Phenolic Compounds from Moroccan *Retama monosperma* L. Boiss, *Berberis vulgaris* L. and *Ricinus communis* L.: Characterization, Antioxidant Activity and Performance Criteria of the Validated Method Using UHPLC/DAD/ESI-MS

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Abstract: *Components of medicinal plants have many pharmacological activities, including antioxidant activity, playing an important role in limiting oxidative stress that can cause several damages. This paper characterizes polyphenols of* Retama monosperma *L.,* Berberis vulgaris *L. and* Ricinus communis *L. plant extracts and evaluates their antioxidant activity by DPPH, conjugated diene and TBARS assay. To ensure the quality of analytical results, this paper presents performance criteria of the validated method using UHPLC/DAD/ESI-MS. Regarding method validation, the results confirm different used tests and evaluate detection and quantification limits. Concerning the characterization and study of antioxidant activity, realized testing showed that* R. monosperma *is rich in isoflavone, flavone and flavonol. For* R. communis*, we notice the presence of rutin as a major compound. Meanwhile,* B. vulgaris *contains significant amounts of gallic acid and* p*-coumaric acid. These plant extracts have high antioxidant activity due to the presence of phenolic compounds.*

Keywords: *polyphenol; antioxidant activity; method validation; UHPLC/DAD/ESI-MS*

■ INTRODUCTION

Since ancient times, many plants have attracted interest as sources of natural products [1]. Various plants have been used not only as a food source but also as medicine. The benefits of these practices are known to be supported by many scientific studies [2-4]. It is known that the vast majority of active ingredients in medicines are obtained from plants. Phytotherapy is once again in the spotlight because of the possible adverse effects of synthetic drugs and the multiple benefits obtained from plant-based medicines [2]. Medicinal plants produce several secondary metabolites like phenols, flavonoids, quinones, and tannins with numerous promising pharmacological activities, such as antioxidant [5-9], anti-inflammatory [10], anti-allergic [11], anticancer, antitumor [12-14], and anti-atherosclerosis [15]. They also provide cardiovascular protection [16]. More specifically, polyphenolic compounds are the most studied plant secondary metabolites due to their abundance and possible positive effects on human health [17]. They have received considerable attention in recent years

because they are considered high-added-value molecules due to their antioxidant and antimicrobial effects [18-19]. Their potential use in the formulation of cosmetics, and as an alternative to chemical food additives, has drawn the attention of a number of researchers. Some of these compounds have also been explored in the packaging and textile industries [20].

In this paper, we will be focusing on antioxidant activity, which acts against oxidative stress. In the human body, environmental radiation splits water to form hydroxyl radicals, and normal metabolism produces oxygen radicals [21]. Oxidative stress is an imbalance between reactive oxygen species formation and antioxidant defense mechanisms. If cellular antioxidants do not eliminate free radicals, the latter can react with different macromolecules [22-23]. At their high concentrations, free radicals attack and damage proteins, lipids, and nucleic acids, thereby causing many health problems. Over time, free radicals can cause a negative chain reaction in the human body, which can block the action of key enzymes, destroy the cell membrane, prevent normal cell division, avoid cellular processes necessary for proper body functioning, and block energy production [24]. Also, these free radicals cause DNA damage and lipid peroxidation, leading to cancerous cells [21]. The human body possesses a natural defense system against these free radicals [21-22]. Once the excessive generation of free radicals attacks the internal antioxidant defense system, external antioxidants are needed to prevent oxidative damage [25]. Antioxidants are considered molecules that prevent the formation of free radicals and seek to neutralize or repair the damage they cause [24].

The biological activities of many phytochemicals are attributed to their antioxidant properties [21]. Experimental and epidemiological evidence shows that dietary antioxidants, such as flavonoids and other phenolic compounds, are also important components of the body's defense [21]. Several studies support a direct link between the antioxidant properties of extracts and the medicinal benefits of plants and their potential use as an alternative to chemical preservatives [26]. Phenolic antioxidants have been reported to inhibit DNA fragmentation. Studies on animal and cell cultures confirm the anti-cancer effects of antioxidants; epidemiological studies show that high consumption of antioxidant-rich foods is inversely related to cancer risk [27]. Evidence from various *in vitro* studies supports a potential protective role for dietary polyphenols in the prevention of cardiovascular disease, neurodegenerative disease, cancer, diabetes, inflammation-related, and infectious diseases [28]. In addition to their importance in the diet, antioxidants can also contribute to the stability and taste of food products. From a plant specialist's point of view, their role in the plant as a defense against biotic and abiotic stresses must also be taken into account [21]. All these benefits justify the considerable interest in researching safe natural antioxidants which are in high demand by the pharmaceutical industry and as food preservatives [29].

Therefore, it is important to study the phenolic composition of various plant extracts and assess their antioxidant activity in order to provide scientific results that would greatly support different sectors such as pharmaceuticals, cosmetics, and food industry. This revelation would be an excellent addition to the scientific literature.

It is within this context that this paper falls. It presents the chemical composition of extracts of three medicinal and aromatic plants: *Retama monosperma*, *Ricinus communis*, and *Berberis vulgaris* followed by an evaluation of the antioxidant activity of their phenolic compounds. For nutritional purposes, many scientific studies have been conducted on the chemical composition of these plant extracts grown in various parts of the world and which are of great interest [30-34]. However, this paper is the first to characterize different extracts of these plants growing in Morocco. Therefore, a lot of effort has been engaged in developing different techniques and methods for the identification of phenolic compounds from natural resources. In most cases, these compounds are analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) and HPLC-diode-array detector (HPLC-DAD). As an important step to ensure the quality of analytical results and to provide researchers with performance criteria, the authors followed a process for

validation of the method chosen for the characterization and quantification of polyphenols before use. The validation process was focused on six polyphenols known as antioxidant standards, such as gallic acid, vanillic acid, *p*-coumaric acid, rutin, quercetin, and genistein [35-40].

This paper details the tests that were carried out, such as specificity, linearity, repeatability, intermediate precision, detection limit, quantification limit, and recovery. It also details the statistical methods used because they are often perceived as a constraint since they are generally poorly used by analysts. The objective of publishing this paper, with supplementary material, is also to provide people wishing to perform a method validation with a document that describes in detail the followed approach, avoiding them to search several references to first understand the experimental plan to be implemented and several others to subsequently find the statistical methods to apply.

■ EXPERIMENTAL SECTION

Materials

Chemical materials

The materials used in this study were methanol (HPLC grade ≥ 99.9% from Honeywell Riedel-de Haen, Germany) used as solvent B and 0.1% formic acid (98% for LC-MS, Merck Germany) aqueous solution (ultra-pure water from Pure Lab) used as solvent A. The gallic acid was purchased from Merck (Germany), and the other phenolic compounds (vanillic acid, *p*-coumaric acid, rutin, quercetin, and genistein) were purchased from Sigma-Aldrich (USA). The 2,2-diphenyl-2'-picrylhydrazyl (DPPH), linoleic acid, ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid, and trichloroacetic acid (Sigma-Aldrich, USA) were used as an antioxidant reagent and the butylated hydroxytoluene (BHT, Merck Germany) as antioxidant standard.

Plant materials

R. monosperma flowers and seeds were collected in February and April 2017, respectively, from Al-Haouzia forest in the region of El Jadida-Morroco. *R. communis* leaves were collected in February 2018 in the region of El Jadida-Morocco. These plant materials were identified by Dr. Fennane from the Scientific Institute of Rabat, Morocco. A voucher specimen (77816 RAB) was deposited in the Herbarium of the Institute. For the *B. vulgaris,* root was collected in March 2019 in the region of Marrakech-Morocco. This plant material was identified by Dr. Ouhamou from the Faculty of Sciences, Cadi Ayyad University, Marrakech, Morocco.

Instrumentation

The instrumentations used in this study were chromatographic separation performed on Dionex Ultimate 3000 UHPLC-DAD system (CA, USA), equipped with a quaternary pump (HPG-3400RS), an autosampler (WPS-3000TSL), and a column oven (TCC-3000). A Vertex plus C18 reversed-phase column (250 × 4.6 mm, Eurospher II 100-5) provided by Knauer was used for the proposed method. The mass spectrometer was a TSQ Endura (Thermo Fisher Scientific) triple quadrupole equipped with heated-electrospray ionization (H-ESI).

Procedure

Extraction

The flowers (600 g) and seeds (400 g) of *R. monosperma* were air-dried for two weeks. The extraction was performed three times by maceration (room temperature, 3 d) with 2 L of *n*-hexane to remove lipophilic compounds. After evaporation of *n*-hexane under vacuum, the resulting mark was extracted three times by maceration with 2 L of ethyl acetate (room temperature, 3 d) for flowers and 2 L of diethyl ether for seeds (room temperature, 3 d). The resulting extract was then evaporated using a rotary evaporator.

R. communis leaves (200 g) and *B. vulgaris* roots (300 g) were air-dried for two weeks. Each sample was extracted using Soxhlet and methanol as solvent. The extracts were evaporated under reduced pressure to give methanol crude extracts. This later was solubilized in water and extracted with ethyl acetate. The resulting extract was then evaporated using a rotary evaporator.

UHPLC-DAD-ESI/MS method

The separation gradient was created using solvent A (0.1% formic aqueous solution) and solvent B (methanol), as shown in Table 1. The mobile phase flow rate was 1 mL/min. The injection volume was 10 µL, and

Table 1. UHPL-DAD separation gradient

Time (min)	% of solvent B	Time (min)	% of solvent B
0	5	18	54
3	25	22	54
6	25	26	95
9	37	29	95
13	37	29.15	5
		31	5

the wavelength was 280 nm. For the LC-MS experiment, negative mode was used. Sheath gas, ion sweep gas, and auxiliary gas were nitrogen (purity > 99.98%) at flow rates of 65, 0, and 40 arbitrary units (a.u.), respectively. The vaporizer temperature and ion transfer tube temperature were set at 350 °C. The electrospray voltage was set at −2.5 kV. Full scan MS acquisition mode (*m/z* 100–1000) in Q1 (mass resolution of 0.7 *m/z* full-width half maximum (FWHM)) with a scan time of 0.5 s was used [41].

Antioxidant activity

DPPH-radical scavenging activity. Free radical-scavenging activity of *R. monosperma*, *B. vulgaris*, and *R. communis* extracts was evaluated using a modified DPPH method. One milliliter of concentrations of samples (5–100 µg/mL) was added to 1 mL of DPPH solution (40 µg/mL), and the mixture was incubated for 30 min [42]. Afterward, the absorbance was measured at 517 nm in a UV spectrophotometer. BHT, rutin, gallic acid, and quercetin were used as a standard antioxidant. Scavenging activity was expressed as IC_{50}, an effective concentration in µg/mL of samples or standard that reduces the absorbance of DPPH by 50 % when compared with negative control. The experiment was carried out in triplicate.

Conjugate diene scavenging activity. Conjugate diene scavenging activity was determined by UV absorbance [43]. A linoleic acid emulsion was prepared in tampon phosphate at pH 7 (10 mM; 10 mL) mixed with the linoleic acid (5.96 µL) and tween 20 (0.1%; 10 mL). Linoleic acid emulsion (1 mL) was added to various concentrations of studies extracts (5–100 µg/mL) and 100 µL of $CuSO_4$ (1.6 g/L). After that, the mixture was incubated at 37 °C in the dark for 1 h. To stop the reaction, 10 µL of EDTA and 10 µL of BHT (1 mg/mL) were added to the mixture. Then, the absorbance was measured at 234 nm. The conjugate diene scavenging activity was calculated using Eq. (1).

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

A_0 is the absorbance of the control (sample without extracts), and A_1 is the absorbance of the sample. The results were expressed as IC_{50}. The experiment was carried out in triplicate.

Thiobarbituric acid-reactive substances assay. The thiobarbituric acid-reactive substances (TBARS) assay determined the inhibition of lipid peroxidation according to Ohkawa method [44] with some modifications [45]. A 1 mL of linoleic acid emulsion was added to various concentrations of extracts (5–100 µg/mL) and 100 µL of $CuSO_4$ (1.6 g/L), and then was left to incubate at 37 °C in the dark for 3 h. The reaction was stopped by putting the mixture of products and reagents in ice and adding 10 µL of EDTA (20 mM). Then, 1 mL of TBA (0.78%) and 1 mL of trichloroacetic acid (TCA 20%) were added to the mixture which was incubated at 95 °C in the dark for 45 min. The *n*-butanol (0.8 mL) was added to the mixture. The absorbance was measured at 532 nm in a UV spectrophotometer. The estimation of TBARS was calculated using Eq. (1). Scavenging activity was expressed as IC_{50}. The experiment was carried out in triplicate.

Method validation

As an important step to ensure the quality of analytical results and to provide researchers with performance criteria, the authors followed a process for validation of the method chosen for the characterization and quantification of polyphenols before use. The validation process was focused on six polyphenols known as antioxidant standards, such as gallic acid, vanillic acid, *p*-coumaric acid, rutin, quercetin, and genistein. Method validation was carried out using the five extracts that contain one or two compounds of interest (Table 2).

The experimental plan of method validation is presented in Table 3. It concerns several studies such as specificity, linearity, repeatability, intermediate precision, detection limit, and quantification limit. As an example, recovery was studied using *R. monosperma*

ethyl acetate extract from the flowers. The choice of the different concentration levels used was made to cover the concentration range of the majority of samples.

■ RESULTS AND DISCUSSION

Characterization of Phenolic Compounds by UHPLC/DAD/ESI-MS

A total of 14 phenolic compounds have been tentatively identified based on their wavelength of maximum UV absorption and mass spectrometry (MS) fragments in the negative mode corresponding to these peaks, alongside literature data on the chemical composition of *Retama*, *Berberis*, and *Ricinus* genus.

The chromatographic profile of *R. communis* methanol extract (RM) showed the presence of phenolic acid (peak 2), ellagitannins, members of the tannin family, are characterized as hydrolyzable conjugates containing one or more hexahydroxydiphenoyl (HHDP) group(s) to esterify a sugar like galloly derivative (peaks 4–6), and flavonoid glycosides like rutin and quercetin (peaks 10 and 11) (Table 4, Fig. 1).

The UHPLC/DAD/ESI-MS results of *B. vulgaris* ethyl acetate showed the predominance of pyrogallic acid (peak 1) followed by vanillic acid and *p*-coumaric acid; however, in methanolic extracts in the same species, we notice the predominance of gallic acid (peak 3) followed by *p*-coumaric acid (Table 4, Fig. 1). Concerning *R. monosperma* extracts, the chromatographic profile of flower ethyl acetate extracts showed the predominance of genistein as isoflavone (peak 13) followed by apigenin (peak 14) as a flavone. For diethyl ether extracts of *R. monosperma* seeds, we can see the taxifolin flavanonols (peak 8) as a major compound, followed by genistein (peak 13) (Table 4, Fig. 1).

Table 2. Information about the matrixes used to validate the method

Matrix	Part of the plant	Nature of the extract	Identified compounds
*Retama monosperma**	Flowers	Ethyl acetate	Genistein
Berberis vulgaris	Roots	Methanol	*p*-Coumaric acid
		Ethyl acetate	Vanillic acid
Ricinus communis	Leaves	Methanol	Gallic acid and rutin
		Ethyl acetate	Gallic acid

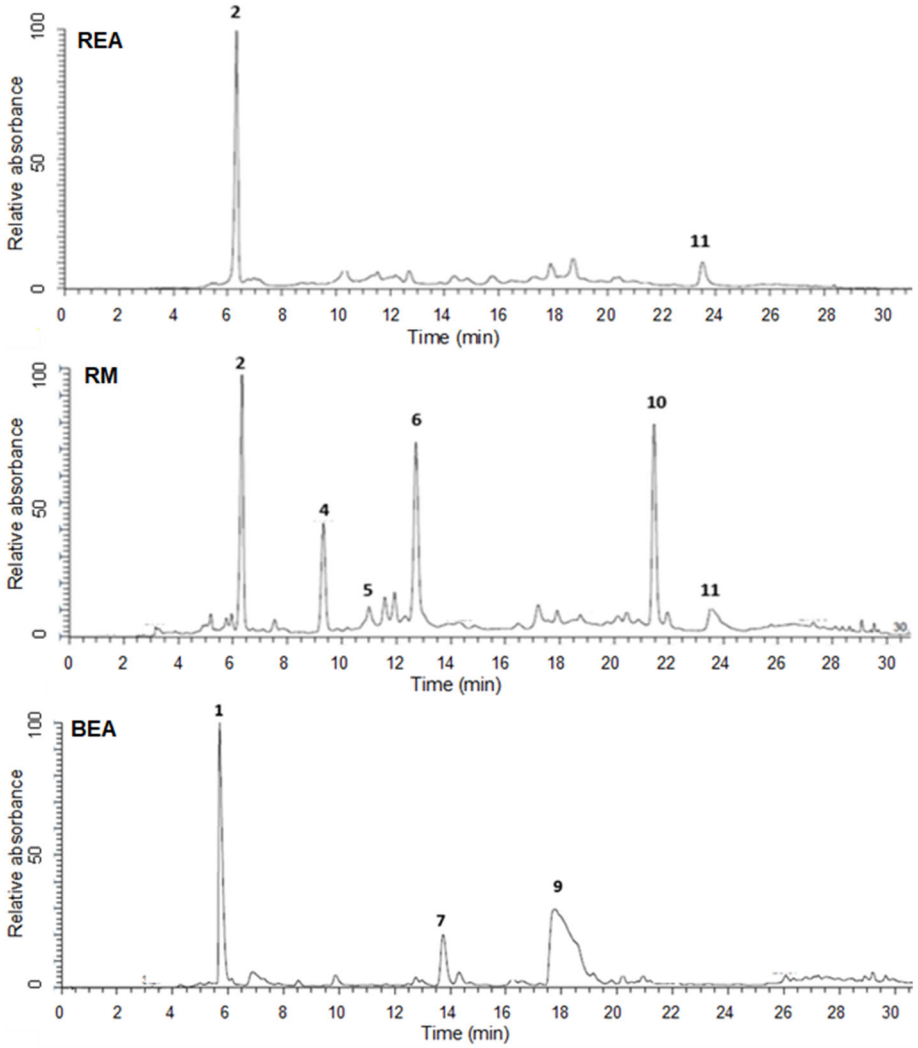
*In Morocco, Zefzoufi et al. [34] revealed that the diethyl ether extract of flowers and ethyl acetate extract of seeds of *R. monosperma* rich in flavonoid compounds such as genistein, quercetin, kaempferol. Other extracts of this plant are used in this paper

Table 3. Experimental plan of method validation

Parameter		Experience
Specificity		Method 1: Each extract sample (Table 2) was analyzed using UHPLC-DAD-ESI/MS [46]. Method 2: Some authors use the recovery [47-48] method to prove the specificity of the method. The experience carried out to perform the recovery test is detailed in the last row of the table.
Linearity		Three series of multi-standard solutions at 5 concentration levels were used (10, 50, 100, 150 and 200 mg/L). Three repetitions for each level of each series were performed by UHPLC-DAD [49].
Detection limit Quantification limit		Ten repetitions for the control sample (methanol) were performed by UHPLC-DAD [48].
Precision	Repeatability	Two concentration levels (50 and 200 mg/L) of multi-standard solution were prepared by the same operator and analyzed by UHPLC-DAD. Ten repetitions were performed for each level on the same day [48].
	Intermediate precision	Every day for 3 d, a series of multi-standard solutions with 5 concentrations levels (10, 50, 100, 150 and 200 mg/L) was prepared by the same operator. Three repetitions for each level of each series were performed by UHPLC-DAD [49].
Recovery		Six samples of the ethyl acetate extract of *Retama monosperma* flowers were used to perform the recovery test. The final concentrations of the standard (Genistein) added are as follows: 0, 10, 50, 100, 150, and 200 mg/L. Three repetitions were performed for each sample using UHPLC-DAD [50].

Table 4. Tentatively identification of phenolic compounds (with their percentage area) from three medicinal plants using UHPLC/DAD/ESI-MS

Peak number	Rt	$(M-H)^-$ m/z	MS fragments	UV_{max}	Identified compound	Molecular formula	Ref.	REA (%)	RM (%)	BEA (%)	BM (%)	RSDE (%)	RFEA (%)
1	5.9	125	-	270	Pyrogallol	$C_6H_6O_3$	-	-		58.74	-	-	-
2	6.1	331	169	270, 310	Galloyl-glucoside	$C_{13}H_{16}O_{10}$	[57]	70.82	20.15	-	-	-	-
3	6.5	169	125	272	Gallic acid	$C_7H_6O_5$	[57]	-		-	38.07	-	-
4	9.3	483	313, 169	256, 310	Digalloyl-glucoside	$C_{20}H_{20}O_{14}$	[57]	-	9.33	-	-	-	-
5	11.0	635	465, 283, 169	270, 310	Trigalloyl-glucoside	$C_{27}H_{24}O_{18}$	[57]	-	2.33	-	-	-	-
6	12.7	633	463, 169	269, 310	Galloyl-HHDP-glucoside	$C_{27}H_{22}O_{18}$	[57]	-	25.83	-	-	-	-
7	13.8	167	-	261, 295	Vanillic acid	$C_8H_8O_4$	-	-		10.50	-	-	-
8	18.1	315	-	228, 290	Taxifolin	$C_{15}H_{12}O_7$	[58]	-		-	-	71.32	-
9	18.6	163	-	310	*p*-Coumaric acid	$C_9H_8O_3$	-	-	-	30.02	45.17	-	-
10	21.8	609	463, 301	257, 357	Rutin	$C_{27}H_{30}O_{16}$	[59]	-	25.55	-	-	-	-
11	23.6	447	301	255, 356	Quercitrin	$C_{21}H_{20}O_{11}$	[59]	10.20	3.10	-	-	-	-
12	26.7	301	-	355, 368	Quercetin	$C_{15}H_{10}O_7$	[34]	-	-	-	-	20.05	-
13	27.1	269	-	261, 302_{sh}	Genistein	$C_{15}H_{10}O_5$	[34]	-	-	-	-	-	74.35
14	27.7	269	-	236, 336	Apigenin	$C_{15}H_{10}O_5$	[51]	-	-	-	-	-	21.02



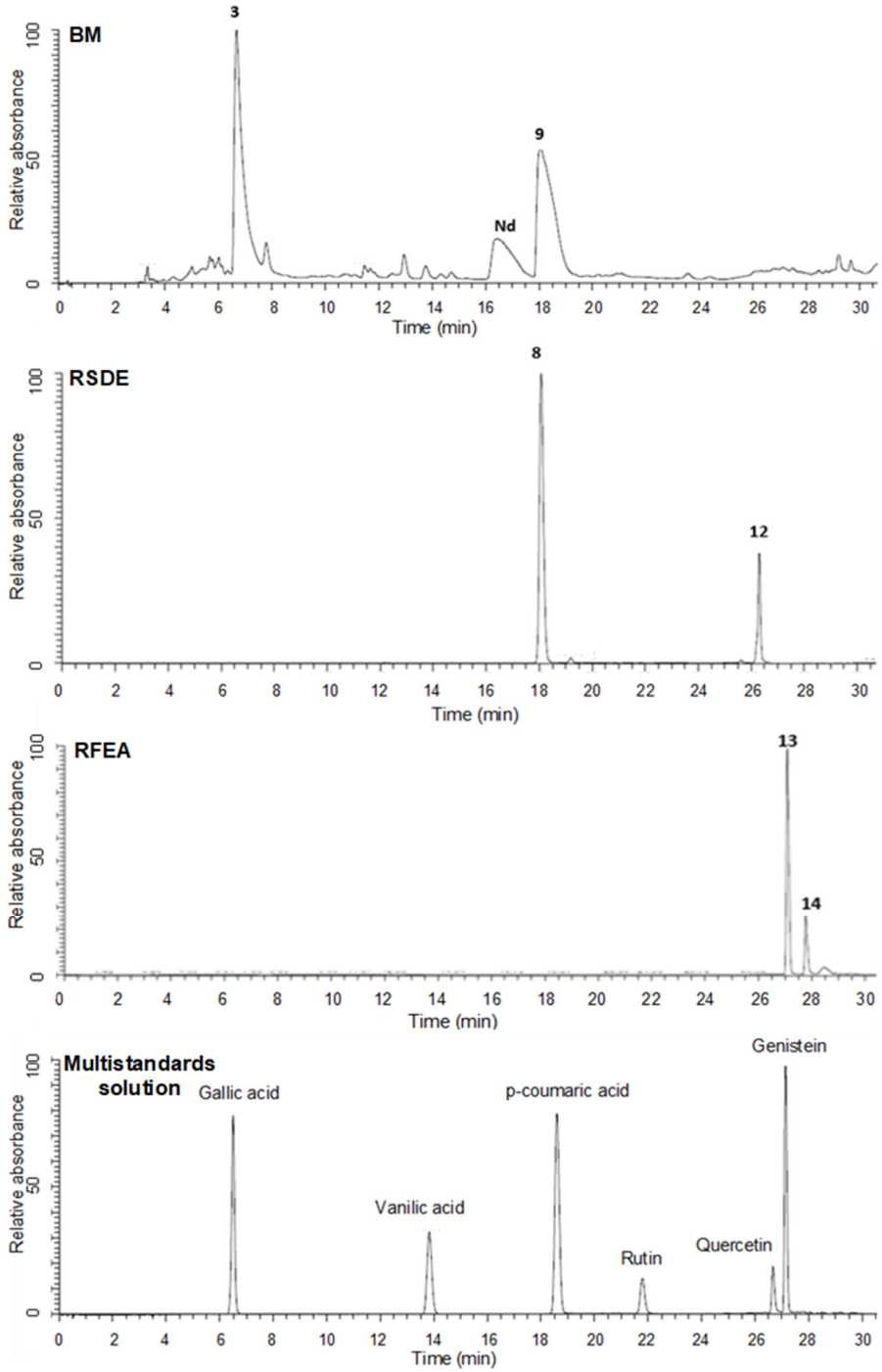


Fig 1. Chromatographic profile of *R. communis* methanolic extract (RM), *R. communis* ethyl acetate extract (REA), *B. vulgaris* methanolic extract (BM), *B. vulgaris* ethyl acetate extract (BEA), *R. monosperma* flowers ethyl acetate extract (RFEA), *R. monosperma* seeds diethyl acetate extract (RSDE) and multi-standards solution at 280 nm

In this study, we tentatively identified in three medicinal plants diverse phenolic compounds like phenolic acid (gallic acid, vanillic acid, *p*-coumaric acid), isoflavone (genistein), flavone (apigenin), flavanonols (taxifolin), flavonol (quercetin), glycoside flavonol (rutin and quercitrin), and ellagitannins (galloyl-HHDP-glucoside).

For *Retama* genus, based on the literature, we found

our results closely similar to other papers. Researchers identified genistein, luteolin, apigenin, and rutin in aqueous extract of *R. monosperma* growing in Spain [51]. Recently, researchers reported the presence of taxifolin and quercetin in ethyl acetate seeds extract and genistein and apigenin in diethyl ether flower extract of Moroccan *R. monosperma* [34]. In our study, we identified genistein as a major compound, followed by apigenin in another extract (ethyl acetate extract) of *R. monosperma* flowers.

For *Ricinus* genus, we found dissimilarities in chemical composition between our results and the literature. For example, researchers reported five phenolic compounds isolated from *R. communis* growing in Vietnam such as gallic acid, vanillic acid, kaempferol-3-*O*-β-D-glucopyranoside, and kaempferol-3-*O*-β-D-xylopyranoside [52]. Another research determined the presence of gallic acid, genistic acid, vitexin, naringenin and rutin in leaves of *R. communis* from Tunisia [53]. In 2016, researchers reported the presence of some alkaloids in the same species as ricinine and bufotenine *O*-glucoside. To our knowledge, this paper is the first to identify digalloly-glucose, trigalloly-glucose, galloly-HDDP-glucose, rutin, and quercitrin in *R. communis* native to Morocco.

For *Berberis*, many studies focused on the identification of *B. vulgaris* alkaloid compounds like berberine, which is known as an important major compound in this plant. In Morocco, researchers reported the presence of some alkaloids in *B. vulgaris* dichloromethane extract such as berberine, palmatine, and epi berberine [54]. To our knowledge, this paper is the first to identify pyrogallol and *p*-coumaric acid in *B. vulgaris* from Morocco. These observed differences in chemical profiles can be explained by the geographic origin of the species [55], the extraction method (maceration in our case), the extraction solvent [56], and the part of the plant used for the preparation of the extracts.

Antioxidant Activity

Natural antioxidants are currently the subject of numerous studies because they can reduce the harmful effects of free radicals in neurodegenerative diseases and cardiovascular, arthritis, cancer, and autoimmune diseases in which oxidative stress is incriminated [60]. Numerous works carried out on the anti-free radical activity of plant extracts have shown that phenolic compounds and, more particularly, flavonoids are recognized as potentially antioxidant substances with the ability to trap free radical species and reactive forms of oxygen. The IC_{50} value represents the concentration of extract that neutralizes or reduces 50% of free radicals. The lower the IC_{50}, the more the extract has a powerful antioxidant potential. Therefore, we evaluated the antioxidant activity of RFEA, RSDE, REA, RM, BEA and BM by three tests, conjugated diene scavenging activity, TBARS assay and DPPH (Table 5). We used rutin, gallic acid, quercetin, and BHT as standards.

DPPH Scavenging Activity

REA showed a higher antiradical activity (IC_{50} = 12.5 µg/mL) followed by RFEA, RM compared to standards BHT, quercetin and gallic acid, but still less than rutin standard. However, the antioxidant capacity of RSDE, BM and BEA were moderate compared to the other extracts and standards (Table 5).

Conjugated Diene Scavenging Activity

The conjugate diene scavenging activity of REA was more effective than BHT, quercetin and gallic acid, but it is similar to rutin. BEA showed moderate antioxidant activity followed by RM, BM, RFEA, and RSDE with IC_{50} values of 59.02, 60.09, 72.47, and 98.08 µg/mL, respectively (Table 5).

TBARS Assay

TBARS assay of RM was significantly greater, followed by BEA and BM but still less than four standards. However, the inhibition of lipid peroxidation of RFEA, RSDE and REA was lower than in other samples (Table 5). Based on the literature, several studies reported the antioxidant activity of these three plants' medicinal extracts. Concerning *R. communis*, researchers revealed that the antioxidant capacity of butanol extract of the aerial part of *R. communis* growing in Pakistan (IC_{50} = 140 µg/mL) is higher than that of ethyl acetate extract (IC_{50} = 190 µg/mL) [61]. In 2009, a paper described that leaves methanolic extract of *R. communis* has a strong antiradical activity with IC_{50} of 4.6 µg/mL

Table 5. Antioxidant activity of standards and samples in terms of IC_{50} (μg/mL) values with $p < 0.05$

	DPPH	Conjugated diene	TBARS
		Standards	
BHT	28.41 ± 0.06	36.55 ± 0.28	40.06 ± 0.15
Gallic acid	30.55 ± 0.02	36.52 ± 0.12	40.08 ± 0.15
Quercetin	35.65 ± 0.25	30.05 ± 0.15	38.42 ± 0.05
Rutin	10.02 ± 0.05	26.02 ± 0.11	30.05 ± 0.05
		Samples	
RFEA	19.59 ± 0.11	72.47 ± 0.25	68.12 ± 0.12
RSDE	84.95 ± 0.16	98.08 ± 0.50	82.26 ± 0.12
REA	12.50 ± 0.11	29.59 ± 0.11	69.02 ± 0.11
RM	20.45 ± 0.11	59.02 ± 0.11	50.30 ± 0.11
BEA	38.05 ± 0.15	44.12 ± 0.02	52.89 ± 0.10
BM	55.32 ± 0.14	60.09 ± 0.22	55. 90 ± 0.15

RFEA: *Retama* flowers ethyl acetate; RSDE: *Retama* seeds diethyl ether; REA: *Ricinus* ethyl acetate; RM: *Ricinus* methanol; BEA: *Berberis* ethyl acetate; BM: *Berberis* methanol

followed by ethyl acetate extract (IC_{50} = 6.04 μg/mL) [62]. For *R. monosperma*, previous work reported that the antiradical activity of ethyl acetate extracts of seeds (IC_{50} = 15.13 μg/mL) was significantly higher than quercetin (IC_{50} = 19.43 μg/mL) and BHT standards (IC_{50} = 30.21 μg/mL) [34]. Regarding *B. vulgaris*, recently, a paper reported higher antioxidant activity using a DPPH assay of ethanol and ethyl acetate extract of *B. vulgaris* roots with IC_{50} of 69.65 and 77.75 μg/mL, respectively [63].

Performance Criteria of the Validated Characterization Method Using the UHPLC-DAD

Use of purity for the calculation of the real concentrations of the standards used

Preparation of the standard solutions required weighing a certain mass of the standards in powder form. Since the purity of standards is different from 100%, the calculation of the real concentrations is necessary. Table 6 shows the different concentrations used, taking their purity into consideration.

Specificity

The plant extracts and multi-standards solutions were analyzed by UHPLC/DA/DESI-MS, and each peak concerned was detected at 280 nm. Table 7 and Fig. 1 show the found results. As can be seen in Fig. 1, the chromatograms show that the separation of all six phenolic compounds was successfully achieved with good resolution. Additionally, no interfering peaks were observed.

Linearity

Linearity is the ability of a method to elicit test results that are proportional to analyte concentration within a given range. The range of the analytical method is the interval between the highest and lowest concentrations in which linearity has been confirmed. Many tests are used to validate method linearity. These tests are presented in Table 8.

The results showed that regression curves of each phenolic compound were found to be linear with R^2

Table 6. Real concentrations (mg/mL) of the standards used

Standards	Gallic acid	Vanillic acid	*p*-Coumaric acid	Rutin	Quercetin	Genistein
Level 1	9.90	9.80	10.00	9.59	9.90	9.90
Level 2	49.49	48.98	49.98	47.94	49.49	49.49
Level 3	98.98	97.97	99.96	95.88	98.98	98.98
Level 4	148.47	146.95	149.94	143.82	148.47	148.47
Level 5	197.96	195.94	199.92	191.76	197.96	197.96

Table 7. Quantitative analysis of phenols in different plant extracts using UHPLC-DAD-ESI/MS at UV 280 nm

Rt	$(M-H)^-$ *m/z*	Molecular weight	UV_{max}	Identified compound	Molecular formula	REA mg/L	RM mg/L	BEA mg/L	BM mg/L	RSDE mg/L	RFEA mg/L
6.50	169	170	272	Gallic acid	$C_7H_6O_5$				29.81		
13.81	167	168	261, 295	Vanillic acid	$C_8H_8O_4$			56.61			
18.57	163	164	310	*p*-Coumaric acid	$C_9H_8O_3$			10.36	11.84		
21.78	609	610	257, 357	Rutin	$C_{27}H_{30}O_{16}$		12.35				
26.66	301	302	355, 368	Quercetin	$C_{15}H_{10}O_7$					69.64	
27.13	269	270	261, 302_{sh}	Genistein	$C_{15}H_{10}O_5$						102.02

Table 8. Tests used to validate method linearity for the six compounds of interest

Standard		Gallic acid	Vanillic acid	p-Coumaric acid	Rutin	Quercetin	Genistein	Conclusion
Slope a*		23967.90	15588.78	34637.73	6532.57	5797.53	24364.42	-
Standard deviation of the slope S_a*		350.71	227.08	545.51	96.98	44.14	400.97	-
Intercept b*		37743.55	24710.59	70174.89	9438.12	5939.70	51282.24	-
Standard deviation of the intercept S_b*		42543.71	27265.38	66829.07	11396.44	5355.25	48640.90	-
Coefficient of determination R^2*		0.9994	0.9994	0.9993	0.9993	0.9998	0.9992	-
Cochran test	C	0.33	0.34	0.34	0.33	0.35	0.34	The homogeneity of variances is confirmed
	$C_{(0.95, 3, 14)}$	Between 0.55 and 0.60 according to the Cochran table						
	Acceptance criteria	There is a homogeneity of variances if $C_{calculated} \leq C_{(1-\alpha, s, nk-1)}$						
Test of intercept [64]	t	0.89	0.91	1.05	0.83	1.11	1.05	The line passes through the origin
	$t_{(0.975, 3)}$	3.18						
	Acceptance criteria	The line passes through the origin if $t_{calculated} \leq t_{(1-\alpha/2, k-2)}$**						
Test of the nullity of the slope [65]	t	68.34	68.65	63.50	67.36	131.32	60.76	
	$t_{(0.975, 3)}$	3.18						
	Acceptance criteria	The slope is null if $t_{calculated} \leq t_{(1-\alpha/2, k-2)}$**						The slope is different from zero, there is a linear relationship between x and y
Test of significance of the slope [64]	F	26011.31	23034.82	23098.18	24269.19	17330.50	22560.96	
	$F_{(0.95, 1, 43)}$	4.07						
	Acceptance criteria	The slope in not significance if $F_{calculated} \leq F_{(1-\alpha, 1, Nk-2)}$						
Test of significance of the regression [64]	F	4670.40	4712.56	4031.72	4536.86	17246.08	3692.10	
	$F_{(0.95, 1, 3)}$	10.13						
	Acceptance criteria	The slope is not different from zero if $F_{calculated} \leq F_{(1-\alpha, 1, k-2)}$						

•Notes: *Calculated using the LINEST function in Microsoft Excel; **One-tail student test; Equation of regression curve: y = (a × x) + b (y: area; x: concentration mg/L)/α: risk = 0.05/s: Number of series/n: number of repetitions per level in the series/k: number of concentration levels/N: number of repetitions per level all series combined. Details of the calculations performed are shown in the supplementary material

greater than 0.999. This value means that 99.9% of the variation in the concentration (within the range of the minimum and maximum concentrations taken into consideration) is expressed by the correlation.

Linearity validation was confirmed by: i) firstly, the Cochran test that confirmed the homogeneity of variances, ii) secondary, the intercept test according to which it can be concluded that the line passes through the origin, and iii) finally, by a number of t and F tests to which it can be concluded that the slope is different from 0 and there is a linear relationship between the concentration of the compound and the peak area.

Repeatability

The repeatability of the method was examined by analysis of two concentration levels by performing 10 repetitions. The results of this test are presented in Table 9.

Intermediate precision

The intermediate precision of the method was examined by analysis of 5 concentration levels three times for three days and calculation of the coefficient of variation and the intermediate precision. The found results are presented in Table 10.

Table 9. Repeatability study results for the six compounds of interest

Compound	Gallic acid		Vanillic acid		*p*-Coumaric acid		Rutin		Quercetin		Genistein	
Concentration (mg/L)	49.49	197.96	48.98	195.94	49.98	199.92	47.94	191.76	49.49	197.96	49.49	197.96
Average of 10 repetitions (mg/L)	50.42	196.51	50.40	195.32	51.32	198.62	48.98	190.45	48.53	190.24	51.09	197.05
Standard deviation of 10 repetitions (mg/L)	0.82	2.21	0.80	2.33	0.78	2.29	0.86	2.24	1.02	2.22	0.82	2.20
Coefficient of variation (CV)	1.63	1.12	1.60	1.19	1.52	1.16	1.75	1.18	2.09	1.16	1.61	1.12
Repeatability r	2.30	6.18	2.25	6.52	2.19	6.42	2.40	6.27	2.85	6.21	2.30	6.16
Acceptance criteria	If the calculated CV is less than 5%, the proposed method is repeatable.											
Conclusion	For the six phenolic compounds, the CV is less than 5%, which is acceptable. These results showed that the current method for quantification of the six phenolic compounds is repeatable.											

•Notes: Coefficient of variation: $CV = \frac{STD}{\bar{x}} \times 100$ Repeatability r [67]: $r = 2.8 \times STD$

STD: Standard deviation of 10 repetitions; $\bar{x}$: Average of 10 repetitions

Table 10. Intermediate precision study results for the six compounds of interest

Gallic acid	Concentration level (mg/L)	9.90	49.49	98.98	148.47	197.96
	Average of 9 repetitions (mg/L)	8.63	49.34	100.48	150.74	195.61
	Standard deviation of 9 repetitions (mg/L)	0.20	1.13	1.86	3.88	2.43
	Coefficient of variation (CV)	2.31	2.30	1.85	2.57	1.24
	Intermediate precision R	0.56	3.17	5.20	10.86	6.80
Vanillic acid	Concentration level (mg/L)	9.80	48.99	97.97	146.96	195.94
	Average of 9 repetitions (mg/L)	8.55	48.84	99.45	149.18	193.63
	Standard deviation of 9 repetitions (mg/L)	0.32	1.34	2.05	4.16	2.69
	Coefficient of variation (CV)	3.73	2.75	2.06	2.79	1.39
	Intermediate precision R	0.89	3.76	5.73	11.65	7.54
p-Coumaric acid	Concentration level (mg/L)	10.00	49.98	99.96	149.94	199.92
	Average of 9 repetitions (mg/L)	8.55	49.89	101.64	152.34	197.37
	Standard deviation of 9 repetitions (mg/L)	0.19	1.33	1.99	4.11	2.52
	Coefficient of variation (CV)	2.26	2.67	1.96	2.70	1.28
	Intermediate precision R	0.54	3.73	5.58	11.50	7.05
Rutin	Concentration level (mg/L)	9.59	47.94	95.88	143.82	191.76
	Average of 9 repetitions (mg/L)	8.42	47.66	97.40	146.05	189.46
	Standard deviation of 9 repetitions (mg/L)	0.27	1.13	1.80	4.02	2.40
	Coefficient of variation (CV)	3.16	2.37	1.85	2.75	1.27
	Intermediate precision R	0.75	3.16	5.05	11.25	6.72
Quercetin	Concentration level (mg/L)	9.90	49.49	98.98	148.47	197.96
	Average of 9 repetitions (mg/L)	9.24	49.35	99.90	149.56	196.75
	Standard deviation of 9 repetitions (mg/L)	0.25	1.20	2.92	4.40	5.41
	Coefficient of variation (CV)	2.72	2.44	2.93	2.94	2.75
	Intermediate precision R	0.70	3.37	8.19	12.33	15.14
Genistein	Concentration level (mg/L)	9.90	49.49	98.98	148.47	197.96
	Average of 9 repetitions (mg/L)	8.23	49.58	100.87	150.76	195.36

	Standard deviation of 9 repetitions (mg/L)	0.31	1.39	1.78	3.89	2.74
	Coefficient of variation (CV)	3.75	2.81	1.77	2.58	1.40
	Intermediate precision R	0.86	3.90	4.99	10.88	7.66
Acceptance criteria	If the calculated CV is less than or equal to 5 %, the intermediate precision of the proposed method is validated.					
Conclusion	It can be seen that all coefficients of variation are less than 5 %, which is acceptable. So, the intermediate precision of the method is validated.					
•Notes:	Coefficient of variation CV $= \frac{STD}{\bar{x}} \times 100$ Intermediate precision [67]: R = 2.8 × STD STD: Standard deviation of 9 repetitions; $\bar{x}$: Average of 9 repetitions					

Detection and Quantification Limits

The detection limit is the lowest concentration of the analyte that can be detected but not necessarily quantified. The quantification limit is the lowest concentration of the analyte that can be quantified at the experimental conditions. In the present paper, the limits were calculated using the graphic approach.

This approach can be applied to analytical methods that provide a graphic recording (e.g., chromatography) with background noise. This method requires the determination of [48]: i) h_{max} is the greatest difference in amplitude on the y-axis of the signal observed between two acquisition points, excluding drift, over a distance equal to twenty times the width at mid-height of the peak corresponding to the analyte, centered on its (analyte) retention time. An explanatory diagram for the calculation of the h_{max} is presented at the level of the reference [66-68]; ii) Factor R is the quantity/signal response factor expressed in height. This factor corresponds to the slope of the regression curve, representing the concentration as a function of the peak height. It was calculated using the same data recorded to perform the intermediate precision test (5 concentration levels analyzed by performing 3 repetitions per day for 3 d). Data used for the calculation of the detection and quantification limits are presented in Table 11.

Table 11. Data used for the calculation of the detection and quantification limits

Standard	Gallic acid	Vanillic acid	p-Coumaric acid	Rutin	Quercetin	Genistein
Retention time (min)*	6.46	13.79	18.55	21.75	26.64	27.11
Width at half height (min)*	0.12	0.22	0.19	0.25	0.16	0.11
h_{max}**	752.64	266.59	381.97	431.90	989.67	885.96
Factor R	3.18×10^{-04}	7.76×10^{-04}	3.35×10^{-04}	1.69×10^{-03}	1.29×10^{-03}	2.69×10^{-04}
Detection limit DL (mg/L)	0.72	0.62	0.38	2.11	3.84	0.71
Quantification limit QL (mg/L)	2.39	2.07	1.28	7.30	12.80	2.38

Notes: *Retention time and width at half height are average values calculated using data from the intermediate precision study (3 repetitions per day for 3 d) for the lowest concentration level; **h_{max}: Average of h_{max} of each repetition of the control sample (10 repetitions in total); Detection and quantification limits were calculated using the following equations [48]: DL = 3 × h_{max} × R, QL = 10 × h_{max} × R

Table 12. Results of the recovery test

Concentration level	Added concentration (mg/L)	Concentration after addition (3 repetitions average) (mg/L)	Recovered concentration (level X - level 1) (mg/L)	Recovery (%)	Recovery average (%)	100.63
Level 1	0.00	100.90	-	-	Standard deviation (%)	1.70
Level 2	9.90	111.04	10.14	102.44	Coefficient of variation (CV)	1.68
Level 3	147.98	247.51	146.62	99.08	$t_{(0.975,2)}$	4.30
Level 4	197.96	299.60	198.70	100.38	Confidence interval ±	4.21

Acceptance criteria	The CV must be less than 2 %, and the confidence interval must include the value 100 % [50]
Conclusion	The coefficient of variation is less than 2%, and the recovery average interval of confidence (CI = 100.63% ± 4.21) includes 100%, so the method is accurate.

•Notes:

Coefficient of variation: $CV = \frac{STD}{\bar{x}} \times 100$

STD: Standard deviation; $\bar{x}$: Recovery average

Recovery: $Recovery = \frac{RC}{AC} \times 100$

RC: Recovered concentration; AC: Added concentration

Confidence interval [64]: $\bar{x} - t(1-\frac{\alpha}{2}, v)STD/\sqrt{n} < m < \bar{x} + t\left(1-\frac{\alpha}{2}, v\right)STD/\sqrt{n}$

m: Confidence interval average

$t_{(1-\alpha/2, k-1)}$: $t_{critical}$ value, it is read on one-tailed Student table (with, α: risk = 0.05); v: degree of liberty, v = k – 1; k: number of recovered concentration levels

Table 13. Results of the specificity study further to the regression curve representing recovered concentrations as a function of added concentrations – Genistein in the EAE of *Retama monosperma* flowers

Slope a*	Standard deviation of the slope S_a*	Intercept b*	Standard deviation of the intercept S_b*	$t_{calculated}$ for the test of the hypothesis of "the slope is equivalent to 1"**	$t_{calculated}$ for the test of the hypothesis of "the intercept b equivalent to 0"***	Critical value of Student test $t_{(0.01, 1)}$
1.00	0.01	-0.08	1.61	0.03	0.05	63.66
Acceptance criteria	The slope of the regression curve is equivalent to 1 if $t_{calculated}$ is less than $t_{(\alpha, k-2)}$ read on a two-tailed Student table (with k: number of concentration levels; α: risk = 0.01) [48]					
	The intercept of the regression curve is equivalent to 0 if $t_{calculated}$ is less than $t_{(\alpha, k-2)}$ read on a two-tailed Student table (with, k: number of concentration levels; α: risk = 0.01) [48]					
Conclusion	The slope of the regression curve is equivalent to 1					
	The intercept of the regression curve is equivalent to 0					

•Notes: y =(a × x) +b (y: recovered concentration (mg/L); x: added concentration (mg/L)); *Calculated using the LINEST function in Microsoft Excel; **Calculated with the following equation [48]: $t = \frac{|a-1|}{S_a}$; *** Calculated with the following equation [48]: $t = \frac{|b|}{S_b}$

Table 14. Results of the specificity study further the two calibration curves (without matrix and with matrix) – Genistein in the EAE of *Retama monosperma* flowers

Calibration curve without matrix		Calibration curve with matrix		$t_{calculated}$**	Critical value of Student test $t_{(0,95,5)}$
Slope a*	Standard deviation of the slope S_a*	Slope a'*	Standard deviation of the slope $S_{a'}$*		
24349.22	156.18	24364.42	400.98	0.04	2.02
Acceptance criteria	The two slopes are equal if $t_{calculated}$ is less than or equal to $t_{(1-\alpha/2, k+k'-4)}$ read on one-tail Student table				
Conclusion	The two slopes are equal				

•Notes: *Calculated using the LINEST function in Microsoft Excel; Calibration curve without matrix y = (a × x) + b (y: peak area; x: concentration (mg/L)); Calibration curve with matrix y = (a' × x) b' (y: peak area; x: added concentration (mg/L)); **Calculated with the following equation [66]: $t_{calculated} = \frac{|a-a'|}{\sqrt{S_a^2+S_a'^2}}$; k is the number of the concentration levels used for the calibration curve without matrix; k' is the number of the concentration levels used for the calibration curve with matrix; α: risk = 0.1

Recovery

In this paper, we will deal with an example of the recovery test which concerns the matrix of *R. monosperma* flowers of the ethyl acetate extract. This test can be used to assess the accuracy of the method (Table 12) but also, by some authors [47], to confirm specificity (Table 13-14).

The concentration after addition was calculated using the calibration curve results for the linearity test. Further to the statistical tests carried out, of which the

results are presented in Tables 13 and 14, we can confirm that the method is specific.

■ CONCLUSION

Extracts characterization using UHPLC/DAD/ESI-MS showed i) the richness of *R. monosperma* of flavonol, isoflavone and flavone, ii) the presence of flavonol glycoside in *R. communis*, iii) the richness of *B. vulgaris* of galloyl-glucose and phenolic acids. These plant extracts have a high antioxidant capacity due to the presence of phenolic compounds. The higher capacity concerns *R. communis* methanolic extract with IC_{50} of 12.5, 29.59, and 50.3 µg/mL for DPPH assay, conjugated diene, and TBARS assay, respectively. As an important step to ensure the quality of analytical results, this paper presented the performance criteria of the validated method using UHPLC/DAD/ESI-MS and focusing on six polyphenols known as antioxidant standards (gallic acid, vanillic acid, *p*-coumaric acid, rutin, quercetin, and genistein). The results of all released tests in the process of method validation are very satisfactory. In short, the polyphenol content of the plants studied in this article makes them an important subject for future research realized by industries, such as pharmaceuticals and cosmetics, seeking to exploit natural products such as plants to develop products with high antioxidant activity.

■ AUTHOR CONTRIBUTIONS

Meriem Outaki, Manal Zefzoufi and Amal Sammama contributed equally to this work. Meriem Outaki prepared the experimental plan for the "method validation" part. Manal Zefzoufi prepared the experimental plan for the "antioxidant activity" part. Meriem Outaki, Manal Zefzoufi, Amal Sammama and Khadija El Gadali carried out the experiments in the laboratory and performed the necessary calculations. All the authors wrote the paper (each a part) and revised it. All authors agreed to the final version of this manuscript.

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