



ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST *Listeria monocytogenes*

Ružica M. Tomičić¹, Ivana S. Čabarkapa², Ana O. Varga², Zorica M. Tomičić²

¹ University of Novi Sad, Faculty of Technology, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia

² University of Novi Sad, Institute of Food Technology, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia

*Corresponding author:

Phone: +381 21 485 3667

E-mail address: ruzica.tomicic@yahoo.com; ruzica.tomicic@uns.ac.rs

ABSTRACT: Food poisoning caused by *Listeria monocytogenes* leads to a 30% rate of mortality among patients. The application of essential oils (EOs) to food products is a suitable strategy to control pathogens and to extend their shelf life by reducing microbial levels. The objective of this study was to evaluate the antimicrobial potential of essential oils (EOs) against *L. monocytogenes*. The EOs used in this study were caraway (*Carum carvi*), cinnamon (*Cinnamomum zeylanicum*), dill (*Anethum graveolens*), clove (*Syzygium aromaticum*), mentha (*Menthae piperitae aetheroleum*), red thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), common sage (*Salvia officinalis*), clary sage (*Salvia sclarea*) and summer savory (*Satureja hortensis*). The minimal inhibitory concentrations (MICs) of EOs were determined using the broth microdilution method. According to the MIC values, all essential oils were effective in the inhibition of *L. monocytogenes* strains, with MICs varying from 256 µg/ml to 4096 µg/ml. The results showed that cinnamon EO had the highest antimicrobial activity, while dill and mentha EOs were the least effective against the *L. monocytogenes*. In addition, two different procedures were carried out to test the effect of antibiotics gentamycin and streptomycin against the *L. monocytogenes* strains, the broth microdilution method and the MIC Test Strip. Our results indicated that the reference strain *L. monocytogenes* ATCC 19111 was much more sensitive to antibiotics than *L. monocytogenes* strains isolated from meat, highlighting that gentamycin was the more effective in comparison to streptomycin.

Key words: *Listeria monocytogenes*, antimicrobial activity, essential oils, antibiotics, broth microdilution method, MIC Test Strip

INTRODUCTION

Listeria monocytogenes is an important gram-positive foodborne pathogen which can cause the serious illness, listeriosis (Magalhães and Nitschke, 2012), which leads to a 30% rate of mortality among patients (Abdollahzadeh et al., 2014). One of its remarkable features is the ability to survive and grow in adverse conditions, such as high salt concentration, and low pH and temperature. These flexible growth conditions enhance its potential as

a contaminant of food products. *L. Monocytogenes* has been found in a wide variety of food products as raw meat, raw vegetables, dairy products and ready-to-eat food (Liu et al., 2017; McLaughlin et al., 2004; Tomičić et al., 2016; White et al., 2002). This bacteria is often linked to ready-to-eat food because it is able to grow at refrigeration temperatures and many outbreaks are associated with the consumption of these products (Gandhi

and Chikindas, 2007; Liu, 2008). In spite of the various modern technologies and safety concepts such as HACCP, the control of this pathogen remains a major problem in food industry (Kramarenko et al., 2016; Liu et al., 2017).

One method for inhibiting or inactivating pathogenic bacteria to improve food safety is the use of antimicrobial food preservatives.

Nowadays, there is a consumer preference for healthy foodstuff without addition of chemical preservatives. Thus, the use of natural substances has shown particular promise, and many natural substances have been found to have antimicrobial properties.

One group of plant-derived natural products that has gained the interests of food industry to meet the consumers' preferences is essential oils (EOs).

Essential oils (EOs) are aromatic oily liquids produced from plant material, like leaves, seeds, flowers, roots and twigs (Burt, 2004).

These products have been widely used as flavouring agents in foods since the earliest recorded history. It is well known that many essential oils have antimicrobial activity against a wide range of spoilage and pathogenic bacteria (Burt, 2004; Chouhan et al., 2017; Nazzaro et al., 2013). However, data on the antimicrobial effect of essential oils on *L. monocytogenes* is still scarce.

Considering the above, the main objective of the present study was performed in order to evaluate the efficacy of EOs against *L. monocytogenes*.

MATERIAL AND METHODS

Strains and growth conditions

Two strains of *L. monocytogenes* 1 and 2 isolated from meat processing industry, and reference strain *L. monocytogenes* ATCC 19111 (lyophilized cultures of microorganisms, American Type Culture

Collection, Kwik-stick™ set, MicroBioLogics) were used in this study. The strains were preserved in Tryptone Soya Broth (TSB, HiMedia) supplemented with glycerol (15%) at – 80 °C and revitalized from frozen stocks by cultivation on the Nutrient Agar (NA, HiMedia) plates for 2 days at 37 °C before performing the assays.

For the antimicrobial activity assays, a loopful of actively growing cells was suspended in Mueller Hinton Broth (MHB, HiMedia) and adjusted to 0.5 McFarland standard turbidity to achieve a final cell concentration of 1×10^6 cells/ml.

Essential oils (EOs)

The EOs used in this study were caraway (*Carum carvi*), cinnamon (*Cinnamomum zeylanicum*), dill (*Anethum graveolens*), clove (*Syzygium aromaticum*), mentha (*Menthae piperitae aetheroleum*), red thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), common sage (*Salvia officinalis*), clary sage (*Salvia sclarea*) and summer savory (*Satureja hortensis*). All EOs were obtained from Institute of Field and Vegetable Crops in Novi Sad and stored in the refrigeration before use.

Antimicrobial susceptibility testing

To determine the minimal inhibitory concentrations (MICs) of plant essential oils (EOs) and antibiotics, the broth microdilution method was used as described before (Klančnik et al., 2009).

Briefly, stock solutions of plant EOs were prepared by dissolving the oils in Mueller Hinton Broth to a concentration of 80.00 mg/ml and further diluted in MHB to a working solution of 32.768 mg/ml.

Afterwards, 200 µl of working solution was pipetted to the first row of a 96-well microtiter plate (Eppendorf, Germany) and serial dilutions were performed to reach final concentrations ranging from 16384 µg/ml to 16 µg/ml. Gentamycin sulfate (AppliChem, Darmstadt, Germany) and Streptomycin sulphate BioChemica (AppliChem, Darmstadt, Germany) were used as antimicrobial standards.

Final concentrations of gentamycin and streptomycin were in the range from 16-0.0156 µg/ml and 32-0.0313 µg/ml, respectively. After twofold serial dilutions by MHB across the plate, each well was inoculated with 100 µl of *L. monocytogenes* activated culture (ca. 1×10^6 cells/ml) and plates were incubated for 24 h at 37 °C.

After incubation, 20 µl resazurin aqueous solution was added to each well. Microplates were incubated for 24 h at 37 °C. The concentration that completely inhibited bacterial growth in the microtiter plate ad oculos (MIC) was the concentration from first nonturbid well at which the blue color did not change into pink.

Wells with culture medium, with the bacterial suspension only, plant essential oils and antibiotics only, were used as control. Four replicates were run for each EO and antibiotic.

MIC Test Strip

The MIC Test Strip was used for determination of the minimal inhibitory concentrations of gentamycin and streptomycin against *L. monocytogenes* strains and performed according to the procedures of manufacturer Liofilchem (Via Scozia, Zona Industriale – Reseto Italy).

Briefly, 200 µl of each *L. monocytogenes* suspension (ca. 1×10^6 cells/ml) was added to a sterile petri dish and 20 ml of Mueller Hinton Agar (MHA, HiMedia) was poured, homogenized and left to tighten.

After drying the surfaces of the plates, a sterile porous strips (Liofilchem, Italy) with a predefined concentration gradient of antibiotics gentamycin and streptomycin was applied on the surface of each inoculated agar surface.

After 24 hours incubation at 37 °C, a symmetrical inhibition ellipse centered along the strip was formed. The MIC was read directly from the scale in terms of µg/ml at the point where the edge of the inhibition ellipse intersects the strip MIC Test Strip.

RESULTS AND DISCUSSION

In recent years there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance.

The antimicrobial activity of plant essential oils (EOs) has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Chouhan et al., 2017; Nazzaro et al., 2013).

An exhaustive review of the literature shows that there is a lack of studies on the antibacterial activity of the EOs against *L. monocytogenes*. This study intends to contribute toward filling some gaps in the current knowledge.

In the present study, the effect of the ten EOs was examined against the foodborne pathogenic bacteria *L. monocytogenes* by the broth microdilution methods (Tables 1).

The application of the broth microdilution method is useful to compare the antibacterial effect between EOs and to identify the minimal concentrations of EO that did not allow bacterial replication (Mazzarrino et al. 2015).

Considerable inhibitory effect of the tested EOs on the *L. monocytogenes* was found, with MICs varying from 256 µg/ml to 4096 µg/ml as presented in Table 1. Among the ten essential oils, caraway, cinnamon and mentha EOs exhibited a similar antimicrobial spectrum on the tested bacteria.

However, cinnamon EO showed the highest activity, inhibiting the strains at the MIC value of 256 µg/ml. The strongest antibacterial activity of cinnamon EO is possibly due to its major constituents.

The most active constituents of many EO with wide spectra of antimicrobial activity are likely the aromatic phenolic compounds, such as cinnamic aldehyde in cinnamon, eugenol in clove and cin-

namon, thymol and carvacrol in thyme (Desai et al., 2012; Shan et al., 2007).

These bioactive principles in the related dietary spices and medicinal herbs were also identified in other studies (Chauhan et al., 2007; Zampini et al., 2005). Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004).

In our contribution, data also indicate that essential oils of red thyme, clove and summer savory exhibited a significant inhibitory effect on the reference strain *L. monocytogenes* ATCC 19111.

Worthy of note is the fact that strains isolated from meat were more resistant to these essential oils as compared to *L. Monocytogenes* ATCC 19111. On the other hand, results clearly demonstrate that dill and mentha EOs (MICs of 4096 µg/ml) showed the lowest antibacterial activity among all tested EOs.

In addition, according to our findings common sage EO was less effective than clary sage EO, although it reduced the bacterial counts.

Generally, most published results demonstrate that the observed variation in antibacterial activities of among EOs is due to at least two factors: 1) the major components of EOs, and 2) the type of bacteria tested (Bakkali et al., 2008; Burt, 2004; Rather et al., 2012).

Interestingly, for some plant oils such as clove and sage, there has been some research and reporting of toxic and irritant properties (Bansod and Rai, 2008; Hammer et al., 1999). In spite of this, most of these oils are available for purchase as whole oils or as part of pharmaceutical or cosmetic products, indicating that toxic properties do not prohibit their use.

Listeria spp. are often exposed to low levels of antibiotics, as these agents are used in large amounts both in human and

animal medicine (Aarestrup 2012). In the present study, the effect of antibiotics gentamycin and streptomycin was examined against the *L. monocytogenes* strains as presented in Table 2.

The results showed that the reference strain *L. monocytogenes* ATCC 19111 was much more sensitive to antibiotics than *L. monocytogenes* strains isolated from meat. Many mechanisms exist by which bacteria can become resistant to antimicrobial agents.

Although mutational events are important in the development of resistance to some agents, by far the most important factor in resistance is extrachromosomal genetic material in the form of plasmids.

Important mechanisms of bacterial resistance to antibiotics are interference with the transport of the antimicrobial agents into the bacterial cell, inactivation of the agent, and alteration of the target site or metabolic pathway by the microorganism (Neu, 1982).

We should mention that resistance of *L. monocytogenes* strains isolated from meat in comparison with *L. monocytogenes* ATCC 19111 undoubtedly reflects inherent physiological differences between strains.

As expected, gentamycin was the most effective against all tested *L. monocytogenes* strains, which is consistent with earlier reports (Li et al., 2006; Al-Nabulsi et al., 2015). *L. monocytogenes* rarely develops acquired resistance to antibiotics.

However, some studies have recently reported an increased rate of resistance of *L. monocitogenes* strains to antibiotics due to mutations or acquisition of mobile genetic elements (Bertsch et al., 2014; Conter et al., 2009; Morvan et al., 2010).

The Test Strip and broth microdilution results for tested antibiotic standards were compared in this study.

Table 1.

Antimicrobial activity of plant essential oils expressed as MIC, determined by the broth microdilution test

Plant species	Common name	<i>L.</i> <i>monocytogenes</i>	<i>L.</i> <i>monocytogenes</i>	<i>L.</i> <i>monocytogenes</i>
		ATCC 19111	1	2
MIC (µg/ml)				
<i>Anethum graveolens</i>	dill	4096	4096	>16384
<i>Carum carvi</i>	caraway	1024	1024	1024
<i>Cinnamomum zeylanicum</i>	cinnamon	256	256	256
<i>Menthae piperitae aetheroleum</i>	mentha	4096	4096	4096
<i>Rosmarinus officinalis</i>	rosemary	2048	2048	512
<i>Salvia officinalis</i>	common sage	4096	1024	1024
<i>Salvia sclarea</i>	clary sage	2048	512	512
<i>Satureja hortensis</i>	summer savory	256	512	512
<i>Syzygium aromaticum</i>	clove	512	1024	1024
<i>Thymus vulgaris</i>	red thyme	256	1024	1024

Table 2.

Antimicrobial activity of antibiotics expressed as MIC, determined by the broth microdilution test and Test Strip

Antibiotics	<i>L.</i> <i>monocytogenes</i>		<i>L.</i> <i>monocytogenes</i>		<i>L.</i> <i>monocytogenes</i>	
	ATCC 19111		1		2	
	Micro-dilution MIC _{mdil}	Test Strip MIC _{ts}	Micro-dilution MIC _{mdil}	Test Strip MIC _{ts}	Micro-dilution MIC _{mdil}	Test Strip MIC _{ts}
Gentamycin	0.25	0.19	0.5	0.38	0.5	0.38
Streptomycin	4	1	8	2	8	6

The results in Table 2 show that the MICs obtained by different methods differed significantly. All isolates tested were sensitive to gentamycin and streptomycin.

However, the minimal inhibitory concentration (MIC_{mdil}) of both gentamycin and streptomycin determined with dilution method was higher than the minimal inhibitory concentration (MIC_{ts}) determined with Test Strip.

All methods were equally suitable for the testing of the sensitivity of *L. monocytogenes* to antibiotics.

Thus, the broth microdilution method appears to be an easy and reliable method for determination of the MICs of antibiotics for *L. monocytogenes*.

CONCLUSIONS

In conclusion, the current study emphasizes a significant potential of essential oils as antimicrobial agents against *L. monocytogenes*. In fact, our findings open up new perspectives on the application of EOs as biopreservatives against food-borne pathogens.

The tested essential oils may contain antimicrobial constituents, and further phytochemical and pharmacological studies will be necessary to isolate the active constituents and evaluate the antimicrobial activity against a wide range of microbial populations.

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АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКИХ УЉА НА *Listeria monocytogenes*

Ружица М. Томичић^{*1}, Ивана С. Чабаркапа², Ана О. Варга², Зорица М. Томичић²

¹ Универзитет у Новом Саду, Технолошки факултет, 21000 Нови Сад, Булевар цара Лазара бр. 1, Србија

² Универзитет у Новом Саду, Институт за прехранбене технологије у Новом Саду, 21000 Нови Сад, Булевар цара Лазара бр. 1, Србија

Сажетак: Тровање храном изазвано бактеријом *Listeria monocytogenes* доводи до 30% стопе смртности међу пацијентима. Примена етарских уља (ЕОс) у прехранбеним производима је погодна стратегија за контролу патогена и продужење њиховог рока трајања смањењем нивоа микроба. Циљ ове студије је био да се процени антимикиробни потенцијал етарских уља (ЕОс) на *L. monocytogenes*. ЕОс коришћена у овој студији била су ким (*Carum carvi*), цимет (*Cinnamomum zeylanicum*), мирођија (*Anethum graveolens*), каранфилић (*Syzygium aromaticum*), нана (*Menthae piperitae aetheroleum*), црвени тимијан (*Thymus vulgaris*), рузмарин (*Rosmarinus officinalis*), зачинска жалфија (*Salvia officinalis*), мускатна жалфија (*Salvia sclarea*) и вртни чубар (*Satureja hortensis*). Минимална инхибиторна концентрација етарских уља је одређена коришћењем микродилуционе методе. Према добијеним вредностима MIC, сва етарска уља су била ефикасна у инхибицији сојева *L. monocytogenes*, са вредностима MIC од 256 µg/ml до 4096 µg/ml. Резултати су показали да је етарско уље цимета испољило највишу антимикиробну активност на сојеве *L. monocytogenes*, док су мирођија и нана били најмање ефикасни. Поред тога, спроведене су две различите процедуре у циљу испитивања утицаја антибиотика гентамицина и стрептомицина на сојеве *L. monocytogenes*, коришћене су микродилуциона метода и MIC Test Strip. Наши резултати указују да је референтни сој *L. monocytogenes* ATCC 19111 био знатно осетљивији на антибиотике у поређењу са сојевима *L. monocytogenes* изолованим из меса, наглашавајући већу ефикасност гентамицина у односу на стрептомицин.

Кључне речи: *Listeria monocytogenes*, антимикиробна активност, етарска уља, антибиотици, микродилуциони метод, MIC Test Strip

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