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Original Article

Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria

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ARTICLE INFO

Article history:

Received 9 March 2016

Received in revised form

20 May 2016

Accepted 11 June 2016

Available online 15 July 2016

Keywords:

antagonistic effect
antibacterial activity
essential oils
food-related bacteria
herbs

ABSTRACT

The aim of this study was to compare the antibacterial effects of several essential oils (EOs) alone and in combination against different Gram-positive and Gram-negative bacteria associated with food products. Parsley, lovage, basil, and thyme EOs, as well as their mixtures (1:1, v/v), were tested against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium*. The inhibitory effects ranged from strong (thyme EO against *E. coli*) to no inhibition (parsley EO against *P. aeruginosa*). Thyme EO exhibited strong (against *E. coli*), moderate (against *S. typhimurium* and *B. cereus*), or mild inhibitory effects (against *P. aeruginosa* and *S. aureus*), and basil EO showed mild (against *E. coli* and *B. cereus*) or no inhibitory effects (against *S. typhimurium*, *P. aeruginosa*, and *S. aureus*). Parsley and lovage EOs revealed no inhibitory effects against all tested strains. Combinations of lovage/thyme and basil/thyme EOs displayed antagonistic effects against all bacteria, parsley/thyme EOs against *B. cereus*, *S. aureus*, *P. aeruginosa*, and *E. coli*, and lovage/basil EOs against *B. cereus* and *E. coli*. Combinations of parsley/lovage and parsley/basil EOs exhibited indifferent effects against all bacteria. The combination of lovage/basil EO showed indifferent effect against *S. aureus*, *P. aeruginosa*, and *S. typhimurium*, and the combination parsley/thyme EO against *S. typhimurium*. Thyme EO has the highest percentage yield and antibacterial potential from all tested formulations; its combination with parsley, lovage, and basil EOs determines a reduction of its antibacterial activity. Hence, it is recommended to be used alone as the antibacterial agent.

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<http://dx.doi.org/10.1016/j.jfda.2016.06.002>

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1. Introduction

Herbs have been used since ancient times for their medicinal or aromatic properties [1]. The increased interest in the use of natural preservatives as an alternative to chemical ones has brought renewed attention to the aromatic plants [2]. Lately, their bioactive compounds, essential oils (EOs), are used in active food packaging formulations for preservation purposes [3,4]. EOs can be extracted from different parts of herbs by several techniques, as water or steam distillation, solvent extraction, expression under pressure, supercritical fluid extraction, and subcritical water extraction [5]. These contain a wide variety of plant secondary metabolites that can inhibit or slow the growth microorganisms [6,7]. The main constituents of EOs are mono- and sesquiterpenes, along with carbohydrates, phenols, alcohols, ethers, aldehydes, and ketones, which are responsible for the biological activity of aromatic and medicinal plants as well as for their fragrance [8]. Oxygenated terpenoids (e.g., alcohols and phenolic terpenes) manifest the highest antimicrobial activity, but some hydrocarbons also display antimicrobial effects. Interactions between these types of compounds may lead to antagonistic, additive, or synergistic effects. The minor components are crucial to these effects [5].

Parsley, lovage, basil, and thyme are few of the aromatic herbs commonly used in Romania. These easy-to-grow plants have low costs of production. Different parts of these herbs (e.g., leaves, flowers, stems, fruits, and seeds) have been used to extract EOs. There are several studies that reveal their antibacterial activities against various bacterial strains (see supplemental online material, Tables S1 and S2). Yet, the efficiency of their mixtures against potential foodborne pathogens and spoilage bacteria has not been as yet fully studied.

In this regard, the antibacterial properties of two species of Lamiaceae (*Ocimum basilicum* and *Thymus vulgaris*) and two of Apiaceae (*Petroselinum crispum* and *Levisticum officinale*) against some Gram-positive and Gram-negative bacteria were studied in the present research. Four EOs (extracted from parsley, lovage, basil, and thyme dried leaves) were evaluated for their antibacterial activities individually and then in combination using five different *in vitro* models. To the extent of our knowledge, this is the first work to investigate the antibacterial potential of these EO mixtures. Particular attention has been paid to their synergistic, additive, indifferent, or antagonistic effects on four potential foodborne pathogens (*Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*) and one spoilage bacteria (*Pseudomonas aeruginosa*). To this intent, two antimicrobial susceptibility tests were used: the Kirby–Bauer disk diffusion test (for measuring zone diameters of bacterial growth inhibition) and the resazurin microtiter plate-based antibacterial assay [to determine the minimum inhibitory concentration (MIC)].

2. Materials and methods

2.1. Plant materials and EO extraction

Dried leaves of parsley, lovage, basil, and thyme were purchased from a Romanian company. EOs were extracted by

hydrodistillation (50 g of dried leaves with 750 mL distilled water) using a Clevenger-type apparatus (for 3 hours). The extracts were dried over anhydrous sodium sulfate and stored at 4°C until analysis. The extraction yield was calculated as the volume of oil (mL) per dried leaves weight (g) and multiplied by 100. EO mixtures were prepared as follows: (1) parsley/lovage EO—parsley EO/lovage EO, 1:1 (v/v); (2) parsley/basil EO—parsley EO/basil EO, 1:1 (v/v); (3) parsley/thyme EO—parsley EO/thyme EO, 1:1 (v/v); (4) lovage/basil EO—lovage EO/basil EO, 1:1 (v/v); (5) lovage/thyme EO—lovage EO/thyme EO, 1:1 (v/v); and (6) basil/thyme EO—basil EO/thyme EO, 1:1 (v/v).

2.2. Bacterial strains

The following microorganisms were tested: *B. cereus* (ATCC 11778), *S. aureus* (ATCC 6538P), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), and *Salmonella typhimurium* (ATCC 14028). Each strain was grown in a test tube containing 45 mL sterile nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 24 hours (except *B. cereus*, which was grown at 30°C for 24 hours). The purity of the inoculum was confirmed by plating on appropriate selective media and microscopic examination of the Gram-stained smear (Optika microscope, B-252, M.A.D; Apparecchiature Scientifiche, Milan, Italy). A loopful of inoculum was transferred by streaking onto a selective medium: (1) MYP agar supplemented with Egg Yolk Emulsion and Polymyxin B (Oxoid Ltd.) for *B. cereus*; (2) Baird–Parker agar base supplemented with Egg Yolk Tellurite Emulsion (Oxoid Ltd.) for *S. aureus*; (3) *Pseudomonas*-agar P, base (Merck KGaA, Darmstadt, Germany) for *P. aeruginosa*; (4) TBX agar (Oxoid Ltd.) for *E. coli*; and (5) XLD agar (Oxoid Ltd.) for *S. typhimurium*. Plates were incubated for 24 hours at 30°C (*B. cereus*) or 37°C (*S. aureus*, *P. aeruginosa*, *E. coli*, and *S. typhimurium*). Bacterial morphology was confirmed by optical microscopy. Several colonies were collected with a sterile inoculating loop, transferred into sterile saline solution, and adjusted to the desired concentration using the McFarland nephelometer standards [9].

2.3. Agar diffusion susceptibility testing

EOs and their mixtures were assessed against all bacteria using the Kirby–Bauer disk diffusion test (9-mm sterile paper disks; ANTF-009-1K0; PRAT DUMAS, Couze-St-Front, France). Gentamicin was used as positive control (0.04 mg/mL in saline solution). One hundred microliters of inoculum (1.5×10^8 CFU/mL) was dispersed over the entire surface of the Mueller–Hinton agar plate (Sifin Diagnostics GmbH, Berlin, Germany) using a Drigalski spatula. A sterile paper disk was placed in the middle of a Petri dish. Then, 40 μ L EO or gentamicin was released on the paper disk. Plates were incubated for 24 hours at 30°C (*B. cereus*) or 37°C (*S. aureus*, *P. aeruginosa*, *E. coli*, and *S. typhimurium*). A digital caliper was used to measure the inhibition zone diameter (in millimeters). Three replicates were run for each EO/mixture.

2.4. Broth microdilution susceptibility testing

The MIC was determined using the resazurin microtiter plate-based antibacterial assay. One part of EO was dissolved in

eight parts 50% ethanol and one part Tween 80 [10]. Into the first well of a 96-well microtiter plate, 100 μ L sterile nutrient broth and 100 μ L diluted EO were added. Serial 11-fold dilutions were performed by transferring 100 μ L from well to well (on row). From the last well of the row, 100 μ L was discarded. To each well, 10 μ L of inoculum (1.5×10^5 CFU/mL) was added. The reached concentrations ranged from 0.01 to 47.62 μ L EO/mL. Gentamicin (0.04 mg/mL in saline solution) was used as a positive control. For the negative control, one part of the saline solution was dissolved in eight parts 50% ethanol and one part Tween 80. Microplates were incubated for 20–22 hours at 37°C (except for *B. cereus*, which was incubated for 20–22 hours at 30°C). To each well, 20 μ L resazurin aqueous solution (0.2 mg/mL) was added. Microplates were incubated for 2 hours at 37°C (except for *B. cereus*, which was incubated for 2 hours at 30°C). The concentration that completely inhibited bacterial growth (MIC) was the concentration at which the blue color did not change into pink. Three replicates were run for each EO.

2.5. Statistical analysis

To perform statistical tests, the Minitab statistical software (version 16.1.0, LEAD Technologies, Inc.) was used. The statistically significant differences between EO formulations were carried out by one-way analysis of variance at 95% confidence level ($p \leq 0.05$). As a posttest procedure, Tukey's honest significance test was used. Correlations among data were calculated using Pearson's correlation coefficient.

3. Results and discussion

An exhaustive review of the literature (see Tables S1 and S2) shows that there is a lack of studies on the antibacterial activity of parsley and lovage EOs. This study intends to contribute toward filling some gaps in the current knowledge. Toward this purpose, EOs of parsley, lovage, basil, and thyme were extracted from dried leaves. The percentage yields of EOs are 0.16% for parsley, 0.28% for lovage, 0.40% for basil, and 2.20% for thyme. Furthermore, individual EOs and their mixtures (1:1, v/v) were evaluated against Gram-positive (*B. cereus* and *S. aureus*) and Gram-negative bacteria (*P. aeruginosa*, *E. coli*, and *S. typhimurium*) using the Kirby–Bauer disk diffusion test. As the results of this test indicate that the antibacterial activity of EO combinations is weaker than that of thyme EO (Table 1), the resazurin microtiter plate-based antibacterial assay was further carried out on individual EOs.

3.1. Antibacterial action on single EOs

Table 1 shows the results of the Kirby–Bauer disk diffusion test. Bacterial strains used in the present study are more or less susceptible to each EO. The size of inhibition zone varies depending on the EO and bacterial strain used. The scale of measurement was as follows (disk diameter included): strong inhibitory effect $\rightarrow$ zone of inhibition ≥ 28 mm, moderate inhibitory effect $\rightarrow 16 \leq$ zone of inhibition < 28 mm, mild inhibitory effect $\rightarrow 12 \leq$ zone of inhibition < 16 mm, and no inhibitory effect $\rightarrow$ zone of inhibition < 12 mm [11]. Thyme EO

exhibits the best inhibitory activity against all bacteria evaluated by the Kirby–Bauer disk diffusion test (range 12.16–36.41 mm), followed by basil, lovage, and parsley EOs. Its zone of inhibition is larger (for *E. coli* and *S. typhimurium*) or similar (for *B. cereus*) to the size of gentamicin zone (the antibiotic used as positive control). Among the tested microorganisms, it produces the largest zone of inhibition against *E. coli* (strong inhibitory effect), followed by *S. typhimurium* (moderate inhibitory effect), *B. cereus* (moderate inhibitory effect), *P. aeruginosa* (mild inhibitory effect), and *S. aureus* (mild inhibitory effect). The inhibition zones of basil EO against tested bacteria vary between 9.91 and 14.85 mm. It shows the best antibacterial activity against *E. coli* (mild inhibitory effect), followed by *B. cereus* (mild inhibitory effect), *S. typhimurium* (no inhibitory effect), *P. aeruginosa* (no inhibitory effect), and *S. aureus* (no inhibitory effect). Lovage and parsley EOs exert no inhibitory effects against all five bacterial strains. Lovage EO causes faint and similar zones of inhibition (range 9.44–10.38 mm) against tested microorganisms. Parsley EO displays the weakest inhibitory activity (range NI–10.07 mm) against all bacteria. It does not show any inhibitory activity against *P. aeruginosa*.

Table 2 summarizes the results of the resazurin microtiter plate-based antibacterial assay. Between the two testing methods, an inverse correlation has been generally noticed; EOs with a large zone of inhibition present a low minimum inhibitory concentration. The strong negative correlations found between the diameter of inhibition zone and MIC of *B. cereus* ($r^2 = -0.776$; $p = 0.003$), *S. aureus* ($r^2 = -0.743$; $p = 0.006$), *P. aeruginosa* ($r^2 = -0.957$; $p = 0.000$), *E. coli* ($r^2 = -0.980$; $p = 0.000$), and *S. typhimurium* ($r^2 = -0.896$; $p = 0.000$) confirm the above remark.

Gram-negative bacteria are more resistant to thyme and basil EOs; however, parsley and lovage EOs manifest similar behaviors against both Gram-positive and Gram-negative bacteria. Results obtained by the resazurin microtiter plate-based antibacterial assay show that thyme EO is the most effective against *S. aureus*, followed by *E. coli*, and evenly by *B. cereus*, *P. aeruginosa*, and *S. typhimurium*. The EO of basil reveals the lowest MIC against *S. aureus*, followed evenly by *B. cereus* and *E. coli*, and evenly by *P. aeruginosa* and *S. typhimurium*. Parsley and lovage EOs have the highest MICs. The activity of parsley EO was more pronounced against *S. aureus* and *E. coli*, followed by *B. cereus*, and to the same extent by *P. aeruginosa* and *S. typhimurium*. The EO of lovage gives the lowest MICs against *S. aureus*, followed by *E. coli*, *P. aeruginosa*, and evenly by *B. cereus* and *S. typhimurium*. Both test results confirm that the antibacterial activity of thyme EO is strong, that of basil EO is moderate, and that of parsley and lovage EOs is weak.

Although there are many investigations on the *E. coli* (ATCC 25923) susceptibility to EOs, this is the first study relating to EOs extracted from dried leaves of parsley, lovage, basil, and thyme. In previous studies (see Tables S1 and S2), the sensitivity of *E. coli* (ATCC 25923) was tested with EOs from fruits of lovage [12], from aerial parts of basil [13–15], and commercially available EOs of basil and thyme [16–25]. Against *B. cereus* (ATCC 11778), commercially available EOs of parsley, basil, and thyme [19,21,23,24,26], and the EO from aerial parts of basil [14] were tested. The sensitivity of *S. aureus* (ATCC 6538P) was tested with commercially available EOs of basil and thyme [26,27]. Against

Table 1 – Antibacterial activity of essential oils (EOs; zone of inhibition including the diameter of the paper disk, mm) by agar diffusion testing.

Test substance	<i>B. cereus</i> (ATCC 11778)	<i>S. aureus</i> (ATCC 6538P)	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. coli</i> (ATCC 25922)	<i>S. typhimurium</i> (ATCC 14028)
Parsley EO	9.46 ^d	10.07 ^{def}	NI	9.60 ^h	9.77 ^c
Lovage EO	9.53 ^d	10.38 ^{cde}	9.44 ^d	9.85 ^{gh}	10.29 ^c
Basil EO	13.58 ^c	9.91 ^{def}	11.12 ^b	14.85 ^e	11.58 ^c
Thyme EO	24.81 ^a	12.16 ^a	14.15 ^a	36.41 ^a	27.44 ^a
Parsley/lovage EO	9.49 ^d	9.43 ^f	9.20 ^d	9.95 ^{gh}	9.74 ^c
Parsley/basil EO	10.05 ^d	9.54 ^{ef}	9.39 ^d	12.19 ^f	10.46 ^c
Parsley/thyme EO	13.89 ^c	11.07 ^{bc}	10.32 ^c	23.03 ^c	24.90 ^a
Lovage/basil EO	10.26 ^d	10.49 ^{cd}	9.41 ^d	11.95 ^{fg}	10.21 ^c
Lovage/thyme EO	13.13 ^c	10.54 ^{cd}	9.84 ^{cd}	20.25 ^d	14.61 ^b
Basil/thyme EO	16.54 ^b	11.64 ^{ab}	11.38 ^b	25.76 ^b	27.27 ^a
<i>p</i>	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
Gentamicin	25.32	22.49	21.28	23.67	24.21

Values are expressed as mean of three replicates. Values with different letters in the same column indicate statistically significant differences (Tukey's test, $p < 0.05$).

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$; $p \geq 0.05$, not significant.

NI = no inhibition (<9 mm diameter).

Table 2 – Antibacterial activity of essential oils (MIC, μ L EO/mL) and gentamicin (MIC, μ g GE/mL) by broth microdilution testing.

Bacterial strain	Parsley EO	Lovage EO	Basil EO	Thyme EO	Gentamicin
<i>Bacillus cereus</i> (ATCC 11778)	22.68	47.62	10.80	0.56	0.05
<i>Staphylococcus aureus</i> (ATCC 6538P)	10.80	2.45	2.45	0.06	0.05
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	47.62	22.68	22.68	0.56	0.50
<i>Escherichia coli</i> (ATCC 25922)	10.80	10.80	10.80	0.27	0.24
<i>Salmonella typhimurium</i> (ATCC 14028)	47.62	47.62	22.68	0.56	0.50

EO = essential oil; GE = gentamicin; MIC = minimum inhibitory concentration.

P. aeruginosa (ATCC 27853), EOs from aerial parts of basil and thyme [8,14,28], as well as commercially available EOs of parsley, basil, and thyme [16,19,20] were tested. The sensitivity of *S. typhimurium* (ATCC 14028) was tested with EOs from aerial parts of basil [14], leaves of basil [29], flowers and leaves of thyme [30], and commercially available EOs of parsley, basil, and thyme [11,19,31].

3.2. Antibacterial action of EO combinations

In a mixture of EOs, the interaction between their compounds can produce a synergistic, additive, indifferent, or antagonistic effect [5]. The antimicrobial efficacy of EOs selected to this study in combination with other EOs is poorly investigated. The few existing studies have focused on the antimicrobial activity of the following: (1) basil/oregano and thyme/oregano EO mixtures against *E. coli* [32]; (2) thyme/oregano EO mixture against *S. aureus* and *S. typhimurium* [6]; (3) thyme/myrtle EO mixture against *S. aureus* and *E. coli* [33]; (4) thyme/Norway spruce, thyme/juniper berry, and thyme/cinnamon EO mixtures against *S. aureus* [22]; (5) thyme/peppermint, thyme/Rosewood, and thyme/lemon balm EO mixtures against *E. coli* [22]; (6) thyme/lavender, thyme/peppermint, and thyme/rosemary EO mixtures against *S. aureus*, *B. cereus*, *P.*

aeruginosa, and *E. coli* [34]; (7) thyme/cinnamon EO mixture against *B. subtilis*, *B. cereus*, *S. aureus*, *E. coli*, and *S. typhimurium* [30]; and (8) parsley/peppermint/coriander EO mixture against *P. vulgaris*, *S. enterica*, and *E. coli* [35]. Among these combinations, only thyme/oregano [6], thyme/myrtle [33], thyme/cinnamon [22], and thyme/peppermint EO mixtures [34] displayed a synergistic effect. The other combinations have shown indifferent, additive, and antagonistic effects.

The results of the Kirby–Bauer disk diffusion test for EO combinations are shown in Table 1. If the value of combined EOs is significantly higher ($p < 0.05$) than the sum of individual values, it is considered to be a synergistic effect; and if it is equal ($p \geq 0.05$), it is an additive effect. An antagonistic effect occurs when the value of one or both EOs is significantly higher than the value of their mixture. A value of combined EOs situated between additive and antagonistic tendency signifies an indifferent effect [5]. The results of the current study show antagonistic and indifferent effects of EO mixtures against tested bacteria.

3.3. *B. cereus* (ATCC 11778)

Four of the EO combinations (parsley/thyme, lovage/basil, lovage/thyme, and basil/thyme EOs) display antagonistic

effects against *B. cereus*. Parsley/thyme, lovage/thyme, and basil/thyme EO mixtures produce increased zones of inhibition with the thyme EO and lovage/basil EO mixture with the basil EO. The other two combinations (parsley/lovage and parsley/basil EOs) exhibit indifferent effects against *B. cereus*. The inhibition zone of parsley/lovage EO mixture does not significantly differ from its individual EOs. Instead, the parsley/basil EO mixture shows a significantly smaller inhibition zone than the basil EO.

3.4. *S. aureus* (ATCC 6538P)

Combinations of parsley/lovage, parsley/basil, and lovage/basil EOs show indifferent effects against *S. aureus*. Parsley/lovage and parsley/basil EO mixtures cause significantly lower antibacterial activities than individual EOs and the lovage/basil EO mixture a significantly higher one. Parsley/thyme, lovage/thyme, and basil/thyme EO mixtures exhibit significantly lower antibacterial activities than the thyme EO, which denote antagonistic effects.

3.5. *P. aeruginosa* (ATCC 27853)

P. aeruginosa is the most susceptible to all EOs and their combinations. Three EO combinations show antagonistic effects against *P. aeruginosa* (parsley/thyme, lovage/thyme, and basil/thyme EOs), and the other three combinations indifferent effects (parsley/lovage, parsley/basil, and lovage/basil EOs). Parsley/lovage, parsley/basil, and parsley/thyme EO mixtures display significantly higher antibacterial activities than the parsley EO. Basil EO does not significantly affect the antibacterial activity of lovage/basil EO mixture. Thyme EO significantly contributes to the antibacterial activity of parsley/thyme and lovage/thyme EO mixtures but does not significantly influence the antibacterial activity of basil/thyme EO mixture.

3.6. *E. coli* (ATCC 25922)

Four EO combinations exhibit antagonistic effects against *E. coli* (parsley/thyme, lovage/basil, lovage/thyme, and basil/thyme EOs), and the other two combinations show indifferent effects (parsley/lovage and parsley/basil EOs). Basil EO significantly contributes to the antibacterial activity of parsley/basil and lovage/basil EO mixtures.

3.7. *S. typhimurium* (ATCC 14028)

Combinations of lovage/thyme and basil/thyme EOs display antagonistic effects against *S. typhimurium* and the other four combinations (parsley/lovage, parsley/basil, parsley/thyme, and lovage/basil EOs) indifferent effects. The antibacterial activity of parsley/lovage, parsley/basil, and lovage/basil EO mixtures do not significantly deviate from those of individual EOs. Thyme EO significantly contributes to the antibacterial activity of parsley/thyme EO mixture.

Parsley/lovage and lovage/basil EO mixtures show no inhibitory effects against all tested microorganisms. Parsley/basil EO mixture exhibits mild (against *E. coli*) or no inhibitory effects. Parsley/thyme EO mixture reveals moderate (against

E. coli and *S. typhimurium*), mild (against *B. cereus*), or no inhibitory effects (against *S. aureus* and *P. aeruginosa*). Instead, basil/thyme EO mixture shows moderate inhibitory effects against *E. coli*, *S. typhimurium*, and *B. cereus* but no inhibitory effects against *S. aureus* and *P. aeruginosa*. Lovage/thyme EO mixture causes moderate (against *E. coli*), mild (against *B. cereus* and *S. typhimurium*), or no inhibitory effects (against *S. aureus* and *P. aeruginosa*).

In summary, all pairwise combinations exhibit lower antibacterial activities than the thyme EO against all five bacteria. Considering that thyme EO has the highest percentage yield and antibacterial potential from all tested formulations, it is therefore recommended to be used alone as the antimicrobial agent.

Conflicts of interest

No potential conflicts of interest were reported by the authors.

Acknowledgments

The authors thank Engineer Mădălina Mentea and Engr. Maria Morar for their valuable help.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jfda.2016.06.002>.

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