In vitro Synergistic Antimicrobial Activity of Romanian Propolis and Antibiotics against Escherichia coli Isolated from Bovine Mastitis

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Abstract

The study was aimed to characterize the chemical composition and the antimicrobial activity of Romanian propolis ethanolic extracts (EEP) against antibiotic-sensitive and antibiotic-resistant E. coli strains isolated from bovine mastitis. The preliminary antimicrobial screening was performed by a disk diffusion method, followed by determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) based on broth microdilution assay; further, the synergistic action of propolis with antimicrobial drugs was assessed by a disk diffusion method on agar containing subinhibitory concentrations of propolis. For the chemical characterisation of EEP, the flavonoids (flavones/flavonols, flavanones/dihydroflavonols) and total phenolics were evaluated by spectrophotometric methods. The phenolic compounds of these extracts were also determined using HPLC. The results indicated for Romanian propolis ethanolic extracts the typical poplar composition profile with flavonoids and phenolic acids as main biological active compounds, with chromatographic analysis data confirmed also spectrophotometrically. In addition, positively correlated with the chemical composition, a strong antimicrobial efficacy was exhibited towards E. coli strains, along with interesting synergistic interaction with antibiotics that can be further investigated to obtain propolis-based formulation with antibacterial properties. Subsequent in vitro and in vivo studies evaluating the safety and efficacy are intended to consider propolis in veterinary therapeutic protocols.

Keywords: antibacterial, antimicrobial resistance, ethanolic extracts, flavonoids, phenolics

Introduction

Bovine mastitis is described as one of the most significant diseases affecting dairy herds, a pathology leading to considerable financial losses to the bovine industry due to the costs associated with diagnostics, treatment, redundancy (milk production losses, discarded milk) and animal culling (Halasa et al., 2007; Viguer et al., 2009; Down et al., 2013; Hegazi et al., 2014). Since the etiology of bovine mastitis involves bacterial pathogens, such as Escherichia coli, Staphylococcus aureus and Streptococcus uberis (Bradley, 2002; Hegazi et al., 2014), the use of antimicrobials (intramammary infusion and systemic administration) represents a fundamental part of the therapeutic protocols. Unfortunately, as one of the most important consequences of the intensive and/or inappropriate use of antimicrobials, elevated levels of antimicrobial resistance are currently reported in cases of mastitis etiological agents, with particular reference to E. coli (Bradley, 2002; Viguer et al., 2009; Hegazi et al., 2014).

Worldwide, the emergence of antibiotic-resistant microorganisms triggered the search for alternatives such as natural products with antimicrobial activity (Nweze and Eze, 2009; Hegazi et al., 2014). The stringent need of research into natural alternatives to antimicrobials is emphasized also by the current farm animal health and welfare policies (Ruegg, 2009).

Propolis may represent a valid choice, in the view of bioavailability and complex therapeutic potential conferred by its rich content in biologically active compounds. Defined as a natural product derived from plant resins collected by honeybees (Apis mellifica L.) (Khalil, 2006), propolis is well-known as a highly valuable natural remedy with a multitude of biological and pharmacological properties, namely antibacterial (Dobrowolski et al., 1991; Marcucci et al., 2001; Kosacek et al., 2005; Seidel et al., 2008; Silva et al., 2012; Stan et al., 2012), antiviral (Gekker et al., 2005; Schnitzler et al., 2010), antifungal (Dobrowolski et al., 1991; Quiroga et al., 2005), antioxidant (De Castro, 2001; Kalogeropoulos et al., 2009), anti-inflammatory (Dobrowolski et al., 1991; Banskota et al., 2001; Lotfy, 2006; Silva et al., 2012),...
immunomodulatory (Kosalec et al., 2005; Lotfy, 2006; Rindt et al., 2009b), wound healing (Pascoal et al., 2014), hepatoprotective (Banska et al., 2001), anti-ulcer (Lotfy, 2006) and anti-tumor activities (Banska et al., 2001; Oršolić, 2010).

Not only the biological properties are complex, but also the chemical composition features, with more than 300 identified compounds such as polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids and inorganic compounds (Kosalec et al., 2005; Khalil, 2006) and content variations depending on the collecting location, time and plant source (Bankova et al., 2002; Melioli and Chinou, 2004; Salomao et al., 2004; Bankova, 2005; Popova et al., 2005; Sahiner and Kaftanoglu, 2005; Uzel et al., 2005; Gonzales et al., 2006; Khalil, 2006; Popova et al., 2007; Barbarić et al., 2011; Mârghităș et al., 2013; Huang et al., 2014).

Scientific data regarding Romanian propolis are documented in the literature, mostly about the chemical composition, quality criteria for standardization and certain biological properties (Laslo, 2007; Rindt et al., 2009a, 2009b; Stan et al., 2011; Stan et al., 2012; Mârghităș et al., 2013). Still, the growing interest in the veterinary apitherapy requires more research studies to substantiate the therapeutic use of propolis. Few studies investigated the synergistic effects between propolis and antibiotics (Stepanovic et al., 2003, Orsi et al., 2006) and no studies regarding the synergistic effect of Romanian propolis with other drugs have been done.

Therefore, taking into consideration the importance of new scientific research relating Romanian propolis, this study was aimed to investigate in vitro antibacterial activity of propolis ethanolic extracts tested alone and in combination with five antibiotics against *E. coli* strains isolated from bovine presenting clinical mastitis.

**Materials and Methods**

**Propolis samples**

Five propolis samples were collected from the following Romanian counties: Satu Mare (sample 1), Maramures (sample 2), Salaj (sample 3), Cluj (sample 4) and Bihor (sample 5) and stored in the freezer (-20 °C) until analysis.

**Extraction of active principles from propolis**

Extraction of active principles from propolis (finely grounded) was performed by maceration with 70% ethanol (final concentration 1:100, v/v) with continuous stirring at 400 rpm for 24 h. The ethanolic extract was further diluted in optimal concentrations needed for quantitative analysis according to methods described by Popova et al. (2004). Extraction was realized in triplicate for all samples. All ethanolic extracts of propolis (EEP 1-5) were kept in the dark until anti *E. coli* activity analysis was performed.

**Quantitative determination of flavonoids and total phenolics**

Spectrophotometric methods were carried out for quantitative determination of flavonoids (flavonoids/flavonols, flavanones/dihydroflavonols) and total phenolics (Folin Ciocalteu method) from all EEP (Popova et al., 2004).

Spectrophotometric method from Popova et al. (2004) was adapted to determine the flavones/flavonols: 1 ml of EEP was added to 0.5 ml of 5% aluminium chloride and adjusted to 25 ml with methanol. The solution was left to stand in the dark for 30 min and the absorbance was measured at 425 nm against blank. Standard solution of galangin (0.5 mg/ml) was prepared for calibration curve. For each calibration curve five concentration levels were prepared and three independent determinations were performed for each concentration (n=3). The equation obtained for flavones/flavonols was $Y = 2.04832 \times X - 0.00233; r^2 = 0.99935$.

The protocol for flavanones/dihydroflavonols evaluation was adapted after Popova et al. (2004): 1 ml of propolis ethanolic extract was added to 2 ml dinitrophenylhydrazine (1 g dinitrophenylhydrazine was mixed with 2 ml H$_2$SO$_4$, 96% and diluted to 100 ml with methanol). The obtained solution was heated at 50 °C for 50 min. After cooling at room temperature, the solution was diluted to 10 ml with 10% KOH in methanol. An aliquot of 0.5 ml was transferred into a volumetric flask and the volume was adjusted to 25 ml methanol. Blank solution was prepared by replacing the amount of sample with methanol and carried out through all steps of the procedure. Standard solution of pinocembrin (1 mg/ml) was prepared for calibration curve (equation $Y = 0.11034 \times X - 0.00416; r^2 = 0.99910$). The absorbance was measured at 486 nm against blank.

Total phenolics were determined by Folin Ciocalteu method (Popova et al., 2004; Laslo, 2007). Briefly, 1 ml of EEP was added to 4 ml Folin-Ciocalteu reagent and 6 ml 20% sodium carbonate and the volume was adjusted to 50 ml with distilled water. The test solutions were kept in the dark for 2 h and then the absorbance was measured at 760 nm against blank. Standard mixture of pinocembrin/galangin (2:1, w/w) was used for calibration curve (equation $Y = 0.00709 \times X - 0.00109; r^2 = 0.99932$).

**Phenolic compound identification (HPLC)**

Chromatographic separations of polyphenolic compounds from EEP were performed after adapted method previously published by Laslo (2007). Separation of phenolic compounds was carried out on HPLC - PDA Shimazu, using a Supelcosil LC-18 column (250 mm x 4.6 mm, particle size 5 μm) with Supelguard LC-18 guard column (20 mm x 2.1 mm, particle size 5 μm), using methanol/acetic acid/water as mobile system in the following ratio 10/2/88 for phase A and 90/3/7 for phase B. Elution was performed at 1 ml/min flow rate and injection volume of 20 μl, using gradient method with the following timetable (% B): (0, 0), (10, 15), (30, 50), (45, 85), (55, 100). Chromatograms were recorded at 280 and 340 nm. 1% EEP prepared in ethanol HPLC were filtered through 0.45 μm filters prior injection. The components of propolis extracts were identified by comparison with retention times of known chemical standards commonly found in propolis. Stock solutions of chemical standards of siringic acid, caffeic acid, vanillin, p-coumaric acid, sinapic acid, ferrulic acid, pinocembrin, chrysin, galangin, pinostrobin were prepared in ethanol HPLC (1 mg/ml, v/v). Concentration of separated compounds from EEP was determined using calibration curves expressed in mg/g propolis.

**Escherichia coli strains**

The EEP were evaluated towards *Escherichia coli* strains (n = 10) isolated from clinical cases of bovine mastitis. Both antibiotic-susceptible strains (n = 5) and strains resistant (n = 5) to amoxicillin/clavulanic acid, tetracycline, gentamicin,
flavonoid groups and total phenolics in analyzed EEP

Table 1. Spectrophotometric quantitative determination of specific flavonoid groups and total phenolics in analyzed EEP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average ± Standard deviation</th>
<th>Sample</th>
<th>Average ± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flavones/Flavonols (mg/g propolis)</td>
<td>Flavones/ Dihydroflavonols (mg/g propolis)</td>
<td>Total phenolics (mg/g propolis)</td>
</tr>
<tr>
<td>1</td>
<td>3.81 ± 0.23</td>
<td>2.75 ± 0.04</td>
<td>30.61 ± 1.16</td>
</tr>
<tr>
<td>2</td>
<td>6.05 ± 0.35</td>
<td>1.96 ± 0.26</td>
<td>30.81 ± 1.16</td>
</tr>
<tr>
<td>3</td>
<td>1.74 ± 0.12</td>
<td>3.32 ± 0.18</td>
<td>34.96 ± 9.36</td>
</tr>
<tr>
<td>4</td>
<td>9.22 ± 0.35</td>
<td>3.92 ± 0.12</td>
<td>45.60 ± 3.27</td>
</tr>
<tr>
<td>5</td>
<td>8.20 ± 0.35</td>
<td>4.01 ± 0.13</td>
<td>48.11 ± 2.76</td>
</tr>
<tr>
<td>Mean</td>
<td>5.80 ± 3.08</td>
<td>3.19 ± 0.86</td>
<td>38.02 ± 8.30</td>
</tr>
</tbody>
</table>

Afterwards, 10 μl of each EEP dilution were cultured on Mueller Hinton agar plates and incubated for 24h at 37 °C, when the MICs values were determined considering the lowest concentrations of EEP able to inhibit the visible growth of bacteria (no turbidity), when compared to the control. The bacterial strains prepared as inoculum were inoculated on Mueller Hinton agar plates; sterile filter paper discs impregnated with 20 μl EEP were applied and the inhibition zone diameters (IZD, mm) were recorded after 24 h incubation at 37 °C. The testing was performed in duplicate. Positive controls (antibiotics) and 70% ethanol (EEP solvent) were included for each determination.

Evaluation of the interaction between the EEP and antibiotics

In order to evaluate the anti E. coli efficacy of EEP and antibiotic combinations, the agar diffusion method was carried out as previously described by Nweze and Eze (2009). Briefly, the bacterial strains prepared as inoculum were cultured on Mueller Hinton agar mixed with sub-inhibitory concentration of EEP and tested against five antimicrobials: amoxicillin/clavulanic acid (AMC, 20/10 μg), tetracycline (TF, 30 μg), gentamycin (CN, 10 μg), enrofloxacin (ENR, 5 μg) and florfenicol (FFC, 30 μg). After 24 h of incubation at 37 °C, the inhibition zone diameters (IZD) were measured. The results of the assay were expressed as percentage change in IZD compared to controls (IZD for antibiotics) and recorded as synergistic effect for IZD ≥ 19%, additive effect for IZD between 0 and 19%, and antagonism for IZD < 0 (Nweze and Eze, 2009).

Statistical analysis of results

Statistical analysis of data was performed using the Microsoft Office Excel 2010 program. The results were expressed as average ± standard deviation. Comparisons between antibiotics and EEP combinations were assessed by analysis of variance (ANOVA). In addition, Pearson’s
The values for Inhibition zone diameters (IZD) (mm) determined for EEP are as follows:

<table>
<thead>
<tr>
<th>EEP</th>
<th>IZD (mm)</th>
<th>MIC % (v/v)</th>
<th>MBC % (v/v)</th>
<th>MBC/MIC</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.5 ± 0.7</td>
<td>1 2 2</td>
<td>1 2</td>
<td>1.0</td>
<td>bactericidal activity</td>
</tr>
<tr>
<td>2</td>
<td>20.5 ± 0.7</td>
<td>1 1 1</td>
<td>1 2</td>
<td>0.5</td>
<td>bactericidal activity</td>
</tr>
<tr>
<td>3</td>
<td>21.0 ± 1.4</td>
<td>0.25 0.5</td>
<td>1 2</td>
<td>1.2</td>
<td>bactericidal activity</td>
</tr>
<tr>
<td>4</td>
<td>24.5 ± 2.1</td>
<td>0.125 0.25</td>
<td>2</td>
<td>0.5</td>
<td>bactericidal activity</td>
</tr>
<tr>
<td>5</td>
<td>25.0 ± 1.4</td>
<td>0.125 0.125</td>
<td>1</td>
<td>1.0</td>
<td>bactericidal activity</td>
</tr>
</tbody>
</table>

The values for Inhibition zone diameters (IZD) (mm) determined for EEP are expressed as average ± standard deviation.

Results and Discussion

Chemical characterization of ethanolic extracts of propolis

The analysed propolis ethanolic extracts had a red reddish-brown color, with a specific taste and smell of aromatic resins from *Populus sp*. To evaluate both total phenolics and flavonoids, spectrophotometric methods were applied according to Popova et al. (2004), Bankova (2005) and Laslo (2007); spectrophotometric methods are fast and reliable methods compared to chromatographic which have the disadvantage to be more expensive. Total phenolics (Table 1) in the studied EEP ranged from 30.61 mg/g propolis to 48.11 mg/g propolis. The lowest amount of phenolics was recorded for EEP 1 and 2, which were collected from a mountain area, where fir is the main vegetal source of resins for propolis. EEP 4 and 5 presented the highest amount of total phenolics (over 45 mg/g propolis) and this is due to abundance of other vegetal sources of resins like pine, poplar and chestnut. These results are in agreement with previous reports on Romanian propolis composition (Laslo, 2007; Stan et al., 2011).

In general, EEP presented high amounts of total flavonoids (9.00 ± 3.94 mg/g propolis), where 1.74 - 9.22 mg/g belonged to the group of flavones/flavanones and 1.96 - 4.01 mg/g were flavanones/dihydroflavonols (Table 1).

The smallest amount of flavonoids was recorded for EEP 3 (about 5 mg/g propolis), although total phenolics were in average range (34.96 ± 9.36 mg/g propolis) compared to the other EEP (38.02 ± 8.30 mg/g propolis). This lead to assumption that EEP 3 had a higher concentration of phenolic acids, hypothesis that was further confirmed by HPLC results (Table 2), where data clearly showed that the highest concentration of p-coumaric acid and ferrulic acid was found in this case.

By HPLC analysis some specific compounds (Table 2) like siringic acid, caffeic acid, vanillin, p-coumaric acid, sinapic acid, ferrulic acid, pinocembrin, chrysin, galangin, pinostrobin were identified and quantified.

These compounds are considered typical for poplar type of propolis, which is mainly found in European temperate zone (Bankova, 2005; Laslo, 2007). All EEP presented most of aforementioned compounds. At the same time, specific phenolics like caffeic acid, galangin and pinostrobin were missing from sample 3.

The most common compounds were represented by p-coumaric acid, ferrulic acid and chrysin. P-coumaric acid was found in all EEP in concentration ranging from 0.46 (sample 2) to 2.45 mg/g propolis (sample 3), while siringic acid and sinapic acid were least present and found only in EEP 3 with concentration of 0.22 mg/g propolis and 0.03 mg/g propolis, respectively.

Flavonoid chrysin was the main compound in propolis EEP in concentration ranging from 0.59 mg/g propolis (sample 3) to 2.75 mg/g propolis (sample 4), while galangin was completely absent in EEP 3, 4 and 5 and in very low amount in EEP 1 and 2 (0.02-0.58 mg/g propolis).

Compared to Croatian propolis analysed by Barbarić et al. (2011) that had the ferrulic acid as the most commonly found phenolic acid (0.03-0.9 mg/g propolis), the EEP in our experiment identified the same compound, but in higher concentration (0.13-2.39 mg/g propolis). Other components identified in Croatian propolis were: p-coumaric acid in 16 samples out of 20, with concentration ranging from 0.0023 mg/g to 0.156 mg/g propolis, chrysin in only 8 samples and in the range between 0.7-4.1 mg/g propolis and galangin in 17 samples and with higher concentration (0.37-4.74 mg/g propolis) (Barbarić et al., 2011).

Previous chemical studies proved the complexity of propolis composition that varies with botanical and geographical origin (Bankova et al., 2002; Melliou and Chinou, 2004; Salomão et al., 2004; Bankova, 2005; Popova et al., 2005; Sahinler and Kaftanoglu, 2005; Uzel et al., 2005; Gonzales et al., 2006; Khalil, 2006; Popova et al., 2007; Barbarić et al., 2011; Huang et al., 2014). The main propolis types are represented by: poplar type (Europe – temperate areal), birch propolis (Russia), green propolis (Brazil), red propolis (Cuba) (Bankova, 2005). Romanian propolis belongs to poplar type, since pine, poplar, chestnut and fir are the main vegetal sources of resins for honey bees to prepare the propolis (Laslo, 2007). According to Bankova (2005) typical poplar type propolis has the following compositional characteristics: 8 ± 4% flavones/flavanones, 6 ± 2% flavanones/dihydroflavonols and 28 ± 9% total phenolics. However, scholars recorded high variation of phenolics content mainly due to various factors such as flora available to bees at collection site, climate and competition of bees over the same area (Laslo, 2007; Mărgitaș et al., 2013).

Antimicrobial activity of ethanolic extracts of propolis

The antimicrobial potential of EEP was expressed in vitro against all tested *E. coli* strains, but with lower inhibition zone diameters in case of the antibiotic-resistant ones. The obtained diameters varied from 20.5 ± 0.7 mm (EEP 2) to 25.0 ± 1.4 mm (EEP 5) in case of antibiotic-susceptible strains and 16.5 ± 0.7 mm (EEP 2) to 19.5 ± 0.7 mm (EEP 4) for the antibiotic-resistant strains, respectively. All five EEP inhibited the growth of *E. coli*; the most intense inhibitory effect was recorded for EEP 4 and 5 (Table 3).
Fig. 2. Inhibition zone diameter (IZD) (mm) determined for EEP and antibiotics combination against E. coli antibiotic-resistant strains (n = 5)

In case of antibiotic-susceptible E. coli strains, the values of IZD were similar to those determined by the antibiotics, the positive controls of the assay, while for the antibiotic-resistant strains significant differences were noticed when comparing to antibiotics – EEP combinations (Fig 2) as follow: F < 0.001 for AMC, CN, ENR and FFC and P < 0.001 for TE (P value determined by ANOVA analysis). The extracts solvent (70% ethanol) had not inhibitory activity on the studied strains indicating the antimicrobial efficacy of propolis against E. coli.

The minimum inhibitory concentrations (MICs) of the EEP ranged between 0.125 and 2% (v/v) when tested against E. coli antibiotic-susceptible strains, while those obtained for antibiotic-resistant strains were between 2 and 4% (v/v). As for the minimum bactericidal concentrations (MBCs), the values were similar or two times higher than MICs for both types of E. coli strains. The EEP effect against E. coli strains was considered bactericidal based on the interpretation previously established (Pavithra et al., 2010) for the ratio MBC/MIC < 4 (Table 3).

A percentage change in the inhibition zone diameter ≥ 19% was noticed for 92% (23/25) of the EEP and antibiotics combinations; thus, according to the interpretation given by Nweze and Eze (2009), in vitro synergistic interactions (Table 4) were established between the EEP and five antibiotics frequently used in bovine pathology, including also E. coli induced mastitis. The synergy between EEP and antibiotics was observed for all the tested antimicrobials, except for florfenicol (Table 4) that in combination with EEP 1 and 3 displayed additive effect against the E. coli strains.

Based on the bacterial growth inhibition zone diameters and values determined as MICs and MBCs an important antimicrobial activity was demonstrated for all tested EEP. The antimicrobial potential of propolis extracts from different geographical locations was indicated by numerous studies (Bankova et al., 1995; Sforzin et al., 2000; Uğur et al., 2000; Banskota et al., 2001; Stepanović et al., 2003; Mellou and Chinou, 2004; Salmão et al., 2004; Popova et al., 2005; Orsi et al., 2006; Scacccozchio et al., 2006; Seidel et al., 2008; Raghukumar et al., 2010; Ramanauškinė et al., 2013). While several review and research articles described a broad spectrum of propolis and propolis compounds, with demonstrated activity against a wide range of organisms (Mrozoeva et al., 1997; Uğur et al., 2000; Lotfy, 2006; Noori et al., 2012; Mărghitaș et al., 2013; Bankova et al., 2014), the information regarding the efficacy against E. coli is slightly contradictory. Generally, a stronger antibacterial effect is presented against the Gram-positive organisms (Banskota et al., 2001; Gonsales et al., 2006; Seidel et al., 2008; Raghukumar et al., 2010) and propolis extracts from different geographical areas appear to be particularly active against Staphylococcus aureus, both reference and clinical strains (Krol et al., 1993; Bankova et al., 1995; Fernandes Júnior et al., 2005; Gonsales et al., 2006; Scacccozchio et al., 2006; Alencar et al., 2007; de Andrade et al., 2009; Santana et al., 2012), including methicillin-resistant Staphylococcus aureus (MRSA) (Onlen et al., 2007; Raghukumar et al., 2010). Bankova et al. (1995) reported an important antibacterial efficacy of Brazilian propolis in relation to the phenolic compounds, confirmed also by Marcucci et al. (2001). The Brazilian red propolis was found active in vitro against reference strains Staphylococcus aureus ATCC 25923 and Staphylococcus mutans UA159, with the biologically active compounds belonging to flavonoids group (Alencar et al., 2007).

With regard to propolis antibacterial activity against E. coli, as opposed to certain data found in literature presenting weak or no activity against this bacterium (Bankova et al., 1999; Kosalec et al., 2005; Gonsales et al., 2006; Seidel et al., 2008), all tested EEP were found active in vitro against E. coli strains, both antibiotic-susceptible and antibiotic-resistant strains isolated from bovine mastitis. The Romanian propolis ethanolic extracts efficacy on E. coli was reported also by Mărghitaș et al. (2013), with inhibition zone diameters ranging between 7 - 12 mm and MIC of 0.625% (v/v). These results are consistent also with those reported by Hegazi et al. (2000), which evaluated European propolis extracts obtained from France, Austria and Germany and observed antimicrobial activity against Staphylococcus aureus, Escherichia coli and Candida albicans. German propolis displayed the highest antimicrobial potential against Staphylococcus aureus and Escherichia coli and possessed significant high concentrations of flavonoids (Hegazi et al., 2000). Thus, the variations noticed in the antibacterial activity of propolis from distinct areas can be explained taking into consideration the chemical composition complexity of this natural product.

The antimicrobial potential of propolis was previously considered as a possible alternative for the bovine mastitis treatment, but such in vitro studies were focused on Staphylococcus aureus (Rindt et al., 2009a; Santana et al., 2012). A recent study pointed out the in vitro efficacy of Egyptian propolis on several Gram-positive bacteria isolated from bovine mastitis (Staphylococcus aureus, coagulase-negative staphylococci, Streptococcus agalactiae, Streptococcus dysgalactiae) and the lack of activity against Gram-negative bacteria (E. coli and Pasteurella spp.) (Hegazi et al., 2014).

To the best of authors’ knowledge, this is the first study aimed to investigate the antibacterial activity of Romanian propolis and antibiotics combinations against antibiotic-sensitive and antibiotic-resistant E. coli strains isolated from bovine mastitis. Synergistic effects of tested EEP and five
The synergistic activity between propolis and antibiotics was reported by other authors (Stepanović et al., 2003; Orsi et al., 2006; Scanzocchio et al., 2006; Orsi et al., 2012), but most of these studies included *Staphylococcus aureus* strains (Krol et al., 1993; Stepanović et al., 2003; Fernandes Júnior et al., 2005; Onlen et al., 2007). From the group of Gram-negative bacteria, *Salmonella Typhi* manifested in *vitro* an enhanced susceptibility towards combinations between both Brazilian and Bulgarian propolis and antibiotics such as amoxicillin, ampicillin and cephalaxin (Orsi et al., 2006; Orsi et al., 2012), with similar MICs (9.90 and 10.0%, respectively), but with different types of action: bacteriostatic activity in case of Brazilian propolis, while the Bulgarian one acted bactericidal (Orsi et al., 2006).

The ability of propolis extracts to potentiate the antimicrobial activity of other substances was reported also in case of essential oils (Probst et al., 2011), honey (Noori et al., 2012), lysozyme (Ramanauksiene et al., 2009).

According to scientific literature, certain mechanisms associated with such synergistic effects may encompass flavonoids from propolis conferring several antibacterial properties: a decreased resistance of the bacterial wall that becomes more susceptible to antibiotics (Pascoal et al., 2014), direct inhibitory effect on ribosomes (Störzin and Bankova, 2011; Orsi et al., 2012; Pascoal et al., 2014), inhibition of several bacterial enzymes (Daglia, 2012), alteration of bacterial protein expression (Daglia, 2012), modulation of β-lactam resistance (Cushnie and Lamb, 2011).

Given the great variability of the chemical composition of propolis and the propolis compounds role in the expression of antimicrobial potential, Popova et al. (2005) stated that a complete characterization of this property should involve qualitative and quantitative chemical analysis. Additionally, scientific data demonstrated that quantification of propolis active principles as groups of compounds correlated better with biological activity, especially the antimicrobial action, than the quantification of individual constituents (Popova et al., 2010). Therefore, following the chemical characterisation of EEP, with the flavonoids and total phenolics quantitative determination and the chromatographic identification of phenolic compounds, and the antibacterial potential evaluation, Pearson correlation coefficients were calculated between these parameters (Table 5).

Flavanones/dihydroflavonols were significantly negatively correlated with MICs for both antibiotic-sensitive *E. coli* strains ($r^2 = -0.922$) and antibiotic-resistant *E. coli* strains ($r^2 = -0.888$). The results also indicated a very strong negative correlation between total phenolics with MICs for antibiotic-sensitive *E. coli* strains ($r^2 = -0.864$) and antibiotic-resistant *E. coli* strains ($r^2 = -0.827$) and a similar pattern in case of total phenolics with MBCs ($r^2 = -0.806$ for antibiotic-sensitive *E. coli* strains and $r^2 = -0.511$ for antibiotic-resistant *E. coli* strains). Previous study (Bankova, 2005) had already demonstrated strong negative correlation between the concentration of total phenolics in propolis and MIC the greater the concentration, the lower the MIC ($P = 0.003$). This data supports the concept that...
measuring the concentrations of groups of active compounds instead of that of individual components is the right approach in the case of propolis (Bankova, 2005; Popova et al., 2007).

Our results showed that combinations between propolis and antibiotics have synergistic effect against *E. coli* strains and the enhanced antimicrobial efficacy was related to the EEP chemical composition (Table 5), with flavone/flavonols strongly positive correlated with flemorical (r² = 0.858) and flavanone/dihydrofuranols strongly positive correlated with enrofloxacin (r² = 0.851). In addition, total phenolics proved strong positive correlation with all tested antibiotics: enrofloxacin (r² = 0.823), gentamycin (r² = 0.825), ofloxacil (r² = 0.685), amoxicillin/cavulanic acid (r² = 0.537), except for tetracycline (r² = 0.162).

Phenolic compounds such as flavonoids represent a key element of propolis samples characterization in connection with the biological activity of this natural product (Bankova et al., 1995; De Castro, 2001; Marcucci et al., 2001; Kosalec et al., 2005; Gonzales et al., 2006; Alencar et al., 2007). Thus, the identification and quantification of phenolic compounds in Romanian propolis ethanolic extracts indicate not only the authenticity and the quality of tested EEP, but also underline the complex antimicrobial potential manifested against *E. coli* and moreover in the form of synergism with antibiotics.

Conclusions

The study indicated for Romanian propolis ethanolic extracts the typical poplar composition profile with flavonoids and phenolic acids as main biological active compounds, with chromatographic analysis data confirmed also spectrophotometrically. Furthermore, a strong antimicrobial efficacy positively correlated to the chemical composition was exhibited against *E. coli* strains isolated from bovine mastitis, along with interesting synergistic interaction with antibiotics that can be further investigated to obtain propolis-based formulation with antibacterial properties. Subsequent in vitro and in vivo studies evaluating the safety and efficacy are intended to consider propolis in veterinary therapeutic protocols.

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References


