CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *VERBENA OFFICINALIS* AND *ALOYSIA CITRODORA* EXTRACTS OBTAINED BY TRADITIONAL AND LABORATORY METHODS

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The present study compares traditional and laboratory extraction methods applied to two plants from the Verbenaceae family, aiming for phenolic compound valorization. The decoction, and maceration were used as traditional methods, while alcoholic and water extractions were accomplished in laboratory conditions. The chemical composition of the Verbena officinalis and Aloysia citrodora extracts was established for the first time, from our knowledge, by Ultra-High-Performance Liquid Chromatography - Electrospray Ionization - High-Resolution Mass Spectrometry. The alcoholic extracts (obtained traditionally and in the laboratory) of both plants had remarkable antimicrobial activity both on planktonic cells and on biofilms.

Keywords: phenolic compounds, UHPLC-MS/MS, antimicrobial activity, *Verbenaceae*

1. Introduction

Verbena officinalis L. and *Aloysia citrodora* Palau (*Aloysia triphylla* Briton)[1] are two of the 250 species of plants in the *Verbenaceae* family, cultivated as ornamental plants, later being recognized as medicinal plants due to their biological activity [2].

The chemical analysis of these plants revealed the presence of several classes of compounds, primarily flavonoids and terpenic compounds [3, 4]. The main bioactive compounds in the composition of *Verbena officinalis* extracts are oleanolic acid, ursolic acid and their derivatives (4-epi-barbinervic acid, 2,3-

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dihydroxyurs-12-en-28-oic acid, and 3, 24-dihydroxyurs-12- en-28-oic acid), luteolin, pedalitin and apigenin[3, 5] while *Aloysia citrodora* has a composition rich in terpenic compounds such as limonene, 1,8-cineole, neral, geranial [6], linalool, α-pinene, β-caryophyllene, and phenolic compounds, as verbascoside salvigenin, eupatorin, eupafolin, luteolin[4, 7–10].

Due to the increased content of phenolic compounds that act on the bacterial cell wall and constituent proteins, literature data have shown that the alcoholic extracts of both plant species show growth inhibitory activity against some pathogenic microbial strains such as *Staphylococcus aureus*, *Salmonella* sp, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida* sp.[2, 11–15].

Conventional extraction methods of phenolic compounds are based on organic solvents or water at atmospheric pressure, while new methods use high pressures. Traditional extraction methods include maceration, percolation, infusion, Soxhlet extraction, decoction, and hydrodistillation. New extraction methods include ultrasonically assisted, microwave, or supercritical gas extraction [16].

In addition, an extract of *Verbena officinalis*' aerial parts showed anti-cryptosporidia activity in the infected immunocompromised mice, a better activity than the nitazoxanide, due to flavonoids, phenolic acids, phenylethanoids, and coumarins identified by LC-ESI-MS/MS [17]. Moreover, in a recent study, it was shown that luteolin-7-diglucuronide, the main constituent of an aqueous extract of *A. citrodora*, inhibited, by itself, the production of aflatoxin B1 by 57% [18].

The aim of the present study is the extraction of bioactive compounds both by traditional methods (maceration, decoction) and by methods applicable exclusively in the laboratory (alcoholic extracts and aqueous extracts) and their chemical characterization by spectrophotometric and chromatographic techniques. The extracts' antioxidant and antimicrobial activity were also assessed.

2. Material and methods

2.1. Plant material

Verbena officinalis was collected from Cobia, Dambovita County (Romania), in July 2016, and an exemplar was deposited in the herbarium of the "D. Brandza" Botanical Garden of the University of Bucharest with the number [BUC 405991]. The plants were manually sorted and dried. *Aloysia citrodora* was purchased from Fares BioVital Laboratories as a dried plant sealed in paper bags. The aerial parts of both plants were manually ground and stored at room temperature.

2.2. Extraction procedure

Alcoholic extracts (AC-ALC and VO-ALC) were obtained by vortexing 5 g of plant with 30 mL of 70% ethanol for 5 minutes, then centrifuged at 3900 rpm at room temperature for 10 minutes.

Aqueous extracts (AC-AQ and VO-AQ) were obtained by mixing 10 g of plant with 90 mL of distilled water. The obtained mixtures were left under magnetic stirring at room temperature for 24 hours and then vortexed at 3900 rpm for 5 minutes.

Macerates (AC-M and VO-M) and decoctions (AC-D and VO-D) were prepared according to Pharmacopeia methods [19]. Briefly, they were obtained by mixing 10 g of plant with 10 mL of 70% ethanol or, respectively, with 10 mL of distilled water.

All the obtained extracts were filtered on cotton and brought to 100 mL final volume.

2.3. Chemical composition

2.3.1. Determination of Total Phenolic Content. Total phenolic compounds content (TPC) was carried out by the Folin-Ciocâlteu method using a Shimadzu UV-1800 UV-Vis spectrophotometer (λ = 746 nm). A calibration curve was drawn with gallic acid standard solutions of concentrations between 5 and 150 mg/L. The equation of the obtained calibration curve was $Y= 0.0059X + 0.0083$, with coefficient of determination $R^2 = 0.9991$.

TPC content was expressed as g gallic acid equivalents in 100 g plant [20].

2.3.2. Determination of Total Flavonoids Content. The total flavonoid content (TFC) was established by the spectrophotometric method with aluminum chloride (λ= 430 nm). The flavonoid content expressed in mg quercetin/100 g dry plant was calculated using the calibration curve for 0-120 µg/mL quercetin concentrations [20]. The equation of the obtained calibration curve was $Y= 0.339X - 0.0008$, with coefficient of determination $R^2 = 0.9954$.

2.3.3. Phenolic Compounds Analysis by UHPLC-DAD-ESI/MS. The analysis was performed using an UltiMate 3000 ultra-high pressure liquid chromatograph (UHPLC) (ThermoFisher Scientific, Bremen, Germany) equipped with quaternary pump, thermostated column compartment, autosampler, DAD detector coupled with a Q Exactive™ Focus Hybrid Quadrupole-OrbiTrap mass spectrometer equipped with heated electrospray ionisation (HESI) probe (ThermoFisher Scientific, Bremen, Germany). Chromatographic and mass spectrometric parameters were set according to Ciucure [21]. Phenolic compounds were identified and quantified according to mass spectra, accurate mass and characteristic retention time, against external standard solutions, covering a calibration range between 0 and 10 mg/L.

2.5. Determination of Trolox Equivalent Antioxidant Capacity (TEAC)

The determination of the antioxidant activity by the TEAC method was carried out by highlighting the neutralization potential of the cation radical ABTS•+ (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate), expressed as Trolox equivalents. The ABTS•+ radical was generated by the oxidation of ABTS by sodium persulfate [22]. A Trolox concentration vs absorbance of ABTS•+ was drown. The equation of the obtained calibration curve was Y= 28.094X, with coefficient of determination $R^2 = 0.9971$.

2.6. Antimicrobial Activity

All strains used were provided by the Microbiology Department Collection, Faculty of Biology, University of Bucharest.

2.6.1. Qualitative Assay. The qualitative method consists of measuring the inhibition zone diameter of the extracts, thus determining the inhibition degree on the solid medium as a result of their action. Microbial suspensions adjusted to 0.5 McFarland for bacteria and 1 McFarland for microfungi, obtained from pure cultures, were used for seeding [23].

2.6.2. Quantitative Evaluation of the Antimicrobial Activity. Quantitative Evaluation of the Antimicrobial Activity. The serial microdilution method was performed in 96-well plates in a liquid medium, in a concentration range between 3.91 and 500 μL extract/mL (binary dilutions). The liquid culture media used were liquid agar (used for the bacteria cultivation) and liquid Sabouraud (used for the microfungi cultivation). Microbial suspensions were prepared as Section 2.6.1. Controls used to determine the minimum inhibitory concentration (MIC) were strain control, solvent control, and media sterility control. After incubating the plates at 37˚C for 24 hours, the results by macroscopic observation and spectrophotometrically (λ = 620 nm) were analyzed.

2.6.3. MBEC (minimum biofilm eradication concentration). The 96-well plates used to determine the minimum inhibitory concentration were washed with sterile water to remove the medium, compound debris, and non-adherent microorganisms and then dried at room temperature. 99.8% methanol was added over the adhered bacteria in order to fix them (15 minutes). After the removal of methanol, biofilms were stained with 1% crystal violet (10 minutes), then the plates were washed with sterile water and dried at room temperature. The absorbances were recorded at 492 nm after the addition of 33% acetic acid. Solvent blank, medium sterility blank, and growth blank were done.

3. Results and discussion

3.1. Determination of TPC, TFC and TEAC

The alcoholic extracts were found to contain a higher quantity of phenolic compounds than water-based extracts in both plants, *V. officinalis*, and *A. triphylla*, regardless of the applied extraction methods, less VO-AQ, with higher phenolic compounds quantity than VO-ALC. As expected, their antioxidant activity was also higher, a result supported by the literature data [24]. The total phenolic compounds and flavonoid content from obtained extracts, together with the antioxidant activity results, are presented in Table 1. It is noted that the macerates of both species have the best total antioxidant capacity, data that are in agreement with those obtained when evaluating TPC and TFC.

Table 1.

Total phenolic content (TPC), total flavonoid content (TFC) and TEAC of *V. officinalis* and *A. citrodora* extracts

Samples	TPC (GAE) [1]	TFC (QE)[2]	TEAC[3]
AC-ALC	70.82	0.34	1.66
AC-AQ	70.48	0.80	0.57
AC-D	224.03	0.39	0.71
AC-M	**282.08**	**0.91**	**4.90**
VO-ALC	26.02	0.24	0.36
VO-AQ	96.05	0.30	0.19
VO-D	150.30	0.83	1.04
VO-M	**204.53**	**1.47**	**2.52**

[1]TPC expressed as mg gallic acid/100mL extract; [2] TFC expressed as mg quercetin/100mL extract; [3]TEAC expressed as mmol Trolox/L.

According to literature data, plant compounds with a phenolic structure [25] are better extracted when using alcoholic solutions, which happened to a large extent in the case of our extracts. It was also expected that a longer extraction time would ensure better efficiency, as observed in the case of *V. officinalis* macerate. It is somewhat surprising that by boiling, it was possible to extract a more significant amount of phenolic compounds than by maceration for the *A. citrodora* sample, although also for *V. officinalis*, the values obtained for TPC, TFC, and TEAC were in second place after maceration. It should also be mentioned that in all samples, as expected, the amount of flavonoids represents a small part of the phenolic compounds.

3.3. Phenolic Compounds Analysis by UHPLC-DAD-ESI/MS

UHPLC-DAD/MS analysis showed that the alcoholic extracts of *V. officinalis* and *A. citrodora* contain various phenolic compounds, including phenolic acids, flavonoids (flavan-3-ols, flavonols, flavones and flavanones), heterosides with a flavonoid nucleus (naringin, rutin, hesperidin) and low amounts

of stilbenoids t-resveratrol. The quantitative results expressed in mg/L extract are presented in Table 2.

Data showed that the most abundant compound identified in *Aloysia citrodora* extracts is p-coumaric acid, followed by 4-hydroxybenzoic acid, ferulic acid, and caffeic acid. Protocatechuic acid, chlorogenic acid, syringic acid, and cinnamic acid were identified in smaller amounts. Traces of gallic acid were present only in the decoction. The obtained results are in agreement with the literature data. In a 2019 study, Rezig et al. identified p-coumaric acid as the major phenolic compound in the alcoholic extract obtained from *A. citrodora* leaves. Caffeic acid and ferulic acid have also been identified [8].

Table 2.

Phenolic content (mg/L) of *Verbena officinalis* and *Aloysia citrodora* extracts

Compounds	*Aloysia citrodora*				*Verbena officinalis*			
	AC-ALC	**AC-AQ**	**AC-D**	**AC-M**	**VO-ALC**	**VO-AQ**	**VO-D**	**VO-M**
Phenolic acids								
p-hydroxibenzoic acid	0.759	1.157	1.254	1.559	1.109	0.669	0.990	1.408
syringic acid	0.044	0.609	0.220	0.402	1.126	1.200	2.968	2.280
protocatechuic acid	0.297	1.147	0.470	0.666	0.091	0.243	0.256	0.313
gallic acid	n.d.	n.d.	0.002	n.d.	0.008	0.781	0.009	0.058
p-coumaric acid	0.873	1.407	1.664	1.767	0.080	0.221	0.397	0.325
ferulic acid	0.725	1.001	0.950	1.754	0.109	0.810	0.535	0.698
caffeic acid	0.268	0.829	0.720	0.787	0.419	0.536	0.411	0.636
cinnamic acid	0.064	0.265	0.099	0.029	0.051	0.040	0.219	0.566
chlorogenic acid	0.137	0.037	0.336	0.455	0.014	0.013	0.011	0.109
Flavan-3-ols and flavonols								
catechin	0.002	0.003	n.d.	0.005	0.001	0.005	0.001	n.d.
epicatechin	0.001	n.d.	n.d.	0.008	n.d.	0.261	n.d.	n.d.
galangin	0.009	0.032	0.004	0.002	n.d.	0.001	0.001	0.003
kampferol	n.d.	n.d.	n.d.	0.005	0.001	0.001	0.002	n.d.
isorhamnetin	0.052	0.006	0.003	0.194	0.003	0.003	0.002	0.005
myricetin	0.005	0.012	0.002	n.d.	n.d.	0.002	n.d.	n.d.
quercetin	0.031	0.024	0.004	0.138	0.003	0.004	0.002	0.022
Flavonoid core heterosides								
naringin	0.184	n.d.	0.058	0.279	n.d.	0.016	0.397	0.305
rutin	0.058	0.004	0.030	0.118	0.005	0.001	0.004	0.006
hesperidin	0.003	0.017	0.009	0.006	0.024	0.015	0.03	0.069
Flavones and flavanones								
crisin	0.010	0.011	0.004	0.005	0.008	0.003	0.005	0.014
apigenin	0.187	0.208	0.059	0.475	5.674	3.815	3.039	13.089
pinocembrin	0.010	0.007	0.009	0.004	0.022	0.009	0.011	0.026
Stilbenoids								
t-resveratrol	0.002	0.001	0.002	0.009	n.d.	n.d.	0.001	0.004

AC-ALC (*Aloysia citrodora* alcoholic extract*);* AC-AQ (ALC (*Aloysia citrodora* aqueous extract*);* AC-D (*Aloysia citrodora* decoction); AC-M (*Aloysia citrodora* macerate); VO-ALC (*Verbena*

officinalis alcoholic extract); VO-AQ (*Verbena officinalis* aqueous extract); VO-D (*Verbena officinalis* decoction); VO-M (*Verbena officinalis* macerate); n.d.-not detected

In the case of *Verbena officinalis* extracts, the most abundant phenolic acid is syringic acid, followed by 4-hydroxybenzoic acid, ferulic acid, caffeic acid, and cinnamic acid. Protocatechuic acid and coumaric acid are in smaller amounts. For the syringic acid, the best extraction method was decoction (2.968 mg/L) and maceration (2.280 mg/L). As the results show, water utilization as solvent favored the extraction of gallic acid.

Regarding flavonoid content, apigenin is the predominant compound, especially in *Verbena officinalis* extracts, such as macerate (13.089 mg/L) and alcoholic extract (5.674 mg/L). The same compound is also the main flavonoid in A. citriodora macerate, which contains 0.475 mg/L apigenin. These results can be attributed to the higher solubility of apigenin in ethanol compared to water. In *Aloysia citrodora* extracts, the amount of apigenin is significantly lower. Other flavonoid compounds identified are chrysin, pinocembrin, catechin, epicatechin, galangin, kaempferol, isorhamnetin, myricetin and quercetin. Pinostrobin was identified in tiny amounts in the alcoholic extract and decoction of *Aloysia citrodora* and *Verbena officinalis*. Three heterosides with a flavonoid core were identified, namely, naringin, rutin, and hesperidin, with important amounts in decoction and macerates of *Aloysia citrodora* and *Verbena officinalis extracts.*

About the phenolic compounds profile, the literature mentions the presence of gallic, syringic, p-coumaric, ferulic and protocatechuic acids in the extracts of the two plant species, more significant amounts of phenolic acids and flavonoids being detected in *Aloysia citrodora* compared to *Verbena officinalis*. Moreover, in most cases, the aqueous extracts of both species were richer in phenolic compounds than the alcoholic extracts, as well as differences in the profile of phenolic compounds [26]. Caffeic acid, quercetin, pinocembrin, and two apigenin's heterosides were previously identified by negative LC-ESI-MS/MS in an n-butanol extract of *V. officinalis* [17]. Tammar, *et al* also quantified apigenin through LC-DAD in methanolic extracts of *Aloysia citrodora* harvested from four Tunisia areas. In addition, they identified caffeoyl-shikimic acid, catechin-gallate, 3,4-di-caffeoylquinic acid, 3,5-di-caffeoylquinic acid, verbascoside, isoacteoside, martynoside, diosmetin [27].

3.4. Antimicrobial activity

The results obtained for the qualitative testing of microbial sensitivity to the extracts of *V. officinalis* and *A. citrodora* are shown in Table 3. The largest diameters of growth inhibition zones were recorded for *Aloysia citrodora* extracts for all tested strains. Aqueous extracts of both plant species were observed to show no inhibitory effect on either strain.

*Table **3.***

The growth inhibition zone diameters (GIZD).

Sample / Bacterial strain	AC-ALC	AC-M	VO-M	VO-ALC	70% ethanol
S. aureus ATCC	±	±	±	±	±
B. subtilis ATCC	±1.0	±1.1	±1.4	±1.4	±
P. aeruginosa ATCC	1.1	1.0	1.0	0.8	0.5
E. coli ATCC	1.0	1.0	1.0	1.0	0.5
C. albicans ATCC 10231	1.2	1.4	1.4	1.3	1.0

± unclear inhibition zone with colony inside of the area

The qualitative evaluation was confirmed by the quantitative method, with the determination of minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) values to correlate with the results of the chromatographic analysis. Table 4 shows the results obtained after testing the minimum inhibitory concentration of the extracts (expressed in µg extract/mL).

*Table **4**.*

Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC), expressed as µg extract/mL

Sample / Bacterial strain	AC-ALC		AC-M		VO-M		VO-ALC	
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
S. aureus ATCC	125.00	125.00	62.50	62.50	62.50	62.50	125.00	125.00
B. subtilis ATCC	62.50	15.63	15.63	15.63	15.63	15.63	62.50	15.63
P. aeruginosa ATCC	31.25	15.63	15.63	15.63	15.63	15.63	31.25	15.63
E. coli ATCC	62.50	7.81	31.25	7.81	31.25	7.81	62.50	7.81
C. albicans ATCC 10231	15.63	3.90	15.63	3.90	15.63	3.90	15.63	3.90

The MIC values varied between 15.63 and 500 µg extract/mL, the lowest values being recorded on the macerate, for many strains. These results correlate with the data obtained by the UHPLC analysis, which revealed a more significant amount of phenolic acids and flavonoids in the macerate compared to the other extracts. Aqueous extracts did not show inhibitory activity on microbial growth for any strains, having the lowest concentrations in phenolic compounds.

Biofilms are represented by microbial communities irreversibly attached to a substrate, the cells in their composition being well protected and resistant to any form of stress. Regarding the activity on bacterial biofilms, the best results were recorded for the extracts obtained from *Verbena officinalis*. The lowest value of the MBEC was recorded in the case of the both plant species macerates.

The data from the literature confirms the obtained results. Studies have shown that the methanolic extract and essential oil of *Aloysia citrodora* show a strong antimicrobial effect on *E. coli* and *B. subtilis* and antifungal on different *Candida* strains: *C. albicans* ATCC 2091, *C. glabrata* ATCC 90030, *C. kefir*, and *C. parapsilosis* [8], but also on strains isolated from clinical samples: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. guillermondii*, *C. parapsilosis* and *C. tropicalis* [11], in agreement with our results.

Regarding *Verbena officinalis*, most of the tests showed that the extracts have an inhibitory effect on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Citrobacter freundii* and *Bacillus subtilis* strains [12–14]. Other studies have shown that the ethanolic extract of *Verbena officinalis* does not inhibit the growth of *Candida* spp and *Gardnerella vaginalis* isolated from vaginal secretions [28]. The antimicrobial activity of the extracts is due to their composition, a significant contribution in this regard being brought by phenolic and flavonoid acids.

The literature mentions that ferulic, gallic, cinnamic and 4-hydroxybenzoic acids have antimicrobial activity on virulent pathogenic strains, such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Candida famata* [29], which confirms and the data obtained in the present study.

Rutin has shown antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Candida famata* strains [30]. Also, in the same study, it is mentioned that epicatechin and catechin present antimicrobial activity against critical pathogenic strains (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans and Candida famata*).

Regarding pinocembrin, quercetin and kaempferol, published data claim antimicrobial activity on *Staphylococcus aureus*, *Escherichia coli and Candida albicans* [31, 32] and isoramnetin has an antibacterial effect on *Staphylococcus aureus* and *Salmonella spp.* [33]. Among the extracts studied, macerates, especially that of *Aloysia citrodora*, with a higher content of quercetin, isoramnetin, and pinocembrin, confirm these data. As a result, it can be stated that the obtained experimental results are consistent with the data synthesized from the literature regarding the compositional relationship in phenolic compounds versus antimicrobial activity.

4. Conclusions

The paper describes a comparative study of the extraction of the active components of *Aloysia citrodora* and *Verbena officinalis* species by four extraction methods: alcoholic and aqueous extractions but also two standard extractions by decoction and maceration. The results in the present paper showed that the most

efficient extraction method of phenolic compounds in both cases was maceration. In the case of the *Aloysia citrodora* species, the aqueous extract, decoction, and alcoholic extract followed, while in the case of the *Verbena officinalis* species, the second method in terms of extraction efficiency was the decoction, followed by alcoholic extract and aqueous extract, results also confirmed by chromatographic analysis (UHPLC-MS/MS).

Regarding antimicrobial activity, we can appreciate that most of the extracts showed growth inhibitory activity on some microfungi, Gram-negative and Gram-positive strains. The macerates of both species, with a 4/7 times higher phenolic content compared with the alcoholic extracts, registered the best antimicrobial activity by qualitative and quantitative antimicrobial assays, both on planktonic and adhered cells but only marginally higher compared with the alcoholic extracts, for instance, which means that antimicrobial activity is not only dependent on the total polyphenolic content but also on the composition.

Nowadays, regarding alternative strategies to antibiotics, polyphenolic compounds (and plant extracts, in general) are targeted as bioinspired compounds and included in modern designs to treat infections based on their antimicrobial activity alone or in association with other agents.

Acknowledgements

E.I. Geana's work was supported by Contract No. 20N/05.01.2023, Project PN 23 15 04 01: "The cascade valorization of agro-industrial waste of plant biomass type in bioproducts with added value in the circular bioeconomy system" and Program 1 - Development of the national research and development system, Subprogram 1.1. Institutional performance - Projects to finance excellence in RDI, Contract No. 19PFE/30.12.2021.

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