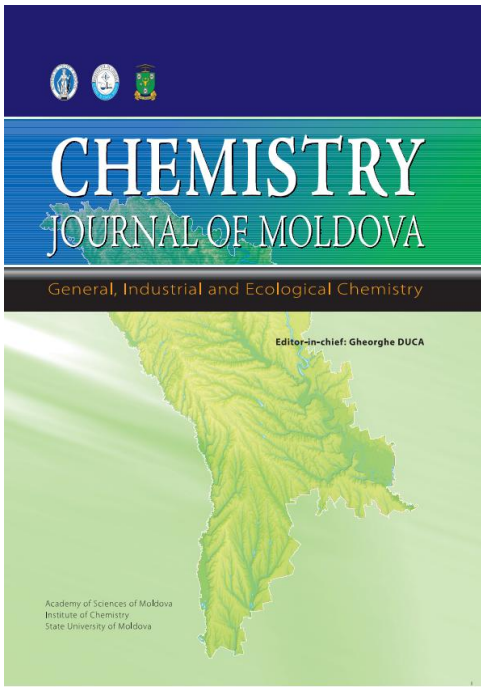


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# CHEMICAL COMPOSITION AND BIOLOGICAL EVALUATION OF TRADITIONAL ALGERIAN PLANTS *MELISSA OFFICINALIS* L. AND *URTICA DIOICA* L.

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**Abstract.** *Melissa officinalis* L. and *Urtica dioica* L. were investigated for their phytochemical profiles as well as their antioxidant and anti-lithiatic properties. LC-MS/MS analysis revealed that *M. officinalis* possessed a more complex and diverse composition, particularly rich in flavonoids (myricetin, and quercetin derivatives) and phenolic acids (caffeic, oleanolic, and salicylic), especially in its ethyl acetate fraction, indicating their lipophilic nature. In contrast, *U. dioica* exhibited a simpler chemical profile, with significant amounts of myricetin, riboflavin, sinapic acid, catechin, and *β*-carotene in its aqueous fraction. These compositional differences correspond to distinct biological activities. Antioxidant assays (DPPH, ABTS, FRAP) indicated that the ethyl acetate fraction of *M. officinalis* exhibited the highest radical-scavenging activity. Furthermore, its aqueous extract showed significant anti-lithiatic efficacy, inhibiting calcium oxalate crystal formation by 87.12% at a concentration of 2 mg/mL.

**Keywords:** *Melissa officinalis* L., *Urtica dioica* L., LC-MS/MS, antioxidant activity, anti-lithiatic activity.

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## Introduction

In recent scientific investigations, growing attention has been devoted to the chemical characterisation of plant extracts, with the aim of elucidating their molecular mechanisms and supporting their traditional uses with scientific evidence. Among these, *Melissa officinalis* L. and *Urtica dioica* L. are two widely used species in traditional medicine, known for their diverse pharmacological activities.

*Melissa officinalis* L., often identified as lemon balm, is classified in the Lamiaceae and is a perennial plant found throughout the Mediterranean region [1]. In addition to its culinary applications, research has increasingly pointed to lemon balm as a source of various potential benefits for health, including antioxidant, spasmolytic, anxiolytic, analgesic, antiviral, precognitive, antimicrobial, anti-inflammatory, and carminative activities [2,3]. Phytochemical screening has led to the identification of multiple bioactive constituents, including myricetin, caffeic acid, protocatechuic acid, rosmarinic acid, ferulic acid, syringic acid, quercetin, *P*-hydroxybenzoic acid, kaempferol, salicylic acid, luteolin, gallic acid, rutin, apigenin, and *p*-coumaric acid [3,4].

*Urtica dioica* L., commonly known as stinging nettle, is a perennial herbaceous species of the Urticaceae family, with a broad geographic distribution across Europe, North America, North Africa, and parts of Asia [5]. It has long been widely used in traditional medicine for rheumatism, sciatica, diabetes, indigestion, eczema, fever, haemorrhoids, kidney stones, and neurodegenerative and cardiovascular diseases [6]. An expanding body of scientific research highlights the phytochemical richness of *Urtica dioica*, which comprises a broad array of bioactive constituents, notably acetohydroxamic acid, *p*-coumaric acid, caftaric acid, gallic acid, 4-hydroxycoumarin, caffeic acid, rutin, *p*-hydroxybenzoic acid, ethyl protocatechuate, ellagic acid, and quercetin [7-9]. On the other hand, *Urtica dioica* is recognised for its considerable content of vitamins (A, C, and E) and minerals [10].

The antioxidant activities of *Urtica dioica* and *Melissa officinalis* are largely associated with

their abundant bioactive compounds, particularly polyphenols, flavonoids, and carotenoids [9,11]. These bioactive molecules are pivotal in scavenging reactive oxygen species, thereby mitigating oxidative damage at the cellular level and reinforcing intrinsic antioxidant defence pathways. Through modulation of redox homeostasis, phytochemical extracts from these species may exert protective effects against the pathogenesis of chronic diseases such as malignancies, metabolic syndromes, and cardiovascular disorders [12].

The present study offers an original contribution by focusing on plant species collected from Algeria. A comparative solvent extraction approach was employed, based on the understanding that selecting an appropriate extraction solvent is essential for maximising the recovery of bioactive compounds aligned with specific pharmacological activities [13]. This research not only confirms the presence of known phytochemicals but also provides a refined characterisation of bioactive fractions that have not previously been described in this regional context.

This study investigates the anti-lithiatic potential of aqueous and ethyl acetate extracts, focusing on their ability to inhibit the formation of calcium oxalate crystals, the most common type of kidney stones [14]. Antioxidant activity was assessed through a multi-assay approach involving DPPH, ABTS, and FRAP methods. Through LC-MS/MS profiling and *in vitro* assays, by combining geographical specificity, detailed compound resolution, and dual bioactivity assessment to offer new insights into the therapeutic potential of Algerian flora and contributes original data to the field of natural product pharmacology.

## Experimental

### *Chemicals*

The experimental procedures were carried out using high-purity reagents, all of analytical grade or higher. The chemicals included hexane (≥99%), chloroform (99–99.4%), activated carbon, methanol (≥99.7%), ethyl acetate (≥99.5%), anhydrous sodium sulphate (≥99%), Folin–Ciocalteu reagent, sodium carbonate (99.5–100.5%), gallic acid (GA, >97.5%), aluminium chloride ($AlCl_3$, ≥98%), catechin (≥98%), vanillin (99%), hydrochloric acid (HCl, 37%), quercetin (≥95%), 2,2-diphenyl-1-picrylhydrazyl (DPPH, ≥95%), ascorbic acid (100%), ferric chloride ($FeCl_3$, 97%), potassium persulphate ($K_2S_2O_8$, ≥99%), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, 98%), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ, 98%), and sodium acetate ($CH_3COONa$, ≥99%). Additional compounds used included sodium chloride (NaCl, 99.7%), sodium oxalate ($Na_2C_2O_4$, ≥95.5%), calcium chloride ($CaCl_2$, ≥99%), sodium dihydrogen phosphate ($NaH_2PO_4$, ≥95%), disodium hydrogen phosphate ($Na_2HPO_4$, 98–100.5%), and calcium oxalate ($CaC_2O_4$, ≥99%). All materials were obtained from Sigma-Aldrich, France.

### *Plant material*

During the months of June and July 2020, leaf samples of *Melissa officinalis* and *Urtica dioica* were collected from the regions of Barbacha and Toudja, respectively, located in Bejaia, Algeria. The plant species were botanically identified at the Department of Natural Sciences at the University of Bejaia. To preserve their chemical integrity, the plant materials were air-dried in the shade at room temperature for 72 hours. Subsequently, they were finely ground (sieve size: 0.5 mm), and storage conditions (airtight containers at 4°C in the dark).

### *Pre-extraction of plant material*

The plant material was pre-treated for lipid elimination with 300 mL of hexane in a Soxhlet apparatus for 48 hours. Excess chlorophyll was eliminated by reflux extraction with 150 mL of chloroform, and activated carbon for 2 hours. The initial air-dried material to activated carbon mass ratio was 1:5 (mass) [15]. The residue was air-dried for 24 hours at room temperature for the complete removal of the solvent.

### *Plant extraction*

A 5 g of the dried residue were further macerated in 100 mL of methanol (80 % *v*/*v*) for 24 hours at room temperature. After filtration through Whatman filter paper, the filtrate was concentrated under vacuum using a rotary evaporator maintained at 40°C. The resulting aqueous phase underwent a liquid-liquid extraction in a separating funnel with a series of organic solvents arranged in ascending order of polarity: hexane (5 × 100 mL) followed by ethyl acetate (3 × 100 mL). After drying with anhydrous sodium sulphate, the organic extract was evaporated at 40°C under vacuum using a rotary evaporator.

### *Determination of total phenolic content*

Phenolic compounds in the plant extract fractions were analysed following the method reported by [16]. A quantity of 100 µL of each plant extract fraction, previously diluted 1:10 in distilled water, was reacted with 500 µL of Folin–Ciocalteu reagent. Following 2 minutes of incubation at room temperature, 2 mL of $Na_2CO_3$ solution (5% *w*/*v*) was introduced. The resulting

solution was incubated in the dark at room temperature for 30 minutes; the absorbance was then measured at 760 nm using a Shimadzu 1601 UV-Vis spectrophotometer. Under identical conditions as above, to generate the standard curve, gallic acid solutions with concentrations ranging from 0.034 to 0.34 g/L were prepared. The quantities were given in mg GAE/g DW, which stands for milligrams of gallic acid equivalent per gram of dry weight.

***Determination of flavonoid content***

The quantification of total flavonoids was carried out according to the method described in [17]. A quantity of 1 mL of a 2% (*w*/*v*) aluminium chloride solution prepared in methanol was added to 1 mL of the appropriately diluted extract. Following a 20-minute incubation period, absorbance readings were taken at 430 nm relative to a blank and quantified based on a standard curve generated with quercetin (0.005 to 0.05 g/L). The quantification was reported as mg QE/g DW.

***Determination of tannin content***

The amount of tannin was quantified following the protocol established by [18] with certain modifications. In test tubes, 200 µL of the plant extract fraction was combined with 1 mL of a freshly prepared vanillin solution in hydrochloric acid (1/4% *v*/*v*), followed by thorough mixing. The reaction mixture was heated at 30°C for 20 minutes. Thereafter, the absorbance was measured at 500 nm. The standard curve for catechin was constructed using a range of concentrations (0.15-1.5 g/L). The tannin contents are expressed in mg CE/g DW.

***LC-MS/MS analysis***

Phenolic compounds were analysed using a Shimadzu 8040 Ultra-High Sensitivity UPLC-ESI-MS/MS system equipped with UFMS technology and a Nexera XR LC-20AD binary pump. The analysis was performed using electrospray ionisation (ESI), which operated in positive ion mode as the ion source. Chromatographic separation was achieved using a Restek Ultra C18 analytical column (3 µm, 150 × 4.6 mm). To overcome suppression effects and obtain optimal separation for thirty-four phytochemical compounds, reversed-phase ultrahigh-performance liquid chromatography was optimised. Elution was performed using a binary solvent system composed of eluent A (water containing 0.1% formic acid) and eluent B (methanol). The gradient programme was applied as follows: from 0 to 0.2 minutes, 98% A; from 0.2 to 2.5 minutes, 25% A; from 2.5 to 4 minutes, 0% A; maintained at 0% A from 4 to 7 minutes; returned to 98% A at 7.1 minutes; and held at 98% A until 12 minutes. The separation process employed a steady flow rate of 0.2 mL/min, with 5 µL of sample injected per run. Mass spectrometry scanning was performed in positive mode. The operating parameters were set as follows: 350°C for the interface, 250°C for the DL, 400°C for the thermal block, 3 L/min for nebulisation gas, and 10 L/min for drying gas.

***Antioxidant activity***

*DPPH radical scavenging test*

Applying the approach outlined by [19] with some modifications, DPPH scavenging activity was determined. An aliquot of 1 mL of each fraction was combined with 1 mL of 0.1 mM DPPH solution in methanol. The reaction system was left to react in the dark for 30 minutes at room temperature. After incubation, absorbance was measured at 517 nm using a blank as the reference. Ascorbic acid served as a standard antioxidant. The calculation of radical scavenging activity followed Eq.(1).

$$Antioxidant\ activity\ (\%) = \frac{A0-Ae}{A0} \times 100 \qquad (1)$$

where, *A0* - the control's absorbance;
*Ae* - the absorbance of the tested extract.

*Ferric reducing antioxidant power assay*

To perform the FRAP test, the experimental protocol used the methodology described by [19]. A quantity of 50 µL of each fraction was mixed with 1 mL of freshly prepared FRAP reagent, which was composed of 20 mM ferric chloride solution, 10 mM TPTZ dissolved in 40 mM HCl, and 300 mM sodium acetate buffer (pH 3.6) in a volumetric ratio of 10:1:1. The reaction mixture was allowed to react for 7 minutes. Absorbance was then measured at 593 nm. The usual antioxidant reference was ascorbic acid. The ferric reducing antioxidant potential (FRAP) was expressed using Eq.(2).

$$PI\ (\%) = \frac{Ae}{A0} \times 100 \qquad (2)$$

where, *Ae* - the absorbance of the tested extract;
$A_0$ - the absorbance of 100% reduction of ferric ions in 1 mL of FRAP reagent.

*ABTS assay*

To evaluate the antioxidant activity of plant samples against ABTS free radicals, a modified methodology as reported by [17] was employed.

To generate the $ABTS^{\bullet+}$ radical cation, a 20 mM ABTS solution was reacted with 70 mM potassium persulfate ($K_2S_2O_8$), followed by an incubation for 24 hours under dark conditions at

room temperature. The ABTS•+ radical cation solution was adjusted to an absorbance of $0.700 \pm 0.020$ at 734 nm by dilution with phosphate-buffered saline (PBS, pH 7.4). The PBS solution was prepared by dissolving 5 mM sodium dihydrogen phosphate ($NaH_2PO_4$), 5 mM disodium hydrogen phosphate ($Na_2HPO_4$), and 153.84 mM sodium chloride (NaCl) in 1 mL of distilled water. For the assay, 100 µL of the diluted extract was combined with 1 mL of the prepared ABTS•+. The solution was vortexed briefly and then incubated in the dark for 5 minutes. Thereafter, absorbance was recorded at 734 nm. Ascorbic acid served as the reference antioxidant compound. The ABTS•+ radical scavenging capacity was calculated according to Eq.(1).

***Evaluation of anti-lithiatic activity with the turbidity method***

The *in vitro* anti-lithiatic potential of the samples was evaluated by examining their ability to inhibit calcium oxalate crystallization in the presence as well as the absence of inhibitory agents. Two stock solutions were prepared in 0.15 M sodium chloride: one containing 40 mM calcium chloride dihydrate and the other 4 mM sodium oxalate. To maintain a stable pH of 5.7, 10 mM sodium acetate was added as a buffering agent. A steady temperature of 37°C was sustained for both solutions throughout the entire experiment [20].

In the control setup (without extracts), equal volumes of sodium oxalate and calcium chloride solutions were mixed, and the resulting turbidity was immediately measured at 620 nm using a Shimadzu UV-Vis spectrophotometer (model 160). Absorbance readings were recorded every 6 seconds over a period of 5 minutes (300 seconds) to monitor the kinetics of forming calcium oxalate crystals. For the experimental groups containing plant extracts, the same protocol was followed. Specifically, to begin, the cuvette was first filled with 500 µL of calcium chloride solution, and then 100 µL of the extract was added at varying concentrations (0.25, 0.5, 1, and 2 g/L). Once a stable baseline absorbance has been established, 500 µL of the sodium oxalate solution was introduced, and absorbance measurements were immediately initiated. The extent of crystallization inhibition was determined according to the Eq.(3).

$$I(\%) = \left(1 - \frac{Si}{Sc}\right) \times 100 \quad (3)$$

where, *Si* - the graph's slope when the inhibitor (extract) is present

*Sc* - the slope in the absence of the inhibitor (negative control).

The ethyl acetate fractions were evaporated using a rotary evaporator under reduced pressure at 40°C. The resulting residues were then dissolved in water and filtered using Whatman filter paper, and various extract concentrations varying from 0.25 to 2 mg/mL were prepared for each sample.

**Statistical analysis**

The mean ± standard deviation of three independent replicates was used to present the results. The linear regression analysis was conducted using the Data Analysis add-in in Microsoft Excel to determine the $IC_{50}$ value. A one-way analysis of variance (ANOVA) was performed, followed by Tukey-Kramer's multiple comparisons test, to perform statistical analyses and calculations using the R software and packages (ggplot2, cowplot, multcompView, dplyr, tidyr). Results with *p*-values less than 0.05 were interpreted as statistically significant.

## Results and discussions

***LC-MS/MS analysis***

LC-MS/MS analysis of *Melissa officinalis*

Polyphenol identification was performed using LC-MS/MS operated in MRM mode, based on a comprehensive characterization of thirty-four authenticated reference compounds. Retention times, compound-specific MRM transitions, and optimized collision energies were established under defined chromatographic conditions. Identification of polyphenols in plant extracts was confirmed solely by strict matching of both retention time and MRM transitions with the in-house standard database.

According to the LC-MS/MS profiling, among the thirty-four examined compounds, the ethyl acetate fraction of *M. officinalis* contains five phenolic acids, ten flavonoids, two terpenoid compounds and their derivatives, four polyphenols, and six other bioactive compounds. This fraction exhibits a high abundance of myricetin, caffeic acid, oleanolic acid, quercetin-3-glucoside, and salicylic acid, while ferulic acid, chrysin, and curcumin are present in lower amounts (Table 1).

*M. officinalis* contains phenolic acids, which are key bioactive compounds. These compounds include caffeic acid, sinapic acid, ferulic acid, *p*-coumaric acid, and salicylic acid. Extensive research has highlighted the antioxidant, antimicrobial, anti-inflammatory, and antimutagenic potential of phenolic acids [21].

The flavonoid compounds identified in the ethyl acetate fraction of *Melissa officinalis* are classified into five major subgroups: flavones

(apigenin, luteolin, and chrysin), flavanones (hispidulin, naringenin), flavanols (myricetin, rutin, and quercetin), flavan-3-ols (epicatechin), and complex flavonoid glycosides (tiliroside). Among the several biological and pharmacological effects of flavonoids are antioxidant, antidepressant, anti-diabetic, anti-inflammatory, immunomodulatory, antibacterial, anticoagulant, antiproliferative, anticancer, antitubercular, and anti-allergic ones. Flavonoids and phenolic acids possess specific chemical structural elements that contribute to their antioxidant properties, which have been extensively validated through biochemical studies [22].

In their phytochemical analysis, Ben Aicha, B. *et al*. found six phenolic constituents in the hydro-alcoholic extract of *M. officinalis*, including rosmarinic acid, caftaric acid, sagerinic acid, hydroxyjasmonic acid glucoside, and caftaric acid glucoside [23]. Zam, W. *et al*. indicated that leaf extracts of *M. officinalis*, obtained using ethyl acetate, and 1-butanol, contain various polyphenolic components, notably caffeic acid, luteolin, *p*-coumaric acid, apigenin, ferulic acid, and caftaric acid [24].

The detection of riboflavin is noteworthy, is not commonly found in typical plant extracts. Riboflavin is a vital vitamin required for maintaining normal metabolic functions. While it is naturally synthesized by various plants and microorganisms, humans and animals must obtain it through their diet [25].

The aqueous fraction of *Melissa officinalis* was characterised by a high content of myricetin, *β*-carotene, 2-methoxybenzoic, and caffeic acid.

The aqueous fraction contains more water-soluble flavonoids (catechin, flavanols, and flavones) and phenolic acids. The ethyl acetate fraction was richer in lipophilic polyphenols such as quercetin-3-glucoside and oleanolic acid. Myricetin is dominant in both fractions but is significantly more abundant in the aqueous fraction.

*Table 1*

**LC-MS/MS profile of ethyl acetate and aqueous fractions of *Melissa officinalis* L.**

| *Peak* | *RT (min)* | *Name* | *Formula* | *[M+H]+ (m/z)* | *Area (%) EA.F* | *Area (%) A.F* |
|---|---|---|---|---|---|---|
| 1 | 6.145–4.959 | Chrysin-6-C glucoside | $C_{21}H_{20}O_9$ | 418 | 0.20 | 0.07 |
| 2 | 6.492–6.567 | Myricetin | $C_{15}H_{10}O8$ | 319 | 37.98 | 63.43 |
| 3 | 6.440 | Catechin | $C_{15}H_{14}O_6$ | 292 | - | 0.09 |
| 4 | 6.039–5.955 | Quercetine-3-glucoside | $C_{21}H_{19}O_{12}$ | 466 | 6.31 | 0.11 |
| 5 | 5.936–5.952 | Tiliroside | $C_{30}H_{26}O_{13}$ | 596 | 0.03 | 0.07 |
| 6 | 6.134–6.167 | Apigenin | $C_{15}H_{10}O_5$ | 272 | 1.32 | 0.20 |
| 7 | 6.686–6.731 | Hispidulin | $C_{16}H_{12}O_6$ | 302 | 3.61 | 0.76 |
| 8 | 5.501–5.530 | 2-Methoxybenzoic acid | $C_8H_8O_3$ | 154 | 3.35 | 9.65 |
| 9 | 6.421 | Chrysin | $C_{15}H_{10}O_4$ | 255 | 0.02 | - |
| 10 | 5.809–5.831 | Curcumin | $C_{21}H_{20}O_6$ | 369 | 0.03 | 0.03 |
| 11 | 7.489–5.907 | Epicatechin | $C_{15}H_{14}O_6$ | 291 | 0.74 | 0.15 |
| 12 | 6.573–5.956 | Ferulic acid | $C_{10}H_{10}O_4$ | 196 | 0.01 | 0.004 |
| 13 | 6.007–6.242 | Luteolin | $C_{15}H_{10}O_6$ | 287 | 0.37 | 1.13 |
| 14 | 7.561–7.723 | Oleanolic acid | $C_{30}H_{48}O_3$ | 458 | 14.16 | 1.23 |
| 15 | 7.413 | Oleuropein | $C_{25}H_{32}O_{13}$ | 541 | 1.65 | - |
| 16 | 6.030–6.008 | Quercetin | $C_{15}H_{10}O_7$ | 304 | 0.26 | 0.05 |
| 17 | 6.119–5.934 | Resveratol | $C_{14}H_{12}O_3$ | 230 | 0.92 | 0.05 |
| 18 | 7.724–7.919 | Riboflavin | $C_{27}H_{30}O_{16}$ | 378 | 2.17 | 1.91 |
| 19 | 5.992–6.102 | Rutin | $C_{27}H_{30}O_{16}$ | 612 | 2.42 | 0.22 |
| 20 | 6.033–5.996 | Sinapic acid | $C_{11}H_{12}O_5$ | 226 | 0.24 | 0.15 |
| *21* | 9.806–9.944 | *β*-Carotene | $C_{40}H_{56}$ | 538 | 0.82 | 10.99 |
| 22 | 6.319–6.117 | Kojic acid | $C_6H_6O_4$ | 143 | 0.01 | 0.01 |
| 23 | 6.387–5.942 | Naringenin | $C_{15}H_{12}O_5$ | 274 | 0.10 | 0.58 |
| 24 | 6.076–6.142 | Thymol | $C_{10}H_{14}O$ | 152 | 0.20 | 0.08 |
| 25 | 5.843–5.871 | Vanillin | $C_8H_8O_3$ | 152 | 0.63 | 0.86 |
| 26 | 5.800–5.832 | Caffeic acid | $C_9H_8O_4$ | 178 | 18.18 | 6.14 |
| 27 | 6.078–6.102 | *p*-Coumaric acid | $C_8H_8O_4$ | 162 | 0.30 | 0.26 |
| 28 | 6.360–6.384 | Salicylic acid | $C_7H_6O_3$ | 136 | 3.96 | 1.78 |

*RT: Retention time, [M+H] + (m/z): Transition pair of mass-to-charge, EA.F: Ethyl acetate fraction, A.F: Aqueous fraction.*

Catechin was exclusively found in the aqueous fraction, whereas epicatechin was identified in both fractions, showing greater abundance in the ethyl acetate fraction. Caffeic acid, apigenin, hispidulin, and chrysin are more prevalent in the ethyl acetate fraction, suggesting better solubility in less polar solvents. The success of extracting phenolic compounds from plant materials largely depends on interaction between solvent polarity and the solubility of the target compounds [19].

*M. officinalis* exhibits a complex phytochemical profile, containing numerous bioactive compounds whose presence and concentration depend on the extraction technique used and the specific plant parts analysed [22].

*LC–MS/MS analysis of Urtica dioica*

The phenolic profiles of the ethyl acetate fraction of *U. dioica* were established by LC-MS/MS analysis, which allowed identification of twenty-seven phenolic compounds as listed in Table 2. This fraction showed a significant presence of myricetin, quercetine-3-glucoside, caffeic acid, riboflavin, sinapic acid, catechin, and vanillin. Several phenolic acids were detected, including caffeic acid, salicylic acid, *p*-coumaric acid, and sinapic acid,

Medicinal plants are known to contain a wide variety of secondary metabolites, among which flavonoids are the most extensively distributed [26]. Three subclasses define flavonoids extracted from the ethyl acetate fraction of *U. dioica*: flavones (apigenin), flavanols (myricetin, quercetin-3-glucoside, and quercetin-3-arabinose), and flavan-3-ols (catechin). The main phenolic compounds found in the aqueous fraction of *U. dioica* include myricetin, *β*-carotene, sinapic acid, and riboflavin.

The higher concentration of flavonoids with glycosides like quercetin-3-arabinose in the aqueous fraction is due to their stronger water solubility. The substantial presence of non-polar compounds, including catechin, and caffeic acid, in the ethyl acetate fraction is consistent with their preferential dissolution in that phase. Carotenoids were more abundant in the aqueous fraction, likely due to their hydrophilic nature or amphipathic solubility in both phases.

*Table 2*

**LC-MS/MS profile of ethyl acetate and aqueous fractions of *Urtica dioica* L.**

| *Peak* | *RT (min)* | *Name* | *Formula* | *[M+H]+ (m/z)* | *Area (%) EA.F* | *Area (%) A.F* |
|---|---|---|---|---|---|---|
| 1 | 7.011–6.798 | Catechin | $C_{15}H_{14}O_6$ | 292 | 2.21 | 0.15 |
| 2 | 6.367 | Chrysine-6-C-glucoside | $C_{21}H_{20}O_9$ | 418 | 0.03 | - |
| 3 | 6.446–6.467 | Myricetin | $C_{15}H_{10}O_8$ | 319 | 62.15 | 65.71 |
| 4 | 5.828 | Quercetine-3-arabinose | $C_{20}H_{18}O_{11}$ | 435 | - | 0.25 |
| 5 | 6.037–6.329 | Quercetine-3-glucoside | $C_{21}H_{19}O_{12}$ | 466 | 9.69 | 0.15 |
| 6 | 6.283–5.715 | Tiliroside | $C_{30}H_{26}O_{13}$ | 596 | 0.09 | 0.01 |
| 7 | 6.131–5.849 | Apigenin | $C_{15}H_{10}O_5$ | 272 | 0.62 | 0.74 |
| 8 | 6.138 | Hispidulin | $C_{16}H_{12}O_6$ | 302 | 0.24 | - |
| 9 | 6.061 | 2-Mythoxybenzoic acid | $C_8H_8O_3$ | 154 | 1.06 | - |
| 10 | 6.377 | Chrysin | $C_{15}H_{10}O_4$ | 255 | 0.01 | - |
| 11 | 5.810–5.811 | Curcumin | $C_{21}H_{20}O_6$ | 369 | 0.04 | 0.25 |
| 12 | 6.543 | Epicatechin | $C_{15}H_{14}O_6$ | 291 | 0.29 | - |
| 13 | 6.154 | Luteolin | $C_{15}H_{10}O_6$ | 287 | 0.58 | - |
| 14 | 7.499–7.522 | Oleanolic acid | $C_{30}H_{48}O_3$ | 458 | 0.90 | 0.67 |
| 15 | 7.009–7.042 | Oleuropein | $C_{25}H_{32}O_{13}$ | 541 | 0.47 | 2.37 |
| 16 | 6.037–5.905 | Quercetin | $C_{15}H_{10}O_7$ | 304 | 0.20 | 0.79 |
| 17 | 6.111–6.506 | Resveratol | $C_{14}H_{12}O_3$ | 230 | 1.57 | 1.62 |
| 18 | 7.648–7.686 | Riboflavin | $C_{27}H_{30}O_{16}$ | 378 | 4.48 | 4.95 |
| 19 | 5.999 | Rutin | $C_{27}H_{30}O_{16}$ | 612 | 1.37 | - |
| 20 | 6.021–5.681 | Sinapic acid | $C_{11}H_{12}O_5$ | 226 | 2.36 | 4.82 |
| *21* | 9.630–7.584 | *β*-Carotene | $C_{40}H_{56}$ | 538 | 1.63 | 11.47 |
| 22 | 6.075 | Kojic acid | $C_6H_6O_4$ | 143 | 0.01 | - |
| 23 | 6.208–6.217 | Naringenin | $C_{15}H_{12}O_5$ | 274 | 0.25 | 0.12 |
| 24 | 6.079–5.726 | Thymol | $C_{10}H_{14}O$ | 152 | 0.03 | 1.91 |
| 25 | 5.976–5.945 | Vanillin | $C_8H_8O_3$ | 152 | 1.51 | 1.72 |
| 26 | 5.787 | Caffeic acid | $C_9H_8O_4$ | 178 | 6.66 | - |
| 27 | 6.066 | *p*-Coumaric acid | $C_8H_8O_4$ | 162 | 0.47 | - |
| 28 | 5.799–5.814 | Salicylic acid | $C_7H_6O_3$ | 136 | 1.07 | 2.31 |

*RT: Retention time, [M+H]+ (m/z): Transition pair of mass-to-charge, EA.F: Ethyl acetate fraction, A.F: Aqueous fraction.*

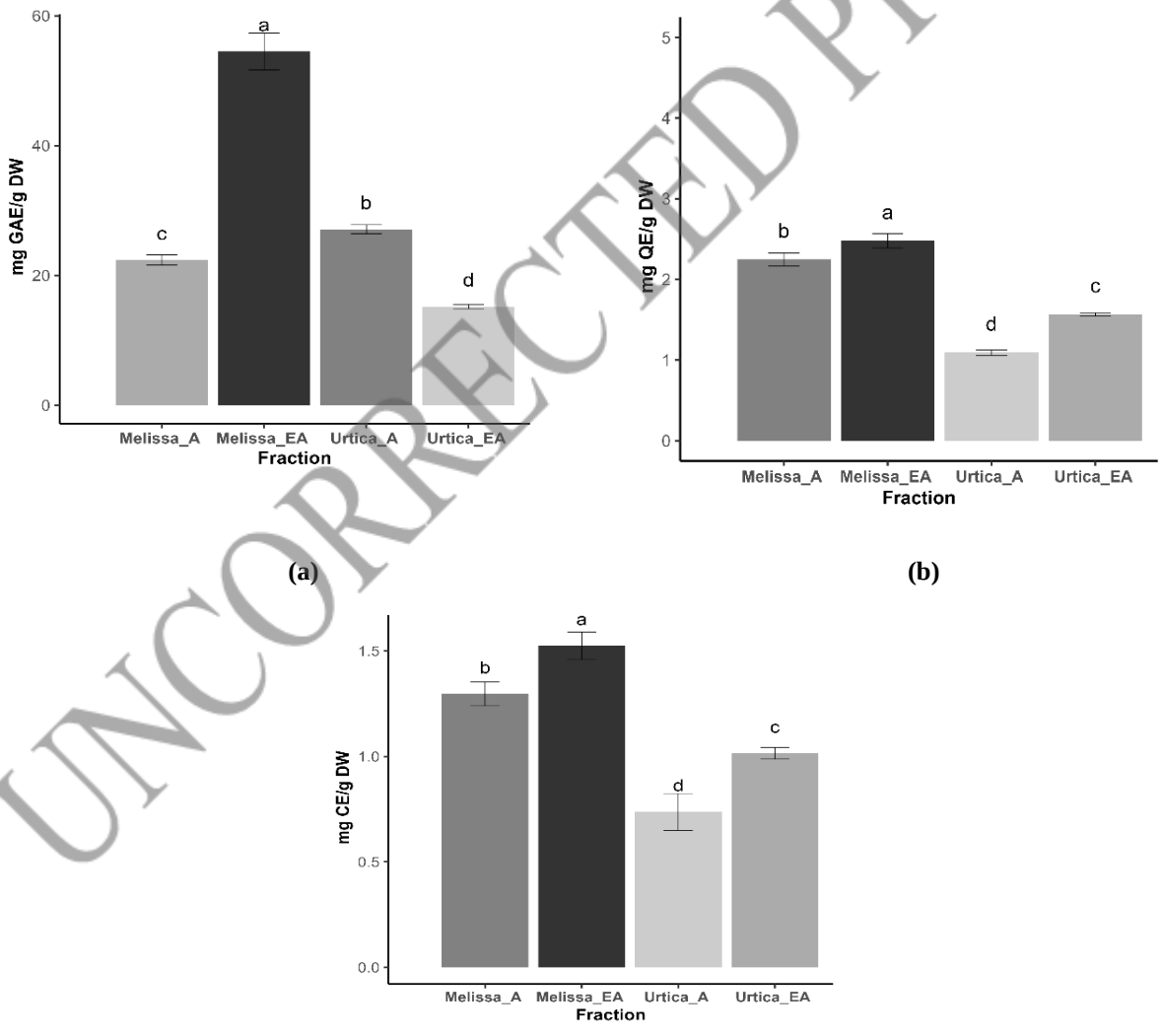
In a related study, Uğur, Y. *et al.* reported the quantification of several phenolic compounds in methanolic extracts of *U. dioica*, including caffeic acid, acetohydroxamic acid, gallic acid, *p*-hydroxybenzoic acid, ellagic acid, and quercetin [7].

*Melissa officinalis* is characterised by a high content of phenolic acids, particularly caffeic acid, and the presence of specific compounds such as oleuropein, while *Urtica dioica* exhibits a greater abundance of flavonoids, including myricetin and quercetin-3-glucoside, as well as a higher concentration of pigments such as *β*-carotene.

***Quantification of total phenolic content (TPC)***

The total phenolic content (TPC), flavonoids, and tannins levels in the aqueous and ethyl acetate fractions of *M. officinalis* and *U. dioica* are separately illustrated in Figure 1.

The ethyl acetate fraction of *M. officinalis* exhibited the highest TPC value, reaching 54.51 ± 2.30 mg GAE/g DW, which greatly exceeding the TPC in the aqueous fraction, measuring only 22.36 ± 0.37 mg GAE/g DW. The obtained result is considerably lower than the 32.76 mg GAE/g DW reported by [27]. Hassan, R.A. *et al*. observed a notably higher total phenolic amount of 143.50 mg GAE/g DW in the ethyl acetate extract [28]. Additionally, according to Ben Aich, B. *et al*., the hydro-alcoholic extract of the same species collected in Algeria contained 118.62 ± 6.57 mg GAE/g DW of polyphenolic compounds [23]. The ethyl acetate fraction of *U. dioica* presents the lowest total phenolic content with (15.21 ± 0.13mg GAE/g DW). The phenolic content values for *U. dioica* are consistent with those found by [29] (23 mg GAE/g DW).




*Melissa_A: Aqueous fraction of M. officinalis; Melissa_EA: Ethyl acetate fraction of M. officinalis; Urtica_A: Aqueous fraction of U. dioica; Urtica_EA: Ethyl acetate fraction of U. dioica.*

**Figure 1. Phytochemical contents: Total phenolic *(a)*, flavonoids *(b)*, condensed tannins *(c)* of *M. officinalis* and *U. dioica* fractions, P< 0.001, n= 3.**

This discrepancy could be attributed to differences in extraction methods, solvent selection, or geographical variations in plant composition. Similarly, this further confirming the substantial presence of phenolic compounds in *M. officinalis* and *U. dioica*. However, variations in total phenolic content across different studies suggest that elements such as the source of the plant, growing conditions, harvesting time, and extraction techniques play a crucial role in determining polyphenol yield [27,30]. These findings highlight the importance of optimising extraction parameters to improve the recovery efficiency of secondary metabolites with bioactive potential from *M. officinalis* and *U. dioica*.

***Total flavonoid content (FC)***

Out of all examined samples, the ethyl acetate fraction of *M. officinalis* demonstrates the richest flavonoid content (2.48 ± 0.05 mg QE/g DW), followed by the aqueous fraction of *M. officinalis* (2.25 ± 0.03 mg QE/g DW). Despite this, both values remain significantly lower than the concentration recorded by [28] which reached 124.96 mg QE/g DW. Conversely, the aqueous extract of *U. dioica* demonstrated the poorest flavonoid presence, with only 0.65 mg QE/g DW detected.

***Total tannin content (TC)***

Total tannin content ranged between 0.61 and 1.52 mg CE/g DW. The most elevated concentration was observed in the ethyl acetate fraction of *M. officinalis* (1.52 ± 0.06 mg CE/g DW), followed closely by its aqueous fraction (1.30 ± 0.06 mg CE/g DW).

The above results illustrate that the solvent has an effect on the extractability of phenolic compounds. Water emerged as the most effective solvent for recovering polyphenolic constituents from *U. dioica* under the tested conditions. The efficiency of phenolic and flavonoid compound recovery can be affected by various parameters: sample size, storage conditions, climatic fluctuations, extraction methods, presence of interfering chemicals, and choice of solvent [31].

***Antioxidant activity***

A study on the antioxidant potential of the plant extract fractions was conducted *in vitro* using three standard assays: ABTS, DPPH, and FRAP. The $IC_{50}$ values (µg/mL) represent the concentration necessary to inhibit 50% of the radical activity; thus, lower $IC_{50}$ values denote greater antioxidant efficacy (Figure S1 in supplementary information).

*DPPH radical scavenging assay*

The DPPH radical, known for its stability, undergoes a colourimetric reduction upon interaction with hydrogen-donating antioxidant compounds, indicating free radical neutralisation capacity [32].

The ethyl acetate fraction of *M. officinalis* displayed the strongest antioxidant effect in the DPPH assay, achieving an $IC_{50}$ of 1.95 µg/mL, comparable to ascorbic acid (1.40 ± 0.26 µg/mL). The efficacy of *U. dioica* extracts was comparatively lower than that of the reference antioxidant, ascorbic acid.

*Ferric reducing antioxidant power (FRAP) assay*

The FRAP assay evaluates the antioxidant strength of a sample by assessing its capacity to reduce the $Fe^{3}$-TPTZ complex to the $Fe^{2+}$ form [19]. The reducing antioxidant power with minimum $IC_{50}$ values was recorded for ethyl acetate fractions of *M. officinalis* (4.97 ± 0.41 µg/mL) which exceeded that of the positive control, ascorbic acid (5.48 ± 0.30 µg/mL), as indicated in Figure 2. The *U. dioica* plant was considerably less effective in reducing capability compared to *M. officinalis* plant extracts.

*ABTS assay*

This approach is based on antioxidants' capacity to decrease the ABTS radical cation [33]. Regarding ABTS assay results, the ethyl acetate fraction of *M. officinalis* exhibited the highest antioxidant activity, with an $IC_{50}$ value of 1.44 ± 0.04 µg/mL. These values were compared to ascorbic acid, used as the reference antioxidant, which showed an $IC_{50}$ of 0.97 ± 0.005 µg/mL. In contrast, the *U. dioica* extract demonstrated significantly lower antioxidant efficacy compared to *M. officinalis*.

Other compounds like polyphenols, which have strong antioxidant potential, are also considered to contribute to plants' antioxidant capacity in addition to vitamins C, E, and *β*-carotene [34].

***Correlation between LC-MS/MS and phenolic content***

The correlation between the quantitative assays and LC-MS-MS analyses highlights that *M. officinalis* has the highest TPC, flavonoid, and tannin contents in its ethyl acetate fraction (54.51 mg GAE/g DW, 2.48 mg QE/g DW, and 1.52 mg CE/g DW, respectively), consistent with the abundance of caffeic acid, quercetin-3-glucoside, and other flavonoids. In contrast, *U. dioica* shows lower overall phenolic levels but is rich in myricetin and *β*-carotene, particularly in

the aqueous fraction. Tannin levels in *M. officinalis* also align with the detection of catechin derivatives in LC-MS.

***Evaluation of anti-lithiatic activity with the turbidity method***

The crystallisation inhibition capacity of *U. dioica* and *M. officinalis* was assessed by comparing the slopes of the test with and without the inhibitor at different concentrations of aqueous and ethyl acetate fractions during the growth stage. Crystal growth, resulting from the aggregation of particles, is an essential stage in the formation of urinary stones. According to Figures S2 and S3, the two fractions of both *U. dioica* and *M. officinalis* significantly reduced the growth of calcium oxalate crystals; this turbidity inhibition was considerably higher in aqueous fractions at 2 mg/mL when compared to ethyl acetate fractions at the same dose. The maximum inhibition of growth (87.12%) was recorded due to *M. officinalis*, followed by *U. dioica* (80.19%), respectively. The least inhibition in growth was seen in the ethyl acetate fraction of *M. officinalis*, which accounted for only 34.65% inhibition at 0.25 mg/mL. Plant extracts can inhibit the formation of calcium oxalate crystals through various mechanisms, including modifying crystal surfaces, binding oxalate ions, or altering urine pH. Hydrophilic compounds like caffeic acid, luteolin, and rutin in aqueous extracts may enhance these effects by promoting diuresis and renal protection [35].

## Conclusion

This study elucidates the chemical profiles and evaluates the antioxidant and anti-lithiatic activities, of *Melissa officinalis* L. and *Urtica dioica* L., two Algerian medicinal herbs with longstanding traditional use. The choice of extraction solvent markedly influenced the yield of phenolic compounds, with water proving more effective for *U. dioica*, whereas ethyl acetate yielded richer extracts from *M. officinalis*.

LC-MS/MS analysis revealed distinct and complex phytochemical profiles in the two species, varying across solvent fractions. The ethyl acetate extract of *M. officinalis* was abundant in myricetin, caffeic acid, oleanolic acid, and quercetin-3-glucoside, whereas the aqueous fraction contained higher levels of water-soluble compounds such as catechin and *β*-carotene. Flavonoids identified in *M. officinalis* encompassed five subclasses, contributing to a range of biological activities, including antioxidant effects. In contrast, *U. dioica* exhibited a different phytochemical spectrum, characterised by higher concentrations of catechins, sinapic acid, and vanillin. *M. officinalis* contained a broader variety of flavonoid subclasses, including flavanones and complex glycosides. These differences in compound solubility influenced their distribution between aqueous and organic phases. Antioxidant activity, assessed by DPPH, ABTS, and FRAP methods, was most pronounced in the ethyl acetate fraction of *M. officinalis*, while its aqueous extract also showed a dose-dependent inhibition of calcium oxalate crystallisation.

These results confirm the traditional uses of both plants and highlight their potential as natural antioxidant and anti-lithiasis agents.

## Supplementary information

Supplementary data are available free of charge at http://cjm.ichem.md as PDF file



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