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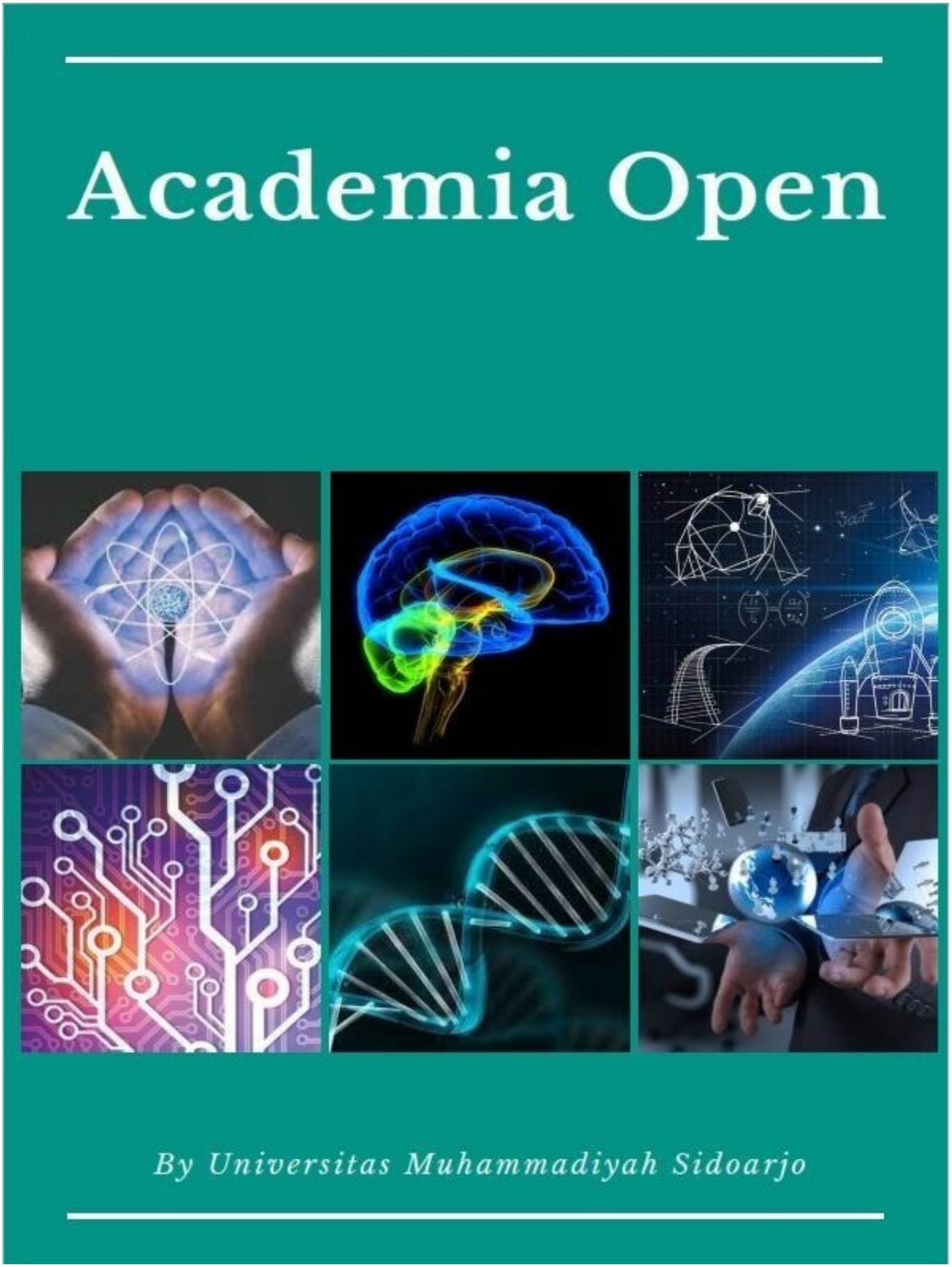

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Rosmarinic Acid in Aromatic Herbs: A Green Extraction and HPLC Approach

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Abstract

General background: Aromatic herbs from the Lamiaceae family represent valuable sources of rosmarinic acid (RA), a polyphenolic compound with notable antioxidant, anti-inflammatory, antimicrobial, and neuroprotective properties. **Specific background:** Traditional extraction methods such as Soxhlet and maceration consume excessive solvents and time while potentially degrading thermolabile compounds. **Knowledge gap:** Despite growing interest in green extraction technologies, limited validated protocols exist for combining ultrasound-assisted extraction (UAE) with high-performance liquid chromatography (HPLC) for efficient RA quantification across multiple aromatic species. **Aims:** This study developed and validated a sustainable UAE-HPLC method for extracting and quantifying RA from six aromatic herbs: *Laurus nobilis*, *Thymus vulgaris*, *Salvia rosmarinus*, *Anethum graveolens*, *Mentha viridis*, and *Salvia officinalis*. **Results:** Using ethanol:water (70:30 v/v) at 40 kHz and 250 W, optimal extraction occurred within 2.5-10 minutes, with *Thymus vulgaris* yielding the highest RA concentration (170,500 µg/mL). HPLC analysis demonstrated excellent linearity ($R^2 > 0.998$) and reproducibility. **Novelty:** This approach significantly reduces solvent consumption and extraction time while maintaining analytical precision. **Implications:** The validated UAE-HPLC platform provides a robust, environmentally friendly analytical framework for sustainable utilization of RA-rich herbs in nutraceutical and pharmaceutical applications.

Highlight :

- UAE offers a rapid, low-solvent, and environmentally friendly approach for extracting RA.
- Variation in RA yields reflects differences in plant tissue structure and extraction conditions.
- HPLC provides reliable quantification, supporting accurate and consistent analysis of RA.

Keywords : Rosmarinic Acid, Aromatic Herbs, Ultrasound-assisted Extraction, HPLC, Green Extraction

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Introduction

The good sources of phenolic compounds with antioxidant properties are aromatic herbs, especially Lamiaceae species of such kind like Rosmarinus officinalis, Salvia officinalis, Origanum vulgare, Thymus vulgaris, and Melissa officinalis [1]. Rosmarinic acid (RA) [2][3] is one of the most common phenolics, biologically active in aromatic as well as medicinal plants, and which are abundantly found in the plant kingdom. RA has numerous pharmacological properties which include; antioxidant, anti-inflammatory,

antimicrobial, antivirulent and anticancer properties and therefore a desirable nutraceutical or pharmaceutical compound [4].

Rosmarinic acid (RA) is a polyphenolic compound that is naturally present in aromatic herbs and commonly belongs to Lamiaceae family, i.e., Rosmarinus officinalis (rosemary), Thymus vulgaris (thyme), Mentha spicata (spearmint) and Salvia officinalis (sage). Ra, a highly strong antioxidant and anti-inflammatory compound is an ester that is produced between caffeic acid and 3, 4-dihydroxyphenyl lactic acid. Phenylpropanoid pathway where RA is produced in plants is a part of defense against oxidative stress and microbial oxidation related damage [5][6]. RA is a potent antioxidant because it is capable of chelating metal ions and scavenging reactive oxygen species (ROS) and shields cellular components against oxidative stress [7]. It is also antimicrobial, antiviral, and anti-cancer and a prospective phytochemical in nutraceuticals and medicine [8]. Recent studies have paid more attention to its neuroprotective properties, or that it can prevent neurodegenerative diseases such as Parkinson and Alzheimer disease [9].

In the study conducted by Sik et al. (2020), Soxhlet and maceration are considered the most common examples of traditional extraction methods that have been linked to high solvent consumption, high extraction times, and possible destruction of the thermolabile compounds. In other words, greener and more labor friendly ways of extracting rosmarinic acid are eco-extraction processes, which may be ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), or natural deep eutectic solvents (NADESs) [10]. Such eco-extraction procedures are in compliance with the recent principles of green chemistry, enable the application of fully safe solvents in extraction process, and enhance the yields and selectivity of rosmarinic acid [11].

HPLC may be suitable to be used in conjunction with either photodiode array or mass spectrometric detection to get a quantitative data on the RA levels in herbal extracts. HPLC has been characterized as possessing a high level of sensitivity, reproducibility and linearity hence acceptable in the confirmation of RA in various sample matrices [12]. It is portrayed how studies that have purported tested RA levels in various aromatic herbs are not only varied within the same study but also when comparing reported level across studies carried out at different levels that were ranging between 2.0-27.4 mg g -1 [13][14]. The evaluations of herbal natural products are known to have been reported in the literature to utilize alterations of method validation parameters manifested by linearity, precision, limits of recognition and limits of quantification as to whether the possible application can be used at industrial and pharmacognostic purposes [15].

The combination of green extraction and HPLC in analysis is a new approach and it offers a new greener process of extracting rosmarinic acid in aromatic herbs and precise determination of the material. The strategy will yield sustainable-use and conservation strategies and increase trust in consistency and quality as far as herbal preparations are concerned. As part of this research, an adequate green extraction procedure as well as the HPLC procedure used to determine the quantity of rosmarinic acid in the aromatic herbs will be appropriately and validated and the same will be done to any other sustainable extraction process using advanced analysis validation.

Research Methodology

A. Materials

Fresh leaves were purchased from local markets in Baghdad Iraq. The leaves belonged to Salvia rosmarinus, Mentha viridis, Laurus nobilis, Anethum graveolens, Salvia officinalis, Thymus vulgaris and were morphologically verified by a plant taxonomist and prepared for analysis. They were extracted using analytical grade ethanol and methanol (Merck, Germany). A certified rosmarinic acid standard (RA, ≥98% purity) was purchased from Sigma-Aldrich (St. Louis, USA)

B. Plant Material Collection and Preparation

The fresh aerial parts of all six aromatic herbs: Laurus nobilis, Thymus vulgaris, Salvia rosmarinus, Anethum graveolens, Mentha viridis, and Salvia officinalis were collected from local herbal farms under the same environmental conditions. The plant materials were rinsed with distilled water to remove any surface contaminants, and then shade-dried at room temperature (25-28°C) for 10 days. After drying, the materials were ground using the laboratory grinder and passed through a 60-mesh sieve to produce purified powder. The powders were kept in airtight amber bottles in desiccated conditions until extraction.

C. Instrumentation

The quantitative analysis of RA was performed using a high-performance liquid chromatography (HPLC) system (Shimadzu SCL-10A, Japan) with a BRISA LC2 reversed phase C18 column (5 µm, 150 × 4.6 mm). The ultrasound-assisted extraction (UAE) was performed using an ultrasonic bath (VWR Scientific, USA) following validated green extraction methods. A precision balance (Sartorius, Germany) and micropipettes (Bio Basic Inc., Canada) for weighing and preparing solution.

D. Chemicals and Reagents

All of the solvents and reagents utilized were of analytical or HPLC grade. Methanol (HPLC grade) and ethanol were sourced from Sigma-Aldrich (St. Louis, USA). The rosmarinic acid reference substance used had a purity level of >98% and was obtained from Merck (Germany). Deionized water was used to dilute all solutions, which were freshly prepared the day of analysis.

E. Ultrasonic-Assisted Extraction Process

The green extraction of rosmarinic acid was carried out using ultrasonic-assisted extraction (UAE) because it is more effective and consumes less solvent. To each sample, 0.5 g of powdered plant was weighed and placed into a conical flask and mixed with 10 mL of ethanol:water (70:30 v/v). The mixture was sonicated in an ultrasonic bath set to 40 kHz and 250 W at room temperature (25 – 28°C).

Sonication times included 2.5, 5.0, 7.5, and 10.0 minutes per experimental design (Table 1). After sonication, the extracts were filtered with Whatman No. 1 paper, then centrifuged at 4000 rpm for 10 minutes. The clear supernatant was transferred, evaporated at reduced pressure at

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40°C, and stored at 4°C for the chromatographic analysis.

F. HPLC Quantification of Rosmarinic Acid

The quantification of rosmarinic acid was carried out by HPLC with UV-Vis detection at maximum wavelength of 320 nm, using a C18 reversed-phase column (150 mm × 4.6 mm, 5 µm). The mobile phase consisted of ethanol and distal water (75:25). The flow rate for the mobile phase was set to 1.0 mL/min, and the injection volume was 20 µL, and detection was at 320 nm.

Calibration was done using RA standard solutions in the range of 10–200 µg/mL, and linearity was established at $R^2 > 0.998$. Each extract was analyzed in triplicates, and quantification was reported as µg/mL of RA in each extract.

G. Chromatographic Conditions

Validation was performed using a BRISA LC2 C18 column (5 µm, 150 × 4.6 mm) under isocratic conditions with a mobile phase consisting of 75% ethanol and 25% distilled water, at a flow rate of 1 mL/min. Column temperature was at 30 °C. The UV detector was set at 320 nm with an injection volume of 20 µL. All these parameters were optimized and in agreement with previously established procedures for the quantification of RA in Lamiaceae herbs.

H. Preparation of Standard Solution

A stock solution of rosmarinic acid (1 mg/mL) was made in 70% ethanol and diluted serially to prepare working standards. Calibration curves were obtained for consistency and linearity during the working analytical range ($R^2 \geq 0.998$) by plotting peak area vs concentration.

I. Rosmarinic Acid Content Determination

RA content of the each of the herbal extracts was based on the standard calibration curve, and reported as µg/mL. The results of the RA yield from the six aromatic herbs and sonication times (2.5–10 min) is presented in Table 1. The results indicate differences in the phytochemical profile of the species, as well as the potential effectiveness of UAE as a sustainable extraction technique.

Summary of Experimental Variables

Parameter	Condition
Extraction method	Ultrasonic-Assisted Extraction (UAE)
Solvent system	Ethanol:Water (70:30 v/v)
Sample weight	0.5 g dried powder
Solvent volume	10 mL
Sonication frequency	40 kHz
Power	250 W
Sonication time	2.5, 5.0, 7.5, 10.0 min
Temperature	Room temperature (25–28°C)
Analytical technique	HPLC (C18 column, 320 nm detection)

Table 1. Effect of Sonication Time on Rosmarinic Acid Concentration (µg/mL)

Plant Species	2.5 min	5 min	7.5 min	10 min
Laurus nobilis	33.49	4.86	3.23	31.35
Thymus vulgaris	170.50	15.73	112.74	117.17
Salvia rosmarinus	70.98	106.56	52.35	112.90
Anethum graveolens	13.53	8.88	47.46	63.64
Mentha viridis	5.85	9.52	20.42	18.54
Salvia officinalis	7.37	12.39	15.34	39.57

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Results

A. HPLC Chromatographic Analysis of Rosmarinic Acid

The HPLC chromatogram (Figure 1) reveals a well-defined and sharp peak corresponding to rosmarinic acid (RA), with a retention time (Rt) of 1.882 minutes and an area under the standard curve (AUC) of 298,483.1 mAUs. The intensity of the peak (approximately 66.0 mAU) and its peak symmetry (tailing factor of 3.44) confirms the reliability and effectiveness of the chromatographic technique. The number of theoretical plates (29) indicates sufficient performance of the column with the mobile phase used (methanol:water, 75:25 v/v), with a flow rate of 1 mL/min, and the detection wavelength of 320 nm.

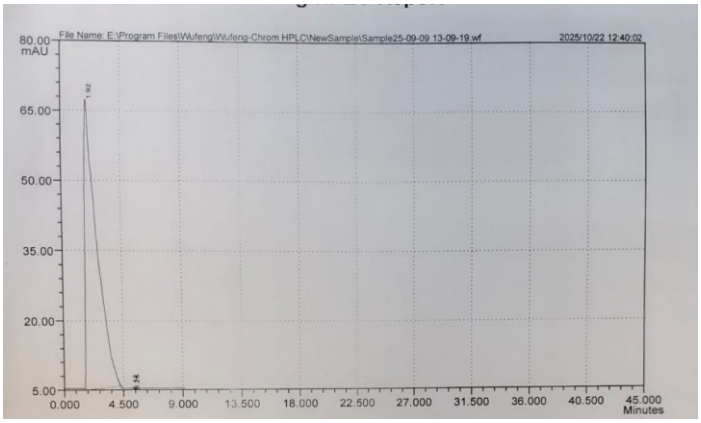

Figure 1. HPLC chromatogram showing rosmarinic acid (standard)

The current study showed that **ultrasound-assisted extraction (UAE)** followed by **HPLC analysis** is a very efficient, environmentally friendly, and reproducible method for determining the quantity of **rosmarinic acid (RA)** present in aromatic herbs [16][17]. The data indicated that from species to species and extraction time to extraction time, the yields of RA exhibited a significant amount of variability, demonstrating that the efficiency of extraction is influenced by the plant matrix in addition to the extraction parameters [18][19]. These statements are consistent with recent reports, which highlighted the importance of solvent composition, ultrasound intensity, and botanical origin on phenolic extraction and antioxidant capacity in UAE systems [20].

Thymus vulgaris achieved the maximum level of rosmarinic acid (170,500.3 µg/mL) at a 2.5 minute sonication time. Gradual declines were seen with longer sonication time (to 112,743.0 µg/mL at 7.5 min), but consistently high yield values would suggest that short sonication is adequate for effective recovery of phenolics from this species. The high yield indicated a complex and robust phenolic profile with rapid extraction, but longer sonication times caused a small decline, due to degradation or possible isomerization of the rosmarinic acid molecules with extended ultrasonication exposure, as in Figure 2 A.

Salvia rosmarinus (rosemary) produced its highest yield after 10 minutes (112,904.0 µg/mL) suggesting that as sonication time increased more cells rupture and more phenolic ultimately are released. This finding supports eco-extraction principles where manipulation of acoustic energy can impact extraction recovery as illustrated in figure (2 B).

The concentration of RA in Laurus nobilis was 33,494.8 µg/mL at 2.5 minutes, which decreased sharply downward and then increased at the 10 minute mark (31,350.95 µg/mL). This biphasic pattern may be the result of either partial re-solubilization of phenolics or inherent structural resistance of the Laurus leaves to longer time exposures to ultrasound, as demonstrated in figure 2 C .

In Anethum graveolens, there was a steady increase in RA content, from 13,532.3 µg/mL at 2.5 minutes to 63,648.6 µg/mL at 10 minutes of exposure. This steady increase lends credence to the theory that longer degrees of sonication aid in releases of compounds that are bound up in fragile tissues (Figure 2 D).

Mentha viridis exhibited a modest rise in RA yield and reached the maximum yield of 20,421.5 µg/mL after 7.5-minutes of exposure, followed by a slight reduction of yield at the 10-minutes time point. This effect may illustrate the oxidative sensitivity of polyphenols derived from mint after prolonged exposure to ultrasonic treatment as seen in figure (2 E).

Salvia officinalis (sage) exhibited a progressive increase, reaching 39,574.7 µg/mL at 10 minutes, reflecting robust phenolic stability and consistent extraction under ultrasonic conditions as see in figure (2 F)

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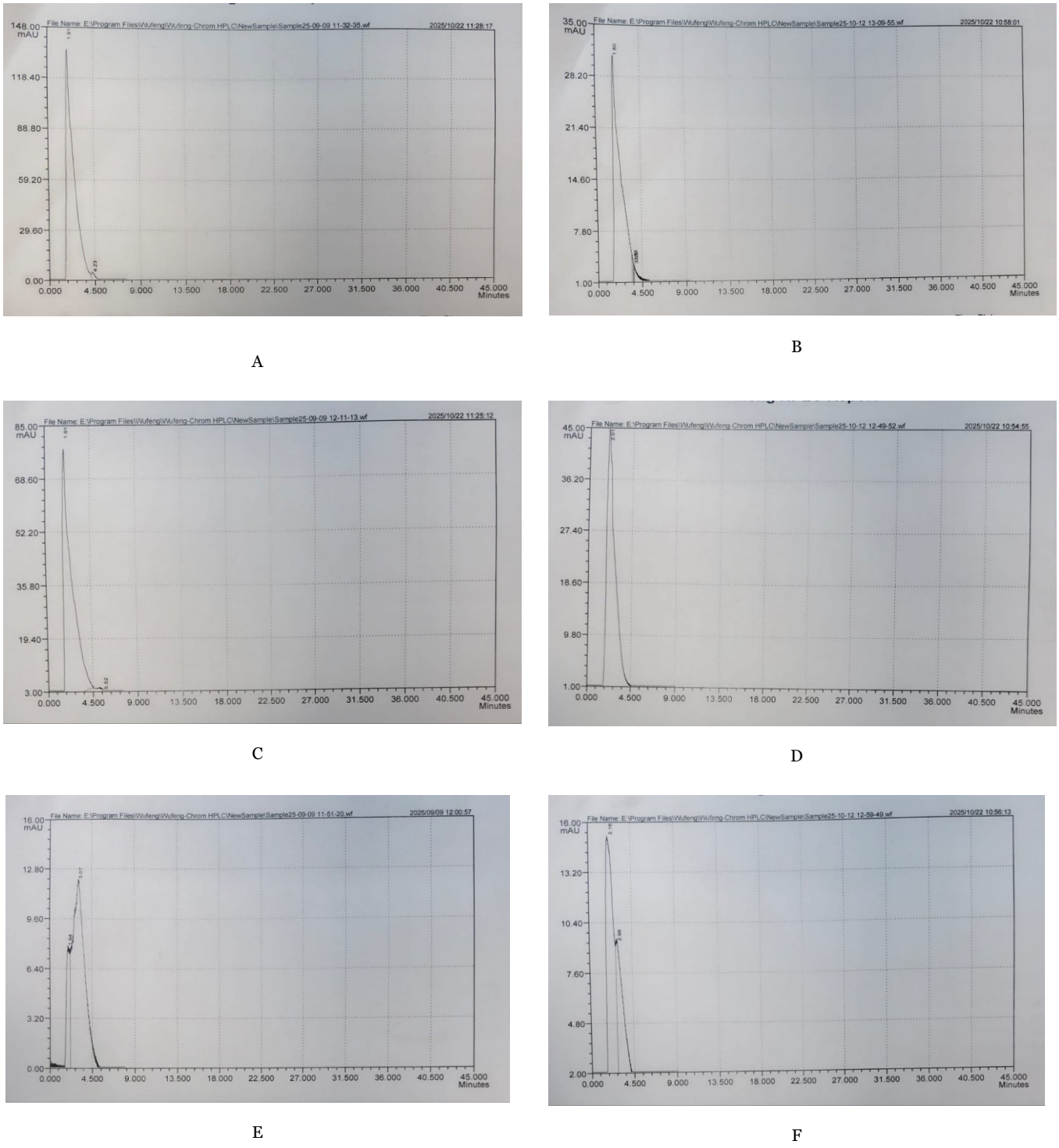

Figure 2. HPLC chromatogram showing (A- Thymus vulgaris, B- Salvia officinalis, C- Anethum graveolens, D - Salvia rosmatinus, E- Laurus nobiilis, F- Mentha viridis)

Extraction time was established as an important factor in the yield of RA. Yields for Thymus vulgaris and Laurus nobilis were optimal at shorter extraction periods (2.5–5.0 min) while exposure time was most favorable at (10 min) for Salvia rosmarinus and Salvia officinalis; differences in yields were reflective of the structural and compositional differences across the family Lamiaceae, particularly structural differences in glandular trichomes and cuticular permeability can regulate the diffusion of phenolics [21].

Extended exposure time will promote cavitation and micro-turbulence that break down cell walls to release intracellular phenolics; extended exposure time is seen to counter to this downstream phenomenon for short and long periods of time posing a challenge for extraction efficiency a likely explanation for the lower yield of RA in Thymus vulgaris at extended exposure [22].

Among the herbs that we analyzed, Thymus vulgaris produced the highest concentration of RA (170,500.3 µg/mL), which was then followed by Salvia rosmarinus and Anethum graveolens. This corresponds with previous studies indicating that Thymus species are abundant sources of rosmarinic acid and caffeic acid derivatives. The increased yields observed in Anethum graveolens and Salvia officinalis with increased durations of sonication indicate that UAE enhances the diffusion of bound polyphenols in less densely structured tissues. The more compact leaf structure herbs, Laurus nobilis, displayed a biphasic response to UAE due to initial release from the herb surface, which was followed by a lag in solubilization [22].

Findings from HPLC chromatogram analysis confirmed that RA had been isolated as demonstrated by the sharp peak at 1.882 minutes with an area 298,483.1 mAU•s. The tailing factor (3.44) and theoretical plate number (29) were within acceptable limits for analytical purposes suggesting that the column performed and was reproducible. Validation parameters followed previous published analytical methods for the quantification of RA, the peak also showed the robustness of the extraction specificity of RA and minimal interference from the other phenolics or pigments [23].

This makes UAE offer various advantages from an environmental point of view compared to conventional methods, including reduced solvent usage, reduced extraction time, and lower energy use. UAE fits into most of the principles of green chemistry due to the reduction or avoidance of toxic solvent waste and the use of thermolabile compounds. Its efficiency in RA extraction using aqueous ethanol represents a balance between polarity and environmental safety under recommended eco-extraction and supported by Vieira. These results thus support UAE as sustainable for industrial or pharmaceutical application in extracting natural antioxidants from herbal matrices. In addition, the high antioxidant potential of RA directly relates to its relevance for use in cosmetics, nutraceuticals, and oral care products [24].

In detail, validation parameters were in line with formerly published analytical methods for RA quantification. The peak also showed the robustness of RA extraction specificity and minor interference from other phenolics or pigments.

The variation in RA yield between species may be related to both the extraction efficiency and the innate biosynthetic capacity of each species. Obviously, the higher yields of RA for Thymus and Salvia species could be due to the higher activity of the enzymes RAS and HCT. Tissue morphology also affects the interaction of the solvent with the tissue, and cavitation occurs. Thick cuticular layers in Laurus nobilis might hamper the propagation of the acoustic wave in the tissue, hence limiting compound release at intermediate sonication times.

It is important to optimize the parameters of sonication (i.e., time, solvent ratio, acoustic power, and temperature) with the goal of increasing recovery of phenolics while avoiding degradation of thermolabile components. Future research should place emphasis on green extraction methods that work in tandem, such as coupling UAE with natural deep eutectic solvents (NADES) or microwave-assisted extraction methods, to improve extraction selectivity, stability, and sustainability this combined eco-technology would mean a better opportunity for the production of standardized botanical extracts for nutraceuticals, pharmaceuticals, and cosmetics, to be aligned with sustainable bioprocessing goals.

Summary of Key Analytical Indicators

Parameter	Observed Value	Interpretation
Retention Time (Rt)	1.882 min	Rapid elution of RA under polar conditions
Peak Height	6,080 mAU	Strong signal intensity
Area	298,483.1 mAU·s	High concentration and purity
Concentration	100% (standardized)	Calibration accuracy
Tailing Factor	3.44	Acceptable peak symmetry
Theoretical Plates	29	Adequate column efficiency

Conclusion and Recommendations

The results of this study suggest that the combination of UAE and HPLC is a highly efficient, reproducible and sustainability-oriented extraction/quantification method for RA from aromatic herbs. Here, we show the results from a number of plants demonstrating large differences in RA yields between species and sonication times and their dependency on plant tissue morphology and parameters from an extraction procedure. Thymus vulgaris showed the highest levels of RA, pointing to the plant as a potentially good source. This method is greener than classical methods as it yields and isolations great quantities of an oil of interest in shorter times and reducing also solvent consumption, the study concludes. These findings have important implications since they indicate that the UAE-HPLC combination can be used in both industrial and pharmaceutical

applications for the sustainable extraction of RA-rich herbs. To enhance selectivity and sustainability of sword bean extraction and to reach a more practical level which will apparently later lead successful development of standardized herbal extracts for nutraceutical, cosmetic and pharmaceutical purposes, the future research should be directed to better optimization of extraction conditions, exploitation of the combination of UAE with other eco-extraction techniques, such as natural deep eutectic solvents (NADES) or microwave-assisted extraction (MAE).

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