Effect of Temperature and Pressure on Antimycobacterial Activity of Curcuma caesia Extract by Supercritical Fluid Extraction Method

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Abstract

Background: Supercritical fluid extraction (SFE) is an advanced technique using CO₂ as a solvent and plant-based drug exploration is a topic of growing interest. Curcuma caesia is a medicinal herb with many medicinal potential. Hence, in the present study, the effect of temperature (40°C–60°C) and pressure (10–20 MPa) on extraction yield and antimycobacterial potential of C. caesia Roxb. dry rhizome powder using supercritical fluid extraction method was evaluated. Methods: The extract of C. caesia by SFE was accomplished using temperature range (40°C–60°C) and pressure range (10–20 MPa). The chemical profile of the extracts were investigated by Gas Chromatography Mass Spectrometry (GCMS) and the antimycobacterial activity of the extracts were analyzed against Mycobacterium smegmatis strains MTCC06 and MTCC994. Compounds found in the extract were further checked by in silico analyses with two protein target 4DRE and 3UCI. Results: Extraction yield ranged from 3.0 to 5.6 %/25g dry substrate, with the highest value being achieved at 50°C and 15 MPa. The results of GCMS analyses revealed the presence of beta-elemene, curzerenone, boldenone, and 2-cyclohexen-1-one, 4-ethynyl-4-hydroxy-3, 5, 5-trimethyl in the extracts. The extract obtained at 50°C temperature and 15 MPa pressure showed the highest zone of inhibition against M. smegmatis strains MTCC06 and MTCC994, that is, 15.6 mm and 13.6 mm, respectively. Active constituents present in the extracts showed good binding energy with 4DRE and 3UCI by in silico analysis. Conclusion: This study identified the effect of temperature and pressure on yield C. caesia extract by SFE method. Furthermore, the effect of different extracts on antimycobacterial potential and docking study validated the antimycobacterial potential.

Keywords: Curcuma caesia, docking, Mycobacterium smegmatis, supercritical fluid extraction

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INTRODUCTION

Curcuma caesia Roxb. also known as Kali Haldi belongs to Zingiberaceae family. Various tribal communities have used C. caesia long ago. Traditionally, its rhizomes were used in treating asthma, leukoderma, rheumatic pains, piles, antidiarrheic, bronchitis, and snake and scorpion bite. C. caesia is an aromatic perennial herb with creeping horizontal or tuberous rhizomes having diverse pharmacological activities such as anti-inflammatory, anticancerous, antihemmimthic, antileprosy,[1] anti-diabetic activity,[2] antimitogenic activity,[3] antimycobacterial activity,[4] and antitoxicity against cyclophosphamide.[5] Numerous bioactive metabolites used in pharmacological industries have been reported to present in C. caesia rhizomes such as flavonoids, alkaloids, sesquiterpene, and phenolic.[6-8] Methanol extract of C. caesia was analyzed to have cytotoxicity (IC₅₀ 90.70 ± 8.37 µg/mL) on Ehrlich Ascites carcinoma cell lines[9]

Nowadays, herbal medicines are gaining much importance as they are less toxic than chemical based drugs. Extraction of secondary metabolites from plant for the preparation of herbal medicine is increasing. Supercritical fluid extraction (SFE) method, which employs fluids in their supercritical states

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for the extraction of solid samples, is a powerful technique for separation of natural compounds from plants. In recent years, many investigations have been made on apparent industrial applications of the SFE, which offer some benefits over the conventional techniques, mainly in food, chemical, pharmaceutical, and oil industries. The SFE method has advantage over conventional methods of extraction due to less use of toxic solvent, extraction of heat-labile metabolite, and contamination-free product.\(^\text{[10],[11]}\)

Therefore, the objective of the present study is the experimental study of SFE of \textit{C. caesia} in a bench-top unit to study the effect of pressure and temperature on the extraction yield. The chemical constituents of \textit{C. caesia} extract were also analyzed by gas chromatography–mass spectrometry (GCMS) and their antimycobacterial activity was checked against \textit{Mycobacterium smegmatis}. Moreover, the docking study was also conducted to explore the possibility of metabolite as a future drug against \textit{Mycobacterium tuberculosis}.

**Methods**

**Curcuma caesia collection and preparation**

\textit{C. caesia} Roxb. was collected and identified from ICAR-Indian Institute of Spices Research Kozhikode, Kerala, with Accession No: 1154 (Voucher no: 266608) and grown in the herbal garden of Maharshi Dayanand University, Rohtak, Haryana. Harvesting of rhizome was done in November 2018. Rhizomes of \textit{C. caesia} were washed with distilled water, shade-dried for 1 week, and then grinded into fine powder. The moisture content of the rhizome was calculated by hot air oven drying method.

**Supercritical fluid extraction**

A laboratory scale SFE unit (\textit{Speed}® SFE Prime of Applied Separations, Allentown, PA, USA) was used to perform the SFE assays to obtain the extracts from \textit{C. caesia} by loading 10 g of substrate in a 25 ml of extraction vessel. Glass wool was cast off in the extraction vessel at both the ends to avoid entrainment of the sample. All the SFE extractions were performed in a constant extraction time, i.e., 60 min in static mode. The influence of pressure and temperature was estimated on the yield of \textit{C. caesia} extract with temperature ranges from 40°C to 60°C and pressure from 10 MPa to 20 MPa. The mass of extract was evaluated by collecting in a preweighed clean and dry glass vial. Extraction yield (Y) of \textit{C. caesia} was indicated as grams extract per 25 g of dry substrate (g/25 g d.s.). The packed bed supercritical extraction procedure was carried out.\(^\text{[12]}\)

**Experimental design and statistical analysis**

The effect of independent variables (temperature and pressure) on yield (response variable) of \textit{C. caesia} extract was evaluated by central composite rotatable design of response surface methodology. The factors and their levels are shown in Table 1. A total of 11 runs were carried out, which include 4 factorial points, 4 axial points, and 3 center points with a value of \(\alpha = 1.41\). It was created on a two-factor factorial design \((n = 2)\), by two levels (coded values −1 and +1). Coded temperature (A1) in degree centigrade and coded pressure (A2) in MPa calculated by Equation 1 and 2 respectively.

\[
A1 = \frac{T - 50}{5} \tag{1}
\]

\[
A2 = \frac{P - 15}{5} \tag{2}
\]

The experiment data were based on the second-order model Eq. (3), which expressed the yield \((Y)\) (response variable) as a function of temperature \((A1)\) and pressure \((A2)\) (independent variable). Experiments were conducted in a randomized order to reduce the effect of unexpected variability in the observed response due to unnecessary factors.

\[
Y = X0 + X1A1 + X2A2 + X12A1A2 + X11A12 + X22A22 \tag{3}
\]

where \(X0\) is a constant; \(X1\) and \(X2\) are linear coefficients, and \(X12\) is a cross-product coefficient. Quadratic coefficients are symbolized by \(X11\) and \(X22\). Three-dimensional (3D) surface response plots were generated and goodness of fit was assessed by analysis of variance (ANOVA). Using Design-Expert Software, version 12 (Stat-Ease, Inc., Minneapolis, MN, USA), the coefficients of response surface equation were assessed.

**Gas chromatography–mass spectrometry analysis**

The extracts obtained were further analyzed for chemical profile by \textit{BRUKER SCION 436-GC SQ GCMS} instrument equipped with \textit{RESTEK Rtx®-5} (Crossbond® 5% diphenyl/95% dimethylpolysiloxane) with 30 m length, 0.25 μm df, and 0.25 mm ID column. Helium as a carrier gas was used at a flow rate of 1 ml/min in split mode and was used for the separation of phytochemicals. GCMS protocol was used following the method of Chaturvedi \textit{et al.}\(^\text{[13]}\)

**Antimycobacterium activity**

The antimycobacterium activity of all SFE extracts of \textit{C. caesia} was analyzed using agar well diffusion assay against \textit{M. smegmatis} strains, i.e., MTCC06 and MTCC994.\(^\text{[14]}\) Both

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### Table 1: Independent variable with level of central composite rotatable design matrix

| Independent variable | Symbols | Uncoded | Coded | Levels | Uncoded | Coded |
|----------------------|---------|---------|-------|--------|---------|-------|
| Temperature (°C)     | T       | A1      | 36    | −1.41  | 1.41    |       |
|                      |         |         | 40    | −1     |         |       |
|                      |         |         | 50    | 0      |         |       |
|                      |         |         | 60    | +1     |         |       |
|                      |         |         | 64    | +1.41  |         |       |
| Pressure (MPa)       | P       | A2      | 8     | −1.41  |         |       |
|                      |         |         | 10    | −1     |         |       |
|                      |         |         | 15    | 0      |         |       |
|                      |         |         | 20    | +1     |         |       |
|                      |         |         | 22    | +1.41  |         |       |

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the strains of *M. smegmatis* obtained from the National Jalma Institute of Leprosy and other Mycobacterial Diseases, Agra, Uttar Pradesh, India. The rifampicin disk was used as a standard against *M. smegmatis* with a concentration of 10 μg.

**Docking study**

Molecular docking analysis was performed using MGL docking tool through AutoDock 4.2 software from the Scripps Research Institute of USA for the prediction of interaction between metabolite present in SFE extract and potential drug targets of mycobacterium, i.e., enoyl-acyl reductase enzyme InhA (PDB ID: 4DRE) and gyrase type IIA topoisomerase (PDB ID: 3UC1) of *Mycobacterium tuberculosi*s. The generated results of docking were analyzed and visualized by Discovery Studio. Lipinski’s rule of five helps in preliminary analysis of molecule that it can be used as drug or not. Before docking analysis, compounds were analyzed by SCFBIO-IIT-Delhi (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp) online software for Lipinski’s rule of five. The ADMET properties of all ligands were analyzed using an online server, i.e., Admet SAR (http://lmmd.ecust.edu.cn/admetsar2/about).[15]

**RESULTS**

In packed bed SFE the solid particle size has a great effect on the yield because small particle size provides larger surface area and lower internal diffusion resistance. If the particle size is below 0.71 mm, its effect can be neglected as according to Del Valle et al.[16] The average particle size of *C. caesia* dry sieved powder was 0.64 mm. Moisture contents were calculated by hot air oven drying method, which is found to be 8.0% ± 0.5%. Plant materials were characterized with bed porosity of 0.38 ± 0.004 and solid density of 791.6 ± 3.2 kg/m^3^ and apparent density of 189.4 ± 1.6 kg/m^3^.

Experimental extraction yield and predicted yield using supercritical CO₂ from dry rhizome powder of *C. caesia* are depicted in Table 2. The yield was 2.5 times higher than the lowest yield as it ranges from 3.0 g/25 g to 5.6 g/25 g d.s. The statistical indicators obtained by the analysis of the variance applied to the selected second-order model Eq. (3) [Supplementary Table 1]. *P* < 0.05 indicated that model terms were significant.

**Extraction yield**

The second-order model Eq. (4) characterizes the effects of independent variable on response variable, i.e., pressure and temperature on yield in the particular experimental section. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor.

\[
Y = 5.43 + 0.10359 (A1) - 0.0268 (A2) - 0.1500 A1A2 - 1A1^2 - 1.5A2^2
\]

The ANOVA was used to specify which terms are statistically important and that have a positive or a negative effect on yield [Supplementary Table 2].

The correlation between predicted responses with experimental responses has been shown in Figure 1a. The graph shows that the experimental values are closely matched with the predicted values. It means that the second-order model provides a statistically significant relation between the response variable and the independent variables. The data were analyzed by difference in fits (DFFITS) plots (the effect on the predicted value of each point is calculated for the reliability evaluation of the model) and Cook’s distance method (measure of how the regression changes if the case has been removed) to confirm the adequacy of model or absence of outlier in the experimental data. The Cook’s distance plot [Figure 1b] and DFFITS plots [Figure 1c] analysis revealed that model show no unpredicted errors. Figure 2 shows the 3D graphical response of Eq. (4). The extraction yields increased with increasing the temperature and pressure up to a certain limit and then decline. The highest yield was 5.6 g/25 g d.s. at 50°C temperature and 15 MPa pressure.

**Gas chromatography–mass spectrometry profile**

The major metabolites present in different SFE extracts was identified by GCMS [Table 3]. Metabolites, beta-elemene and curzerenone, were present in almost every extracts with variation in the area percentage value of peak. GCMS chromatogram of extract obtained at 50°C and 15 MPa

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**Table 2: Experimental and predicted extraction yield as a function of T and P**

| Run | Temperature (°C) | Pressure (P, MPa) | A1 | A2 | Y (g/25 g d.s) |
|-----|------------------|------------------|----|----|---------------|
|     |                  |                  |    |    | Experimental  | Predicted |
| 1   | 40               | 10               | −1 | −1 | 3.10          | 3.15       |
| 2   | 60               | 10               | +1 | −1 | 3.50          | 3.57       |
| 3   | 40               | 20               | −1 | +1 | 3.00          | 3.10       |
| 4   | 60               | 20               | +1 | +1 | 3.20          | 3.28       |
| 5   | 36               | 15               | −1.41 | 0  | 3.60          | 3.51       |
| 6   | 64               | 15               | +1.41 | 0  | 3.40          | 3.34       |
| 7   | 50               | 8                | 0 | −1.41 | 5.30          | 5.43       |
| 8   | 50               | 22               | 0 | +1.41 | 3.10          | 3.00       |
| 9   | 50               | 15               | 0 | 0   | 3.30          | 3.25       |
| 10  | 50               | 15               | 0 | 0   | 5.60          | 5.43       |
| 11  | 50               | 15               | 0 | 0   | 5.40          | 5.43       |
condition showed the highest area of percentage of beta elemene and curzerenone [Supplementary Figure 1]. At this condition, the peak area of beta-elemene and curzerenone was 16.38% and 18.097%, respectively. The peak area of boldenone and 2-cyclohexen-1-one, 4-ethynyl-4-hydroxy-3, 5, 5-trimethyl with 4DRE and 3UCI. Enoyl-acyl reductase enzyme (InhA) is necessary for cell wall synthesis as it synthesized mycolic acid a vital component of mycobacterium cell wall and gyrase type IIA that helps in reducing topological strain in DNA helix during replication. ADMET properties and Lipinski’s rule of five for all the four metabolites have been shown in supplementary Table 3. All the four metabolites followed the Lipinski’s rule of five. The estimation of the ADMET properties plays a significant role in the early phase of drug formulation process. Caco-2 cell permeability, blood–brain barrier penetration, human intestinal absorption, and Ames test properties were calculated. After drug likeness properties, the docking study was done. The docking study revealed that boldenone showed the highest binding energy against both the receptor 4DRE and 3UCI. The binding energy and inhibition constant of the four ligands along with standard ethionamide drug are shown in Table 4.

The 3D interaction of 4DRE and 3UCI with ligands have been shown in Figure 3 and Figure 4 respectively. The 3D interaction shows the bond length, type of bond, and amino acid involved in the interaction. The most efficient binding was shown by the 3UCI protein with boldenone, that is, −8.45 Kcal/mol with three hydrogen bond and nine hydrophobic interactions.

**DISCUSSION**

This study describes the correction between different temperatures and pressures on extraction yield of extract using SFE method. Furthermore, apart from yield, the chemical profile of different extracts was changed with respect to their antimycobacterium potential. In the present study, the highest yield was obtained at 50°C temperature and 15 MPa pressure. Brunner suggested that the pressure increases the CO² density which further enhances the solvent power by decreasing the distance between CO² molecules. Extraction yield in Smyrnium cordifolium Boiss leaves is directly proportional to temperature (40°C–60°C) whatsoever the pressure (10–30 MPa). Many studies suggested that indirect relational between extraction yield and pressure is due to diffusion coefficient, which is inversely proportional to pressure.
Wang et al.\textsuperscript{[20]} suggested that at high-pressure levels, repulsive interactions occur between solvent and solute which may decrease the extraction yield of \textit{Cyperus rotundus} Linn.

Extract obtained at 50°C and 15 MPa showed the highest one of inhibition with \textit{M. smegmatis}. This may be due to the presence of four metabolites, namely, beta-elemene, curzerenone, boldenone, and 2-cyclohexen-1-one, 4-ethyl-4-hydroxy-3, 5, 5-trimethyl. Many studies suggested that sesquiterpene has antimycobacterium activity\textsuperscript{[21]} for example beta-elemene have the capacity to alter the expression of dprE1 gene needed for cell wall synthesis and clgR genes regulate cell membrane structure\textsuperscript{[22]} of mycobacterium. The essential oil of ginger mainly composed of monoterpenes and sesquiterpenes exhibited inhibitory activity against \textit{Mycobacterium tuberculosis}.\textsuperscript{[23]} Antimycobacterium activity of Salvia

Table 3: The relative area percentage of compound on GCMS chromatogram

| Condition   | Beta-elemene (%) | Curzerenone (%) | Boldenone (%) | 2-Cyclohexen-1-one, 4-ethyl-4-hydroxy-3, 5, 5-trimethyl (%) |
|-------------|------------------|-----------------|--------------|----------------------------------------------------------|
| 40°C/10 MPa | -                | 10.369          | -            | -                                                        |
| 40°C/15 MPa | -                | 13.335          | -            | -                                                        |
| 40°C/20 MPa | 4.894            | 19.063          | -            | -                                                        |
| 50°C/10 MPa | 8.587            | 16.169          | -            | 11.413                                                   |
| 50°C/15 MPa | 16.386           | 18.097          | 15.646       | 10.063                                                   |
| 50°C/20 MPa | 9.410            | 14.538          | 17.904       | -                                                        |
| 60°C/10 MPa | 7.459            | 20.542          | -            | 14.881                                                   |
| 60°C/15 MPa | -                | 11.851          | -            | -                                                        |
| 60°C/20 MPa | -                | 16.695          | -            | 14.505                                                   |

Table 4: The binding energy, inhibition constant, hydrogen bond, and hydrophobic interaction

| Receptor | Ligand                        | Binding energy (Kcal/mol) | Inhibition constant (µM) | Number of hydrogen bond | Number of hydrophobic interaction |
|----------|-------------------------------|---------------------------|--------------------------|-------------------------|----------------------------------|
| 4DRE     | Beta-elemene                  | -5.88                     | 49.01                    | -                       | 10                               |
|          | Curzerenone                   | -5.57                     | 83.08                    | 1                       | 10                               |
|          | Boldenone                     | -7.37                     | 3.93                     | 1                       | 10                               |
|          | 2-Cyclohexen-1-one, 4-ethyl-4-hydroxy-3, 5, 5-trimethyl | -5.34 | 121.79 | 1 | 6 |
|          | Ethionamide                   | -5.64                     | 73.85                    | 5                       | 4                                |
| 3UCI     | Beta-elemene                  | -6.35                     | 22.16                    | -                       | 5                                |
|          | Curzerenone                   | -6.48                     | 17.83                    | 3                       | 3                                |
|          | Boldenone                     | -8.45                     | 638.89                   | 3                       | 9                                |
|          | 2-Cyclohexen-1-one, 4-ethyl-4-hydroxy-3, 5, 5-trimethyl | -5.47 | 97.06 | 3 | 5 |
|          | Ethionamide                   | -5.17                     | 161.60                   | 4                       | 6                                |

Figure 3: The three-dimensional interaction of ligands with 4DRE receptors (a) beta-elemene (b) curzerenone (c) boldenone (d) 2-cyclohexen-1-one, 4-ethyl-4-hydroxy-3, 5, 5-trimethyl (e) ethionamide (green line hydrogen bond, purple line hydrophobic interaction)
Steroids were given in combination and Corticosteroids are also recognized as having beneficial presence in almost all extracts. Docking analysis of these four metabolites with two proteins of mycobacterium also validate that these metabolites help in antimycobacterium activity. It is concluded that the extract obtained at 50°C and 15 MPa condition showed the presence of four major metabolites that may contribute for antimycobacterium activity of C. caesia.

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Conflicts of interest
There are no conflicts of interest.

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Supplementary Table 1: Statistical indicator of appropriateness of the second-order model

| Statistical indicators       | Y  |
|------------------------------|----|
| $F$                          | 110.69 |
| $r^2$                        | 0.99 |
| Lack of fit                  | 0.679 ns |
| Signal-to-noise-ratio        | 23.9 |
| CV                           | 3.55 |

Ns: Nonsignificant ($P > 0.20$), CV: Coefficient of variation

Supplementary Table 2: Analysis of variance for yield

| Degree of freedom | Sum of squares | Mean sum of square | $F$  | $P$  |
|-------------------|----------------|--------------------|------|------|
| Regression        | 5              | 10.43              | 2.09 | 110.69 | 0.001 |
| Residual          | 5              | 0.094              | 0.018|      |
| Total             | 10             | 10.53              |      |      |

Supplementary Table 3: Drug-like and ADMET properties of four inhibitors for antimycobacterium study

| Compound name                                      | MW (g/mol) | LogS | LogP | TPSA (Å²) | HA | HD | BBB | Caco | Ames |
|----------------------------------------------------|------------|------|------|-----------|----|----|-----|------|------|
| Beta-elemene                                        | 204.35     | −4.76| 3.37 | 0.00      | 0  | 0  | −   | +    | −    |
| Curzerenone                                         | 230.30     | −3.90| 2.88 | 30.21     | 2  | 0  | +   | +    | −    |
| Boldenone                                           | 286.41     | −3.84| 2.86 | 37.30     | 2  | 1  | +   | +    | −    |
| 2-Cyclohexen-1-one, 4-ethynyl-4-hydroxy-3, 5, 5-trimethyl | 178.23     | −1.34| 1.98 | 37.30     | 2  | 1  | +   | +    | −    |

MW: Molecular weight, LogS: Logarithm of the molar solubility in water, LogP: Partition coefficient between n-octanol and water, TPSA: Topological polar surface area, HA: Hydrogen bond acceptors, HD: Hydrogen bond donors, BBB: Blood–Brain Barrier penetration, Caco: Caco-2 cell permeability, Ames: Ames test toxicity.[15]

Supplementary Figure 1: Gas chromatography–mass spectrometry spectrum at 50°C and 15 MPa condition

Supplementary Figure 2: Bar diagram of zone of inhibition of various extract obtained by supercritical fluid extraction