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ORIGINAL ARTICLE

Optimized ultrasonic-assisted deep eutectic solvents extraction of *Clematis flammula* L. leaves, phytochemical screening, biological activities and the characterization of its volatile compounds

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Abstract

Lately, green chemistry has pushed researchers to investigate alternative solvents for the recovery of phenolic compounds (PC). Therefore, the optimization of PC extraction from *Clematis flammula* L. leaves (CFL) using ultrasound-assisted deep eutectic solvents (UAE-DES) was investigated. Additionally, phytochemical screening, biological activity, and characterization of the bioactive molecules using gas chromatography coupled with mass spectrometry (GC–MS) were performed. Choline chloride-acetic acid (ChCl-Acet) was explored as a potential extractant of TPC (110 ± 5.98 mgGAE/Gdw) and the optimal extraction conditions were as follows: 18.2% of water, 49.4 °C, and 40 min of extraction time. Also, the in vitro antioxidant assays revealed that CFL extract was a potent antioxidant agent with a high inhibition compared to the control with 97.23 ± 1.72% and 94 ± 4.21% inhibition for ABTS and DPPH, respectively. CFL extract showed antimicrobial effects against fungus and two bacterial strains, namely Candida *albicans* (CA), *Methicillin-resistant staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* (PA), respectively, with a minimal inhibitor concentration (MIC) of 390 µg/mL. For the first time, GC–MS was used to assess the characterization and quantification of phenolic profiles. Protoanemonin (63.94%), phenol (8.76%), decamethyl (7.00%), linalool (5.47%), salicylic acid (4.66%), dodecamethyl (4.41%), and *n*-dexadecanoic acid (4.22%) were the major compounds identified. The obtained results highlight the effectiveness of choline chloride-based DES as an alternative extraction solvent for phenolic compounds for industrial and pharmaceutical applications.

Keywords *Clematis flammula* L. · GC–MS · Identification · Antimicrobial · Deep eutectic solvents

Highlights
- The identification of 10 volatile compounds from CFL was investigated for the first time.
- An effective, eco-friendly approach was established using UAE-DES for the recovery of TPC from CFL.
- The optimal extraction conditions were obtained as follows: 18.2 % of water, 49.4 °C, and 40 min of extraction time.
- CFL showed good anti-microbial activity against *Candida albicans*, *Methicillin-resistant staphylococcus aureus*, and *Pseudomonas aeruginosa*.

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1 Introduction

Clematis flammula L. (CF) (*Ranunculaceae* family) is a native herb from southern Europe and northern Africa. It is a botanical source of various pharmaceutical active components widely used in traditional medicine. CF was used as a treatment for sexually transmitted infections, rheumatoid arthritis, chronic skin diseases, gout, and varicosity [1, 2]. Moreover, *Clematis flammula* L. leaves (CFL) were used to treat arthritis and superficial burns [3].

Polyphenols are the most abundant special bioactive substances ubiquitous in plants, vegetables, and fruits with remarkable antioxidant properties and nutritional benefits [4]. They are used in many fields including food [5], pharmaceutical [6], and cosmetics [7]. The increasing awareness of the potential commercial value of polyphenols pushes industries to search for new sustainable techniques and solvents for their extraction.

Conventional extraction methods have been used for a long time, presenting many disadvantages such as requiring a long time for extraction, the tremendous amount of toxic volatile solvents, low selectivity for the target molecule, and low yield. Over the last years, the use of green methods and solvents has been dramatically expanded [8]. From an environmental and economic perspective, deep eutectic solvents (DES) offer many advantages including biodegradability, low cost, simple preparation, and availability. They are composed of hydrogen bond acceptors (HBA) such as salts and hydrogen bond donors (HBD) like alcohols, organic acids, sugars, amino acids, and amines [9]. Recently, there has been a growing interest in studying DES since they present very interesting properties for diverse applications [10–13]. DES adheres to green chemistry principles such as reducing energy consumption and the use of toxic flammable solvents [14]. They have been used as enzyme reaction media [15], biotransformation [16], biomass processing [17], and as natural pigment stabilizers [18]. Moreover, the extraction of biological molecules from plants using DES solvents has been the subject of many reports [5, 10, 19, 20]. Therefore, new extraction methods with reduced time and solvent consumption and increased attention to pollution prevention must be involved. Ultrasound-assisted extraction (UAE) uses high-intensity waves which disrupt cells during acoustic cavitation and help the release of extractable components which enhance mass transfer [21]. Different solvents such as water, alcohols, and acetone have been used for the extraction of different compounds in UAE. However, the complete elimination of chemical solvents in extraction with satisfactory yield needs to be achieved. In addition, studies on green extraction of phenolic compounds from *Clematis flammula* L. leaves are still lacking.

The objectives of this study are:

- Establishing an effective, eco-friendly approach for the optimization of extraction factors of CFL extract using the DES-UAE system.
- Studying the phytochemical screening and general characterization of DES extract.
- Investigating the antimicrobial activity of CFL against fungus and microbial strains.
- The identification of volatile compounds in CFL extract using GC–MS for the first time.

2 Materials and methods

2.1 Instrumentation

Ultrasound (TIERRATECH LT-100 PRO, 100 W), magnetic stirrer (VELP SCIENTIFICA), refrigerated centrifuge (SIGMA 2–16 PK), balance (AS 220/CI2), vortex (V-1 plus BOECO), water bath (MAMMERT GmbH), electric mill (KIKA Labortechnik, Staufen, Germany), UV–visible spectrophotometer (SHIMADZU UV-1800), microplate reader (Synergy-HTX, Biotek), and FT-IR spectrophotometer (SHIMADZU FTIR 8400 s).

2.2 Chemical products

Coomassie blue brilliant G-250 ($C_{47}H_{44}N_3O_7S_2Na$), Mayer's reagent (K_2HgI_4), phosphoric acid (H_3PO_4) (> 99%), Folin Ciocalteu reagent ($C_{10}H_5NaO_5S$), sodium carbonate (Na_2CO_3) (> 99%), sulfuric acid (H_2SO_4) (95–97%), and bovine serum albumin (BSA) (> 96%) were purchased from BIOCHEM Chemopharma (USA). 2,2-Diphenyl-1-picrylhydrazyle (DPPH) ($C_{18}H_{12}N_5O_6$), 2,2'- azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) ($C_{18}H_{18}N_4O_6S_4$), potassium persulfate ($K_2S_2O_8$) (99%), sodium dodecyl sulfate (SDS) (> 99%), ferric chloride hexahydrate ($FeCl_3$) (> 97%), copper sulfate ($CuSO_4$) (> 98%), hexamine ($(CH_2)_6N_4$) (99%), potassium ferrocyanide ($K_4[Fe(CN)_6]$) (99%), sulfurique acid (H_2SO_4) (98%), 5-thio-2-nitrobenzoic acid (TNB) (98%), iron(II) sulfate heptahydrate ($FeSO_4$) (99%), sodium phosphate monobasic monohydrate (NaH_2PO_4) (99%), sodium phosphate dibasic (Na_2HPO_4) (99%), ascorbic acid ($C_6H_8O_6$) (99%), ethylenediaminetetracetic (EDTA) (> 98%), choline chloride ($C_5H_{14}CINO$) (98%), acetic acid ($C_2H_3O_2$) (98%) sodium chloride (NaCl) (99%), chloroform ($CHCl_3$) (99%), Fehling's reagent (CuH_2O_4S), urea (CH_4N_2O) (> 98%), glycerol ($C_3H_8O_3$) (> 99.5%), fructose ($C_6H_{12}O_6$) (> 99%), and lactic acid ($C_3H_6O_3$) (> 99%) were purchased from Sigma Aldrich. Hydrogen peroxide (H_2O_2) (30%), hydrochloric acid (HCl) (37%), sodium hydroxide (NaOH) (96%), and tris(hydroxymethyl)aminomethane (Tris–HCl) (99%) were purchased from Chimoza (France). Muller Hinton Agar (M173-500G) was purchased from HIMEDIA. Muller Hinton Broth (70,192-500G) was purchased from FLUKA Analytical and Sabouraud dextrose agar was purchased from CONDA.

2.3 Plant material

The collected plant was identified in the laboratory of Botany, University of Bejaia, Algeria (June 2019), from the forest of Azru'n Bechar in the county of Amizour Bejaia. The global positioning systems (GPS) location is 36.644° N, 4.921° E, according to a listed voucher specimen deposited in the National Institute of Agronomy, Algiers, Algeria (voucher number: 114_13).

The collected plant material was dried in darkness at room temperature and ground and sieved into a fine of < 63 µm.

2.4 Extraction procedure

2.4.1 DES preparation

Deep eutectic solvents used in this study were prepared by adding hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) at a ratio of (1:2) (mole/mole). The resulting mixture was placed in a round-bottom flask and heated to 80 °C in a water bath with agitation until a homogeneous liquid was formed [22].

2.4.2 CFL extract preparation

The extraction of polyphenols using ultrasound was investigated in this study. Briefly, the powdered plant material (5 g) of CFL was added to 100 mL of DES, the mixture was agitated and then immersed in the flask, and the mixture was placed in the ultrasonic water bath. After the extraction was finished, the flask was removed and cooled to room temperature before the mixture was centrifuged at 8000 g for 20 min to ensure a complete separation. The clear solution above was transferred in 15-mL tubes and collected and analyzed [23].

2.4.3 Single-factor experiment

To investigate the critical-factor influence on the extraction of CFL extract, a single-factor experiment as a preliminary study was used to determine approximate ranges of independent variables including X_1 (water percentage), X_2 (temperature), and X_3 (time) on total phenolic content (TPC) yield.

2.4.4 Optimization and model validation

Based on single-factor experiment results, major factors influencing the extraction process were selected for designing experiments using response surface methodology (RSM). Three level three-factorial Box-Behnken experimental design (BBD) was employed to determine the best combinations of extraction variables for CFL. Three levels were coded as − 1 (low), 0 (middle or center point), and 1 (high). The factors including water percentage (5–40%), extraction temperature (40–55 °C), and extraction time (20–40 min) were selected. The general equation to predict the linear quadratic model is described as follows:

$$Y = \beta_0 + \Sigma_i^4 = 1\beta_i X_i + \Sigma_{i=1}^4 \beta_{ii} X^2 + \Sigma_{i>j}^4 \beta_{ij}\, X_{ij}.$$

where Y represents the response function (TPC); B_0 is constant coefficient; B_i, B_j, and B_{ij} are coefficients of linear, quadratic, and interactive terms, respectively; X_i and X_j are the coded independent variables. The analysis of variance (ANOVA) was used to evaluate the significance of the obtained data.

2.5 Phytochemical screening

To highlight the presence or absence of certain compounds belonging to the chemical families of special metabolites, we carried out specific phytochemical tests based on staining, turbidity, or precipitation reactions, using the methods described in the literature [24–26].

2.5.1 Detection of alkaloids

Briefly, 2 mL of 1% HCl was mixed with 1 mL of DES extract and heated in a water bath at 60 °C. Mayer's reagent was added after cooling. Meyer's reagent produces a yellowish white precipitate, indicating the presence of alkaloids.

2.5.2 Detection of terpenoids

First, 1 mL of chloroform was added to 1.4 mL of concentrated sulfuric acid solution and a known volume of DES extract. The mixture was then treated with few drops of anhydride acetic acid. The blue coloration is indicative of the presence of terpenoids in the extracts.

2.5.3 Detection of sugars

Reducing sugars were detected in crude DES extract by Fehling's reagent. Briefly, 5 mL of Fehling's reagent was mixed with the same volume of DES extract; the reaction mixture was heated at 70 °C for 3 min. Brick red precipitate indicates the presence of sugars.

2.5.4 Detection of proteins

Proteins were detected by dissolving an amount of DES extract from CFL in 2 mL of NaOH (20%) in a test tube. Thereafter, drops of aqueous $CuSO_4$ (2%) were added. Violet coloration signifies the presence of proteins in the extract.

2.5.5 Detection of coumarins

In a test tube, 2 mL of DES solution was added to 0.5 mL of NaOH (10%) in a boiling water bath. After cooling, 4 mL of distilled water was added to the mixture. Coumarins were determined by comparing the test tube with the control tube (replacing the alkaline solution with distilled water). The reaction remains positive if the test tube was more transparent than the control.

2.5.6 Detection of saponosides

The protocol was used with minor modifications. One milliliter of DES extract was poured into a test tube along with 2.5 mL of distilled water, agitated for 30 s, and then left to stand for 20 min to detect saponosides. A persistent foam indicates the presence of saponosides.

2.5.7 Detection of tannins

Briefly, a few drops of diluted $FeCl_3$ solution were added to 1 mL of DES extract. Upon standing at room temperature for 1 min, the mixture developed a greenish color indicative of catechic tannins and a blue color indicative of gallic tannins.

2.6 Yield and chemical composition

2.6.1 Total phenolic content determination

The total phenolic content was determined by colorimetric analysis with Folin-Ciocalteu reagent according to Atanassova et al. [27] with minor modifications. The results were given as milligram acid gallic equivalent per gram of dry weight (mg-GAE/gdw). Briefly, 20 µL of DES-diluted extract was deposed in 96-well microplate with 100µL of Folin-Ciocalteu reagent and 80 µL of Na_2CO_3. After 10 min of incubation at room temperature, the absorbance was performed at 765 nm.

2.6.2 Total flavonoid determination

The analysis of total flavonoid determination (TFC) for CFL extract was determined using the protocol of Atanassova et al. [27]. Based on the addition of aluminum chloride reaction to the solution containing the extract, the absorbance of the obtained complex was measured at 430 nm. From a quercetin standard curve, flavonoids were calculated and expressed as mg-Q/gdw.

2.6.3 Total anthocyanins analysis

The total anthocyanin content (TAC) was conducted by the pH differential method of Lee et al. at 518–700 nm [28]. The TAC was calculated using the following equation:

$$TAC(mg/kg) = A_t \times M_w \times D_f \times 1000/\varepsilon \times L.$$

where A (A_{518}nm–A_{700}nm) pH1.0–(A_{518}nm–A_{700}nm) pH4.5, Mw (molecular weight of delphinidin-3-sambubioside), D_f (dilution factor established), L (path length in cm), and $\varepsilon = 26{,}900$ molar extinction coefficients, in $L \times mol^{-1} \times cm^{-1}$, for delphinidin-3-sambubioside.

2.6.4 Total sugar content

The method of Saha et al. [29] was used with minor modifications. Briefly, 50µL of CFL DES diluted solution was added to 150 µL of H_2SO_4; the mixture was allowed to stand for 3 min at 25 °C. Also, 30µL of phenol solution 5% (m/v) was added to each well and the mixture was heated at 90 °C for 5 min then the absorbance was read at 485 nm.

2.6.5 Total protein content

This assay was performed using 50 µL of the sample and 200 µL of Bradford reagent in a microplate well; the content was mixed and incubated for 5 min at room temperature and the absorbance value of all the samples was measured at 595 nm [30].

2.7 Spectroscopic analysis CFL extract

2.7.1 UV–visible analysis

In the present work, the sample solution was scanned by UV–Vis spectrophotometer in the range of 200–800 nm to inspect their characteristic absorption regions.

2.7.2 Fourier transform-infrared spectroscopy

The CFL extract was analyzed using a Fourier transform-infrared spectrophotometer. One drop of the liquid extract was deposed on a solid KBr window, and then scanned from wavenumber 4000 to 400 cm^1.

2.8 Evaluation of the antioxidant potential of CFL

2.8.1 ABTS radical scavenging ability

An ABTS solution was prepared 16 h before the test by mixing 7 mM ABTS with 2.45 mM potassium persulfate in distilled water. Prior to use, ABTS stock solution was diluted with ethanol to obtain 0.7 ± 0.05 absorbance of ABTS measured at 734 nm [31]. The scavenging percentage was calculated as follows:

$$Scavenging(\%) = [(A_o - A_1)/A_o] \times 100.$$

where A_0 is the absorbance of the blank without samples, and A_1 is the absorbance of the test samples.

2.8.2 DPPH-free radical scavenging assay

The free radical scavenging activity of CFL DES extract was evaluated. Briefly, 20µL of DES extract was added to 180 µL of DPPH solution (100 mM); after 30 min, the absorbance was recorded at 515 nm [32]. The scavenging activity was calculated using the same equation:

$$\text{Scavenging}(\%) = [(A_o - A_1)/A_o] \times 100.$$

where A_0 is the absorbance of the blank without samples, and A_1 is the absorbance of the test samples.

2.9 Evaluation of the antimicrobial potential of CFL

Candida albicans, *Methicillin-resistant staphylococcus aureus* ($gram^+$), and *Pseudomonas aeruginosa* ($gram^-$) were used to evaluate the antimicrobial effect of CFL. The referenced fungal and bacteria strains were transferred into the surface of nutritive gelose plates using platinum ance, left for 24 h at 37 °C to allow their growth. The colonies were suspended in physiological water and controlled according to McFarland norms. By using a swab, the strains were transferred in a Muller Hinton (for bacteria) and Sabouraud (for fungus) agar plate after well creation. About 50 µL of CFL (50 mg/mL) extract was deposed in the wells and left for 1 h at 4 °C to allow better diffusion and then incubation overnight at 37 °C. The control well contained only DES solvent; also, tetracycline and ciprofloxacin were used as positive controls. After incubation, the inhibition zones around the wells were measured using a caliper. Furthermore, the minimum inhibitory concentration (MIC) was determined as the lowest concentration that ended with no growth.

2.10 Volatile profile of CFL using GC–MS

The recovery of volatile compounds present in CFL-DES extract was performed using a gas chromatograph-mass spectrometer (GC–MS). CFL extract was directly injected into the column (30.0 m × 530 µm) and analyzed under optimal conditions. The carrier gas and make-up gas were high pure helium and nitrogen respectively and the flow rate was set at 10 mL min^{-1}. The inlet temperature was 200 °C with a split ratio of 10:1 and the pressure was 11.6 psi. The temperature was initially set at 60 °C for 5 min and then increased gradually to 200 °C and 300 °C for another (5 min × 2) in the column oven. For MS, the full scan mode from *m/z* 30 to 600 at 1562 amu/s quadrupole and ion source temperatures were 150 °C and 230 °C, respectively. The identification was based on a comparison of their GC retention time and MS retention index of n-alkane saturated alkanes and the reference spectra [33].

2.11 Statistical analysis

All experiments were conducted in triplicate, and data were expressed in means ± standard deviations. Statistical analysis was performed using GraphPad Prism 8. Statistical significances were carried out by one-way analysis of variance (ANOVA) followed by Tukey's test. Values of $p < 0.05$ were considered as statistically significant. For optimization, 3D surface by the JMP (version 13.0 SAS) was used.

3 Results and discussion

3.1 Optimization of DES extraction conditions

The main objective of this work is to evaluate the feasibility of choline chloride-based DES to recover CFL extract. The effect of various coded independent factors of experimental points X_1 (water percentage), X_2 (temperature), and X_3 (time) was studied using a one-variable at a time approach for the optimization of TPC as a response (Y) (Table 1). Regarding the influence of the studied parameters, the selection of extraction solvent is important and will determine the yield of phenolic compounds. Several DES including urea-glycerol (Ure-Gly), choline chloride-lactic acid (ChCl-Lac), choline chloride-glycerol (ChCl-Gly), glycerol-lactic acid (Gly-Lac), fructose-acetic acid (Fru-Acet), glycerol-acetic acid (Gly-Acet), and choline chloride-acetic acid (ChCl-Acet) at 1:2 ratio (mole/mole) were tested to screen for the best solvent for TPC recovery from CFL. The obtained results showed that ChCl-Acet DES was the most efficient for the extraction of TPC with significant difference recorded (Supplementary materials, Fig S1). Same results were obtained according to Ma et al. for the extraction of phenolic compounds from *Camellia oleifera* [34]. Generally, conventional solvents such as methanol and acetone are toxic solvents dangerous to human health and the environment. The effect of conventional extraction methods for polyphenols recovery from CFL in terms of total phenolic content (TPC) and antioxidant activity was explored previously [3]. The yield in TPC was significantly inferior to that obtained with DES in this study. Therefore, the ChCl-Acet combination was selected for the extraction of TPC from *Clematis flammula* L. leaves.

3.1.1 Effect of water addition

A single-factor experiment was carried out to evaluate the influence of a single factor on DES-based extraction of

Table 1 Coded Box-Behnken design for CFL with the analytical and experimental response

	Coded values	X_1	X_2	X_3	Y	Y predicted
1	− −0	5	40	30	28.75	25.95
2	0	22.5	47.5	30	103.69	103.06
3	+0+	40	47.5	40	57.47	59.41
4	0	22.5	47.5	30	100.86	103.06
5	−0−	5	47.5	20	25.73	23.78
6	+ −0	40	40	30	38.79	33.41
7	0+ −	22.5	55	20	59.23	54.32
8	−0+	5	47.5	40	92.01	91.26
9	0− −	22.5	40	20	38.79	45.03
10	0+ +	22.5	55	40	98.41	92.16
11	− +0	5	55	30	45.00	49.91
12	+ +0	40	55	30	48.03	52.32
13	0	22.5	47.5	30	107.64	103.06
14	0− +	22.5	40	40	59.91	62.08
15	+0−	40	47.5	20	65.02	68.00

X_1 water percentage, X_2 temperature, X_3 time, Y total phenolic content (TPC)

TPC from CFL and to determine the appropriate range of each parameter. According to several reports, viscosity is one of the most important properties for the application of DES in many fields [11]. The physicochemical properties of DES are determined by the hydrogen-bond acceptor (HBA) to hydrogen-bond donor (HBD) molar ratio [11]. The majority of the reported DES have densities higher than water (1.0–1.63 g/cm^3) and are highly viscous at room temperature (> 100 mPa s) [8]. The viscosity (39 mPa s) and density (1.11 g/cm^3) of ChCl-Acet have been reported to be low [35, 36]. A low viscosity assisted in the movement of charges within the network presenting interesting properties compared to the other DES at the same ratio [35, 36]. The addition of water affects the extraction efficiency of DES solvents; increasing in water content improves the extraction of TPC until it reaches a certain range; after that, the extraction decreases with the increase of water percentage. Water lessens surface tension and viscosity of the extraction solvent, increasing its diffusivity of the latter in the matrix tissue. As a result, adding a large amount of water dilutes the extraction solvent and may affect the hydrogen bonds between DES components [37]. When adding water, the density and viscosity of ChCl-Acet decreased with increasing temperature until it reached 70–80 °C, where no difference in viscosity was observed. However, a high amount of water weakens hydrogen bonds and modifies the solvent's polarity [35]. In this study, the optimization of water percentage was carried out with varying percentages of water while temperature and extraction time were fixed. The results indicate no significant difference in TPC yield outside this range (5–40%) (Supplementary materials, Fig S2).

3.1.2 Effect of temperature

Temperature is an important factor to be considered during the optimization process. From an economic standpoint, temperature optimization is required for the efficient phytochemical extraction. High temperatures can cause molecular rearrangements by increasing the vibrations of anions and cations. These structural variations give rise to weak interactions between ions resulting in a decrease in solvent viscosity and density making it easily penetrable in the plant matrix during the extraction process [38]. Furthermore, temperature improves solute solubility, electrical conductivity, and diffusion from the solid phase to the liquid phase by disrupting the solute-matrix interactions [38]. However, an increase in temperature beyond certain limits might weaken the interactions between the target and the extraction solvent. Moreover, the plant tissue may be softened, influencing biomolecule bioactivity and inducing their degradation [39]. The effect of temperature and water content on ChCl-Acet was investigated. The results showed that at 50 °C, ChCl-Acet DES reached its lowest viscosity (6.83 ± 0.15 mPa s) [35]. In our study, TPC yield from CFL increased by the increase of temperature until it reached 55 °C, then the tendency was reversed after this value. Therefore, extraction temperature was ranged (from 40 to 55 °C) for RSM trials (Supplementary materials, Fig S3).

3.1.3 Effect of extraction time

It is known that UAE accelerates the mass transfer and increases the extraction yield. In this study, UAE was used to optimize the extraction of phytochemical compounds of CFL. Time plays an important role to minimize energy, and the cost

of the extraction process. CFL extract was extracted over a 5–70-min period at a constant temperature, ratio, and water percentage. TPC increased gradually with the extraction time until it reached 40 min, then decreased. As a result, 20–40 min range was selected for RSM trials (Supplementary materials, Fig S4). The increase in TPC yield with the increase of extraction time might be due to the "slow extraction stage" during which the solvent dissolves the cell's components by diffusion and osmotic process. Additionally, in Fick's second law of diffusion between the solid mass and extraction solvents, when the final equilibrium is reached after a certain time, no possible extraction will occur [40]. Moreover, prolonged exposure is not advisable because it may result in phenolic compound oxidation in the presence of light and air. Furthermore, previous research has shown that the best extraction ranges for polyphenols are between 30 and 180 min [39], with the specific extraction time being strongly dependent on the physicochemical properties of bio resources and DES as well as the thermal stability of the target compound [38].

3.2 Model analysis

Water addition, extraction time, and temperature have proved to be key parameters for the optimization of *Clematis flammula* L. leaves extract based on the data presented above.

Results of regression analysis of data in terms of the actual factors for the responses such as TPC values for CFL (1) respectively as a function of additional water (X_1), temperature (X_2), and time (X_3) were obtained as follows:

$$TPC = 104.06 + 10.55X_2 + 14.87X_3 - 18.45X_1X_3 - 33.97X_1^2 - 29.94X_2^2 - 10.03X_3^2$$

TPC from CFL was ranged from 25.73 to 107.64 mg/Gdw; the highest TPC was detected in run 13 (X_1 22.5%, X_2 47.5 °C, and X_3 30 min) while the lowest values were recorded at run 5 (X_1 5%, X_2 47.5 °C, and X_3 20 min) as shown in Table 1.

The results of ANOVA show the effect of different parametric values on the responses; the model developed for CFL total phenolic compounds provided R^2 of (0.98) with $P < 0.0001$, which was close to R^2 adjusted (0.95) which recommends the fitness of the RSM. Regarding the lack of fit of this model, P value was greater than 0.05 Prob > F 0.1953 and means that the model fits well and there is a significant effect parameter on response. Great precision was recorded with a coefficient of variation (CV%) which was inferior to 10% (see Table 2).

The interaction between (X_2, X_3) and (X_1X_3) (X^2_2, X^2_3) were significant on TPC yield while (X_1) and (X_1X_2, X_2X_3) had no obvious effect on the model terms.

Figure 1(a–c) represents three-dimensional graphs that depict the quadratic polynomial model used to describe the effects of independent variables on the extraction yield of CFL extract. TPC improves when low water percentage is added to DES, as shown Fig. 1a. When the temperature rises to 50 °C, the extraction decreases. However, Fig. 1b shows that the longer the extraction time, the higher the TPC yield. The interaction of temperature and time on TPC is shown in Fig. 1c, the temperature increased the yield in TPC over time. However, after 50 °C, the yield started to decline which is explained by the thermal degradation of bioactive compounds [41]. According to the maximum response in the defined range, the optimal extraction conditions were as follows: 18.2% of water, 49.4 °C, and 40 min of extraction time. Furthermore, to confirm the obtained values, a verification experiment was carried out under optimal conditions, and the TPC was as follows: 110 ± 5.98 mgGAE/Gdw, the obtained value was so close to the predicted response of 112.85 mgGAE/Gdw (Supplementary materials, Fig S5) proving that the model was suitable for optimization of extraction of CFL extract. The results of the optimization in this study showed that DES-Acet optimal temperature for the extraction of TPC from CFL was 49.4 °C and this is when the DES achieved its lowest viscosity according to a previous report [35].

Table 2 Analysis of variance (ANOVA) for response surface quadratic model for the TPC of CFL

	Estimated coefficients	Standard error	Prob > *t*
Model B_0	104.0633	3.3864	< 0.0001*
X_1	2.2275	2.0737	0.3318
X_2	10.5537	2.0737	0.0038*
X_3	14.8787	2.0737	0.0008*
X^2_1	− 33.9741	3.0524	0.0001*
X^2_2	− 29.9466	3.0524	0.0002*
X^2_3	− 10.0316	3.0524	0.0218*
X_1X_2	− 1.7525	2.9327	0.5762
X_1X_3	− 18.4575	2.9327	0.0015*
X_2X_3	4.5150	2.9327	0.1843
Lack of fit			Prob > F 0.1953
Model *P* value			0.0005
R^2			0.9850
R^2 adjusted			0.9580
CV%			9.0680

* indicates significance ($P < 0.05$)

X_1 water percentage, X_2 temperature, X_3 time, Y total phenolic content (TPC), R^2 determination coefficient, CV variation coefficient

3.3 Chemical composition and yield

Investigations on the phytochemical screening of CFL under optimum conditions revealed the presence of catechic tannins, proteins, and sugars (see Table 3). These compounds are known to be biologically active. CFL extract was obtained by ultrasound optimized extraction using ChCl-Acet as

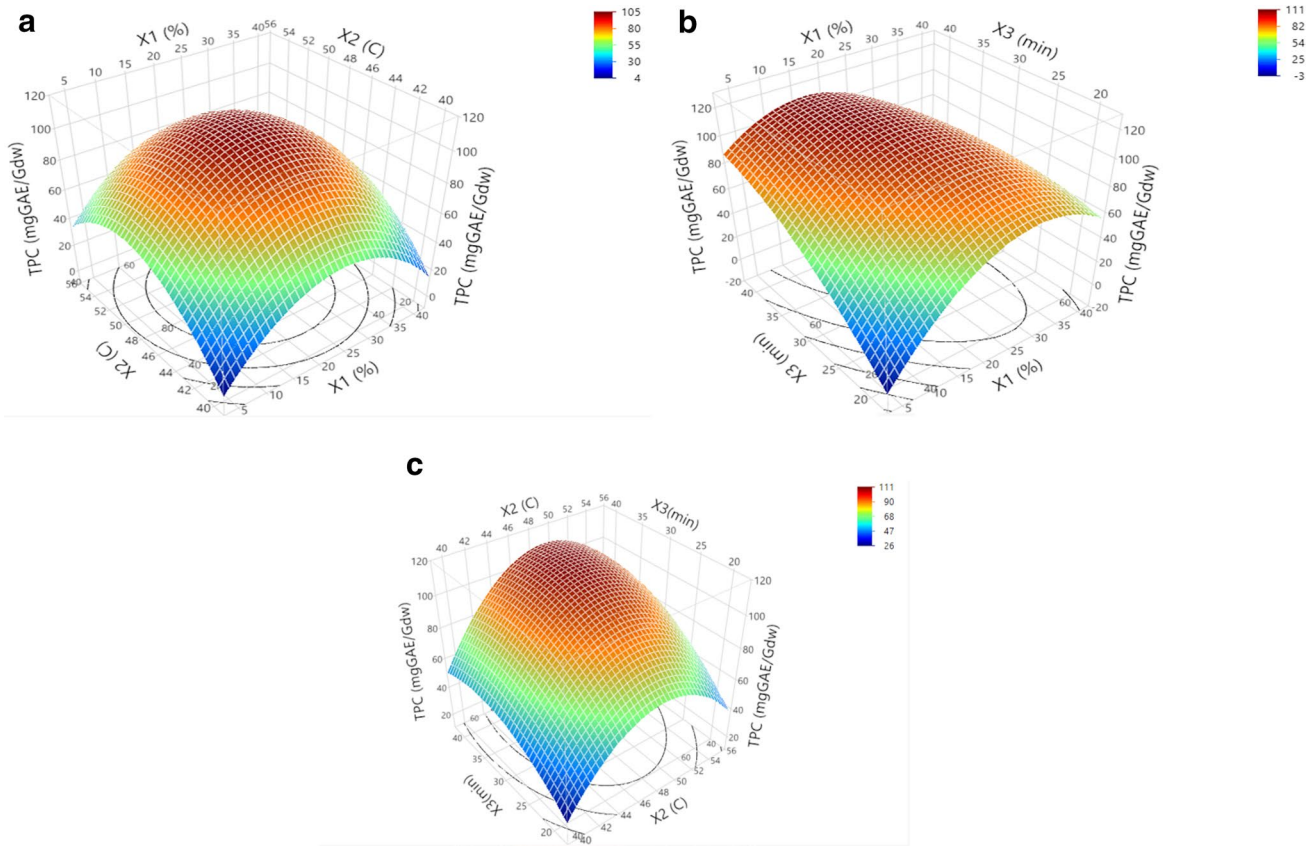

Fig. 1 The response surface plot (X_1 water addition, X_2 temperature, X_3 time) for CFL. **a** Water percentage and temperature interactions (X_1X_2), **b** water percentage and time interactions (X_1X_3), and **c** temperature and time interactions (X_2X_3)

DES solvent; total phenolic compounds for CFL extract are expressed as gallic acid equivalent milligrams per gram of the dried extract (mgGAE/Gdw), and the obtained results showed that extraction yield of polyphenols was high with a value 110 ± 5.98 mgGAE/Gdw. Also, this extract had a good flavonoid content: 37.78 ± 2.16 mg EQ/g, and the yield of TPC and TFC using DES was higher than those obtained using conventional solvents compared to literature [42]. The results for the determination of total sugars on the other hand showed that this extract had a good amount of sugars (61 ± 3.5 mg G/g). However, ChCl-Acet poorly extracted proteins.

Table 3 Phytochemical screening and yields

Phytochemical screening	Results
Alkaloids	-
Catechic tanins	-
Terpenoids	-
Cardiac glycosids	-
Proteins	-
Coumarins	-
Saponins	-
Sugars	-
Yields	Results
TPC (mg/g ± SD)	110 ± 5.98
TFC (mg/g ± SD)	37.78 ± 2.16
TAC (mg/kg ± SD)	Traces
TSC (mg/g ± SD)	61 ± 3.50
TPnC (mg/g ± SD)	5.84 ± 0.16

(+): presence, (-): absence. TPC: total phenolic content, TFC: total flavonoids content, TAA: total anthocyanin content, TSC: total sugar content, TPnC: total proteins content.

3.4 Spectroscopic analysis of DES and CFL extract

3.4.1 UV–visible screening

To inspect the absorption characteristics of CFL sample, a diluted solution of the sample was scanned using a UV–visible spectrophotometer. As Fig. 2 shows, the strong absorption peaks detected at 230–270 nm indicate the presence of conjugated dienes and trienes of unsaturated fatty acids and benzoic acid derivatives. Also, the peak observed at 280 nm provides a qualitative information regarding the presence of aromatic rings of phenolic compounds and/or some amino acids [43]. Flavanol and flavonol have a maximum absorbance at 280 nm and 350 nm, respectively [44]. The absorption

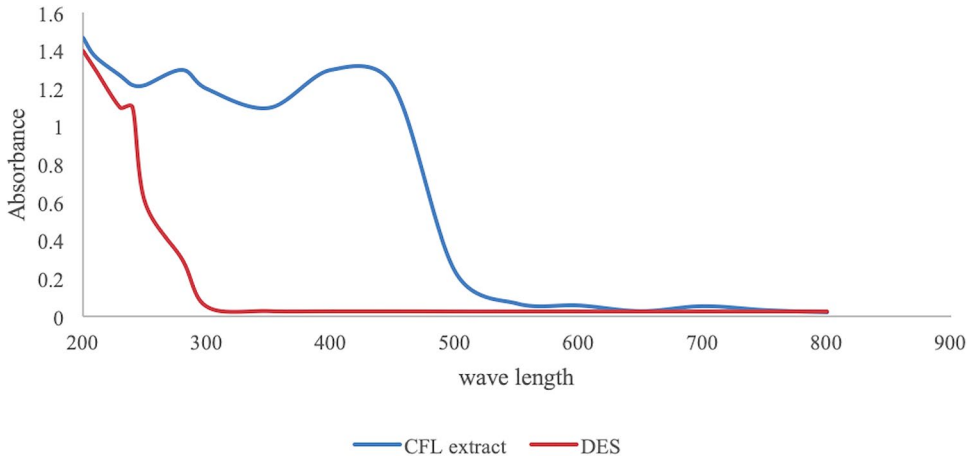

Fig. 2 UV–visible scanning for CFL extract

recorded between 400 and 490 nm may be due to the presence of colored compounds such as carotenoids and chlorophyll b [45]. Furthermore, FTIR analysis was carried out to confirm the results of the phytochemical and UV–Visible screenings.

3.4.2 Fourier transform-infrared spectroscopy

FTIR spectra of CFL extract were recorded at 4000–400 cm^{-1} region (Fig. 3). An OH large stretching vibration at 3000–3500 cm^{-1} was obviously broadened and another peak was observed at 1370–1380 cm^{-1} which is attributed to phenolic OH stretching vibrations and CH deformation in methyl groups. Also, a small peak was detected at 1115 cm^{-1} which is due to aromatic CH bending vibrations. Furthermore, another large peak was recorded at 1040 cm^{-1} attributed to CO stretch vibration. C = O stretching vibration absorption was estimated within wave-number 1700–1750 cm^{-1} [46]. Guatam et al. discussed the FTIR of ChCl-Acet, ChCl, and acetic acid.

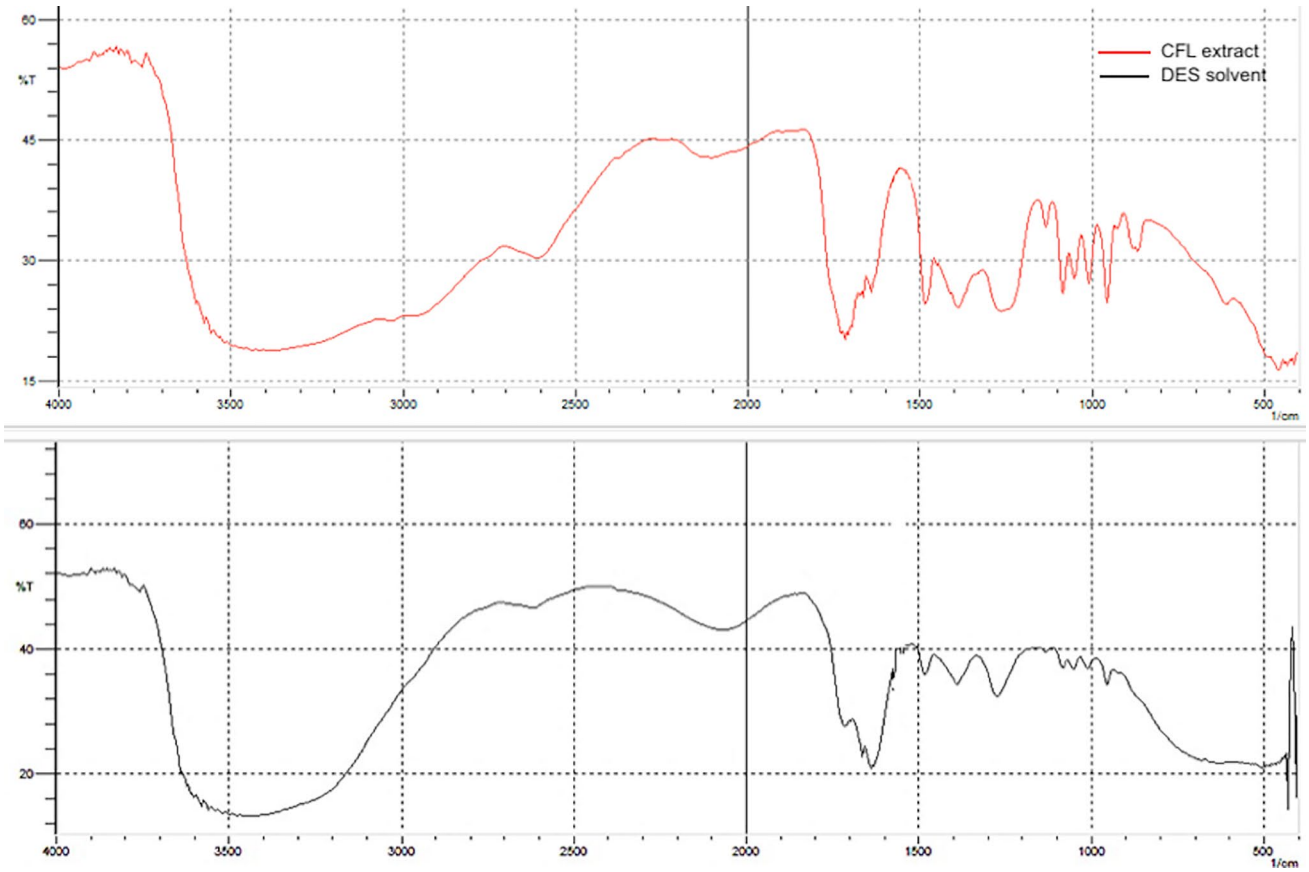

Fig. 3 FTIR analysis of CFL extract (from top, CFL extract, DES extraction solvent)

Significant shifts in vibrational frequencies from ChCl and acetic acid to ChCl-Acet were observed [36], indicative of the formation of hydrogen-bonding interaction (O = C-O–H···N). The C-O–H in-plane bending vibration at 1250 cm^{-1} was evident and bands observed from 850 to 950 cm^{-1} are characteristic of the absorption of Amide III, indicating the presence of proteins in the extract. Also, carbohydrates were identified by the presence of a distinctive peak at 1105 cm^{-1}. Moreover, 1500 cm^{-1} is a specific band to CH vibration of phenyl rings and another peak at 1000 cm^{-1} of the C–OH bond of oligosaccharides [47]. Gabriele et al. studied the effect of water addition on several DES, and according to the author, DES formation depends on intermolecular bonds between HBA and HBD and bonds existing between the individual components. When adding water to the mixture, hydrogen bonds between DES are broken and new ones are formed between them and water majorly with chloride anions; nonetheless also, in minor extension with choline cation and acid molecules [36, 41], at low percentages, water molecules are adsorbed in the molecular matrix of the DES and hydrogen bonds with ions and HBD are established. However, strong interactions with the components of the DES are created at high concentrations of water, reducing inter- and intramolecular interactions in the liquids and preventing them from interacting with each other [41]. Also, Lanjekar et al. (2020) analyzed the effect of water addition on acid-based DES with and without adding water using FTIR. And as a result, the author confirmed that there is no change in OH stretching peak at 3300 wavelength even at 30% of aqueous acid-based DES [35, 48]. The dissociation of carboxylic acid due to the addition of water to the mixture was indicated. Hence, the hydrogen bonds between ChCl and acetic acid have been reduced due to the increase in hydrogen bonds with water molecules [35]. Water molecules can act as plasticizer agents, disrupting the network of hydrogen bonds formed between DES components; introducing a small amount of water can result in their conductivity increment. More water, however, causes the dilution of electrolytes and consequent decrease in conductivity and thereafter increasing its polarity. According to the data obtained above (Sections 3.3 and 3.4.2) in addition to FTIR results, DES proved to extracted more compounds *from a wider range of functional groups and classes of molecules such as oligosaccharides,* proteins, and fatty acids in addition to phenolic compounds.

3.5 Antioxidant activities

Phenolic compounds have received a significant attention due to their bioactive properties; an excellent antioxidant capacity was recorded for CFL extract using DES as extraction solvent.

3.5.1 ABTS scavenging activity

In order to make the phenolic compound extraction ecofriendlier, a novel class of solvents known as DES was proposed as an alternative to conventional organic solvents. ABTS assay is used to evaluate the free radical scavenging ability of the molecules. As Fig. 4 shows, ABTS scavenging activity of CFL after optimization was achieved; the scavenging percentage of CFL DES extract was 97.23 ± 1.72% with no significant difference observed compared to the positive control (ascorbic acid). In addition, the diluted extract (1:10) had the same radical scavenging activity (96.2 ± 1.29%) as the crude extract. These findings highlight the potency of DES extracts over the conventional ones [34]. Also, choline chloride combined to HBD presents many advantages including the preservation of the target compound bioactivities [45], biodegradability, low cost, and low toxicity [17]. The effectiveness of choline chloride-based DES in TPC extraction was proved compared to conventional solvents due to their cell disruption ability [5], the interaction between molecules and DES (dissolution of compounds in the solvent), and the ability to extract a wide range of bioactive molecules additionally to phenolic compounds [49]. Water is an important factor for the extraction of phenolic compounds by DES. According to the literature, the largest drop in viscosity is observed with 20–30% of water addition. This lessens the viscosity of the reaction mixture and improves the extraction of target molecules which enhances the antioxidant potential. However, 50% of water can lead to the destruction of hydrogen bonds between DES components and as a result decrease of extraction efficiency [50].

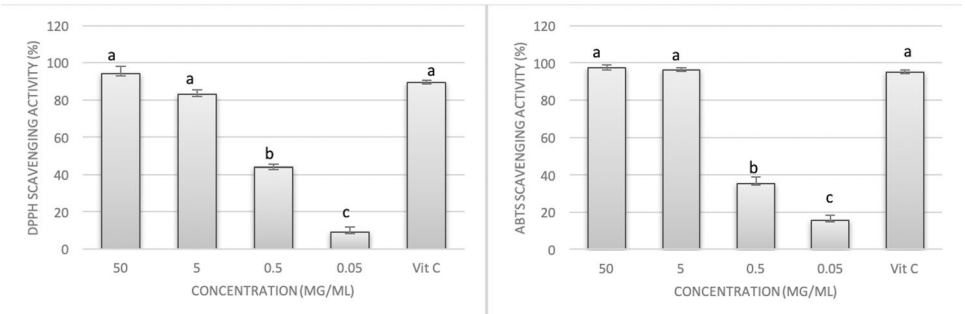

Fig. 4 Antioxidant activity of CFL extract. SS, stock solution (50 mg/mL); D1, dilution 1:10 (5 mg/mL); D2, dilution 1:20 (0.5 mg/mL); D3, dilution 1:30 (0.05 mg/mL); and standard ascorbic acid (Vit C)

3.5.2 DPPH scavenging activity

DPPH is an in vitro antioxidant assay which is often used to evaluate the effectiveness of extracts in scavenging free radicals; the extract will react with DPPH to donate hydrogen and reduce it to diphenyl-picrylhydrazine which will be observed by the change of color from violet to yellow. As shown in (Fig. 4), the DPPH-free radical inhibition ability of CFL at various concentrations was also tested and the results demonstrated that this extract could inhibit 94 ± 4.21% of this radical which was comparable to ascorbic acid as standard. This supports the findings of the ABTS assay. As a result, the antioxidant activity of CFL extract inhibits/can reduce the formation of reactive oxygen species (ROS) which causes damage to DNA, RNA, and macromolecules [51]. DES have been applied to both liquid and solid samples including plants and herbs [52], beverages [53], and honey [54] to evaluate a wide range of compounds with excellent results. Acid based choline chloride DES are known to behave similarly to ionic liquids; the free H^+ in the mixture could induce hydrolysis of cell walls and as a result the diffusion of phenolic compounds to the solvent [55]. A previous report indicated that the higher acid-based DES in COOH and OH groups the lower the extraction yield in TPC [55], making acetic acid an excellent HBD to choline chloride for TPC extraction. Furthermore, UAE has been shown to significantly improve the recovery of bioactive molecules [21] which could be attributed to the acoustic cavitation phenomenon causing pressure and temperature and, therefore, facilitating the release of the target compounds. However, polyphenol degradation may occur and affect their biological activities. DES have promised to overcome the limitations of conventional organic solvents; previous reports showed that DES can stabilize several compounds against decomposition [18] which is an important parameter for preserving longer the biological activities of the extracted molecules.

3.6 Antimicrobial activity of *Clematis flammula* L. leaves

Phytochemicals as antioxidant eliminate free radicals and protect the cell's material from damage. Numerous studies have demonstrated that phytochemicals can produce antimicrobial agents. CFL was tested for antimicrobial activity against *Candida albicans* and two pathogenic bacteria including *Methicillin-resistant staphylococcus aureus* and *Pseudomonas aeruginosa* were assessed. CFL showed activity against CA and both MRSA and PA (gram$^-$ and gram$^+$) strains. The inhibition zone of CFL against MRSA (48.08 ± 1.5 mM) and PA (39.05 ± 1.02 mM) and CA (30.00 ± 0.8 mM) were more important than the control used. Furthermore, the minimum inhibitory concentrations (MCI) by micro-dilution method of CFL against both bacteria and fungus were also determined and the results are summarized in Table 4. CFL showed a great MCI values against all strains used in this study (390 µg/mL). However, previous studies reported the antimicrobial activities of organic acids against bacteria; acetic acid exhibited the weakest antimicrobial activity among the tested organic acids but produced a high ratio of injured cells [56]. Therefore, the blank (ChCl-Acet) was deposed in wells to determine whether the antimicrobial activity was only due to the DES solvent used since it contained acetic acid as an HBD. The results showed a smaller inhibition zone compared to the extract with 25 ± 0.8 mM, 28 ± 0.41 mM, and 20 ± 1.3 mM for MRSA, PA, and CA, respectively. Volatile compounds derived from various plant extracts have been showed to exhibit antimicrobial activity against a wide range of bacteria and fungi. This is due to the presence of biologically active compounds including phenolic acids, alcohols, aldehydes, esters, and terpenes [57]. The phytochemical screening showed that DES extract contains not only phenolic compounds and organic molecules but also sugars and proteins, which means that it extracts a wider range of biological molecules with various effects, explaining its high potential compared to extracts obtained by traditional solvents.

3.7 Volatile profile of CFL using GC–MS

The characterization of volatile compounds from CFL under optimized conditions was performed using GC–MS. Table 5 shows different compounds present in DES extract; a total of 10 compounds were identified. The major compounds were as follows: protoanemonin (63.94%), phenol (8.76%), decamethyl (7.00%), linalool (5.47%), salicylic acid (4.66%), dodecamethyl (4.41%), and *n*-dexadecanoic acid (4.22%). However, borneol (0.70%), octadecanoic acid (0.42%), and thymol (0.42%)

Table 4 The minimal inhibitory concentration (MIC) of CFL against fungus and bacteria strains

Concentration (mg/mL)	0.195	0.390	0.781	1.562	3.125	6.25	12.5	25	50
Candida albicans	−	+	+	+	+	+	+	+	+
Methicillin-resistant staphylococcus aureus	−	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	−	+	+	+	+	+	+	+	+

(+) presence of inhibition, (−) absence of inhibition

Table 5 Quantitative analysis of molecules from CFL identified by GC–MS

Pic number	Molecule	Chemical formula	Rt (min)	KI	Area	Cencentration (%)
1	Salicylic acid	$C_7H_6O_3$	6.31	980	235,641	4.66
2	Decamethyl	$C_{10}H_{30}O_5Si_5$	11.68	2120	354,227	7.00
3	Phenol	C_6H_6O	21.27	1080	443,338	8.76
4	Dodecamethyl	$C_{12}H_{36}O_6Si_6$	22.21	1250	222,946	4.41
5	Linalool	$C_{10}H_{18}O$	27.29	930	276,740	5.47
6	Protoanemonin	$C_5H_4O_2$	35.05	510	3,234,610	63.94
7	Borneol	$C_{10}H_{18}O$	37.66	1120	35,370	0.70
8	*n*-Dexadecanoic acid	$C_{16}H_{35}O_4P$	43.22	950	213,546	4.22
9	Octadecanoic acid	$C_8H_{18}O$	50.1	800	21,354	0.42
10	Thymol	$C_{10}H_{14}O$	51.2	710	21,354	0.42
					Total	100%

were found in traces (see Fig. 5). The urgent need for a new alternative to chemical drugs pushed researchers to search for bio-based molecules extracted from plants. Salicylic acid (SA) was found in CFL extract; SA is characterized by its anti-oxidative and anti-inflammatory properties since it was reported to reduce tissue damages and could inhibit the expression of pro-inflammatory genes [58]. Decamethyl, *n*-octadecanoic acid, *n*-hexadecanoic acid, protoanemonin, and dodecamethyl are well known to be antimicrobial agents isolated from different plant species. Also, several reports have identified linalool in the essential oil of many plant extracts. It was proved to be an effective antioxidant and antimicrobial agent against many bacteria [59, 60]. Additionally, linalool has shown promising activity against parasites including *Plasmodium falciparum* and *Leishmania amazonensis*. Moreover, it was reported that linalool exhibits a peripheral analgesic effect [59]. Thymol is an interesting substance due to its different properties and potential applications in varied fields; it is a biologically active component in the pharmaceutical field. It has been proved that thymol is an excellent immunomodulatory, anti-inflammatory, and antimicrobial agent [61]. This could explain the potent antioxidant and anti-microbial activity of CFL extract against PA, CA, and MRSA. Hence, Borneol, *n*-octadecanoic acid, and *n*-hexadecane acid were previously reported to be potent anti-inflammatory molecules [62]. DES are more effective in extracting phytochemicals than ethanol and water for example, and this is explained by the differences in their polarity. Even though ethanol and water are green solvent, the hydrogen bond network in them is easily disintegrated and the hydrogen bonds between choline chloride and acetic acid improve the extraction of phenolic compounds from CFL.

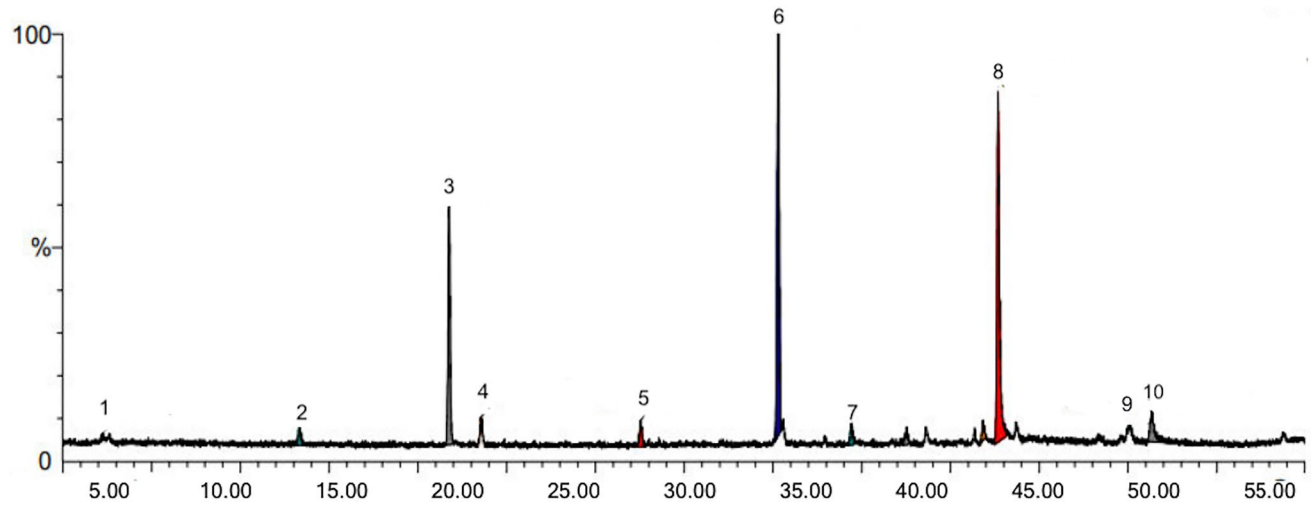

Fig. 5 The chromatogram of the identified volatile compounds from CFL. (1) Salicylic acid, (2) decamethyl, (3) phenol, (4) dodecamethyl, (5) linalool, (6) protoanemonin, (7) borneol, (8) *n*-dexadecanoic acid, (9) octadecanoic acid, (10) thymol

4 Conclusion

In this study, the antioxidant and antimicrobial effect of CFL extracted by green alternative technology (UAE-DES) was assessed. The data obtained showed that CFL exhibited not only a high scavenging ability against free radicals but also a good bactericidal effect. Phytochemical screening and the UV–visible and infrared analysis and GC–MS qualitative and quantitative analysis indicated that the extract was a rich source of phytochemicals with various biological effects. Proving that the UAE-DES is an excellent approach for the extraction of phenolic compounds which could replace the use of toxic organic solvents and time and energy-consuming methods, the presence of antimicrobial substances in CFL is well established in this study, providing a source of inspiration for novel drug formulations which could treat several diseased related to pathogenic microbes. However, studies including toxicity evaluation and purification of active anti-microbial constituent are highly recommended looking toward pharmaceutical use.

Abbreviations *CA*: *Candida albicans*; *CFL*: *Clematis flammula* L. leaves; *ChCl-Acet*: Choline chloride-acetic acid; *ChCl-Gly*: Choline chloride-glycerol; *ChCl-Lac*: Choline chloride-lactic acid; *DES*: Deep eutectic solvents; *Fru-acet*: Fructose-acetic acid; *GC-MS*: Mass tendam coupled gas chromatography; *Gly-Acet*: Glycerol-acetic acid; *Gly-Lac*: Glycerol-lactic acid; *HBA*: Hydrogen bond acceptor; *HBD*: Hydrogen bond donor; *MIC*: Minimal inhibitor concentration; *MRSA*: *Methicillin-resistant staphylococcus aureus*; *PA*: *Pseudomonas aeruginosa*; *TPC*: Total phenolic content; *TFC*: Total flavonoid content; *TSC*: Total sugar content; *TPnC*: Total proteins content; *UAE-DES*: Ultrasound-deep eutectic-assisted extraction; *Ure-Gly*: Urea-glycerol

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Author contribution S.O. TEBBI: aim, ideas, design of methodology, performing the experiment, data analysis, reproducibility of results; N. DEBBACHE: ideas, data analysis, management, and coordination responsibility for the research; K. MOULAOUI: verifications; R. KADI: formulation; S. ZAIDI: formulation.

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Data availability All original data related to this study will be provided on demand.

Declarations

Ethics approval Not applicable.

Consent for publication All authors of this paper are aware of the submission and agree to its publication.

Competing interests The authors declare no competing interests.

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