Received: 20 September 2023 | Revised: 18 November 2023 | Accepted: 22 November 2023

DOI: 10.1002/jssc.202300695

JOURNAL OF SEPARATION SCIENCE

RESEARCH ARTICLE

An analytical framework combining online high-performance liquid chromatography methodologies and biological properties of different extracts of *Leonurus cardiaca*

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Little or no information is available concerning online high-performance liquid chromatography (HPLC) antioxidants and the antibiofilm effect of *Leonurus cardiaca*. Five distinct extractions of methanolic, ethyl acetate, dichloromethane, hexane, and water were obtained from *L. cardiaca*. In the online-HPLC-antioxidant analysis of all examined samples, rosmarinic acid emerged as the primary antioxidant, registering concentrations ranging from 6 to 15 ppm at wavelengths of 517 and 734 nm. Notably, the water extract exhibited robust antioxidant activity In vitro. Regarding acetylcholinesterase and butrylcholinesterase inhibition, the n-hexane extract exhibited superior inhibition with values of 3.08 and 5.83 galanthamine equivalent, respectively. Except for the water extract, all tested extracts (at a concentration of 20 μg/mL) exhibited substantial inhibitory activity against biofilm formation, in many cases superior to 80%, and reached even 94.52% against *Escherichia coli*. Although less vigorous, the extracts also acted against the mature biofilm (inhibition up 76.50% against *Staphylococcus aureus*). They could work against the metabolism inside an immature and mature biofilm, with inhibition percentages up to 93.18% (vs. *Pseudomonas aeruginosa*) and 76.50% (vs. *Acinetobacter baumannii*), respectively. Considering its significant antioxidants, enzyme inhibition, and antimicrobial activity, *L. cardiaca* emerges as a promising candidate for therapeutic potential.

KEYWORDS
biofilm, biological potential, chemical profiles, *Leonurus*, natural agents, rosmarinic acid

Article Related Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CUPRAC, cupric reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; GALAE, galantamine equivalent; HPLC-DAD, high-performance liquid chromatography with diode-array detection; IE, infective endocarditis; KAE, kojic acid equivalent; MCA, metal chelating; MIC, minimal inhibitory concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; RE, rutin equivalents; TE, trolox equivalent; TFC, total flavonoid content; TPC, total phenolic content; UV, ultraviolet.

https://doi.org/10.1002/jssc.202300695

1 | INTRODUCTION

Leonurus cardiaca, commonly known as motherwort, is a perennial herb that belongs to the mint family (Lamiaceae) [1, 2]. It is native to regions in Asia and southeastern Europe but has now achieved widespread distribution globally [2]. Throughout history, this plant has served various purposes, including its use as a cardiotonic agent and for addressing gynecological and abdominal issues [2–4]. While comprehensive documentation exists regarding the utilization of motherwort in both oriental and occidental medical practices, its scope extends beyond traditional medicinal purposes. Motherwort finds application as a culinary ingredient in certain cuisines, notably as a flavor enhancer in various vegetable soup formulations, particularly those featuring lentils or split peas. Additionally, it imparts its distinct essence to beverages like beer and tea, thereby augmenting the potential for wider public consumption of this botanical remedy [2]. Scientific studies confirm its antioxidant, antimicrobial [5, 6], angiogenic [7], anti-inflammatory [8, 9], and anticancer [10] properties; furthermore, since the ancient time, *L. cardiaca* was beneficial for treating nervous [4] and cough disorders [5]. In the genus *Leonurus*, various secondary metabolites have been unearthed, with *L. cardiaca* being short out in particular. Among these, flavonoids [9], phenylethanoid glycosides [11], iridoids, labdane diterpenes [12], guanidine pseudoalkaloids [13], alkaloids [2, 7], and essential oils [7, 14] are prominent. Fleming et al. showed that *L. cardiaca* effectively increases lactoperoxidase's ability to produce hypothiocyanite, which plays a vital role in inflammation [8]. Due to its broad spectrum of biological activities, *L. cardiaca* has achieved its place in the European Pharmacopoeia [4].

Recently, combining high-performance liquid chromatography with diode-array detection (HPLC-DAD) separation with antioxidant testing, either online or offline, has become known as an effective and fast method for studying the antioxidant capacity of specific compounds within complex mixtures [15, 16]. By isolating particular substances and assessing their activity, the approach provides a significant advantage in analyzing antioxidants [14, 17]. This method involves liquid chromatography combined with established antioxidant tests using 2′-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and cupric reducing antioxidant capacity (CUPRAC) to assess the plants' capability to neutralize harmful radicals. In a single operation, the developed online method can extract compounds directly from the extract, separate and analyze bioactive, and screen individual antioxidants by post-column DPPH, FRAP, CUPRAC, and ABTS HPLC. As a result, it can be accomplished to figure out the isolated compounds and evaluate the antioxidant strengths simultaneously [18].

The present investigation examined the chemical composition and biological characteristics of diverse extracts obtained from *L. cardiaca*. Phenolic and flavonoid compounds were assayed through colorimetric and chromatographic analysis. Although several studies have shown that *L. cardiaca* extracts contain substances such as phenolics, flavonoids, and polysaccharides, which have high antioxidant and other therapeutic activities, the profiling of the plant's antioxidant compounds using online-coupled HPLC molecular basis for these activities has not yet reported. This study also investigated enzyme inhibition against cholinesterases, tyrosinase, α-amylase, α-glucosidase, and in vitro scavenging/reducing properties were evaluated as well. The current research uses an optimized online HPLC antioxidant capacity assay to identify and assess antioxidant components in *L. cardiaca*, laying the scientific basis for future applications.

2 | MATERIALS AND METHODS

2.1 | Plant materials

During the summer of 2022, Dr. Ismail Senkardes, a botanist, collected aerial parts of *L. cardiaca* in Istanbul (Turkey). The information is provided below. One sample from the plants was preserved in Marmara University herbarium (Voucher number: 19049). Before being taken out, the plant materials were meticulously cleaned using both tap and purified water to remove any dirt and pollutants. After being left to air-dry for a period of 10 days, specifically in a shaded environment at room temperature, the aerial portions of the plant were subsequently finely ground.

2.2 | Preparation of extracts

Utilizing the maceration method, n-hexane, dichloromethane, ethyl acetate, and methanol extracts were obtained by combining 10 g of plant material with 200 mL of each respective solvent and letting it steep for 24 h at room temperature. After that, we filtered the mixtures using Whatman 1 filter paper and eliminated the solvents with the help of a rotary evaporator. To make the water extract, 10 g of plant material was mixed with 200 mL of boiled water for 15 min before being filtered and lyophilized with a Syclon-10 N (Ningbo Haishu Sklon Electronic Instrument) for 48 h. All extracts were carefully

stored at a temperature of 4°C, ensuring they were kept under suitable conditions until the analyses.

2.3 | Total phenolic and flavonoid contents

The Folin–Ciocalteu and $AlCl_3$ assays, respectively, were utilized to determine the total phenolic and flavonoid contents, and the procedures are reported in our earlier work [19].

2.4 | HPLC analysis

LC-HPLC Agilent-1100 modular compact system (USA) with autosampler unit, ultraviolet (UV)-DAD, thermostable column cabinet with three trays utilized for the gradient analysis included combined a secondary syringe pump post-flown with reagent programmable quaternary channel injection (Inovenso IPS 13-RS laboratory model). The experimental determinations were conducted utilizing Purospher star, C18 column with guard-column (5 μm, 4.6 × 250 mm) (Merck). The injection volume of all samples was 20 μL. The analysis time was 30 min. Data investigations (retention time, peak area, and detection limits) were performed utilizing with Chem-station of the Agilent Program. The mobile phase contained quaternary solvents in harmony: solvent A was methanol, solvent B was a mixture of formic acid/acetonitrile/aqua (3.5/48.25/48.25, v/v/v), solvent C was a mixture of 1.5% formic acid: aqua (v/v) and solvent D was HPLC-grade acetonitrile [20].

2.5 | Online HPLC assays

The antioxidant potential of all the extracts was evaluated using online HPLC-based assays (FRAP, DPPH, ABTS, and CUPRAC). Throughout the study, we maintained consistent parameters, including principal and secondary pump flow rates, column temperature settings, solvent gradient ratios, and sample injection concentrations across all HPLC-FRAP, HPLC-DPPH, HPLC-ABTS, and HPLC-CUPRAC methodologies [21–24]. Four distinct HPLC methodologies—HPLC-FRAP, HPLC-DPPH, HPLC-ABTS, and HPLC-CUPRAC—were employed separately for antioxidant detection and activity determination. In HPLC-FRAP, fresh FRAP reagent was used with detection signals at 280 nm for DAD phenolic peaks and 595 nm for UV antioxidant peaks. At the same time, HPLC-DPPH and HPLC-ABTS utilized negative post-column detection with fresh DPPH and ABTS radical reagents, respectively, resulting in UV peak chromatograms at 517 and 734 nm alongside DAD 280 nm peak chromatograms. Additionally, HPLC-CUPRAC allowed negative post-column detection and involved the reduction of Cu(II)-Neocuproine reagent in a redox reaction, with simultaneous UV peak chromatograms at 450 nm and DAD 280 nm peak chromatograms. The detailed methodologies for all assays can be found in the supplementary materials.

2.6 | In vitro antioxidant and enzyme inhibitory assays

A set of six complementary in vitro spectrophotometric tests were performed to assess the antioxidant potential of the extracts. These included the ABTS and DPPH assays, which examine the antioxidants' ability to neutralize free radicals, and FRAP and CUPRAC assay, which evaluates the extracts reduction capabilities, as well as metal chelating ability (MCA) and phosphomolybdenum assays. Each assay, except for MCA, was evaluated using the Trolox standard (TE). The comparison for MCA was made in terms of equivalent EDTA equivalent per gram of extract. All used procedures are given in our previous work [25]. To assess the inhibitory effects of the tested extracts on various enzymes, we employed acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, amylase, and glucosidase. Details of the experimental procedures can be found in our prior publication [25]. We quantified AChE and BChE inhibition as milligrams of galantamine equivalents (GALAE) per gram of extract, tyrosinase inhibition as milligrams of kojic acid equivalents (KAEs) per gram of extract, and α-amylase and α-glucosidase inhibition as millimoles of acarbose equivalents per gram of extract. These measurements provide a standardized assessment of the inhibitory potential of the extracts on these enzymes.

2.7 | Antibacterial activity

2.7.1 | Microorganisms and culture conditions

Acinetobacter baumannii (ATCC 19606), *Escherichia coli* (DSM 8579), *Pseudomonas aeruginosa* (DSM 50071), *Listeria monocytogenes* (ATCC 7644), and *Staphylococcus aureus* subsp. *aureus* Rosebach (ATCC 25923) was the bacterial tester strain used in our experiments. They were cultured in Luria Broth for 18 h at 37°C (except *A. baumannii* which was cultured at 35°C) and 80 rpm (Corning LSE).

2.7.2 | Minimal inhibitory concentration

The resazurin microtiter-plate assay evaluated the minimal inhibitory concentration (MIC) [26]. The tests were performed in flat-bottomed 96-well microtiter plates incubated at 37 or 35°C (depending on the strain) for 24 h. The MIC value was revealed by the color change from dark purple to colorless. Determinations were performed in triplicate, and results were expressed as the arithmetic mean ± standard deviation.

2.7.3 | Antibiofilm activity

The *L. cardiaca* extracts' capability to inhibit bacterial biofilm formation and mature biofilm was assessed in flat-bottomed 96-well microtiter plates [27], using two concentrations of each extract. In evaluating the inhibitory activity of *L. cardiaca* extracts on the biofilm formation, 10 uL of the overnight bacterial cultures, standardized to 0.5 McFarland (1.5×10^7 cells/mL) with fresh culture broth, were put in each well. Then, 10 and 20 μL/mL (corresponding to 10 and 20 μg/mL of each extract, respectively) and Luria–Bertani broth were added to reach a final volume of 250 μL/well. Microplates were closed with parafilm to prevent evaporation; they were incubated for 48 h at 37°C (except *A. baumannii*, incubated at 35°C). The extracts were added at the same concentrations after 24 h of the bacterial growth, after discarding the planktonic cells and adding fresh culture medium, to evaluate the inhibitory effect of the *L. cardiaca* extracts on the mature biofilm. The plates were then incubated for another 24 h.

In both cases, wells were washed with sterile phosphate-buffered saline (PBS) once the planktonic cells were removed, and we added 200 μL of methanol to fix the adhered cells. After 15 min, we removed the methanol and left the microplates to dry. We stained the adhered cells by adding 200 μL of 2% w/v crystal violet solution, which was removed after 20 min. The wells were gently washed with sterile PBS and left to dry under the flow laminar cap. 200 μL of glacial acetic acid 20% w/v allowed for the release of the bound dye. The absorbance was measured at $\lambda = 540$ nm (Cary; Varian). The percent of inhibition was calculated with respect to the control; an inhibition of 0% was considered for cells without treatment. The tests were carried out in triplicate, and the average results were taken for reproducibility.

2.7.4 | Antibiofilm metabolic activity

3-[4,5-dimethylthiazol-2-yl]−2,5 diphenyl tetrazolium bromide (MTT) assay led to the evaluation of the metabolic changes occurring in the bacterial cells within immature and mature biofilms [27]. After 48 h of bacterial growth in the 96-well microtiter plates, we removed the planktonic cells and added 150 μL of PBS and 30 μL of 0.3% MTT. Then, microtiter plates were kept at 37°C. After 3 h, the MTT solution was removed, and 200 μL of dimethyl sulfoxide (DMSO)/well was added, to allow the dissolution of the formazan crystals formed in the wells. After 3 h of incubation at 37°C, purple formazan derivatives were dissolved in DMSO and measured at $\lambda = 570$ nm (Varian Cary). Triplicate tests were done, and the average results were taken for reproducibility.

2.8 | Statistical analysis

Statistical analysis was performed using Xl Stat (Version 16). All analyses were performed in triplicate ($n = 3$) and presented as means and standard deviations (mean ± SD). The significance level for the One-way analysis of variance (ANOVA) and Tukey's post-hoc test was set at $p \leq 0.05$ when comparing sample differences.

3 | RESULTS AND DISCUSSION

3.1 | Total phenolic, flavonoid contents, and chemical composition of the tested extracts

The presence of phenolic compounds in plant-based extracts plays a crucial role in determining their pharmacological ability in specialized metabolic products. Spectrophotometric methods may not provide precise phenolic levels due to potential interference from non-phenolic compounds [28], emphasizing the demand for more reliable online chromatographic approaches to assess plant extract chemical profiles. We evaluated the total concentrations of phenolics and flavonoids in this current study. Results are shown in Figure 1. The water-based extract had the highest total phenolic content (TPC) concentration, measuring 52.74 mg gallic acid equivalent (GAE)/g of extract. This is consistent with an earlier study on methanolic extract, which revealed a value of 53.4 mg GAE/g of extract [29]. Our recently published research [30] found that the water extract had higher TPC values than the hydroalcoholic extract from the same plant, which had a TPC of 14.168.

The methanolic extract exhibits the second-highest concentration of phenolic compounds with 31.91 mg GAE/g of extract. It is followed by dichloromethane (28.37 mg

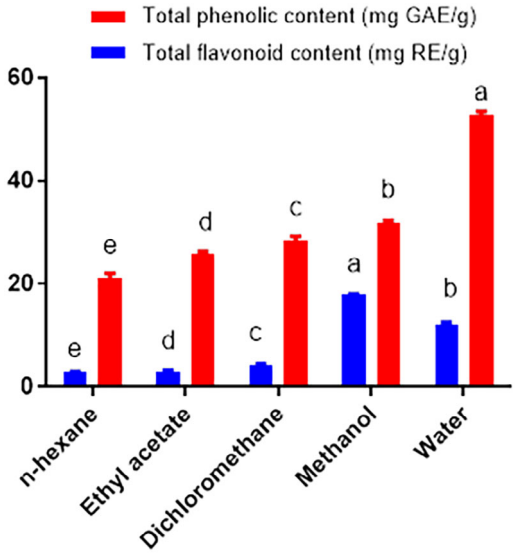

FIGURE 1 Total phenolic and flavonoid content of the tested extracts. Values are reported as mean ± SD of three parallel measurements. GAE: gallic acid equivalent; RE: rutin equivalent; Different letters indicate significant differences in the tested extracts ($p \leq 0.05$).

GAE/g), ethyl acetate (25.76 mg GAE/g), and n-hexane (21.08 mg GAE/g) extracts. It is noteworthy that the methanolic extract's phenolic composition was lower than that of the methanolic extract compared to the study on *L. cardiaca* that was previously mentioned [29]. Variations in the extraction techniques might cause this discrepancy. Furthermore, Bernatoniene et al. found 227 mg/100 mL of total polyphenols in the extract of *L. cardiaca* [31]. Nevertheless, the techniques used in this research varied, so the results are not reasonably comparable. In terms of total flavonoid content (TFC), the sequence of concentrations observed was as follows: the methanolic extract yielded the highest flavonoid content with 17.98 mg of rutin equivalents per gram (mg RE/g) of extract, followed by water extraction with a TFC of 12.20 mg RE/g extract, and dichloromethane extraction exhibited the lowest TFC at 4.27 mg RE/g extract. Additionally, the flavonoid contents of the dichloromethane extract matched that of the reported water extract of *Leonotis leonurus* [32]. The ethyl acetate and n-hexane extract exhibited the lowest TFC, which was relatively the same (2.91 mg GAE/g extract and 2.77 mg GAE/g extract, respectively). However, it is important to highlight that the TFC of *L. cardiaca* documented in [29, 30] was higher than the current research. This disparity could be due to differences in the extraction methods and solvents used in the respective studies. Furthermore, flavonoids were found to be the most important component in motherwort ariel part and leaves by Zhao et al. [33].

3.2 | Online-HPLC-antioxidants analysis

The novel HPLC-antioxidants methodology allows for the simultaneous use of HPLC separation and the evaluation of antioxidant activity. In scientific literature concerning spectrophotometric analysis, it is commonly documented that the DPPH-sample reaction time is 30–50 min. However, in the context of online HPLC post-column systems, this reaction time has been significantly reduced to 1.5 min [34]. In this analytical approach, a DAD was set at 280 nm to detect phenolic compounds, while UV spectroscopy set at 595, 517, 734, and 450 nm assessed the antioxidant activity after post-column reactions (FRAP, DPPH, ABTS, and CUPRAC), enabling real-time measurement of antioxidants in a sample based on their absorption patterns at specific wavelengths. For all tested samples, eight antioxidants, catechin, quercetin, ferulic acid, caffeic acid, ellagic acid, chlorogenic acid, rutin, and rosmarinic acid, were detected at 280, 595, 517, 734, 450 nm. In the analyzed samples, when measuring at wavelengths of 280 and 595 nm, the most prominent compound detected was catechin, present in concentrations ranging from 6 to 12 parts per million (ppm). At wavelengths of 517 and 734 nm, the primary antioxidant identified was rosmarinic acid, found in concentrations between 6 and 15 ppm across all samples. In contrast to the findings of Kosar et al., the Lamiaceae plants exhibited a notably lower level of rosmarinic acid, with a minimum detectable concentration of 0.02, observed in the water extract using the online HPLC-DPPH System [35]. HPLC–ABTS and HPLC-CUPRAC assay screened higher concentrations of antioxidants by comparison with HPLC–DPPH and HPLC–FRAP, conversely to the study of Burnaz et al. [34]. In post-column FRAP (595 nm), all extracts had lower baseline noise. However, DPPH (517 nm), ABTS (734 nm), and CUPRAC (450 nm) assays of all extracts showed that the baseline noise was highly similar to the previous literature (15 online > 29). These findings are summarized in Figure 2. Consequently, the post-column FRAP assay is superior in accurately determining the concentration of antioxidant compounds.

In all tested samples, chlorogenic acid dominated at 450 nm, with concentrations of 9, 10, 11, 12, and 13 ppm, respectively, similar to previous literature where they found chlorogenic acid showed a strong positive response to the CUPRAC reagent [36]. However, chlorogenic acid exhibited the highest concentration compared to other phenolics in the online HPLC-DPPH assay (7.50 μg/mL) (32). For all samples labeled at the same wavelength (450 nm), catechin was the second predominant compound, while ellagic acid showed a lower concentration. These findings are summarized in Tables S1–S5. Rosmarinic acid was the principal compound at 517 nm in all

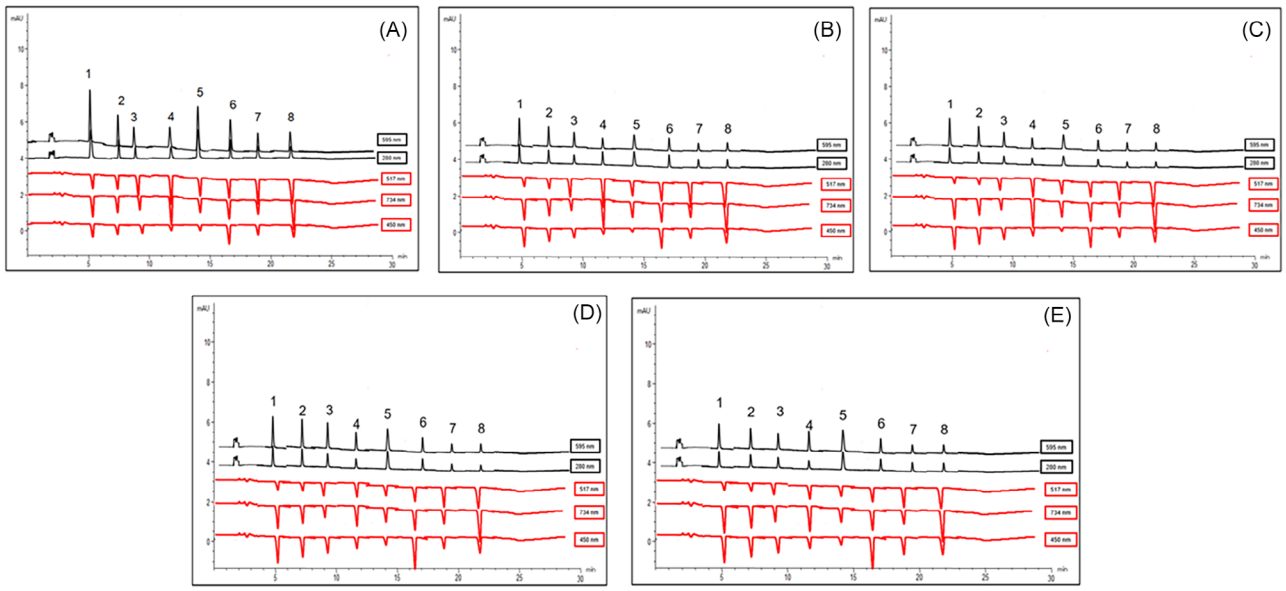

FIGURE 2 Chromatogram peaks profile defined at 595, 280, 517, 734, and 450 nm for methanol (A), ethyl acetate (B), dichloromethane (C), *n*-hexane (D), and water (E) extracts of *Leonurus cardiaca*.

tested extracts. Likewise, Rosmarinic acid was dominant within the studied Lamiaceae plant species examined by the Online HPLC-DPPH System [35].

3.3 | In vitro antioxidant activity

Free radicals are accountable for oxidative damage to vital biomolecules. Keeping an equilibrium between oxidants and free radicals is a prerequisite for good health [8, 37]. Excess free radicals must be neutralized, and antioxidants must be consumed to sustain redox homeostasis.

Here, the water extract showed potent antioxidant activity in free radical scavenging assays (DPPH and ABTS), CUPRAC, and FRAP tests (45.13, 63.11, 138.25, and 73.90 mg TE/g, respectively), followed by the methanolic extract having value 38.16, 45.42, 94.51, and 48.50 mg TE/g, respectively (Table 1). The higher total phenolic compound concentration level in the water and methanolic extracts may readily account for the assessments. In PDB, all the tested extracts show relatively similar activity. However, in the case of MCA, the dichloromethane extract showed higher activity (25 mg EDTAE/g). In the case of DPPH, the n-hexane, ethyl acetate, and dichloromethane extract did not show radical scavenging action, and there was a positive relation with the HPLC-online-DPPH of these extracts. Methanol extract exhibited a notably reduced DPPH scavenging activity, in agreement with the studies of Pereira et al., which found an EC50 value of 18.3 μg/mL in the DPPH test performed on *L. cardiaca* hydroethanolic extracts [37]. Furthermore, a study carried out by Jafari et al. revealed that the hydroalcoholic extract of *L. cardiaca* had higher antioxidant activity (IC50 53.79 μg/mL) than ethyl acetate extract (IC50 107.16 ug/mL) [38]. Besides *L. cardiaca*, the 95% ethanolic extract of *Leonurus sibiricus* demonstrated an IC50 value of 267.6 μg/mL for DPPH radical reduction and 7894.3 μg/mL for ABTS radical reduction [39].

The In vitro antioxidant activity outcomes align with the levels of phenolic and flavonoid compounds present. Notably, the *n*-hexane extract demonstrated the lowest values across nearly all spectrophotometric assays.

3.4 | Enzyme inhibitory effects

We investigated the enzyme inhibitory activity of *L. cardiaca* extracts against AChE, BChE, tyrosinase, α-amylase, and α-glucosidase (Table 2). Regarding the inhibition of AChE, no differences were observed in all tested samples except ethyl acetate extract, which was inactive to this enzyme. These results differed from our previous research, where the *L. cardiaca* extract showed AChE inhibition and BChE inhibition [30]. In BChE, n-hexane, ethyl acetate, and dichloromethane extracts showed similar inhibitory action (5 mg GALAE/g). The ethyl acetate inhibition of BChE was different from previous research [40], which showed another inhibition than the present research (2.59 and 5.53 mg GALAE/g, respectively). However, in the same article [40], the methanolic and water extract did not show an inhibitory effect, while we observed that only water was inactive to the inhibition of BChE. The methanolic extract exhibited BChE inhibitory activity similar to AChE (2.90 and 2.39 mg GALAE/g, respectively).

TABLE 1 Antioxidant properties of the tested extracts.

Extracts	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	PBD (mmol TE/g)	MCA (mg EDTAE/g)
n-Hexane	na	4.29 ± 0.52^{e}	54.56 ± 2.15^{e}	14.56 ± 1.22^{d}	1.67 ± 0.09^{c}	23.40 ± 0.36^{b}
Ethyl acetate	na	9.38 ± 1.47^{c}	67.97 ± 4.78^{d}	20.24 ± 1.46^{c}	2.00 ± 0.03^{b}	22.83 ± 0.32^{c}
Dichloromethane	na	7.06 ± 0.21^{d}	80.27 ± 1.37^{c}	13.80 ± 0.40^{e}	2.22 ± 0.13^{a}	25.73 ± 0.78^{a}
Methanol	38.16 ± 0.34^{b}	45.42 ± 0.18^{b}	94.51 ± 1.09^{b}	48.50 ± 1.68^{b}	0.92 ± 0.06^{d}	22.07 ± 0.18^{c}
Water	45.13 ± 0.10^{a}	63.11 ± 0.06^{a}	138.25 ± 3.78^{a}	73.90 ± 1.44^{a}	0.95 ± 0.03^{d}	21.58 ± 0.17^{d}

*Values are reported as mean ± SD of three parallel measurements.

Different letters (a-e) indicate significant differences in the tested extracts ($p \leq 0.05$).

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid; CUPRAC, cupric reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTAE, EDTA equivalent; FRAP, ferric reducing antioxidant power; MCA, metal chelating activity; na, not active; PBD, phosphomolybdenum; TE, Trolox equivalent.

TABLE 2 Enzyme inhibitory effects of the tested extracts.

Methods	AChE (mg GALAE/g)	BChe (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
n-Hexane	3.08 ± 0.01^{a}	5.83 ± 0.74^{a}	54.31 ± 1.00^{b}	0.55 ± 0.03^{b}	1.17 ± 0.01^{a}
Ethyl acetate	na	5.53 ± 0.19^{b}	56.74 ± 1.15^{a}	0.63 ± 0.03^{a}	1.19 ± 0.02^{a}
Dichloromethane	2.46 ± 0.19^{c}	5.88 ± 0.71^{a}	53.94 ± 0.38^{bc}	0.68 ± 0.02^{a}	1.18 ± 0.02^{a}
Methanol	2.90 ± 0.23^{b}	2.39 ± 0.71^{c}	56.59 ± 0.46^{a}	0.32 ± 0.01^{c}	0.98 ± 0.03^{b}
Water	2.17 ± 0.03^{d}	na	5.10 ± 0.98^{d}	0.06 ± 0.01^{d}	0.03 ± 0.01^{c}

**Values are reported as mean ± SD of three parallel measurements. Different letters (a-d) indicate significant differences in the tested extracts ($p \leq 0.05$).

Abbreviations: ACAE, acarbose equivalent; AChE, acetylcholinesterase; GALAE, galantamine equivalent; KAE, kojic acid equivalent; na, not active.

Fleming et al. observed that *L. cardiaca* has the remarkable ability to effectively restore hypothiocyanite production by the action of lactoperoxidase, contributing to the modulation of inflammatory reactions [8]. However, the water extract did not inhibit BChE.

Tyrosinase constitutes the principal enzymatic catalyst in the melanin synthesis process, and inhibiting its activity is one of the most effective strategies for addressing hyperpigmentation issues [40]. Methanolic and ethyl acetate extracts had more potent and similar inhibitory properties (56 mg KAE/g), followed by n-hexane (54.31 mg KAE/g) and dichloromethane (53.94 mg KAE/g). Water extract showed less inhibition. In previous research, the *L. cardiaca* extract exhibited higher inhibition of tyrosinase; however, compared to this present research, its inhibition was lower (28 mg KAE/g) [40]. Based on these results, we believed *L. cardiaca* could be used in dermatoprotective agents. The water-based extract showed inadequate inhibition in all tested enzyme inhibitory assays. Scientific efforts are focused on improving human health and reducing obesity and diabetes. In this sense, α-amylase and α-glucosidase enzymes are selected key enzymes to check carbohydrate metabolism, which in turn controls diabetes mellitus [41].

All the tested extracts show less but almost similar inhibitory effects regarding the glucose digestive enzyme (α-amylase and α-glucosidase). The results were similar to our previous research [40]. However, in another study, the purified *L. cardiaca* extract displayed stronger α-glucosidase and α amylase inhibitory activity [28]. In addition to *L. cardiaca*, *L. japonicus* also plays a role in enzyme inhibition. Their results showed that compounds extracted from *L. japonicus* had moderate to high inhibitory activity on AChE and low inhibitory activity on α-glucosidase [42].

3.5 | Antibiofilm activity

Although the antibiofilm potential of *L. cardiaca* is not reported in the scientific record, numerous studies have tried to determine the impact of various other botanical extracts. After the resazurin test, which allowed us to evaluate the MIC (never inferior to 30 μg/mL) (Table 3), we decided to study the potentiality of the *L. cardiaca* extracts in inhibiting the biofilm at the initial step of formation and the mature biofilm, (indicated in Table 4 with "Time 0″ and "Time 24″, respectively), and to act against the

TABLE 3 Minimal inhibitory concentration (MIC, μg/mL) of the extracts evaluated against *Acinetobacter baumannii* (AB), *Escherichia coli* (EC), *Listeria monocytogenes* (LM), *Pseudomonas aeruginosa* (PS), and *Staphylococcus aureus* (SA), through the resazurin test.

	AB-	EC-	PS-	LM+	SA+
MeOH	32 ± 1	34 ± 2 [b]	35 ± 1	38 ± 4 [c]	35 ± 2
EA	33 ± 2[a]	32 ± 3 [b]	33 ± 1	30 ± 1 [b]	36 ± 1
DCM	33 ± 3	32 ± 1 [b]	32 ± 1	32 ± 2 [a]	35 ± 1
Hexane	32 ± 2	32 ± 2 [b]	32 ± 2	30 ± 3	36 ± 3
Water	40 ± 2 [c]	42 ± 2 [d]	42 ± 2 [c]	30 ± 2	38 ± 2 [a]
Tetracycline	30 ± 2	25 ± 2	30 ± 1	28 ± 1	34 ± 1

Results are the average of three independent experiments ± SD.[a] $p \leq 0.5$; [b]$p \leq 0.01$; [c]$p \leq 0.001$; [d]$p \leq 0.0001$ compared with the positive control. (ANOVA followed by Dunnett's multiple comparison test).

metabolism of bacterial sessile cells (also herein at the initial step of the biofilm formation and in the mature biofilm, indicated in Table 5 with "Time 0″ and "Time 24″, respectively). Concerning the activity of the extracts to fight the biofilm formation, our results indicate that all extracts (at a concentration of 20 μg/mL), except for the water extract, displayed significant inhibitory activity against biofilm formation (as shown in Table 4). The inhibition percentages span a remarkable range from 8.09% to 94.52%. Compared to the TPC of the water extract and the results of the online HPLC-antioxidants assay, this intriguing result highlights a certain level of discrepancy between these specific findings.

Among the tested extracts, it is noteworthy that both ethyl acetate and dichloromethane doses exhibited pronounced effectiveness against *E. coli*, displaying an impressive inhibition rate ranging from 89.35% to 94.52%. Following closely, the methanolic extract at a concentration of 20 μg/mL also demonstrated significant inhibitory activity, recording a notable 93.55% inhibition rate. The effectiveness in preventing biofilm formation also depended on the specific bacterial strain, with *E. coli* showing the most significant inhibition rate (2). Similarly, the inhibition observed against the *E. coli* strain, the methanolic extract at a concentration of 20 μg/mL inhibited both *P. aeruginosa* and *S. aureus*, reaching 93.47%. Concerning *E. coli*, both doses of the hexane extract displayed a nearly identical inhibitory effect of 77.24%. Similarly, the methanolic extracts at 20 μg/mL concentration for ethyl acetate and dichloromethane demonstrated a comparable inhibition rate of 92% against *S. aureus*, close to the inhibition achieved by the 10 μg/mL hexane extract. This result is consistent with previous work carried out by Micota et al. (2014), which reported the antibiofilm activity exhibited by the *L. cardiaca* extracts against the adhesive properties of *S. aureus* NCTC, expressing virulence factors important in the pathogenesis of infective endocarditis (IE) [43].

The infusion extract exhibited lower inhibitory activity, with the 10 μg/mL concentration only achieving an inhibition of 8.88% against *S. aureus*. Notably, the most substantial inhibitory effect of the infusion extract was observed against *Pseudomonas aeruginosa*, reaching an inhibition rate of 50.51%. Regarding *Listeria monocytogenes*, the methanolic, ethyl acetate, and dichloromethane extracts at a concentration of 20 μg/mL exhibited a uniform inhibition rate of 92%. This result is exciting, confirming that *L. cardiaca* can exert antibacterial activity against this foodborne pathogen and inhibit its virulence, so the extracts could be entrapped not only to avoid the *L. monocytogenes* contamination [44] but also, if present, to limit its virulence. On the other hand, across all five microbial strains, *L. cardiaca* had an excellent ability to prevent biofilm development. The antibiofilm activity exhibited in many ways so incisively by the extracts on the bacterial biofilm formation process remained, albeit more or less weak, also on the mature biofilm. Once again, the MetOH, EA, Hexane, and DCM extracts proved effective in combating mature biofilm, with inhibition percentages reaching 76.50% (testing 20 mg/mL vs. *Acinetobacter baumannii*). *L. monocytogenes*, whose biofilm formation was inhibited by up to 28.56% when placed in contact with the infusion extract, proved to be doubly sensitive in the test conducted on mature biofilm, recording, again in the presence of this extract, an inhibition percentage that it reached double (53.67%). *S. aureus* was found to be sensitive to the action of the hexane extract, recording inhibition percentages which, at the maximum concentration of extract used, were practically in line with what we observed in the antibiofilm activity test conducted by adding the extracts at zero time. The effectiveness exhibited against the mature biofilm of *S. aureus* is remarkable, *L. cardiaca* extracts can be of particular appeal in the preparation of *L. cardiaca*-based herbal products not only to prevent but also for supportive purposes in case of staphylococcal IE [45]. *E. coli*, whose biofilm formation was sensitive to the action of the various extracts, retained this sensitivity, albeit less strongly, even in the test conducted by adding the extracts after 24 h.

3.6 | Antibiofilm metabolic activity

Considering the metabolic perspective, the inhibitory action of all extracts exceeded 50% at the concentration of 20 μg/mL (Table 5). Notably, after time zero, the hexane extract revealed the highest inhibition metabolic activity, with a remarkable 91.46% inhibition at 20 μg/mL. In contrast, against *L. monocytogenes*, the inhibition reached

TABLE 4 Inhibitory activity of the extracts on the biofilm formation capacity by the pathogenic bacterial strains *Acinetobacter baumannii* (AB), *Escherichia coli* (EC), *Listeria monocytogenes* (LM), *Pseudomonas aeruginosa* (PS), and *Staphylococcus aureus* (SA)., at 0 and 24 h.

Time (0)	**Concentration (μg/mL)**	**AB -**	**EC -**	**PS -**	**LM +**	**SA +**
MeOH	10	65.36 ± 2.05^{d}	1.73 ± 0.04	64.37 ± 2.63^{d}	90.17 ± 2.39^{d}	47.74 ± 1.95^{d}
	20	84.53 ± 6.22^{d}	93.55 ± 3.45^{d}	93.47 ± 1.48^{d}	92.31 ± 2.35^{d}	93.47 ± 1.77^{d}
EA	10	59.50 ± 2.54^{d}	94.16 ± 3.30^{d}	87.88 ± 5.29^{d}	91.58 ± 2.29^{d}	24.79 ± 0.21^{c}
	20	86.41 ± 11.2^{d}	94.52 ± 1.64^{d}	90.56 ± 6.70^{d}	92.07 ± 1.88^{d}	75.60 ± 4.23^{d}
DCM	10	16.99 ± 0.49^{b}	89.35 ± 4.97^{d}	82.55 ± 0.81^{d}	0.66 ± 0.02	46.59 ± 0.46^{d}
	20	82.89 ± 3.61^{d}	94.07 ± 2.29^{d}	90.52 ± 1.16	92.61 ± 5.85^{d}	72.43 ± 2.15^{d}
Hexane	10	9.39 ± 0.45^{a}	77.24 ± 4.75^{d}	90.14 ± 6.8^{d}	92.03 ± 1.27^{d}	62.36 ± 4.55^{d}
	20	81.55 ± 8.94^{d}	77.24 ± 4.7^{d}	92.42 ± 2.61^{d}	93.18 ± 3.79^{d}	65.20 ± 4.07^{d}
Water	10	na	na	0.00	na	8.88 ± 0.25^{a}
	20	8.03 ± 0.20^{a}	27.71 ± 1.12^{c}	50.51 ± 2.24^{d}	28.56 ± 2.42^{c}	46.53 ± 1.57^{d}
Time (24)	Concentration (μg/mL)					
MeOH	10	AB -	EC -	PS -	LM +	SA +
	20	43.73 ± 0.60^{d}	20.56 ± 1.50^{b}	27.18 ± 0.10^{c}	15.74 ± 1.50^{b}	51.14 ± 0.071^{d}
EA	10	67.61 ± 1.31^{d}	29.92 ± 0.67^{c}	33.57 ± 1.22^{c}	15.60 ± 0.067^{b}	52.39 ± 0.057^{b}
	20	52.35 ± 0.051^{b}	22.27 ± 0.032^{b}	11.42 ± 0.70^{a}	15.14 ± 0.77^{b}	na
DCM	10	54.93 ± 0.051^{d}	38.51 ± 0.067^{c}	12.69 ± 1.77^{a}	18.77 ± 1.21^{b}	22.89 ± 1.06^{b}
	20	na	na	na	na	na
Hexane	10	76.50 ± 1.34^{d}	22.51 ± 1.67^{b}	26.92 ± 1.24^{c}	24.23 ± 0.67^{b}	52.61 ± 1.01^{d}
	20	36.10 ± 1.57^{c}	7.21 ± 1.81^{a}	2.99 ± 0.17	na	63.47 ± 1.67^{d}
Water	10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	36.11 ± 2.67^{c}	0.00 ± 0.00
	20	0.00 ± 0.00	9.69 ± 1.13^{a}	0.00 ± 0.00	53.67 ± 3.44^{d}	0.00 ± 0.00

Results are the average of three independent experiments ± SD. [a] $p \leq 0.5$; [b] $p \leq 0.01$; [c] $p \leq 0.001$; [d] $p \leq 0.0001$ compared with the positive control (for which the inhibition was assumed as zero). (ANOVA followed by Dunnett's multiple comparison test).

68.18 % at the same concentration. Among the tested extracts at 20 μg/mL, apart from water extract, which showed 16.38% metabolic inhibition, the others demonstrated a substantial 89% inhibition of metabolic activity against Staphylococcus aureus. In the test carried out using *A. baumannii*, acetyl acetate, dichloromethane, and methanolic extract had the highest inhibition against bacterial metabolism (87.04%, 86.97%, and 85.78%, respectively); hexane, at 53.98%, and infusion, at 48.92%, were weaker. The water extract at a concentration of 20 μg/mL demonstrated ineffectiveness against *P. aeruginosa*, while at 10 μg/mL, it proved ineffective against *E. coli*, *P. aeruginosa*, and *S. aureus*. Consistently with previous reports, it was observed that also in the case of *L. cardiaca*, the impact of the extracts on bacterial biofilm formation did not always align with a comparable effect on bacterial cell metabolism [46]. The effect of the water extracts on the metabolism of *P. aeruginosa* seemed to be contrary to the findings observed in the crystal violet test. Specifically, the concentration of 20 μg/mL, which effectively curtailed the biofilm metabolism of this bacterium, proved to be ineffectual in influencing its overall metabolism. The ethyl acetate extract reduced biofilm formation significantly against *E. coli*, reaching a remarkable 94% reduction at both concentrations. However, it had a much weaker effect on preventing the metabolic activity of these microbial cells, recording a reduction of 14.84% at 10 μg/mL and 51.35% at 20 μg/mL.

4 | CONCLUSION

The study's findings reveal distinct profiles of phenolic compounds and flavonoids in different extracts of *L. cardiaca*. Rosmarinic and chlorogenic acids were identified as the primary antioxidants, with water extract showing notable antioxidant activity in various tests. The n-hexane extract demonstrated superior AChE and BChE inhibition, while the ethyl acetate and methanolic extracts exhibited comparable tyrosinase enzyme inhibition. All tested extracts, except for the water extract, displayed substantial inhibitory activity on biofilm and the metabolic activity of sessile cells. Considering its remarkable antioxidant, enzyme inhibition, and antimicrobial properties, *L. cardiaca* emerges as a promising candidate for therapeutic applications.

TABLE 5 Percent inhibition of two doses of the extracts on the sessile cells metabolism of *Acinetobacter baumannii* (AB), *Escherichia coli* (EC), *Listeria monocytogenes* (LM), *Pseudomonas aeruginosa* (PS), and *Staphylococcus aureus* (SA), at 0 and 24 h.

Extracts	Concentration (μg/mL)	AB	EC	PS	LM	SA
Time: 0						
MeOH	10	36.24 ± 0.60^{c}	5.61 ± 0.04	29.74 ± 0.30^{b}	24.06 ± 0.21^{b}	52.12 ± 0.88^{d}
	20	85.78 ± 2.37^{d}	84.06 ± 1.27^{d}	79.00 ± 1.45^{d}	82.90 ± 3.85^{d}	89.21 ± 2.89^{d}
EA	10	37.77 ± 1.73^{c}	14.84 ± 0.13^{a}	22.28 ± 0.07^{b}	41.56 ± 1.00^{c}	49.19 ± 0.68^{c}
	20	87.04 ± 1.38^{d}	51.35 ± 1.36^{d}	85.12 ± 1.81^{d}	87.85 ± 4.29^{d}	89.73 ± 5.45^{d}
DCM	10	42.21 ± 0.82^{c}	4.86 ± 0.05	7.54 ± 0.14	13.85 ± 0.37^{a}	41.37 ± 0.42^{c}
	20	86.97 ± 2.21^{d}	84.80 ± 5.89^{d}	82.89 ± 1.54^{d}	86.75 ± 5.29	89.27 ± 9.94^{d}
Hexane	10	51.98 ± 0.84^{d}	3.21 ± 0.02	3.15 ± 0.04	68.18 ± 1.39	61.63 ± 1.17^{d}
	20	53.98 ± 0.77^{d}	74.39 ± 1.84^{d}	85.21 ± 15.37^{d}	91.46 ± 1.90^{d}	89.20 ± 5.48^{d}
Water	10	21.95 ± 1.15^{b}	na	na	5.43 ± 0.14	na
	20	48.92 ± 1.41^{c}	5.38 ± 0.06	na	32.15 ± 0.65^{c}	16.38 ± 0.29^{a}
Time (24 h)						
MeOH	10	3.53 ± 0.01	2.74 ± 0.03	12.24 ± 0.56^{a}	11.58 ± 0.15^{a}	2.82 ± 0.82
	20	5.43 ± 0.05	5.75 ± 0.04	15.55 ± 0.20^{a}	25.88 ± 0.38^{b}	36.57 ± 1.14^{c}
EA	10	3.25 ± 0.02	5.23 ± 0.16	33.82 ± 2.62^{c}	8.21 ± 0.05^{a}	52.78 ± 1.74^{d}
	20	3.00 ± 0.02	14.10 ± 0.21^{a}	45.62 ± 1.81^{c}	20.89 ± 0.29^{a}	84.94 ± 1.06^{d}
DCM	10	4.35 ± 0.07	3.91 ± 0.06	25.11 ± 0.11^{b}	17.51 ± 0.12^{a}	29.91 ± 0.92^{b}
	20	5.33 ± 0.03	18.56 ± 0.22^{a}	46.44 ± 0.36^{c}	38.13 ± 0.22^{c}	77.30 ± 5.50^{d}
Hexane	10	1.38 ± 0.02	2.85 ± 0.03	4.36 ± 0.07	22.46 ± 0.46^{b}	33.22 ± 1.63^{c}
	20	3.46 ± 0.05	41.38 ± 0.63^{c}	48.19 ± 1.39^{c}	54.49 ± 0.43^{d}	46.18 ± 2.15^{c}
Water	10	na	na	na	na	na
	20	$54.03^{c} \pm 1.07$	65.99 ± 1.30^{d}	na	na	6.82 ± 0.10

Results are the average of three independent experiments ± SD. [a] $p \leq 0.5$; [b] $p \leq 0.01$; [c] $p \leq 0.001$; [d] $p \leq 0.0001$ compared with the positive control (for which the inhibition was assumed as zero). (ANOVA followed by Dunnett's multiple comparison test).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nilofar N, Eyupoglu OE, Nazzaro F, Fratianni F, Ahmed S, Ferrante C, et al. An analytical framework combining online high-performance liquid chromatography methodologies and biological properties of different extracts of *Leonurus cardiaca*. J Sep Sci. 2023;2300695.
https://doi.org/10.1002/jssc.202300695