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EDITED BY
Prashant Upadhyay,
IFTM University, India

REVIEWED BY
Rachid Touzani,
Mohamed Premier University, Morocco
Doha Berraaouan,
Mohamed Premier University, Morocco

*CORRESPONDENCE
Bruna Laratta
✉ bruna.laratta@cnr.it

†These authors have contributed equally to this work

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Chemical composition of wild *Rosmarinus officinalis* essential oil: bioactivity evaluation, and valorisation strategies for the spent biomass after essential oil extraction

Bruna Laratta[1*†], Filomena Monica Vella[1†], Anna Giulia Castaldo[2], Immacolata Fiume[1], Francesco Siano[3] and Domenico Cautela[4]

[1]Institute of Biosciences and BioResources (IBBR), National Research Council (CNR), Napoli, Italy, [2]Department of Architecture and Urban Studies (DASTU), Politecnico di Milano, Milano, Italy, [3]Institute of Food Science (ISA), National Research Council (CNR), Avellino, Italy, [4]Department of Theoretical and Applied Sciences, E-Campus University, Milano, Italy

Minimising waste generation is at the heart of sustainable management policies in the agri-food sector, with a focus on the valorisation of by-products. In this scenario, solid residues from the essential oil (EO) industry are a rich source of bioactive phenolic compounds for use in food and pharmaceuticals. This study explores the potential exploitation of essential oil (EO) and the solid residue produced after the distillation of a wild variety of *Rosmarinus officinalis*. The EO from the leaves was extracted by hydro-distillation and, afterwards, antioxidant activity and sun protection factor were evaluated, as well the chemical composition was established using gas chromatography. The results of this characterisation reveal a new EO chemotype with the major component being α-pinene. The bioactive compounds in rosemary residue after essential oil (EO) distillation were explored. The presence of polyphenols, *ortho*-diphenols and flavonoids was determined, followed by antioxidant characterisation. The results were analysed using the statistical tool 'Response Surface Methodology' to identify the optimal parameters for maximising the yield of bioactive compounds (time, temperature and solvent concentration) and, so that, the best value of this rosemary by-product could be ascertained. The recovery of terpenes and polyphenols has been demonstrated in this wild variety of *R. officinalis* for the first time, which allows the full utilisation of its by-products and may help to make the EO industry more sustainable. Furthermore, these research outcomes provide confirmation of the worth of this aromatic plant for application in food, beverages, cosmetics, pharmaceuticals and medicine.

KEYWORDS

rosemary, *prostratus* variety, essential oils, waste valorisation, antioxidant activity, response surface methodology

1 Introduction

The Circular Economy Action Plan launched by the EU promotes a climate-neutral and resource-efficient economy by minimizing inputs, waste, and emissions through reuse and recycling strategies (Velasco-Muñoz et al., 2022; Chiaraluce et al., 2021).

Managing waste in the agri-food sector is widely recognised as a crucial aspect of the circular economy system, providing a feasible way to handle the waste and by-products generated in this sector. It proposes actions and solutions that allow the reintegration of these materials into production processes and their valorisation in the production of high-value-added biomolecules (Chiaraluce et al., 2021).

In the agri-food sector, essential oils (EOs) from aromatic and medicinal plants (AMPs) are important products (De Elguea-Culebras et al., 2022; Skendi et al., 2022). The EOs global market are extensively employed in fragrances and cosmetics sector (perfumes, skin creams, body lotions, soaps, shampoos, make-up products), as well as in food and beverages (herbs, spices, and additives) and medicinal field (pharmaceutical industry, aromatherapy, dentistry and medicinal supplements). In particular, the worldwide EOs production has passed 70,000 tons per annum and it is estimated that about 65% is supported by developing countries (De Elguea-Culebras et al., 2022; Kant and Kumar, 2022). In depth, USA (40%), Western Europe (30%), and Japan (7%) are the main consumers of EOs, with a continuously increasing requirement of these natural products (Kant and Kumar, 2022).

On the other hand, the growing demand for EOs causes a major problem in terms of managing the waste produced by the distillation process. Saha and Basak (2020) estimate that 200,000 tons of solid residues are generated worldwide each year from the EO extraction process from AMPs. EO yields are typically low (0.5%–5% of dry weight), meaning that most plant biomass is discarded, creating environmental and economic concerns. This scenario shows how recovering active molecules could be valuable alternative (Saha and Basak, 2020).

Lamiaceae family is one of the most important involved in the EOs production, playing a central role in people lifestyle, health and wellbeing (Nieto, 2017). Approximately 236 genera and 7,200 species make up this botanical family, all of which are native to the Mediterranean basin. Oregano, sage, rosemary and thyme are the main ones from a commercial point of view (Kant and Kumar, 2022).

In particular, rosemary (*Rosmarinus officinalis* L.) is one of the best-known herbs used since ancient times, as wild or cultivated, ornamental and aromatic shrub (González-Minero et al., 2020). Currently rosemary leaves are employed as spice in many food preparations and dishes and have been traditionally used as a medicinal herb for their many biological properties, such as anti-inflammatory, analgesic, astringent, antimicrobial, anti-rheumatic, carminative, anti-fungal, and antioxidant (González-Minero et al., 2020; Nieto, 2017). The antioxidant activity of leaves has been extensively investigated, and many recent studies have demonstrated that this property is mainly attributable to bioactive compounds present in EOs and in alcoholic extracts (González-Minero et al., 2020; Nieto, 2017; Erkan et al., 2008; Borges et al., 2019).

Since 2008, the European Food Safety Authority (2008) (EFSA) has recognised rosemary extract as a food additive (European Food Safety Authority, 2008). Moreover, Directive 2010/67/UE of 2010 approved the use of rosemary extracts as a new food additive, attributing the label E392. Nowadays, rosemary extracts are authorised to be added to food and beverages at levels of up to 400 mg/kg, considering the sum of carnosic acid and carnosol, which are the most abundant antioxidants present in the rosemary extract (European Food Safety Authority, 2018).

From a chemical point of view, rosemary EO contains about 90%–95% of monoterpenes and monoterpenes derivatives and a lower quantity of sesquiterpenes (2%–5%). The main compounds are 1,8-cineole, α-pinene, limonene, verbenone, camphor, borneol, and camphene, as reported by many studies (Jamshidi et al., 2009; Giacometti et al., 2018; Borges et al., 2019). The chemical composition depends not only from the plant species but also from age, variety, part utilized, origin, climate, soil, and extraction procedure (Jamshidi et al., 2009; Hernández et al., 2016; Giacometti et al., 2018; Borges et al., 2019; González-Minero et al., 2020). The polyphenolic compounds in rosemary are also renowned and are mainly phenolic diterpenes, such as carnosol, carnosic acid, rosmanol, epirosmanol and isorosmanol, and phenolic acids such as rosmarinic and caffeic acids (Erkan et al., 2008; Terpinc et al., 2009; Hosseini et al., 2018).

To the best of our knowledge, no studies have simultaneously characterised rosemary EO and valorised the post-distillation residue, particularly in wild ornamental varieties. A particularly exquisite and aromatic variety of *R. officinalis* L. (hereafter referred to as ROP) is a wild species of rosemary that is distinguished by its creeping and prostrate growth habit (Hammer and Junghanns, 2020). The plant is known to thrive in the Mediterranean climate, in gardens, in pots and along walls. It is frequently used for its scent in cooking or as a decorative plant. In the present study, the EO was extracted from the leaves of ROP by using hydro-distillation. Following, the EO was characterized for chemical composition, antioxidant activity and sun protection factor (SPF). ROP biomass residue, after EO distillation, was studied for polyphenolic compounds, varying some experimental parameters to optimize the protocol extraction. Specifically, extraction time (15 min, 30 min, and 60 min), temperature (25 °C, 40 °C, 50 °C, 60 °C, and 70 °C) and ethanol concentration (50%, 60%, 70%, and 80%) were tested and the results were analysed by using the statistical tool of Response Surface Methodology. Finally, chromatographic profile and quantification of the main phenolics on the ROP residue extract were carried out with the aim of a whole valorisation and chemical characterization of this rosemary by-product.

The results obtained from this study will contribute to promote the sustainable development of the AMPs industry through the implementation of circular economy and the recovery of novel inputs from these resources, in particular from rosemary species.

2 Materials and methods

2.1 Reagents and standards

Folin–Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sodium molybdate (Na_2MoO_4), sodium acetate, iron (III) chloride hexahydrate ($FeCl_3 \times 6H_2O$), aluminium chloride hexahydrate ($AlCl_3 \times 6H_2O$), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, caffeic acid, (+)-catechin hydrate, L-ascorbic acid, quercetin, rosmarinic acid and butylated hydroxyl-toluene (BHT) were supplied by Sigma-Aldrich (St. Louis, MI, USA). Further, acetic acid, hydrochloric acid (HCl), sodium hydroxide (NaOH), ethanol, and methanol were of analytic grade and were obtained from Carlo Erba Reagents (Milan, Italy). MS grade ethanol and heptane, and HPLC-grade acetonitrile, trifluoroacetic acid (TFA) and water were purchased from Merck KGaA (Darmstadt, Germany).

2.2 Plant sampling and EO distillation

Wild rosemary (*Rosmarinus officinalis* L. var. *prostatus*) was harvested in a field situated in Agerola (Latitude: 40°38′19″32 N; Longitude: 14°32′22″92 E) in Naples province (Italy) in the spring of 2024, before the main flowering season. The plant material was transported to the laboratory, where the leaves were removed from the branches and stored at 4 °C, until the EO extraction. The EO hydrodistillation was performed by using a Clevenger-type apparatus, in accordance with the European pharmacopeia method (2005). Briefly, 70 g of shredded rosemary leaves and 350 mL of distilled water (ratio 1:5 w/v) were placed in the 1 L spherical flask of the Clevenger at 100 °C for 3 h. Rosemary EO was collected in a glass vial, dried with anhydrous sulphate and stored in the dark at 4 °C, until further analyses.

The yield (Y) of process was expressed as a percentage value (volume of the essential oil in 100 grams of fresh leaf) and determined according to Equation 1:

$$Y\left(\%_{V/w}\right) = {}^{V_{EO}}\!/_{ms} \times 100 \quad (1)$$

where V_{EO} was the EO volume reported in mL and m_s was the weight mass of rosemary expressed in grams.

2.3 GC-FID and GC–MS analysis of rosemary essential oil

The volatile composition of the essential oil was determined using a gas chromatography-flame ionization detection (GC-FID) analysis, and the identification of EO composition was confirmed by a gas chromatography–mass spectrometry (GC–MS) analysis.

GC-FID analysis was performed using a Trace GC-FID system (Thermo Fisher Scientific, USA) with detector set at 280 °C, while a Trace 1300 GC system coupled to the TSQ DUO triple quadrupole mass spectrometer (Thermo Fisher Scientific, USA), equipped with an electron impact (EI) ion source, was used for mass spectrometry (GC–MS) analysis. The parameters used for GC–MS analysis were the following: ionization energy of 70 eV, mass range between 50 and 550 m/z and interface temperature of 250 °C. Both systems were equipped with an automatic sampler (AS 3000, Thermo Fisher Scientific, USA) with a 10 µL syringe set to a delivery volume of 1 µL at high injection speed (split ratio 1:30). The injector temperatures were 250 °C. Samples (diluted 1:100 in ethanol) were injected without derivatization into the DB-5 column, 30 m × 0.25 mm with 0.25 µm film (Thermo Fisher Scientific, USA) using the 1:10 split mode.

The carrier gas utilized was helium, with a flow rate of 1.0 mL/min, while the make-up gas was nitrogen, at a flow rate of 30.0 mL/min. The initial oven temperature was set at 60 °C for a duration of 5 min. Subsequently, the temperature was increased to 190 °C at a rate of 5 °C per minute and maintained at this temperature for 5 min. Following this, the temperature was decreased to 190 °C at a rate of 15 °C per minute and held for an additional 15 min.

The data were acquired and processed using Chromeleon Chromatography Data System (CDS) software (Thermo Fisher Scientific, USA). The identification of GC-FID peaks was achieved through two distinct methodologies. Firstly, a direct comparison of retention times with those of standard compounds was conducted. Secondly, a comparison was made with GC–MS using mass spectral data of standard compounds and the National Institute of Standards and Technology (NIST) library. The results were presented as a relative percentage of the normalized peak area and were reported as the average values of three injections.

2.4 Sun protection factor of rosemary essential oil

For the determination of the sun protection factor (SPF), EO solution was prepared in ethanol (0.1% v/v). The absorbance of the sample was measured spectrophotometrically at 5 nm intervals in the range of 290–320 nm using a LAMBDA 365 + UV/Vis Spectrometer (PerkinElmer, Italy). The SPF determined *in vitro* was calculated by using Equation 2 previously reported by Vella et al. (2021):

$$SPF = CF \times \sum_{290}^{320} EE\left(\lambda\right) \times I\left(\lambda\right) \times Abs \quad (2)$$

where CF = correction factor (= 10), EE (λ) = erythemal effect spectrum, I (λ) = solar intensity spectrum, and Abs = absorbance of samples. The EE (λ) × I (λ) values were determined according to Sayre et al. (1979).

2.5 Extraction of bioactive compounds from rosemary post-distillation residue

Post-distillation ROP leaves were recovered, placed at −20 °C and freeze-dried. Afterwards, 250 mg were extracted, in a 1:20 (w/v) ethanolic solution, by varying three different parameters: time (15 min, 30 min, and 60 min), temperature (25 °C, 40 °C, 50 °C, 60 °C, and 70 °C) and ethanol concentration (50%, 60%, 70%, and 80%, v/v). The extractions were ultrasound-assisted, performed applying a sonication power of 120 W with a frequency of 40 Hz in several cycles until the programmed extraction time was reached. The sonication cycles comprised 30 s of ultrasonic extraction, followed by a 5-min interval between cycles. All the extracts were recovered by centrifugation at 13,000 g for 10 min at 4 °C and dried via rotary evaporator.

2.6 Determination of total phenolic content and assessment of antioxidant activities on rosemary post distillation extract

Polyphenols were determined through the spectrophotometric method Folin–Ciocalteu as described by Singleton and Rossi (1965). Briefly, diverse quantities of ROP extracts were added to 750 µL of Folin–Ciocalteu reagent and 600 µL of Na_2CO_3 at 7.5% (w/v). After 2 h of incubation in the dark, the absorbance was read at 765 nm. Gallic acid was used as standard, and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW) biomass.

Ortho-diphenols were determined with the test as estabilished by Arnow (1937). In brief, various amounts of extracts were mixed with

400 µL of 0.5 M HCl, 400 µL of 1.45 M $NaNO_2$–0.4 M Na_2MoO_4, and 400 µL of 1 M NaOH. The absorbance was determined at 500 nm and the *ortho*-diphenols were reported as µg of caffeic acid equivalents (CAE) per mg of DW biomass.

Flavonoids were determined according to the colorimetric test based on aluminum complex formation (Zhishen et al., 1999). Different amounts of extracts were added to 1.25 mL of distilled water and 75 µL of $NaNO_2$. After incubation for 5 min, 150 µL of $AlCl_3 \times 6H_2O$ were supplemented and the reaction was stopped by 1 M NaOH. The absorbance was recorded at 510 nm and results were expressed as µg of catechin equivalents (CE) per mg of DW biomass.

The antioxidant properties were evaluated through two assays, the Ferric Reducing Antioxidant Power (FRAP) and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity.

As reported by Benzie and Strain (1996), freshly prepared FRAP reagent (25 mL of 300 mM sodium acetate buffer, pH 3.6; 2.5 mL of 10 mM TPTZ in 40 mM HCl; 2.5 mL of 20 mM $FeCl_3 \times 6H_2O$) was joined to several quantities of extracts. The absorbance was recorded after 4 min at 593 nm. The antioxidant power was calculated from the calibration curve of L-ascorbic acid and the results were expressed as mg of ascorbic acid equivalents (AAE) per g of DW biomass (or per g of EO).

The radical scavenging activity of the extracts was assessed according to the procedure of Blois (1958). Briefly, 1.35 mL of 60 µM DPPH methanolic solution was combined with different concentrations of extracts (or EO). The reduction in absorbance was continuously recorded at 517 nm and the radical scavenging activity percentage (%RSA) of DPPH discoloration was calculated by using the following Equation 3:

$$\%\mathrm{RSA} = \left(A_{DPPH} - A_s\right) \Big/ A_{DPPH} \times 100 \quad (3)$$

where A_{DPPH} is the absorbance of the DPPH solution and A_S is the absorbance of the sample tested.

The concentration required to achieve 50% of radical DPPH inhibition (EC_{50}) was graphically calculated by plotting the %RSA *vs* the concentrations (expressed as µg/mL for extracts or for EO).

Antioxidant activities of the synthetic butylated hydroxyl-toluene (BHT) and quercetin were also determined as positive control in both antioxidant assays (FRAP and DPPH).

2.7 HPLC-DAD analysis on ROP post distillation extract

Phenol composition was determined by reversed phase-high performance liquid chromatography (RP-HPLC), adapting a previously reported procedure (Siano et al., 2023). To this purpose, aliquots of ethanolic extract were 10-fold diluted with 0.1% TFA, and 100 µL of the resulting solutions was separated by RP-HPLC using a modular HP 1100 chromatographer (Agilent, USA) equipped with a diode array detector (DAD). The stationary phase was a Jupiter C18 reverse phase column 250 × 2.1 mm, 4 µm particle diameter (Phenomenex, USA), kept at a 40 °C using a thermostatic oven. Phenolic compounds were separated applying a 5%–65% gradient of solvent B (acetonitrile/TFA 0.1%) in 65 min, following 5 min of isocratic elution at 5% B, at a 0.2 mL/min constant flow rate. After the column was extensively washed with 100% B and then equilibrated with 5% B. Solvent A was 0.1% TFA in HPLC-grade water. Separations were monitored at λ = 280, 320, 360, 520 nm wavelengths and peaks were integrated using the HPLC ChemStation software A.07.01 (Agilent, USA). Rosmarinic acid was quantified using a calibration curve ($y = 50.496x + 88.246$, $R^2 = 0.9976$) built with five different concentrations of standard, (25–100 µg/mL) in methanol and diluted 10-fold with 0.1% TFA prior to injection.

2.8 Statistical analysis

All samples were analysed in triplicates ($n = 3$) and the results were expressed as mean ± standard deviation (SD). Means, SDs, calibration curves and linear regression analyses (R^2) were performed by using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). Correlation analyses were carried out by using CORREL function in Microsoft Excel 2013. Pearson correlation coefficients (r) were calculated and followed by t-Student test, with two-sample equal variance and two-tailed distribution. Differences at $p < 0.01$ were considered highly significant.

2.9 Response surface methodology

The extraction of phenolic compounds from solid waste mass was achieved through the utilization of an extraction ratio equal to 1:20 (w/v, 250 mg of waste mass). This extraction ratio was selected based on preliminary tests to ensure the maximum sample quantity that could be used without causing solid aggregates to form in the flask during the extraction process.

The optimization of the extraction process was achieved by implementing a Box–Behnken design with regard to three variables: extraction temperature (x_1) ranging from 25 to 70 °C, extraction time (x_2) ranging from 15 to 60 min, and ethanol percentage (x_3) ranging from 50% to 80% v/v.

The experimental data were analysed using the response surface regression procedure to fit the following second-order polynomial equation used to study the interactions between the independent variables, their optimal levels, and their effects on the responses:

$$Y = \beta 0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \sum_{j=1}^{2} \beta_{i,j} x_i x_j + \sum_{i=1}^{3} \beta_i x_i^2$$

where Y was the response (total polyphenolic content, flavonoids content, FRAP and DPPH scavenging activity); β0, $\hat{a}_i$, and $\hat{a}_{i,j}$ were constant coefficients of interaction, and x_i and x_j were the independent factors which influenced the response of variable Y.

The collected data were then analysed using MINITAB 15 software (Minitab Inc., State College, PA, USA) to ascertain the interaction between the variables and the responses.

To identify the impact of the variables and their interrelations on the observed responses, an analysis of variance (ANOVA) was conducted. The model's fitness was ascertained by employing several statistical metrics, including the coefficient of determination (R^2), the adjusted R^2, the significance of the lack of fit, and the regression coefficient (β).

The *F* value was employed to make a comparison between the mean value obtained from the experimental data and that which had been predicted. It was also used to validate the model that had been designed.

The results were considered statistically significant at $p < 0.05$.

Response surface plots and contour plots were obtained using the fitted model, with the independent variables maintained simultaneously.

3 Results and discussion

3.1 Rosemary EO characterization

EO from ROP leaves was extracted by hydro-distillation using a Clevenger-type device. The most common industrial technique is steam distillation, which is faster and suitable for large quantities. In this study, hydrodistillation (at a temperature of lower than 100 °C) was employed to extract AMPs, thereby preserving thermolabile compounds that might otherwise be degraded by steam distillation (Borges et al., 2019; Meziane et al., 2025; Vella et al., 2020). Furthermore, in the hydro-distillation process, the EO obtained is shielded from the surrounding water phase, which functions as a barrier to prevent degradation from potential overheating (Kant and Kumar, 2022).

In this study, the resulting EO yield was 1.57% (± 0.14), which is higher than the yields reported in other studies. For example, Boutekedjiret et al. (2003) reported a yield of 0.44%, whereas Conde-Hernández et al. (2017) and Bousbia et al. (2009) recorded yields of 0.35 and 0.30%, respectively. However, our results are in accordance with those of Flamini et al. (2002), Angioni et al. (2004), and Jamshidi et al. (2009), who reported comparable EO total yields from *R. officinalis*, of 1.44%, 2.13%, and 2.60%, respectively. These differences could be attributed to diverse variety and environmental condition, such as climate, soil, altitude, water availability (Jamshidi et al., 2009; Hernández et al., 2016; Borges et al., 2019; González-Minero et al., 2020).

In this study, only the rosemary leaves were used to distillate EO, obtaining a pale-yellow transparent liquid, with an intense, refreshing and herbal aroma (Borges et al., 2019). ROP EO was analysed by GC-FID and the results are presented in Table 1 and Supplementary Figure S1. These data identified 26 compounds, of which 25 confirmed by GC–MS. The most abundant component is *α*-pinene (43.43%) and moderate amounts of α-terpineol (13.87%) and 1,8-cineole (8.12%) are reported. Furthermore, lower amounts of camphor (4.36%), followed by myrcene (3.08%), camphene (2.80%), and bornyl acetate (2.08%) are present. All other compounds are below 2% as shown in Table 1.

Chemical composition of rosemary EO has been the subject of numerous studies. The main constituents reported in the current literature include camphor, 1,8-cineole, *α*-pinene, borneol, verbenone, and *β*-pinene (Jamshidi et al., 2009; Hernández et al., 2016; Andrade et al., 2018; Borges et al., 2019; González-Minero et al., 2020). Literature data show a close connection between the geographic origin of the *R. officinalis* plants and their chemical composition. Three main essential oil (EO) chemotypes have been described for *R. officinalis* (Hernández et al., 2016; Satyal et al., 2017; Napoli et al., 2010).

TABLE 1 Composition of rosemary EO analysed by GC-FID.

Peak n.	RT (min)	Component	Area % (relative percentage)
1	5.1	α-Thujene	0.08
2	5.4	α-Pinene	43.43
3	5.8	Camphene	2.80
4	6.0	β-Mircene	0.54
5	6.7	Sabinene	0.08
6	6.8	β-Pinene	1.33
7	7.4	Myrcene	3.08
8	8.1	α-Phellandrene	0.23
9	8.7	α-Terpinene	0.51
10	9.1	*p*-Cymene	1.35
11	9.4	D-Limonene	3.57
12	9.5	1,8-Cineole	8.12
13	11.3	γ-Terpinene	1.21
14	13.1	Terpinolene	1.01
15	13.9	Linalool	1.83
16	15.6	Verbenone	0.43
17	16.3	Camphor	4.36
18	17.5	Unknown	1.25
19	18.2	Citronellal	1.04
20	19.0	Terpinen-4-ol	1.61
21	19.9	α-Terpineol	13.87
22	21.6	Neral	0.26
23	22.4	Geraniol	0.95
24	23.2	Geranial	0.10
25	23.8	Bornyl acetate	2.08
26	29.8	β-Caryophyllene	0.30

The results shown are the average of three replicates and in all cases relative standard deviation was lower than 5% and it is not reported (RT, retention time).

These are the cineoliferum type, which contains over 40% 1,8-cineole and is mainly found in Morocco, Tunisia, Turkey, Yugoslavia, Greece, Italy and France. The camphoriferum type, which contains approximately equal ratios of 1,8-cineole, *α*-pinene and camphor and is chiefly reported in Spain, France, Italy, Bulgaria and Greece; and the verbenoniferum type, which contains over 15% verbenone and is mainly found in France and Egypt. Taking this classification into account, the data on ROP EO in this research is quite different from the above chemotypes because it shows a high amount of α-pinene (43.43%) and a moderate quantity of 1,8-cineole (8.12%) and camphor (4.36%), as shown in Table 1.

The gas chromatographic profile of the analysed EO demonstrates elevated concentrations of both α-pinene and 1,8-cineole, thus classifying it as a less prevalent chemotype defined as the α-pinene/1,8-cineole chemotype.

The following constituents were identified: monoterpenes (59.22%), sesquiterpenes (0.30%), alcohols (18.26%), ketones (4.79%), aldehydes (1.40%), esters (2.08%), and ethers (8.12%), in addition to oxygenated ethers (8.12%), representing a total of 95.42%. The gas chromatographic composition of EO is consistent with that

documented in the study by Serralutzu et al. (2020), which characterizes a wild population of Mediterranean *Rosmarinus officinalis* with high concentrations of both α-pinene (up to 75.4%) and 1,8-cineole. This chemical profile is characteristic of an α-pinene/1,8-cineole chemotype (Serralutzu et al., 2020).

The chemical composition of *R. officinalis* essential oil (EO) affects its biological activities. The main components, 1,8-cineole, α-pinene and camphor, are responsible for the pharmacological activities attributed to rosemary EO. The most abundant compound in our EO the terpene α-pinene, has been reported in the literature to exhibit antioxidant, antifungal, antibacterial, and anti-inflammatory activities (Hernández et al., 2016; Borges et al., 2019; González-Minero et al., 2020). For this reason, the antioxidant activity of ROP EO was evaluated using two *in vitro* assays (FRAP and DPPH) and by measuring SPF (Vella and Laratta, 2023). All the results are shown in Table 2. This table also shows the FRAP antioxidant capacity and the DPPH radical scavenging activity for the main chemical compounds that are commonly used in the food industry. In particular, BHT (the most widely used synthetic compound) and quercetin (one of the main natural, plant-derived antioxidants) were used as positive controls in both the FRAP and DPPH assays (see Table 2).

Although FRAP assay was originally developed to measure plasma antioxidant capacity, it can be used to quantify the antioxidant capacity from a wide variety of biological samples ranging from pure compounds to herbal and plant extracts, essential oils, beverages and pharmaceuticals (Christodoulou et al., 2022; Munteanu and Apetrei, 2021; Katalinic et al., 2006).

The antioxidant power of the ROP EO showed a FRAP value of 60.55 ± 1.11 mg AAE/g., resulting higher than quercetin (75.79 ± 1.45 mg AAE/g) and lower than BHT (58.12 ± 2.05 mg AAE/g), as reported in Table 2.

The radical scavenging activity DPPH assay is based on the ability of a bioactive compound to donate electrons in order to reduce and stabilize the DPPH radical (Christodoulou et al., 2022; Munteanu and Apetrei, 2021). The rosemary ROP EO showed an EC_{50} of 240.39 ± 2.27 μg/mL (Table 2) and a comparable EC_{50} value of 337.23 μg/mL was recorded for *R. officinalis* EO in literature data (Ouknin et al., 2021). Higher results of EC_{50}, and a lower antioxidant activity, were reported by Olszowy and Dawidowicz (2016) and Viuda-Martos et al. (2010), with 433.05 mg/mL and 17.00 mg/mL, respectively. The very different outcomes reported in literature were mostly due to the employment of rosemary belonging to diverse plant age, variety, and environmental condition. These parameters exert a significant influence on the chemical composition of essential oils (EOs) and the associated biological activities (Jamshidi et al., 2009; Hernández et al., 2016; Nieto, 2017; Giacometti et al., 2018; González-Minero et al., 2020).

Considering the in vitro SPF measurement of ROP EO, a calculated SPF value of 2.55 was obtained (see Table 2). The higher the SPF value, the greater the protection offered by biomolecules against UV light. In particular, when added to foods or cosmetic formulations, EO confers the ability to absorb UV radiation, thereby preventing oxidation and reducing the formation of free radicals caused by sun exposure, which can lead to food alteration or skin damage (Vella et al., 2021; Vella and Laratta, 2023).

Sun Protection Factor (SPF) is a fundamental tool for the assessment of the sun protection and oxidative potential of this EO. The chemical components of essential oils are the determining factor in this value, with the growing conditions and harvest period of the plants and different rosemary cultivars, or even the same variety grown in different geographical areas, having a significant impact (Jamshidi et al., 2009; Hernández et al., 2016; Nieto, 2017; Giacometti et al., 2018; González-Minero et al., 2020).

TABLE 2 Antioxidant activity (FRAP and DPPH assays) and SPF for *R. officinalis* EO, quercetin and BHT.

	R. officinalis EO	Quercetin	BHT
FRAP (mg AAE/g)	60.55 ± 1.11	75.79 ± 1.45	58.12 ± 2.05
DPPH – EC_{50} (μg/mL)	240.39 ± 2.27	15.41 ± 0.28	132.82 ± 1.03
SPF	2.55 ± 0.29	–	–

3.2 Post-distillation solid waste valorisation

To find out the real biological value of rosemary solid residue after EO distillation, three different parameters were varied during the extraction: ethanol concentration, time, and temperature (complete set results are shown in Supplementary Tables S1–S5). In this study, the amounts of polyphenols, ortho-diphenols and flavonoids, as well as the antioxidant power (FRAP) and activity (DPPH), were considered response variables. The increase in phytochemicals content and antioxidant activity was found to be directly proportional to the increase in ethanol concentration (from 50 to 80%), extraction temperature (from 25 °C to 70 °C), and time (from 15 min to 60 min), when taking into account the overall data. As reported, the utilization of a solvent containing water and ethanol promotes phytochemicals extraction because water swells up the plant material and ethanol can penetrate more easily to disrupt the bonds between the bioactive compounds and plant matrix (Cujić et al., 2016; Ghitescu et al., 2015; Hosseini et al., 2018). In addition, ethanol employed for chemical extractions is a generally recognized as safe (GRAS) solvent, attributing to this recovered by-product a less negative impact on the environment, thus making the procedure recommend and accepted as green approach (Giacometti et al., 2018).

Additionally, the polyphenols extraction is improved with the rising temperature due to an increase in phenolic solubility. According to the literature data, the diffusion rate and the mass transfer, as well as the reduction in solvent viscosity and surface tension, resulted improved (Hosseini et al., 2018; Juntachote et al., 2006). Moreover, the extraction rate of polyphenols is greatly influenced by the extraction time. Polyphenol extraction generally results in higher amounts when a longer extraction time is used, but degradation could occur at high temperatures (over 70 °C) and, for this reason, the extraction time was tested ranging from 25 °C to 70 °C in this study.

Ultrasound-assisted extraction of bioactive compounds from plants is enhanced due to wall disruption and mass transfer (Rodríguez-Rojo et al., 2012; Hosseini et al., 2018; Juntachote et al., 2006). Furthermore, ultrasound facilitates solvent penetration and augments contact between solid and liquid (Rodríguez-Rojo et al., 2012; Hosseini et al., 2018; Juntachote et al., 2006). With the goal to

better evaluate the relationships among total polyphenols, *ortho*-diphenols, flavonoids, and antioxidant properties, Pearson correlation analysis was performed. The data obtained from spectrophotometric assays (Supplementary Tables S1–S5) were analyzed to obtain the triangular matrix of Pearson correlation coefficients (r), as shown in Table 3.

All the bioactive compound classes showed highly significant positive correlations (at a $p < 0.01$ level of significance). The correlation analyses revealed a highly significant correlation between the content of polyphenols, *ortho*-diphenols and flavonoids and the antioxidant activity (measured by FRAP assay), with *r*-values of 0.790, 0.910, and 0.792, respectively.

In addition, a negative correlation was observed between the *r*-values and the biomolecules classes, and the EC_{50} calculated in the DPPH assay. This finding indicates that an increase in bioactive compounds is associated with a decrease in extract concentration required to achieve 50% DPPH inhibition, thereby suggesting a higher antioxidant activity.

Finally, the *r*-value obtained from FRAP and DPPH assay data was −0.848 ($p < 0.01$), thus confirming the highly significant correlation between these two antioxidant assays, both based on antioxidant compound tested the action of electrons donor.

3.3 Response surface methodology

A Response Surface Methodology (RSM) analysis was conducted with the aim of a retrospective analysis and modeling of the data obtained from experiments. Through this analysis was achieved an in-depth understanding of the relationships between independent variables (process factors) and dependent variables (process responses). In this ways, it was enabled the identification of the optimal conditions for a given response and understanding of the interactions between the factors, including time, temperatures, and solvent concentration. Additionally, it facilitated the development of a reliable predictive model and predictive validation of the model.

A Box–Behnken design was used for RSM analysis to explore the individual and combined effects of ethanol concentration (50%–80% v/v), treatment time (15–60 min) and process temperature (25–70 °C) on the extraction yield of total polyphenols (TPC). The responses were expressed as TPC in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW) and as antioxidant activity (FRAP and DPPH). Table 4 presents the data for the one-way analysis of variance (ANOVA) and the statistical significance of the three experimental responses that were modelled using the response surface methodology (RSM) with a Box–Behnken design. According to ANOVA, the quadratic model obtained for each response was highly significant ($p < 0.001$), with R^2 values greater than 0.81 indicating that the models had an excellent ability to explain the variability in the dataset.

The *F*-value for all the models was greater than 28, which suggest the models adequately represented the data. There is only a 0.01% chance that an *F*-value this large could occur due to noise.

Figure 1 shows the response surface plots (3D) which explain the relationships between the three factors (ethanol concentration, extraction time and process temperature) and the two responses (polyphenol content and antioxidant activity). The increase in temperature, as demonstrated in Figures 1a–d, has been observed to facilitate the solubilization of phenolic compounds and their subsequent recovery.

The interactions between temperature and ethanol concentration, as well as between time and temperature, were found to be statistically significant. The maximum recovery of polyphenol, yield of 24.14 mg/g DW, was observed at 80% ethanol, 60 min of treatment, and 70 °C. The maximum ferric reducing antioxidant potential (4.19 mg/g DW) was observed under the same conditions (Figures 1e–h), while the minimum DPPH reagent value (88.95 μg/mL) indicates greater antioxidant activity (Figure 1i–m).

Aboulghazi et al. (2022) reported that the combination of temperature and time has a synergistic effect on polyphenol solubilization and the activation of antioxidant compounds. The impact of ethanol concentration on polyphenol extraction exhibited a positive effect up to approximately 70%–80%, which is consistent with the findings reported by Cano-Lamadrid et al. (2023).

Moreover, the response surfaces exhibited non-linear characteristics, manifesting a clearly delineated optimal zone. This finding aligns with the observations made by Box and Behnken (1960), who noted that the BBD facilitates effective estimation of quadratic terms without the need for extreme conditions. These results are consistent with those of previous studies on the optimisation of antioxidant compound extraction from plant matrices (Cvetanović et al., 2014; Moundib et al., 2023).

Our study demonstrated that using RSM analysis made it easy for us to develop a reliable predictive model and validate the operative condition. The test showed that the model worked well, which means it can be used in other studies and might even be used in industry.

3.4 Extraction and HPLC analysis of spent ROP

Considering data analysed by using the statistical approach RSM, the 80% ethanol at 70 °C for 60 min resulted the best parameters set for bioactive compounds extraction from rosemary post-distillation

TABLE 3 Triangular matrix of Pearson correlation coefficients (*r*) calculated considering polyphenols, *ortho*-diphenols, flavonoids, FRAP, and DPPH.

	Polyphenols	*Ortho*-diphenols	Flavonoids	FRAP	DPPH
Polyphenols	1	0.815	0.908	0.790	−0.837
Ortho-diphenols	–	1	0.773	0.910	−0.893
Flavonoids	–	–	1	0.792	−0.754
FRAP	–	–	–	1	−0.848
DPPH	–	–	–	–	1

10.3389/fsufs.2025.1677990

TABLE 4 One-way analysis of variance (ANOVA) and statistical significance of the three experimental responses* [total polyphenols, antioxidant activity expressed as FRAP and DPPH modelled using response surface methodology (RSM) with a Box–Behnken design].

Source of variation	Total polyphenols compounds				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	350.94	9	38.99	28.46	<0.0001
Temperature (X_1)	142.71	1	142.71	104.18	<0.0001
Extraction time (X_2)	21.49	1	21.49	15.69	0.0002
Ethanol concentration (X_3)	139.79	1	139.79	102.04	<0.0001
Interaction $X_1 \times X_2$	1.18	1	1.18	0.8648	0.3569
Interaction $X_1 \times X_3$	2.15	1	2.15	1.57	0.2166
Interaction $X_2 \times X_3$	0.0539	1	0.0539	0.0393	0.8436
Quadratic X_1^2	18.45	1	18.45	13.47	0.0006
Quadratic X_2^2	3.55	1	3.55	2.59	0.1139
Quadratic X_3^2	15.64	1	15.64	11.42	0.0014
Residual error	68.49	50	1.37		
Total	419.43	59			
R^2 coefficient			0.8367		
R^2 adjusted			0.8073		
Source of variation	FRAP				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	21.96	9	2.44	121.22	<0.0001
Temperature (X_1)	5.29	1	5.29	262.73	<0.0001
Extraction time (X_2)	1.70	1	1.70	84.49	<0.0001
Ethanol concentration (X_3)	13.42	1	13.42	666.66	<0.0001
Interaction $X_1 \times X_2$	0.0813	1	0.0813	4.04	0.0498
Interaction $X_1 \times X_3$	0.3862	1	0.3862	19.19	<0.0001
Interaction $X_2 \times X_3$	0.0021	1	0.0021	0.1021	0.7506
Quadratic X_1^2	0.0711	1	0.0711	3.53	0.0660
Quadratic X_2^2	0.2161	1	0.2161	10.74	0.0019
Quadratic X_3^2	0.0795	1	0.0795	3.95	0.0524
Residual error	1.01	50	0.0201		
Total	22.96	59			
R^2 coefficient			0.9384		
R^2 adjusted			0.9483		
Source of variation	DPPH				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	24151.20	5	4830.24	173.26	<0.0001
Temperature (X_1)	12213.41	1	12213.41	438.09	<0.0001
Extraction time (X_2)	3214.14	1	3214.14	115.29	<0.0001
Ethanol concentration (X_3)	8498.58	1	8498.58	304.84	<0.0001
Interaction $X_1 \times X_2$	–		–	–	
Interaction $X_1 \times X_3$	550.57	1	550.57	19.75	<0.0001
Interaction $X_2 \times X_3$	–		–	–	
Quadratic X_1^2	–		–	–	

(Continued)

TABLE 4 (Continued)

Source of variation	DPPH				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Quadratic X_2^2	183.83	1	183.83	6.59	0.0130
Quadratic X_3^2	–		–	–	
Residual error	1505.44	54	27.88		
Total	25656.63	59			
R^2 coefficient			0.9137		
R^2 adjusted			0.9364		

*The following second-order polynomial equations were employed to depict the relationship between independent factors and responses in terms of coded factors: $Y_{Polyphenols} = 21 + 2{,}24 \times_1 + 0{,}73 \times_2 + 2{,}07\ X_3 - 0.24 \times_1 \times_2 - 0.36 \times_1 \times_3 - 0.04 \times_2 \times_3 - 1.28\ X_1^2 - 0.59\ X_2^2 - 1.1 \times_3^2$, $Y_{FRAP} = 2.8 + 0.43 \times_1 + 0.21 \times_2 + 0.73 \times_2 + 0.06 \times_1 \times_2 + 0.15 \times_1 \times_3 - 0.01 \times_2 \times_3 - 0.08\ X_1^2 - 0.14\ X_2^2 + 0.8 \times_3^2$, $Y_{DPPH} = 129 - 20 \times_1 - 8 \times_2 - 15 \times_2 + 5.8 \times_1 \times_3 - 0.01 \times_2 \times_3 + 4.2\ X_2^2$.

solid residue. Polyphenols, ortho-diphenols, and flavonoids amounts, antioxidant power (FRAP) and activity (DPPH) results obtained applying these optimized extraction conditions were listed in Table 5 and the results are expressed both per g dry weight (DW) of biomass and per g dry weight (DW) of extract.

EO hydro-distillation extraction mainly aims to recover volatiles; therefore, the majority of non-volatile compounds present in the tissues of AMPs are not extractable using this method. Consequently, they remain in the solid residue biomass.

We have therefore used the rosemary residue left over after distillation to measure the total polyphenols, ortho-diphenols and flavonoids. We found 149.23 ± 3.34 mg GAE/g, 100.67 ± 0.63 mg CAE/g and 103.36 ± 1.21 mg CE/g, respectively, considering g of extract (DW). These results are consistent with those reported by Sánchez-Vioque et al. (2015) and Bouloumpasi et al. (2024) for different rosemary varieties after distillation. However, for the purpose of better valorising the spent and underutilised biomass, our results were also expressed per gram (dw) of biomass. Specifically, one gram of rosemary post-distillation solid residue was found to contain 24.14 ± 0.54 mg GAE/g of polyphenols, 16.28 ± 0.10 mg CAE/g of ortho-diphenols, and 16.72 ± 0.20 mg CE/g of flavonoids (see Table 5).

Considering the EC_{50} results, the optimised rosemary residue extract was less active than quercetin (15.41 ± 0.28 μg/mL) but more active than BHT (132.82 ± 1.03 μg/mL), the most widely used synthetic antioxidant compound. This suggests its potential use as a natural antioxidant in the food, beverage and cosmetics industries, offering an eco-friendly approach. Among antioxidant compounds, polyphenolic acids are the widely occurring natural products in almost herbal plant, and rosmarinic acid is one of the most present in the *Lamiaceae* family, above all in *R. officinalis* (Harindranath et al., 2025; Guan et al., 2022).

HPLC analysis of the solid residue obtained from the distillation of *R. officinalis prostratus* revealed the presence of rosmarinic acid (Supplementary Figure S2).

Due to the growing attention on natural products, a large number of pharmacological studies have been carried out in order to prove and validate the various biological activities of rosmarinic acid (Harindranath et al., 2025; Guan et al., 2022).

As a result, it is now part of a wide range of plant-based medicines, including extracts, elixirs and ointments (Harindranath et al., 2025; Ghasemzadeh Rahbardar and Hosseinzadeh, 2020).

Bouloumpasi et al. (2024) investigated the potential utilisation of rosmarinic acid, a major non-volatile phenolic compound in *R. officinalis* extracts, extracted from post-distillation solid residue of various AMPs (Bouloumpasi et al., 2024; Ribeiro-Santos et al., 2015; Skendi et al., 2022). In this study, for the first time rosmarinic acid quantification was performed in post-distillation ROP. The amount of rosmarinic acid in the polyphenolic extract is reported in Table 6, where it is compared to the main results present in the literature.

Rosmarinic acid amount in post-distillation solid residue of wild ROP was 0.71 mg/g of extract DW, is perfectly comparable to literature data on wild rosemary, accordingly Almela et al. (2006). Further, rosmarinic acid content resulted variable between 0.36 and 125.2 mg/g (Table 5) as shown in different studies conducted on cultivated *R. officinalis* varieties (Bouloumpasi et al., 2024; Ziani et al., 2025; Irakli et al., 2023; Tzima et al., 2020; Psarrou et al., 2020; Oreopoulou et al., 2018). These differences may be related to several factors: *R. officinalis* variety and plant age studied, as well as to the environmental and geographical conditions (climate, soil composition, altitude, water availability) are reported to deeply influence the plant phytochemical composition (Giacometti et al., 2018; Borges et al., 2019; González-Minero et al., 2020). Lastly, the phytochemicals recovered and the rosmarinic acid amount from the solid residue are also influenced by EO extraction technique employed. In fact, based on the distillation method and the related temperatures used, a bioactive compounds partial degradation as well as a solubilization in the distillation water and subsequent elimination via the wastewater stream may be caused (Bouloumpasi et al., 2024; Irakli et al., 2023).

Concluding, the spent biomass after EO distillation is a valuable natural source of bioactive phytochemicals like polyphenolics antioxidants. In particular, the rosmarinic acid content of wild *R. officinalis* var. *prostratus* with its diverse biological activities and their potential in health implications, may be suitable for utilization in pharmaceutical, cosmetic and perfumery industry. In this way the residual biomass of EO distillation industry is not be considered a waste but a resource: the adoption of new ways of processing and end-of-life options for raw materials, allows to valorize and to reduce waste volume, so alleviating the global related managing environmental concerns.

10.3389/fsufs.2025.1677990

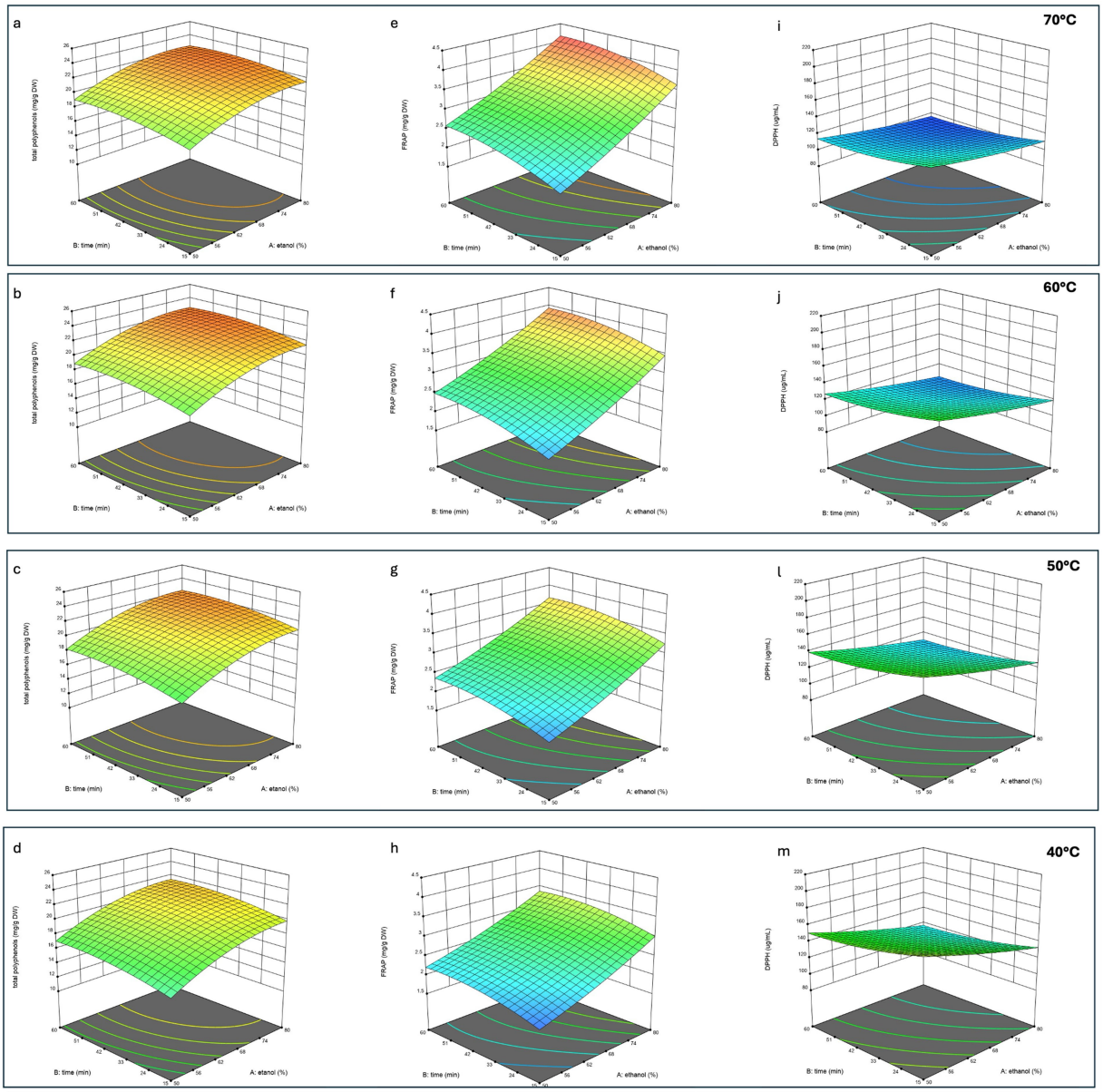

FIGURE 1
Response surfaces and corresponding contour curves showing the effect of ethanol concentration, extraction time, and temperature on total polyphenol extraction (left column), FRAP antioxidant activity (middle column), and DPPH radical scavenging activity (right column). Left column: Variation in total polyphenol content (mg/g DW) as a function of ethanol concentration and extraction time at different temperatures: 70°C **(a)**; 60°C **(b)**; 50°C **(c)** and 40°C **(d)**. Middle column): Variation in antioxidant activity measured using the FRAP assay (mg/g DW) as a function of ethanol concentration and extraction time at different temperatures: 70°C **(e)**; 60°C **(f)**; 50°C **(g)** and 40°C **(h)**. Right column: variation in antioxidant activity measured using the DPPH assay (mg/mL) as a function of ethanol percentage and time, at different temperatures: 70°C **(i)**; 60°C **(j)**; 50°C **(l)** and 40°C **(m)**.

4 Conclusion

This study demonstrates the significant bioactive content of a wild variety of *R. officinalis* (*prostratus*), which acts as a promising source of natural compounds.

The terpenic fraction of rosemary essential oil (EO) was analysed in depth using gas chromatography (GC), which allowed the identification of 26 compounds in total. Out of these, 25 were identified using mass spectrometry (MS) analysis. α-Pinene was found to be the most abundant component, whereas α-terpineol and 1,8-cineole were present in smaller amounts. Furthermore, lower quantities of camphor, followed by myrcene, camphene and bornyl acetate, are also characterizing the EO profile. Moreover, the antioxidant potency of the EO surpassed that of quercetin.

According to RSM, non-linear characteristics were exhibited, manifesting a clearly delineated optimal zone and confirm the ethanol concentration for the best polyphenol extraction to approximately 70%–80%. HPLC analysis of the solid residue from the distillation of *R. officinalis prostratus* revealed the presence of antioxidant biomolecules, including the typical rosmarinic acid.

TABLE 5 Polyphenols, *ortho*-diphenols, and flavonoids amounts, antioxidant power (FRAP) and activity (DPPH), in rosemary post-distillation solid residue extracted applying the best extraction parameters (80% ethanol at 70 °C for 60 min).

	/g Biomass (DW)	/g (or mL) extract (DW)
Polyphenols (mg GAE/g ± SD)	24.14 ± 0.54	149.23 ± 3.34
Ortho-diphenols (mg CAE/g ± SD)	16.28 ± 0.10	100.67 ± 0.63
Flavonoids (mg CE/g ± SD)	16.72 ± 0.20	103.36 ± 1.21
FRAP (mg AAE/g ± SD)	4.19 ± 0.13	25.92 ± 0.80
DPPH – EC_{50} (μg/mL ± SD)	–	88.95 ± 3.35

TABLE 6 Rosmarinic acid content in *R. officinalis* post-distillation solid residue.

Rosmarinic acid content (mg/g of extract DW)	Reference
0.71	Present study
53.31	Bouloumpasi et al. (2024)
40.2	Ziani et al. (2025)
24.7	Irakli et al. (2023)
0.36	Tzima et al. (2020)
8.24	Psarrou et al. (2020)
125.2	Oreopoulou et al. (2018)
0.9	Almela et al. (2006)

Our study of wild rosemary revealed that the compounds recovered from the EOs could be effectively used in several industrial sectors, including food, cosmetics, and pharmaceuticals. Moreover, valorizing distillation waste, as proposed here, could support sustainable development by providing environmental benefits through a bioeconomy approach.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

BL: Supervision, Writing – review & editing, Investigation, Methodology, Data curation, Conceptualization, Resources. FV: Methodology, Writing – original draft, Investigation, Data curation, Formal analysis. AC: Formal analysis, Writing – original draft, Methodology, Software. IF: Writing – original draft, Formal analysis, Methodology. FS: Methodology, Writing – original draft, Formal analysis. DC: Writing – review & editing, Investigation, Methodology, Supervision, Data curation.

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Conflict of interest

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