biology and life sciences forum

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Proceeding Paper

Rosemary Essential Oil Extraction and Residue Valorization by Means of Polyphenols Recovery †

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† Presented at the 4th International Electronic Conference on Foods, 15–30 October 2023; Available online: https://foods2023.sciforum.net/.

Abstract: Increasing demand for natural bioactive ingredients extracted from Aromatic and Medicinal Plants (AMPs) has produced disposal problems associated with residual solid waste. One of the main sectors interested in the exploitation of AMPs is the Essential Oils (EOs) industry. Nevertheless, EO is the main commodity and represents only a small part of the AMPs, generally less than 5% (*w*/*w*). This results in the production of a remarkable quantity of biomass that has no apparent commercial value and is therefore underestimated and underutilized by the EOs industry. Among AMPs, *Rosmarinus officinalis* L., commonly known as rosemary and belonging to the *Lamiaceae* family, is an aromatic plant endemic to the coastal area of the Mediterranean region but worldwide spread. Rosemary can be cultivated or grow wild as an ornamental evergreen shrub. Their leaves are usually used fresh or dried to flavor foods, mostly in traditional Mediterranean gastronomy and nowadays rosemary extracts are approved as food additives in Europe.. The antioxidant activity of the leaves is acknowledged and is ascribed to EOs and polyphenolic compounds. To the best of our knowledge, the optimization of polyphenols recovery from rosemary residues after EO extraction has not yet been investigated. Hence, in the present study, the EO extraction from rosemary leaves was performed by using the hydro-distillation method, and the antioxidant (EC_{50}) and sun protection (SPF) activities were evaluated. The polyphenolic fraction was extracted from rosemary residue acting on some experimental variables. In particular, the extraction time (15 min, 30 min, and 60 min), the temperature (25 °C, 40 °C, 50 °C, 60 °C, and 70 °C), and the ethanol concentration (50%, 60%, 70%, and 80%) were tuned. In this reserch, an EO yield of 1.57% was obtained with an EC_{50} value of 240.39 µL/mL and a SPF of 2.55. The maximum amount of polyphenols extracted from rosemary residue was 24.14 mg GAE/g DW, achieved by using an 80% ethanolic solution at 70 °C for 60 min. This preliminary study reveals how exploitation and consequential valorization of AMPs solid waste may represent new answers to circular economy strategies adopted by European countries.

Keywords: rosemary; essential oil; waste valorization; polyphenols; green extraction; by-products

Citation: Vella, F.M.; Laratta, B. Rosemary Essential Oil Extraction and Residue Valorization by Means of Polyphenols Recovery. *Biol. Life Sci. Forum* **2023**, *26*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s): Name

Published: date

1. Introduction

Essential oils (EOs) production has passed 70,000 tons per annum and it is estimated that about 65% of global production is supported by developing countries [1,2]. Furthermore, USA (40%), Western Europe (30%), and Japan (7%) are the main consumers of EOs, with a continuously increasing requirement of natural products to employ in different human activities [2]. In fact, in the global market EOs are extensively used 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).

Some of the EOs and their constituents are applied as alternatives to the synthetic compounds broadly used in the chemical industry. In fact, natural substances are safer and more sustainable than chemical ones that owns some drawbacks: their possibly connection to toxicity problems, the use of organic solvents, or the release of carbon dioxide and other greenhouse gases during their production [3,4].

On the other hand, the growing demand for EOs extracted from Aromatic and Medicinal Plants (AMPs) causes a chief problem linked with the management of residual wastes from the distillation process. Considering that the first market of AMPs is the production of EOs, which rarely yields more than 0.5–5% *w*/*w* of dry biomass, and in the processing of EOs often a single herb part is employed, this means that most biomass remains discarded and therefore become waste [5]. It is estimated that annually near about 200,000 tons of solid residues are generated worldwide during EO extraction from AMPs [5].

In this scenario, the transition from a linear to a circular management of AMPs residues may drive the development of new strategies to produce high value-added biomolecules, valorizing agricultural and industrial wastes and reducing the volume of residues to be treated. The use of this model where the costs and the energy could have fallen rapidly, agree with the aims of the European Union's Circular Economy Action Plan [6].

Lamiaceae family is probably one of the most important in the EOs production since play a vital role in health and wellbeing of people [7]. This botanical family consists of approximately 236 genera and 7200 species native to the Mediterranean basin, where oregano, sage, rosemary, and thyme are the main ones from a commercial point of view [8]. In particular, rosemary (*Rosmarinus officinalis* L.) is one of the best-known herb used since ancient times, as wild or cultivated, ornamental and aromatic shrub [9]. It has traditionally been used as a medicinal herb because it holds many properties, such as anti-inflammatory, analgesic, astringent, antimicrobial, anti-rheumatic, carminative, antifungal, and antioxidant [7,9]. Rosemary leaves is used as spice in many food preparations and dishes, often in the form of ground powder. The antioxidant activity of leaves are well-known and many recent studies have demonstrated that this biological property is mainly attributable to bioactive compounds present in rosemary EO and in polyphenolic extracts [7,9,10].

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 foremost compounds are 1,8-cineole, α-pinene, limonene, verbenone, camphor, borneol, and camphene, as reported by many studies [11–13]. The chemical composition depends not only from the plant species but also from age, variety, part utilized, origin, climate, soil, stocking time, preparation [7,9,12]. 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 [10,14,15].

The European Food Safety Authority (EFSA) has been evaluating rosemary extract as a food additive since 2008, because of its numerous compounds with significant biological functions [16]. The European Commission with Directive 2010/67/EU approved the use of rosemary extracts as a new food additive attributing the label E392 [17]. Nowadays, in the European Union, rosemary extracts are added to food and beverages at levels of up to 400 mg/kg, considering the sum of carnosic acid and carnosol, the most powerful antioxidants contained in the rosemary extract [18].

To the best of our knowledge, polyphenol recovery from the rosemary residue of EO extraction has never been explored. For this reason, in the present study, the EO was extracted from rosemary leaves by using hydro-distillation. Following, the EO extraction yield, antioxidant activity, and sun protection factor (SPF) were evaluated. Rosemary 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.

2. Materials and Methods

2.1. Reagents and Standards

Folin-Ciocalteu reagent, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and gallic acid, were supplied by Sigma-Aldrich (St. Louis, Missouri, USA). Analytic grade ethanol and methanol were bought from Carlo Erba Reagents (Milan, Italy).

2.2. Plant Sampling and EO Extraction

Wild rosemary (*Rosmarinus officinalis* L.) was harvested in a field where it grows spontaneously in Agerola (Latitude: 40°38′19″32 N; Longitude: 14°32′22″92 E), Naples province (Italy). The plant material was transported into the laboratory where the fresh leaves were removed from the branches and stored at 4 °C until the EO extraction.

The extraction of EO was performed according to the European Pharmacopeia method 2005.2812 [19], by hydro-distillation in Clevenger type apparatus.

Briefly, 70 g of fresh rosemary leaves (slightly blended) and 350 mL of distilled water (ratio 1:5 *w*/*v*) were placed in a 1 L spherical flask. The balloon was connected to the Clevenger apparatus and was placed in a thermostatic bath at 100 °C for 3 h. After the extraction time, rosemary EO was collected in a glass vial, dried under anhydrous sulphate and stored in the dark at 4 °C, until further analyses.

The yield (Y) of process was calculated according to the Equation (1):

$$\mathbf{Y}\ (\%) = \frac{\boldsymbol{V_{EO}}}{\mathbf{m_s}} \times \mathbf{100} \quad (1)$$

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

2.3. Polyphenols Extraction and Quantification

After EO extraction, the residual leaves were recovered, frozen at −20 °C and liophilized. Subsequently, 250 mg of this biomass were utilized to evaluate polyphenols content by adding 5 mL of ethanol extraction solution (ratio 1:20 *w*/*v*). Polyphenolic compounds extractions were carried out varying three different parameters: 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%). Ultrasound-assisted extraction (UAE) were carried out applying a sonication power of 120 W with a frequency of 40 Hz. All the extracts were recovered by centrifugation at 13,000× *g*, at 4 °C for 10 min, and dried using a rotary evaporator.

Polyphenols were determined by means of the spectrophotometrical method Folin-Ciocalteu, according to Singleton and Rossi [20]. Briefly, 150 µL of each rosemary extract 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 determined at 765 nm. Gallic acid was used as standard and the results were reported as mg of gallic acid equivalents (GAE) per g of DW biomass. All extracts were analyzed in triplicates ($n = 3$).

2.4. Antioxidant Activity Assay

The antioxidant activity of rosemary EO and polyphenolic extract was assessed in vitro through the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay [21]. In particular, 1.35 mL of 60 µM DPPH methanolic solution was blend with different samples amounts. The reduction in absorbance was continuously recorded at 517 nm. The radical scavenging activity percentage (%RSA) of DPPH discoloration was obtained with the following the formula:

$$\%\mathbf{RSA} = \frac{(\mathbf{A_{DPPH}} - \mathbf{A_s})}{\mathbf{A_{DPPH}}} \times \mathbf{100} \quad (2)$$

where A_{DPPH} is the absorbance of the DPPH solution and A_S is the absorbance of the solution when the sample was added. The EC_{50}, the extract concentration required to achieve 50% of radical DPPH inhibition, was calculated graphing the RSA percentage vs. the concentrations. The results were expressed as mg/mL, as reported by Vella et al. [22].

2.5. Sun Protection Factor Determination

The sun protection factor (SPF) was determined in vitro by measuring the percentage of transmittance in the range of 290–320 nm, considering the known erythemal factors at each wavelength, as reported in the equation (3):

$$\mathbf{SPF} = \mathbf{CF} \times \sum_{\mathbf{290}}^{\mathbf{320}} \mathbf{EE}\ (\boldsymbol{\lambda}) \times \mathbf{I}\ (\boldsymbol{\lambda})\ \times \mathbf{Abs} \quad (3)$$

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, determined by Sayre et al. [23], were previously reported by Vella et al. [24].

For the determination of the SPF, EO solution was prepared in ethanol (0.1% *v*/*v*). The absorbance of the sample was spectrophotometrically acquired at intervals of 5 nm in the range of 290–320 nm [24].

2.6. Statistical Analysis

Means, standard deviations (SD), calibration curves and linear regression analyses (R^2) were carried out through Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

3. Results and Discussion

3.1. Rosemary EO

Initially, the study was focused on extracting EO from rosemary leaves by hydrodistillation using a Clevenger-type device. Due to the need for a temperature below 100 °C, the distillation process has become the most common method for extracting EOs from plant material. Two phases are produced at the end of distillation, the upper organic one. The EO obtained is protected from the surrounding water phase, which acts as a barrier to prevent it from overheating in this way.

The resulting rosemary EO yield was 1.57%, which is higher than reported in some literature studies. Boutekedjiret et al. [25] reported a yield of 0.44%, while Conde-Hernández et al. [26] and Bousbia et al. [27] recorded a yield of 0.35%. Our results are in agreement with Flamini et al. [28], Angioni et al. [29], and Jamshidi et al. [11] that reported comparable total yields of *R. officinalis* EO, which were 1.44%, 2.13%, and 2.60%, respectively. These little differences could be attributed to plant age, variety, and environmental condition of origin country such as climate, soil, altitude, water availability [7,9,11,12].

In this study, the in vitro SPF measurement was applied as a rapid and suitable test, for screening of the potential ingredients to employ as a natural additives in foods and cosmetics. The higher value SPF is, the more is the protection offered by biomolecules against UV light. Particularly, EOs added in foods or in cosmetic formulations, confers the ability to absorb UV radiations, preventing and reducing skin damage and other health problems related to the formation of free radicals caused by sun exposure [24].

The wavelength values obtained and the related SPF calculation were reported in Table 1.

Table 1. Wavelength values and sun protection factor (SPF).

Wavelength (nm)	Absorbance (Abs)
290	0.1619 ± 0.022
295	0.1902 ± 0.013
300	0.2186 ± 0.010
305	0.2412 ± 0.017
310	0.2821 ± 0.024
315	0.3754 ± 0.035
320	0.6075 ± 0.029
SPF	2.55

In this study, the SPF value of rosemary EO was 2.55. This value is determined by the chemical components of EOs, depending on the growing conditions and harvest time of the plants [7,9,11,12]. Modified values can be recorded for EOs extracted from diverse cultivars of rosemary, or even in the same variety grown in various geographic places. Despite the great variability experienced in the SPF value, it is important this assay with the aim to preliminarily assess the potential use of this EO in foods and cosmetics as a sun and oxidative protection.

3.2. Polyphenols Extraction from Rosemary EO Residue

The residue remaining after rosemary EO distillation was extracted using UAE method at different concentration of ethanol (50%, 60%, 70%, and 80%). Further, extraction time (15 min, 30 min, and 60 min) and temperature (25 °C, 40 °C, 50 °C, 60 °C, and 70 °C) were tuned in order to identify the best polyphenolics yields. The procedure is recommend and accepted as green approach by the food industry [15].

The results of extractions of polyphenols from rosemary residue in different conditions were showed in Table 2.

Table 2. Polyphenols amounts (mg GAE/g DW ± SD) extracted by rosemary residue after EO distillation.

Extraction Parameters	25 °C	40 °C	50 °C	60 °C	70 °C
50%–15 min	10.14 ± 0.38	15.04 ± 0.51	18.56 ± 0.53	18.01 ± 0.46	18.12 ± 0.60
60%–15 min	15.37 ± 0.62	16.31 ± 0.46	19.22 ± 0.54	20.18 ± 0.65	21.19 ± 0.52
70%–15 min	17.62 ± 0.81	20.96 ± 0.47	20.32 ± 0.48	20.59 ± 0.52	21.21 ± 0.50
80%–15 min	15.14 ± 0.82	19.67 ± 0.40	21.11 ± 0.57	21.01 ± 0.28	21.60 ± 0.54
50%–30 min	15.14 ± 0.32	15.78 ± 0.65	18.69 ± 0.51	18.35 ± 0.57	17.75 ± 0.51
60%–30 min	16.31 ± 0.62	19.65 ± 0.86	18.81 ± 0.38	18.05 ± 0.63	21.27 ± 0.77
70%–30 min	18.90 ± 0.61	21.24 ± 0.68	21.46 ± 0.69	20.83 ± 0.81	22.11 ± 0.58
80%–30 min	19.02 ± 0.63	22.89 ± 0.42	20.74 ± 0.62	22.15 ± 0.48	23.34 ± 0.71
50%–60 min	13.12 ± 0.61	17.64 ± 0.69	19.73 ± 0.65	19.12 ± 0.61	18.31 ± 0.55
60%–60 min	16.83 ± 0.49	18.17 ± 0.56	20.75 ± 0.58	21.99 ± 0.64	21.29 ± 0.42
70%–60 min	18.63 ± 0.58	20.96 ± 0.42	21.39 ± 0.57	22.22 ± 0.42	21.50 ± 0.65
80%–60 min	19.47 ± 0.57	19.99 ± 0.62	21.62 ± 0.64	22.30 ± 0.40	24.14 ± 0.54

The conventional extraction procedures using solvents and temperatures have some drawbacks, including high heat, time consuming, and often lead to low extraction yields. Therefore, it is suggested to employ other assisted extraction methods, such as those that utilize sonication. It has been reported that ultrasounds increase the extraction efficiency of active compounds from plants, as consequence of noteworthy disruption of wall cells, and also for enhancement of mass transfer induced by cavitation bubble collapse in the solvent [30]. Moreover, the mechanical effect of ultrasound waves facilities the penetration

of solvent into the matrix and enhances the contact surface between the solid and liquid phases [15,30–33].

Taking into account the overall data, 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), led to an increase in polyphenol content. The maximum amount of polyphenols was found to be 24.14 mg GAE/g DW using the following parameter, 80% ethanolic solution at 70 °C for 60 min.

The utilization of a solvent containing both water and ethanol is reported to facilitate polyphenols 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 [32,33]. The polyphenol extraction is improved with the growing temperature due to an increase in phenolic solubility. As reported in literature, the diffusion rate, the mass transfer, as well as the reduction in solvent viscosity and surface tension, is enhanced [15,34]. 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).

3.3. Antioxidant Activity

The growing interest in bioactive compounds to be devoted to the food and cosmetic markets, in line to the emerging demands of new applications, could be explored by means of routine tests of biological activities.

In this view, the antioxidant activity evaluation of the best polyphenolic extract and of the rosemary EO was carried out by using a direct method based on the radical scavenging capacity. Assays that use linoleate or ABTS cation radical are known for their turbidity and interference with hydrophobic samples. Almela et al. [35] recommended the use of the free radical DPPH assay for this reason. The assay is based on the ability of a bioactive compound to reduce and stabilize the DPPH radical, aging as a hydrogen donor. In particular, the assay is based on the ability of a bioactive compound to reduce and stabilize the DPPH radical, aging as a hydrogen donor.

In this study, antioxidant activity of the rosemary EO and of the best polyphenolic extract (PE; 80% ethanolic solution at 70 °C for 60 min) from rosemary residue was evaluated and the results were reported in Table 3.

Table 3. Antioxidant activity (expressed as EC_{50}) of the best polyphenolic extract (PE) and of essential oil (EO) from rosemary.

	EC_{50} (μg/mL)
PE	143.90
EO	240.39

The results of the activities were expressed as EC_{50}, defined as the concentration of the EO needed to scavenge 50% of the DPPH present in the test solution.

In this work, it has been observed an EC_{50} value of 240.39 μL/mL in EO, which is greater than that reported by Almela et al. [35]. Mostly, the difference in the results is due to plant age, variety, and environmental condition [7,9,11,12].

The sample PE showed an EC_{50} of 143.90 μg/mL, a value lower than that reported by Almela et al. [35], suggesting a higher antioxidant activity of PE extract.

This important outcome demonstrates that by-products of EO distillation can be considered a low cost and interesting candidates to obtain natural biomolecules, proposing the suitability of rosemary wastes as an alternative to synthetic antioxidants.

4. Conclusions

Utilization and recycling of AMPs biomass wastes after distillation of EOs represent new and interesting subjects to be exploited from a point of view of circular economy.

Consumers, due to the reduced side effects of polyphenols compared with their synthetic counterparts, generally recognize them as valuable antioxidants to employ in the food sector as natural preservatives. In fact, they could be functional for the shelf life of foodstuffs, or in the cosmetic industry as antiaging agents, in order to prevent natural oxidation and deterioration.

In this scenario, this preliminary research conducted on rosemary residues from EO industry, may be a significant improvement on the knowledge of extraction of bioactive phytochemicals, thus valorizing a by-product discarded from distillation process. Further chemical investigation will be planning in order to obtain a whole identification of polyphenols pattern.

Author Contributions: Conceptualization, F.M.V. and B.L.; investigation, F.M.V.; data analysis, F.M.V. and B.L.; writing-review and editing F.M.V. and B.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors gratefully thank Ornella De Somma for carrying out some experiments during her master thesis.

Conflicts of Interest: The authors declare no conflict of interest.

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