO have been explored, bringing it closer to commercialization and optimizing the extraction conditions to ensure both maximum yield and consistent chemical quality are critical factors for its commercial viability and therapeutic efficacy. Without systematic optimization, extraction protocols may underutilize plant material, resulting in reduced oil recovery and higher raw material costs, or degrade sensitive constituents due to excessive heat, prolonged exposure, or suboptimal solvent ratios, compromising the oil's chemical integrity and bioactivity.

Since different extraction methods and conditions significantly influence the quantity and quality of essential oils, refining the process is essential to enhance its commercial potential. To achieve this, the present study focuses on optimizing the extraction conditions of CTEO using Response Surface Methodology (RSM). This statistical approach enables systematic evaluation of multiple variables to identify optimal conditions for maximum yield. This study examines key factors such as extraction time, material-to-liquid ratio (MLR), and drying period, all of which play a critical role in determining oil yield and analyzing its chemical composition through Gas Chromatography-Mass Spectrometry (GC-MS). Effective optimization safeguards economic and functional value, ensuring that *C. torulosa* oil can be produced at scale with predictable quality and maximal resource efficiency.

Materials and Methods

Collection of plant material

Approximately 30 kg of *C. torulosa* needles were gathered from Ogla, Uttarakhand, India (29°44'1.2" N, 80° 18'26.9" E, 1561 m) in January 2024. The identity of the plant material was confirmed by Dr. P. K. Verma from the Systematic Botany Branch, Botany Division of ICFRE-Forest Research Institute, Dehradun, India.

Experimental design and statistical analysis

A three-factor, three-level Box-Behnken Design (BBD) was employed to optimize the extraction process by hydrodistillation. There independent variables *viz.* extraction time, MLR, and drying period were chosen with their central points and ranges based on results from initial experiments. Samples were dried at room temperature (~25°C) with 50% relative humidity under shaded, well-ventilated conditions to preserve volatile compounds. Each parameter was tested at three levels, coded as -1 for the lower level, +1 for the higher level, and 0 for the central value, as presented in Table 1.

A total of 45 experimental runs were designed using BBD, as shown in Table 2. The experiments were carried out, and the response variable, essential oil yield (%; v/w), was determined using the equation 1:

Table 1 — Independent variables and range of levels used for experimentation

Factors	Levels		
	-1	0	+1
Time (hours)	3	4	5
MLR	11	15	19
Drying period (days)	0	3	6

Table 2— Experimental design matrix and the extraction yield evaluated at various experimental settings

Run	Factor			Yield %
	Extraction time	MLR	Drying period	
1	4	19	0	0.67
2	5	15	6	0.93
3	5	15	0	0.60
4	4	19	0	0.70
5	4	19	6	0.77
6	4	11	0	0.63
7	4	15	3	1.00
8	4	19	6	1.07
9	3	19	3	0.84
10	5	19	3	1.07
11	4	15	3	0.90
12	4	15	3	1.00
13	4	19	6	1.27
14	5	11	3	0.97
15	5	15	0	0.97
16	4	11	0	0.63
17	5	15	6	1.27
18	3	11	3	0.83
19	4	15	3	0.97
20	5	15	0	0.97
21	5	15	6	1.03
22	4	11	0	0.60
23	4	11	3	0.97
24	3	15	6	0.67
25	3	15	6	0.84
26	3	11	3	0.73
27	4	15	3	1.00
28	4	15	3	1.07
29	5	11	3	0.97
30	3	15	0	0.57
31	3	15	0	0.57
32	4	15	3	0.97
33	3	15	0	0.83
34	5	19	3	0.93
35	3	11	3	0.73
36	3	19	3	0.57
37	4	19	0	0.70
38	3	19	3	0.93
39	3	15	6	0.77
40	4	15	3	0.97
41	4	11	6	0.93
42	5	11	3	0.83
43	4	11	6	0.93
44	4	11	6	1.03
45	5	19	3	1.00

$$\text{Yield (\%)} = \frac{Volume\ of\ extracted\ essential\ oil\ (mL)}{Initial\ Mass\ of\ sample\ (g)} \times 100 \quad \ldots (1)$$

Regression analysis was performed to fit the data into a Second-Order Quadratic Model. The model's statistical significance was assessed using analysis of variance (ANOVA) and the F-test to establish the relationship between input factors and extraction yield. The significance of each model term was determined based on *p*-values (considered significant if $p \leq 0.05$). Response surface and contour plots were generated to visualize the effects of individual variables and interactions on essential oil yield, helping to identify the optimal extraction conditions.

Extraction of essential oil

After finalizing the 45-run experimental design, essential oil extraction was carried out according to the specific conditions outlined for each experiment by hydrodistillation using the Clevenger apparatus. Once extracted, the essential oil was carefully dried using anhydrous Na_2SO_4 to remove any residual moisture, and the dried essential oil was transferred into amber-colored vials. The vials were then stored at 4°C.

GC-MS analysis of the essential oil

GC-MS analysis was performed using an Agilent Technologies GC 7890B system coupled with a 5977A Mass Spectrometer (EI 70 eV) and a DB-5 MS UI column (30 m × 0.25 mm × 0.25 µm) following the protocols established in our previous studies[5,6]. The GC oven temperature program began at 40°C, holding for 4 minutes, then increased at a rate of 4°C per minute until reaching 220°C, where it was held for a final 15 minutes. The injector port and detector temperatures were set and maintained at 250°C and 280°C, respectively. Helium was used as the carrier gas, flowing at a rate of 1 mL per minute, and a 1 microliter sample was injected in splitless mode. The compounds were identified by matching their mass spectra against the Wiley11-NIST17 and Wiley FFNSC2 spectral libraries, as well as by comparing their retention indices (RIs) with values from the NIST database and published literature. Mass spectra were scanned over a *m/z* range of 40 to 300. RIs were calculated using a series of *n*-alkanes ranging from C6 to C25.

Results and Discussion

Optimization of essential oil yield

The yield of CTEO obtained from the 45 experimental runs ranged from 0.57% to 1.27%, as shown in Table 2. The optimal extraction conditions, determined using RSM, were an extraction time of 5.355 hours, a MLR of 1:16.351, and a drying period of 5.285 days. Under these optimized conditions, the

predicted yield of CTEO was 1.121%, with a 95% confidence interval ranging from 0.971% to 1.270%.

The second-order quadratic model for predicting yield can be expressed as:

$$YIELD=\beta0+\beta1(TIME)+\beta2(MLR)+\beta3(DRYING\ PERIOD)+\beta4(TIME^2)+\beta5(MLR^2)+\beta6(MLR\times DRYING\ PERIOD)+\beta7(TIME\times DRYING\ PERIOD)+\beta8(TIME\times MLR)$$

where β_0, β_1, β_2, β_3, β_4, β_5, β_6, β_7, and β_8 are the regression coefficients for the constant term and interaction effects of extraction time, MLR, and drying period. The coefficients obtained are listed in Table 3.

By substituting the estimated coefficients into the model, the final regression equation for predicting the essential oil yield (%) can be expressed as:

$$Yield = -1.303 + 0.450Time + 0.123MLR + 0.081Drying\ Period - 0.050Time^2 - 0.004MLR^2 - 0.010\ Drying\ Period^2 + 0.004Time*MLR + 0.000MLR*Drying\ Period + 0.004Time*Drying\ Period$$

To validate the model, ANOVA was performed (Table 4). The results showed that the regression F-ratio was 6.858, exceeding the tabulated F-ratio, confirming the model's validity. The study analyzed linear and quadratic effects, with the linear effects demonstrating significant variations in essential oil yield due to changes in independent variables. In contrast, the quadratic effects highlighted non-linear relationships. The interaction effects among variables were statistically insignificant, indicating that their combined influence did not significantly impact yield beyond their contributions. The quality and accuracy of the regression model were assessed using multiple statistical parameters. The Multiple R value of 0.799 indicates a strong positive correlation between the independent variables (TIME, MLR, and DRYING PERIOD) and the dependent variable (YIELD). The Squared Multiple R value of 0.638 suggests that these independent variables account for approximately 63.8% of the variance in essential oil yield. The Adjusted R-squared value of 0.545, which accounts for the number of predictors, further supports the model's adequacy while acknowledging a slight reduction in predictive power due to the limited number of independent variables. Although the R^2 and Adjusted R^2 values are moderate, this reflects that essential oil yield is influenced by a broader range of factors beyond the three variables optimized in this study. Previous research on other plant species has identified additional influencing parameters such as distillation temperature, particle size, and salt (NaCl) addition, which were not incorporated in the present optimization model[12,13]. Consequently, the limitation of the current study lies in its focus on only three independent variables, which collectively explain 63.8% of the total variability in yield. Nonetheless, the model's reliability is further substantiated by a

Table 3 — Regression coefficients for uncoded factors

Effect	Coefficient
CONSTANT	-1.303
TIME	0.450
MLR	0.123
DRYINGPERIOD	0.081
TIME*TIME	-0.050
MLR*MLR	-0.004
DRYINGPERIOD*DRYINGPERIOD	-0.010
TIME*MLR	0.004
MLR*DRYINGPERIOD	0.000
TIME*DRYINGPERIOD	0.004

Table 4 — Analysis of variance table

Source	df	Type I SS	Mean squares	F-ratio	*p*-value
Regression	9	0.842	0.094	6.858	0.000
Linear	3	0.699	0.233	17.089	0.000
Quadratic	3	0.138	0.046	3.372	0.029
Interaction	3	0.005	0.002	0.112	0.953
Residual error	35	0.477	0.014		
Total error	44	1.319			
Lack of fit	4	0.094	0.023	1.888	0.137
Pure error	31	0.384	0.012		
Residual error	35	0.477	0.014		
Multiple R	0.799				
Squared multiple R	0.638				
Adjusted squared multiple R	0.545				
Standard error of estimate	0.117				

standard error of estimate of 0.117, indicating minimal average deviation between observed and predicted values, and hence, a good model fit. Moreover, the lack-of-fit test yielded a *p*-value of 0.137, above the 0.05 threshold, indicating that the model does not suffer from a significant lack of fit and is statistically robust for the defined parameter space.

Two-dimensional contour plots were used to visualize the interactions between independent variables and their impact on essential oil yield. Fig. 1 illustrates these interactions. The contour plot of YIELD vs. TIME and MLR (Fig. 1a) reveals that the EO yield increases with both TIME and MLR, peaking at approximately 5.0 hours of extraction and an MLR of around 16. The concentric nature of the contours suggests a well-defined optimal region, indicating that prolonged extraction and adequate solvent availability enhance oil recovery. However, excessive levels may not further increase the yield due to saturation. Similarly, the plot of YIELD vs. TIME and DRYING PERIOD (Fig. 1b) shows a marked rise in yield with increasing TIME and DRYING PERIOD, reaching an optimum when drying is performed for about 5 to 6 days and extraction is carried out for roughly 5 hours. This trend emphasizes the role of proper drying in reducing moisture content, which facilitates better oil release. The third plot, YIELD vs. MLR and DRYING PERIOD (Fig. 1c), also highlights an optimal zone, with the highest yields observed when MLR is around 16–17 and DRYING PERIOD ranges between 5 and 6 days. The elliptical contours indicate that both variables significantly influence the yield, with MLR playing a more dominant role in this interaction. This can be attributed to improved diffusion and solvent contact with plant material at higher MLR values, in conjunction with moisture reduction through optimal drying.

Chemical composition of essential oil

The chemical composition of CTEO was analyzed using GC-MS, which identified 31 compounds constituting 91% of the total oil. The major constituents included *β*-caryophyllene (10.91%), *Δ*-3-carene (10.21%), terpinen-4-ol (9.81%), and caryophyllene oxide (9.15%). The composition was predominantly made up of monoterpenoids (49.59%), followed by sesquiterpenoids (37.91%) and diterpenes (3.5%) (Table 5).

Several studies have investigated the chemical composition of CTEO. The findings of this study align with previous research while also revealing notable differences in extraction efficiency, chemical composition, and influencing factors. This study optimized the extraction process using RSM, identifying optimal conditions to maximize oil yield (1.121%). In contrast, earlier studies primarily focused on comparing the effects of different extraction techniques, seasonal variations, tree age, and plant parts on oil composition[2,8,14]. The chemical composition of CTEO in this study identified *β*-caryophyllene, *Δ*-3-carene, terpinen-4-ol, and caryophyllene oxide as the major constituents.

In contrast, previous studies reported variations in dominant compounds, such as terpinen-4-ol and umbellulone in hydro-distilled oil, and *α*-pinene and *δ*-3-carene in steam-distilled samples[2]. Furthermore, *α*-pinene-rich compositions have been reported from three locations in Uttarakhand (Kalsi, Joshimath, and Jeharikhal)[7]. Notably, a separate study conducted at the same location revealed compositional differences, with caryophyllene, identified as a major compound in the present study, being absent[15]. This variation may be attributed to differences in the timing of plant material collection, as seasonal changes significantly influence essential oil composition. Additionally, the drying duration prior to extraction can alter the

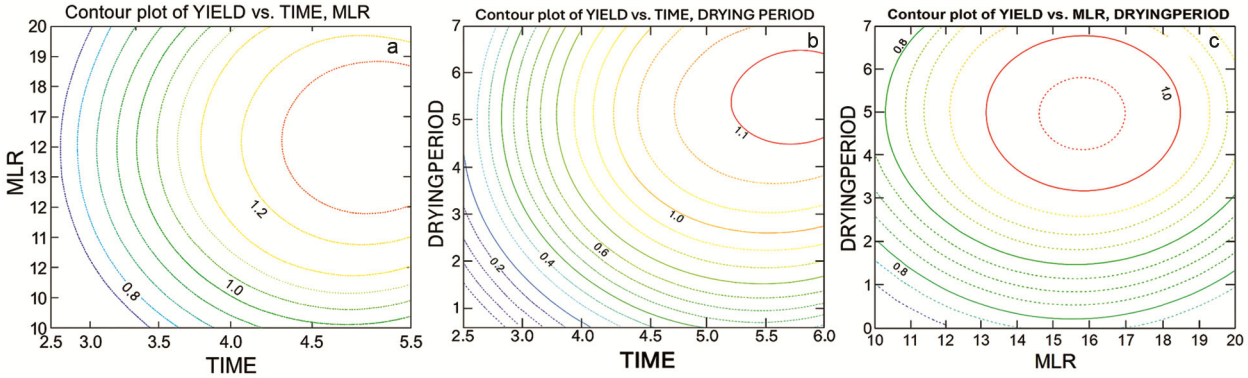

Fig. 1 — Two-dimensional contour plots by RSM analysis.

Table 5 — Chemical composition of the essential oil extracted from *Cupressus torulosa* needles

S. No.	Rt. (Min.)	Compounds	RI (Cal.)	RI (Lit.)	Area (%)	Class of compounds
1	11.65	*α*-Pinene	938.	937	3.13	MH
2	13.305	4-Thujene	978	980	7.23	MH
3	14.045	*β*-Myrcene	996	997	1.49	MH
4	14.757	*Δ*-3-Carene	1014	1010	10.21	MH
5	15.118	*α* -Terpinene	1023	1023	2.67	MH
6	15.433	Cymene	1031	1030	0.73	MH
7	15.613	Limonene	1030	1030	3.98	MH
8	16.791	*γ*-Terpinene	1060	1060	4.30	MH
9	17.852	*α*-Terpinolene	1092	1093	1.72	MH
10	18.469	Linalool	1107	1110	0.64	MH
11	21.075	Umbellulone	1171	1171	1.34	MH
12	21.529	Terpinen-4-ol	1189	1190	9.81	MH
13	22.054	*α*-Terpinenol	1203	1203	1.10	MH
14	27.341	*α*-Terpenyl acetate	1355	1352	1.24	MH
15	28.774	(-)-*β*-Elemene	1391	1391	1.73	SQ
16	29.765	*β*-Caryophyllene	1430	1429	10.91	SQ
17	30.051	*γ*-Elemene	1439	1435	0.64	SQ
18	30.884	*α*-Humulen	1466	1462	1.17	SQ
19	31.088	*cis*-Muurola-4(14),5-diene	1464	1464	3.05	SQ
20	31.683	Germacrene D	1483	1483	1.77	SQ
21	32.161	Epizonarene	1506	1501	2.41	SQ
22	32.446	2,4-Di-tert-butylphenol	1508	1509	1.48	SQ
23	32.814	*δ*-Cadinene	1528	1526	0.28	SQ
24	34.737	Caryophyllene oxide	1593	1593	9.15	SQ
25	35.069	Cetene	1596	1593	1.02	DT
26	35.530	Humulene oxide II	1610	1612	0.81	SQ
27	36.043	Isospathulenol	1630	1630	0.37	SQ
28	36.824	*α*-Cadinol	1666	1669	2.52	SQ
29	38.695	*cis*-Nuciferol	1725	1724	1.62	SQ
30	47.712	Abitadiene	2082	2083	1.30	DT
31	48.773	Nezukol	2132	2133	1.18	DT
	Monoterpenoids (MH)				49.59	
	Sesquiterpenoids (SQ)				37.91	
	Diterpenoids (DT)				3.5	
	Total				91	

relative proportions of monoterpenoids and sesquiterpenes. Prolonged drying may lead to the evaporation of highly volatile compounds, such as *α*-pinene and limonene, thereby increasing the concentration of less volatile oxygenated monoterpenes, such as terpinen-4-ol. Bhalla *et al.* recently reported several chemotypes for CTEO from the Uttarakhand region of India, including terpinen-4-ol/limonene, terpinen-4-ol/sabinene, terpinen-4-ol, terpinen-4-ol/umbellulone, and terpinen-4-ol/totarol[6]. Although terpinen-4-ol was one of the major compounds in the present study, the high concentrations of *β*-caryophyllene and *Δ*-3-carene suggest the presence of a distinct chemotype beyond those previously reported. A more comprehensive investigation, including seasonal variations, is required to fully characterize *C. torulosa* for commercial applications and maximize its potential utility. Overall, this study provides a detailed assessment of extraction optimization while reaffirming the chemical variability observed in *C. torulosa* essential oil across different studies.

Conclusion

The present study is the first to apply a BBD-based RSM for optimizing three extraction parameters (extraction time, MLR, and drying period) to maximize the yield of essential oil from the needles of *Cupressus torulosa*. Among the three variables, extraction time emerged as the most influential factor

affecting oil yield. While the optimization significantly improved the extraction process, it was also observed that several other parameters, such as distillation temperature, particle size, and the addition of salt (e.g., NaCl), influence the final yield. These findings emphasize the need for further comprehensive studies incorporating additional variables to refine the extraction protocol. Furthermore, identifying *β*-caryophyllene, terpinen-4-ol, and *Δ*-3-carene as major constituents, reported in high concentrations for the first time, suggests seasonal variability in the essential oil composition. This highlights the importance of considering chemotypic and seasonal influences for the standardization and targeted development of composition-linked end-use products, particularly in the fragrance, pharmaceutical, and nutraceutical industries. Despite the current study's scope being limited to three key parameters, the findings provide a critical foundation for academic research and industrial applications. The optimized conditions offer practical guidance to industries aiming to commercialize *C. torulosa* needle essential oil by enabling more efficient extraction processes and better resource utilization. Ultimately, this study contributes valuable insights for maximizing yield and setting the stage for broader investigations into the commercial potential of *C. torulosa* essential oil.

Acknowledgement

The authors acknowledge the National Authority CAMPA, Ministry of Environment, Forest & Climate Change, GOI, for their financial support through the Indian Council of Forestry Research & Education under a project (AICRP 16) (Sanction no. 13-28/2018-CAMPA dated 02-01-2020) as part of the scheme "Strengthening Forestry Research for Ecological Sustainability and Productivity Enhancement". The authors thank Dr. Raman Nautiyal for his assistance in the RSM analysis.

Conflicts of interest

The authors declare no conflicts of interest.

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