Assessment of rosmarinic acid content in six *Lamiaceae* species extracts and their antioxidant and antimicrobial potential

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Abstract: In the present study, six indigenous species of Lamiaceae family (*Origanum vulgare* L., *Melissa officinalis* L., *Rosmarinus officinalis* L., *Ocimum basilicum* L., *Salvia officinalis* L. and *Hyssopus officinalis* L.), have been analyzed to assess the rosmarinic acid, phenyl propane derivatives and polyphenolic contents and their antioxidant and antimicrobial potential. HPLC-MS method has been used for the analysis ofrosmarinicacid. The phenyl propane derivatives and total phenolic contents were determined using spectrophotometric method. The ethanolic extracts were screened for antioxidant activities by DPPH radical scavenging, HAPX (hemoglobin ascorbate per oxidase activity inhibition), and EPR (electron paramagnetic resonance) methods. The ethanolic extracts revealed the presence of rosmarinic acid in the largest amount in *O. vulgare* (12.40mg/g) and in the lowest in *R. officinalis* (1.33 mg/g). *O. vulgare* extracts exhibited the highest antioxidant capacity, in line with the rosmarinic acid and polyphenolic contents. The antimicrobial testing showed a significant activity against *L. monocytogenes, S. aureus* and *C. albicans* for all six extracts.

Keywords: Rosmarinic acid, polyphenols, *Lamiaceae*, antioxidant, antimicrobial, HAPX.

INTRODUCTION

Rosmarinic acid (RA) is a natural phenolic substance in numerous Lamiaceae species, used frequently as food plants among which oregano, rosemary, basil, sage, savory and mint. Fromchemical point of view, RA is a caffeic acid ester of 3, 4-dihydroxyphenyllactic acid, being a phenyl propanoid derivative commonly found in the plant kingdom (Clifford, 1999; Petersen and Simmonds, 2003). Numerous biological properties of RA were described, namely antimicrobial, antidepressive, cytoprotective, antiviral, anti-allergic, anti-angiogenic, antitumor activities (Abedini *et al*., 2013; Petersen and Simmonds, 2003; Boonyarikpunchai *et al*., 2014; Osaka be *et al*., 2005; Hossan *et al*., 2014). Also is known that the RA shows an important antioxidant activity as a reactive oxygen species scavenger and lipid per oxidation inhibitor, which can promote health (Basappa Mahaewarappa *et al*., 2014; Fadel *et al*., 2011; Luis and Johnson, 2005).

The Lamiaceae familyis highly widespreadinare as of the Mediterranean, Central Asia, America, Africa, and China. It contains over 230 generaand 7000 flowering plants that which were considered closely linked to Verbenaceae. After 1990s, the phylogenetic studies included the some Verbenaceae genera in Lamiaceae family. It contains numerous medicinal and aromatic plants: *Lavandula* sp., *Mentha* sp., *Marrubium* sp., *Hyssopus* sp., *Ocimum* sp., *Origanum* sp., *Rosmarinus* sp., *Salvia* sp., *Satureja* sp., *Thymus* sp. etc. used since early times. In terms of chemical composition, several species of this family have been the subject of many studies centered on: essential oils, flavonoids, iridoids, sterols, diterpenoids, for the pharmaceutical, food and cosmetics industries. The secondary metabolites from Lamiaceae species have revealed important activities antispasmodic, antiviral, stimulant digestive, antiseptic, antimicrobial, anti-inflammatory, antioxidant, hepatoprotective, insecticide, aromatic etc. Some species are cultivated as ornamentals plants: *Ajuga* sp., *Coleus* sp., *Lavandula* sp., *Nepeta* sp., *Rosmarinus* sp., *Salvia* sp., *Stachys* sp. etc (Esquivel *et al*. 2000; Basappa Mahaewarappa *et al*., 2014).

In the spontaneous flora of Romania, the Lamiaceae family is represented by 33 genera, 128 species and above 37 subspecies, hybrids and varieties (Ciocarlan, 2009). The flowers and leaves of Lamiaceae species contain flavonoids, triterpenoids, essential oils etc. Specific to this family is the higher amount of the phenolic compounds (tannins, flavonoids, hydroxy cinnamic acids). One of the major phenolic compounds in this family is the rosmarinicacid (Capecka *et al*., 2005; Petersen and Simmonds, 2003).

The research objective has been to quantify the RA in six ethanolic extracts of *Origanum vulgare* L. (oregano), *Hyssopus officinalis* L. (hyssop), *Ocimum basilicum* L. (basil), *Rosmarinus officinalis* L. (rosemary), *Melissa*

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officinalis L. (lemon balm) and *Salvia officinalis* L. (sage) by HPLC-MS method and to assess their antimicrobial and antioxidant potentials.

MATERIALS AND METHODS

Plant materials and preparation of extracts and standard solution

The studied medicinal plants have been *H. officinalis* (Voucher No. 781), *S. officinalis* (Voucher No. 791), *O. basilicum* (Voucher No. 792), *O. vulgare* (Voucher No. 793), *R. officinalis* (Voucher No. 794), and *M. officinalis* (Voucher No. 795). The aerial parts of these species were harvested in the blossom period (2014) from cultures and spontaneous flora (Cluj and Alba). *Powdered* plant aerial parts (5.0g) were refluxed with 70% ethanol (50mL) and then were filtered. Stock standard solution: 10 mg of RA was dissolved in methanol into 10mL volumetric flask (Benedec *et al*., 2013; Vlase *et al*., 2014).

HPLC-MS method

The identification and quantification of RA from the extracts have been made using an Agilent 1100 HPLC Series system (Agilent, Santa Clara, CA, USA) equipped with G1322A degasser, G13311A binary gradient pump, column thermostat (operating at 48^{o}C), G1313A auto sampler and VL Ion Trap and UV. The separation has been achieved on a reverse-phase analytical column (Zorbax SB-C18 100 x 3.0mm i.d., 3.5µm particle). The mobile phase has been prepared with acetonitrile and ammonium acetate in water (1mM), the gradient elution: start with 5% acetonitrile, at 3.3min 25% acetonitrile. The flow rate of mobile phase has been 1mL/minutes. The autosampler injection volume has been set on25 µL (Vlase *et al*., 2014).

Quantification of phenylpropanoid derivatives

The phenyl propane derivatives and the completely polyphenolic contents (TPC) have been determined by spectrophotometric methods described in Romanian Pharmacopoeia and European Pharmacopoeia 5th Edition (Romanian Pharmacopoeia, 1993; Benedec *et al*., 2013; *Ph. Eur.,* 2005; Singleton *et al.*, 1999; Uddin *et al*., 2015). The percentages of phenylpropane derivatives and total polyphenolic compounds have been expressed as RA equivalents.

Measurement of in vitro antioxidant activity

DPPH antioxidant method: Two milli litres of DPPH ethanolic solution (0.1g/L) was added to 2.0ml of each extracts to various concentrations (12.5-100µg/mL herbal extracts, and 0.75-5.25µg/mL RA solution, respectively). Measuring of absorbance was made using a UV-VIS Jasco V-530 spectrophotometer. The solution of RA (0.012mg/mL) been used as standard DPPH scavenging capacity of was expressed as IC_{50}; lower IC_{50} values indicate a higher DPPH scavenging capacity (Benedec *et al*., 2013; Prior *et al*., 2005; Singleton *et al*., 1999: Gharbani and Javazi, 2015).

HAPX assay

The reaction has been initiated with methemoglobin added to peroxide, sodium ascorbate, samples, in acetate buffer, pH 5.5; the absorbance being measured at 405 (nm), where the ferryl formation inhibition by tested samples in competition with sodium ascorbate can be monitored. The antioxidant ability was reflected in the increase of the inhibition time. The percentage of the inhibition time has been converted into RAE (Cooper *et al*., 2008; Mot *et al*., 2015).

EPR spectroscopy method

EPR-DPPH experiment. EPR measurements for the DPPH test have been made on a Bruker EPR spectrometer which is equipped with X-band (9.54 GHz) Microwave Bridge; the method being described by Mocan and Sgherri (Mocan *et al*., 2015; Sgherri *et al*., 2011). EPR spectra have been registered at various time points. The relative concentration changes of the para-magnetic species have been achieved with double integration of the spectra (Integral intensity) using X EPR software.

Direct detection of free radicals. For free radical generation experiment, the extracts were diluted 10 times in 90 % ethanol, and treated with 5mM NaOH (yielding a pH of 11.7). The concentration of the pure compound, rosmarinic acid, was 2mM in 90% ethanol. 100µl were rapidly transferred to a glass capillary EPR tube. The capillary was placed in the holder of a Bruker ELEXSYS E-580 spectrometer with continuous wave at X band (~ 9.4 GHz). The measurements were performed at room temperature with the following parameters: frequency modulation, 100kHz, microwave power, 9.6mW, modulation amplitude, 1 G, center field 3514 and sweep field 100G (Mot *et al*., 2015). Absolute area for each spectrum was determined through integration of the experimental spectra using Origin Pro 8.

Antimicrobial activity method

The six samples were tested against *Salmonella typhimurium*, *Escherichia coli, Staphylococcus aureus*, *Listeria monocytogenes* and *Candida albicans*, using the disk-diffusion method, which was previously described (Benedec *et al*., 2013; Reeves and White, 1983). Gentamicin has been used as standard antibiotics. Fluconazole was placed as a reference antifungal.

STATISTICAL ANALYSIS

The averages of triplicate measurements are tabulated together with standard deviations (SD). The statistical analysis was carried out using Excel software package.

RESULTS

HPLC analysis of rosmarinic acid

The mass spectrometer with ESI source was operated in negative mode and was set for isolation and fragmentation of deprotonated RA molecule with m/z=359 (fig. 1A). Quantification of RA was based on the deprotonated molecule with m/z=359 from the MS spectrum (fig. 1B).

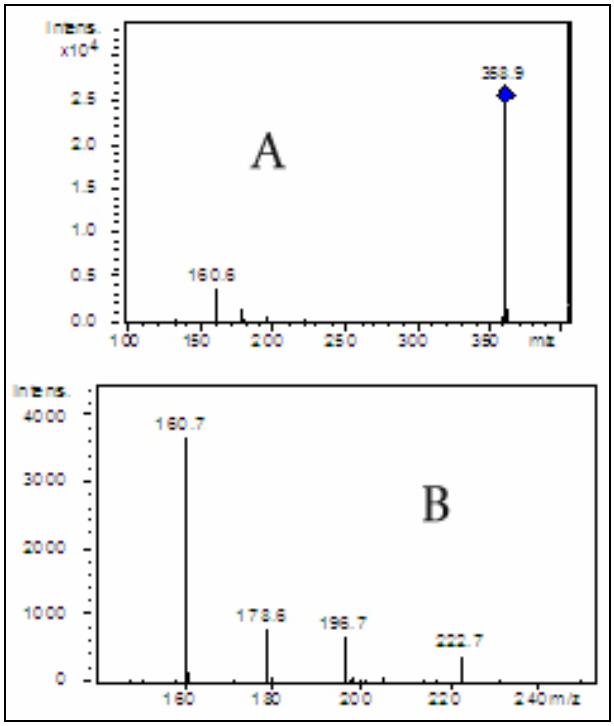


Fig. 1: A. Full-scan ESI-MS spectra of RA in mobile phase; B. MS/MS spectra of RA in mobile phase.

Absorption spectra and mass spectra that were obtained from HPLC of the *O. vulgare* extract are shown in fig. 2. For the other extracts, similar chromatograms were obtained and the RA was identified.

The quantification of RA in the extracts was achieved with a retention time of 2.2 min. The calibration curve was linear in a range40-640ng/mL (R^2=0.999). RA content of the six extracts is listed in table 1. The concentration ranged from 1.33-12.40mg of RA/g material plants.

Polyphenolic content

The comparative data about phenyl propane derivatives and total polyphenols content in the six Romanian species are presented in table 1. The amount of phenyl propane derivatives ranged from 0.50 to 3g/100g dry material plant (table 1). TPC has been determined with the Folin-Ciocalteu reagent. The TPC values in the extracts ranged between 4.91 and 12.48 g/100 g plant material.

Antioxidant activity

Results antioxidant capacities obtained by DPPH bleaching method are shown in table 2. RA has been used as a standard. *O. vulgare* extract showed the greatest radical scavenging activity (IC_{50}=35.03µg/mL), while *H. officinalis* extract showed the lowest capacity (IC_{50} 135.89 µg/mL).

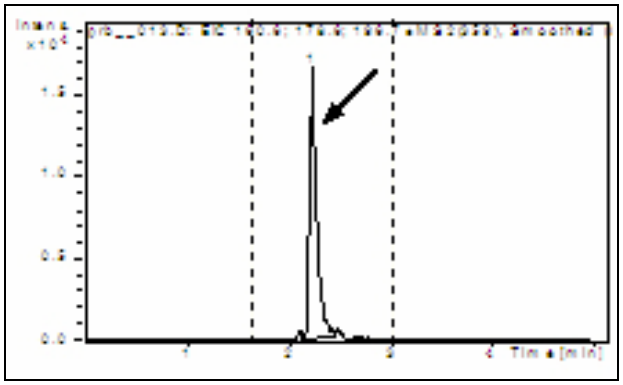
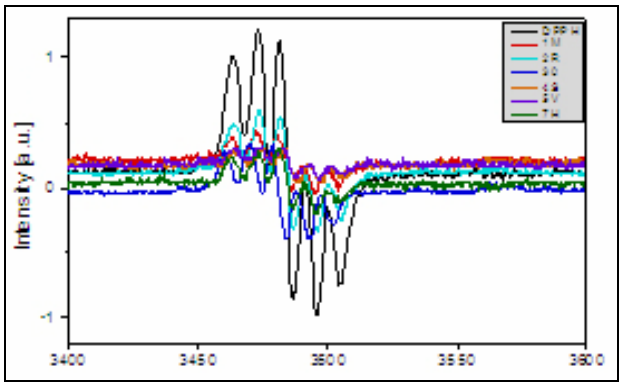


Fig. 2: Chromatograms of RA from *O. vulgare* extract (MS/MS signal).



Notes: *O. vulgare* -5V, *R. officinalis* -2R, *M. officinalis* -1M, *O. basilicum* -3O, *H. officinalis* -7H and *S. officinalis*-4S.

Fig. 3: The EPR spectra of reaction between antioxidant compounds and DPPH radical.

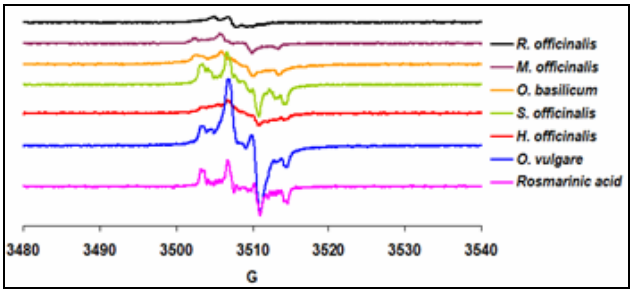


Fig. 4: The EPR spectra of the analysed extract diluted 10 times and treated with NaOH, in ethanol 90%, recorded in the first 2 minutes after mixing.

HAPX method measures the ability of the vegetal extract compounds to quench the free radicals generated in hemoglobin after exposure to peroxide (table 2). More information can be brought about by this test because it

involves the interaction of antioxidants with ferryl hemoglobin. The antioxidant properties of the samples were investigated by means of spectroscopy using DPPH radicals (EPR method). In this test the reactions of DPPH· with the extracts of *O. vulgare*, *R. officinalis, M. officinalis, O. basilicum, H. officinalis,* and *S. officinalis* were examined. The rate of reaction of the natural antioxidants and DPPH· was monitored by integral intensity, which is linked to the number of the paramagnetic species (fig. 3).

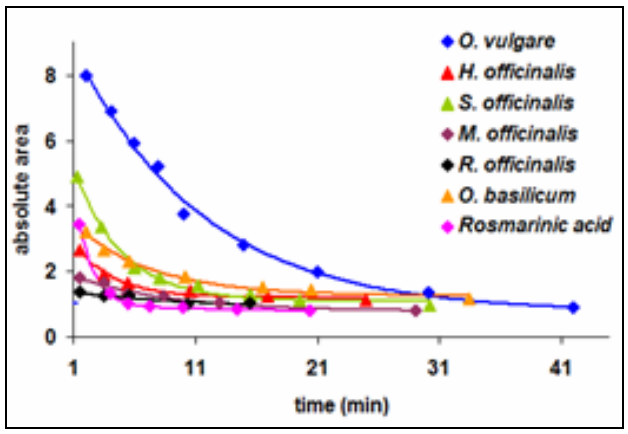


Fig. 5: Radical decay kinetic curves of the 5mM NaOH treated Lamiaceae extracts in 90% ethanol

In vitro antimicrobial activity

The results of disk-diffusion assay obtained using the measurement of the diameter of the inhibition zones, were tabulated (table 4).

The polyphenolic content and to some extent details about individual components can be detected in EPR spectra of extracts treated with a base, where free radical signals such as those in fig. 4 can be detected. A direct relation between the intensity of the signal and the content of phenols was previously observed in propolis extracts using similar experiments (Mot *et al*., 2009). The mechanism of radical formation has not been elucidated yet in detail, but it is expected to be based on the generation of semiquinone anion radical during the auto oxidation of polyphenolic compounds treated with alkali compound in the presence of molecular oxygen (Mot *et al*., 2015). Probing the phenolic content of a natural extract by such alkaline treatments, EPR spectra would have the advantages of directly detecting free radicals pertaining to the sample (as opposed to indirectly detecting them via reaction with ABTS, DPPH, ascorbate, etc.) including structural information (as each radical is expected to display a different EPR line shape) - while also allowing for the kinetic approaches.

DISCUSSION

RA was quantified by an HPLC-MS method in six indigenous Lamiaceae species. The expected molecular ion signal, according to the rosmarinic acid's molecular weight (M=360.2) respectively according to the ionisation way (negative) is the signal at m/z=359, a deprotonated RA molecule. To increase the analysis method's selectivity was performed also the ion fragmentation of the deprotonated RA molecule. It can be observed that the ion fragmentation lead to four main fragments having m/z of 160.7, 178.6, 196.7 respectively 222.7. As shown in fig. 2, the major compound in all extracts showed the intensity of the mass signal at 359 m/z. The fragments that are similar in the standard and extracts comprise 160.7, 178.6 and 196.7. These results confirm the identity of RA. The results showed that among the analyzed plants, the high RA contents were found in *O. vulgare* and *M. officinalis* (12.40, and 7.84 mg/g, respectively). The RA amounts in the extracts of *S. officinalis, H. officinalis* and *O. basilicum*, presented similar values (2.12-3.59mg/g). Although it is known that rosemary is the main source of RA in many countries, our extract contained the smallest amount of this acid (1.33mg/g); low levels were also recorded for the Iranian species (7.2mg/g) (Shekarchi *et al*., 2012). This variability concerning the RA content can be explained bypedo-climatic and storage conditions that may influence concentration of active principles in plants (Shekarchi *et al*., 2012). Therefore, it was important to analyze the Romanian species in order to identify the richest antioxidant natural source of RA.

Polyphenolic content

The highest level of phenyl propane derivatives was found in *O. vulgare* (3.04%), while the lowest was in *M. officinalis* (2.12%). *R. officinalis* (1.90%), *O. basilicum* (1.84%) *S. officinalis* (1.38%) and *H. officinalis* (0.68%) presented lower levels of phenolic compounds. The levels of TPC in the six samples have dropped in the order: *O. vulgare>M. officinalis>R. officinalis>O. basilicum >S. officinalis >H. officinalis,* the oregano being a major source of polyphenolic compounds. Capecka reported inoregano high content of phenolics, particularly RA (Capecka *et al*., 2005).

In vitro antioxidant activity

The following performance order of the six ethanolic extracts by DPPH•, expressed as IC_{50}, was: RA *>O. vulgare>M. officinalis>R. officinalis>S. officinalis>O. basilicum>H. officinalis*. Comparing the antioxidant activities of hyssop and basil, similar results were obtained earlier for another Romanian samples (Vlase *et al*., 2014). Kaurinovic reported previously that the extracts of *O. vulgare* and *O. basilicum* showed stronger antioxidant (IC_{50} value was between 7.28 and 17.21 µg/mL) (Kaurinovic *et al*. 2011).

Hemoglobin interacts with hydrogen peroxide, a physiological reaction which occurs normally in the body especially and is accelerated by certain stress factors, yielding the formation of a high valent species - ferryl.

Table 1: RA and phenolic contents in the studied extracts

Species name	RA (mg/g)	Phenylpropane derivatives (g RAE/100 g)	TPC (g RAE/100 g)
O. vulgare	12.40±0.08	3.04±0.18	12.48±0.31
M. officinalis	7.84±0.07	2.12±0.06	9.58±0.40
R. officinalis	1.33±0.01	1.90±0.09	8.67±0.33
O. basilicum	3.59±0.01	1.84±0.15	7.36±0.19
S. officinalis	2.12±0.02	1.38±0.21	7.57±0.26
H. officinalis	2.85±0.004	0.68±0.01	4.91±0.10

Note: Values are the mean ± SD (n = 3).

Table 2: Antioxidant activity of the studied extracts

Samples	DPPH IC_{50} (µg/mL)	EPR Integral intensity	HAPX (mg RAE/g)
O. vulgare	35.03±1.57	24.75±0.86	791.67±138.32
M. officinalis	65.14±2.74	195.20±2.67	0
R. officinalis	70.26±1.73	179.13±2.88	14.53±6.02
O. basilicum	113.47±4.52	106.71±3.74	421.97±73.24
S. officinalis	81.12±1.87	48.21±1.79	237.61±26.35
H. officinalis	135.89±3.10	342.49±4.62	421.97±62.12
Rosmarinic acid	3.30±0.11	-	-
DPPH		578.85±7.60	-

Note: RAE: Rosmarinic acid equivalents.

Table 3: The absolute area and k_{rd} values obtained for the EPR signals generated byalkalinetreatments of the studied extracts

Samples	Absolute area	k_{rd}
O. vulgare	8	10.40
M. officinalis	1.8	7.83
R. officinalis	1.35	3.89
O. basilicum	3.22	6.33
S. officinalis	4.89	3.89
H. officinalis	2.68	3.16

The kinetic curves of the radical decay (fig. 5) for the alkaline-pH EPR signals in the studied extracts were fitted to an exponential function (decay 1).

Table 4: Antimicrobial activity of the studied extracts

Samples	Zone of inhibition (mm)				
	S. aureus	*L. monocytogenes*	*E. coli*	*S. typhimurium*	*C. albicans*
O. vulgare	12±0.50	10±1.50	8±0.00	6±0.00	12±1.00
M. officinalis	11±0.40	10±2.00	6±0.00	6±1.00	16±2.00
R. officinalis	22±1.00	20±2.00	8±0.50	10±0.00	28±3.00
O. basilicum	12.0±0.00	10.0±0.50	6±0.50	6±1.00	14±0.50
S. officinalis	16±1.50	15±0.00	6±0.00	6±0.00	28±1.50
H. officinalis	12.0±0.00	10.0±0.00	6±1.00	6±1.00	14±0.30
Gentamicin	19±0.60	18±1.00	22±0.50	18±0.00	-
Fluconazole	-	-	-	-	25±0.20

Notes: [a]The values represent the average of three determinations ± standard deviations. Gentamicin (10µg/well) and Fluconazole (25 µg/well) were used as a positive control.

This highly reactive intermediate can be reduced by antioxidants, such as ascorbate, urate or exogenous compounds found in the vegetal products (e.g. polyphenols). Involving a protein found in a large concentration in the blood (hemoglobin), this method is proposed to have a more physiological relevance than other well known methods described for the evaluation of antioxidant capacity (Mot *et al*., 2015). Here, one may

note a significant correlation of the HAPX results with the rosmarinic acid content (R^2=0.810) (except for *M. officinalis*).

Regarding EPR spectroscopy, as expected, the integral intensity of DPPH is notably reduced by the antioxidant extracts with the integral intensity values of the six extracts were summarized in table 2. There by *O. vulgare* and *S. officinalis* exhibited a greater antioxidant effect than the other samples. *H. officinalis* showed the lowest antioxidant capacity. In the present study the shapes of the signals generated by alkaline treatments (fig. 4) appear to be very similar, for all samples, with the spectrum of RA generated under similarly alkaline conditions. *O. vulgare* extract has the highest intensity of the signal, followed by *S. officinalis*. Absolute areas obtained by integration of the signal are listed in the table 3 and are well good correlated with HAPX (R^2=0.840) as well as with the EPR-DPPH experiment (R^2=0.920, excepting *H. officinalis*) and with rosmarinic acid content (R^2=0.990, excepting *M. officinalis* and *S. officinalis*). The kinetic constant k_{rd}, listed in table 3, is significantly correlated with HAPX (R^2=0.880) (excepting *M. officinalis*), RA (R^2 =0.940) and caffeic acid (0.890).

Concerning the antimicrobial activity, *O. vulgare, M. officinalis, O. basilicum, H. officinalis* have shown low antibacterial effect on *S. aureus* and *L. monocytogenes*. *S. officinalis* was found to have a *moderate antibacterial*c apacity. Nevertheless, the *R. officinalis* extract has shown a profound antibacterial activity with respect to Gram-positive, even stronger than Gentamicin. These extracts were not active on: *E. coli* and *S. typhimurium.* All the tested samples inhibited fungal growth (*Candida albicans*). Additionally, *S. officinalis* and *R. officinalis* showed a stronger activity against this fungal strain, than Fluconazole used as antifungal control. From above results it can be concluded that these extract showed effectiveness against the subjected *Staphylococcus aureus*, *Listeria monocytogenes* and *Candida albicans* strains, *R. officinalis* showing the most intense antimicrobial activity. Close values were previously presented to hyssop, lemon balm and basil from Serbia, Romania, Turkey or Germany (Adıguzel *el al*., 2005; Canadanović-Brunet *et al*., 2008; Vlase *et al*., 2014). Concerning *O. vulgare*, our results were consistent with previous data for oregano from Pakistan, thus the gram positive bacteria (*S. aureus*) showed more susceptibility than gram negative (Ashraf *et al*., 2011).

CONCLUSIONS

There *have been* determined the rosmarinic acid, phenylpropane derivatives and polyphenolic contents and the antioxidant and antimicrobial activities from six Romanian Lamiaceae medicinal plants: *O. vulgare*, *M. officinalis*, *H. officinalis, R. officinalis, O. basilicum* and *S. officinalis*, this study provides complete up-to date information. Regarding the amount of RA, phenylpropane derivatives and TPC, this research highlighted significant differences among the six plants. *O. vulgare* and *M. officinalis* were found to be the richest species in RA. The antioxidant capacity measured by DPPH, HAPX and EPR spectroscopy methods has found that *O. vulgare* has proved every strong antioxidant effect in accordance with the amount of RA, phenylpropane derivatives and TPC. The antimicrobial study highlighted a remarkable activity against *L. monocytogenes, S. aureus* and *C. albicans* for all samples. The phytochemical and biological potential shown in this study will help to maximize the desired therapeutic benefits of these well known medicinal plants.

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