IN VITRO ANTIMICROBIAL ACTIVITY OF PLANT ACTIVE COMPONENTS AGAINST *PSEUDOMONAS LUNDENSIS* AND *LISTERIA MONOCYTOGENES*

Running title: *Antimicrobial activity of active components against P. lundensis* and *L. monocytogenes*

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ABSTRACT

The aim of my work was to study the antimicrobial activity of eight various components of plant origin on the growth of *Pseudomonas lundensis* and *Listeria monocytogenes.* For this aim different *in vitro* methods were used: agar plate diffusion, micro atmosphere, agar hole diffusion, micro-dilution, and gradient-plate method. In the first agar plate assay, p-cymene and γ-terpinene were not active in inhibiting the growth of the tested bacteria hence they were not used in further experiments. Both α-pinene and limonene were only partially effective, but these were screened only for their partial inhibition. The other four components show complete inhibition. Using the agar-hole diffusion method showed that carvacrol and thymol were found to be the most effective components and low concentration was sufficient to effectively inhibit the growth of bacteria. Additionally, eugenol and camphor show the same results but in higher concentrations. Gradiens plate method was used to determine minimum inhibitory concentration (MIC) values, in which it has been proved the most active components were thymol and carvacrol low MIC value (1.887 mg/ml) obtained for thymol. However, carvacrol was inhibited even at lower concentration. Further experiments could have determined the concentrations required.

Keywords: active components, antimicrobial activity, pathogenic bacteria

1. INTRODUCTION

Nowadays, even in developed countries, many foodborne infections and poisonings can pose a threat to human health, despite advanced hygiene regulations, stringent regulations, and precautions. One reason for this is that pathogenic bacteria have protective mechanisms or resistance to old and new antibiotic formulations causing serious food-borne infections, which in many cases are fatal. According to statistics, more infectious diseases have occurred in recent years, and the number of pathogenic bacteria and fungi has increased. This is prevalent in developing countries, but according to a 2012 statement by the WHO (2012), it can be a problem for all human worldwide. *P. lundensis* a gram-negative bacterium that is generally characterized by a cell wall consisting of a thin layer while *L. monocytogenes* a gram-positive bacteria gas thicker but simpler cell of peptidoglycan. They often cause deterioration the dairy products, fresh vegetables, meat and fish (Yazdankhah et al., 2001). The use of various natural antimicrobial components of plant origin and combinations thereof against various pathogens may be a better solution to this problem compared to synthetic additives. These substances have long been used for a variety of therapeutic and religious purposes, but they are also well recognized in the food, cosmetic and medical applications. Numerous studies and experiments address their beneficial or antimicrobial, antiseptic or antifungal properties. Whereas, it has the disadvantage that when used in larger amounts, they can negatively affect the organoleptic properties of different foods, and change their taste, smell and aroma. For these reasons, it would be important to determine the minimum concentration of various antimicrobial agents that are sufficiently effective against various pathogens, but do not significantly alter the properties of the food (Burt, 2004). The active constituents of essential oils are mostly terpenes and terpenoids: monoterpenes or sesquiterpenes and may also be diterpenes and triterpenes. The monoterpenes can be classified based on their diverse functional groups: terpinene, pinene; alcohols e.g. geraniol; aldehydes e.g. citral; ketones e.g. camphor or phenols e.g. thymol and carvacrol (Bakkali et al., 2008). Some of these active components such as carvacrol capable of breaking down the lipopolysaccharide membrane of gram-negative bacteria, disrupting active cell transport, and may also cause coagulation. It can interact with the double lipid layer of the cytoplasmic membrane of planktonic cells, which inhibits proton permeability, leading to cellular leakage, which later results in cell death (Luz et al., 2012; Ilham et al., 2014). This study was conducted with using different methods to illustrate the *in vitro* antimicrobial activity of different active components against food spoilage and food pathogenic bacteria *P. lundensis* and *L. monocytogenes*.

2.0 MATERIALS AND METHODS

2.1. Material and strains

In this experiments *Pseudomonas lundensis* CCP5 and *Listeria monocytogenes* ATCC 4699 were selected as targeted strains from the Department of Microbiology and Biotechnology, Szent István University. The antimicrobial components (carvacrol, thymol, eugenol, camphor, α-pinene, limonene, p-cymene and γ-terpinene) were obtained from SIGMA (Germany).

2.2 Measurement procedure

For each of the assays, freshly inoculated cultures on Tryptone Glucose Extract (TGE) agar were incubated for 24 hours. *P. lundensis* was incubated at 30 °C and *L. monocytogenes* was incubated at 37 °C. The culture suspension was adjusted to 10^8 cells by 0.5 optical density (OD) by using a densitometer (DEN-1B, McFarland), and the density of the diluted working culture was set to approximately 10^6 CFU ml^{-1}. Three replicates performed of each component per microbe.

2.2.1. Agar diffusion method

The cell concentration of *P. lundensis* and *L. monocytogenes* was adjusted to 10^8 cells / ml by OD and diluted to 10^6 cells / ml. Subsequently, the plate was cast with 1 ml of culture in petri dishes with 20 ml TGE agar, and a sterile disc-shaped filter paper was placed in the centre of the plate with a flaming tweezer. For this small disk 4 µl of the diluted active component was pipetted in a suitable ratio. In the case of a control, the components dissolved in ethanol and pipetted to the disc. The stock suspension concentration was 200 mg camphor / 200 µl ethanol and for thymol 30 mg thymol / 600 µl ethanol. The petri dishes were packaged to prevent evaporation of volatile components and incubated upside-down for 24 and 48 hours at the appropriate temperatures. Zones of inhibition (mm) were measured using a digital Vernier calliper (Workzone-calliper).

2.2.2. Micro atmosphere method

Similar to agar diffusion method, 10^6 microbial suspensions were sued. The filter paper discs were 2 cm in diameter and placed on top of the petri dishes upside-down. 30-30 µl of antimicrobial component was pipetted onto these. Ethanol was also used as a control. Inhibition zones around the disk were also measured with a digital caliper after incubation at 30 or 37 °C.

2.2.3. Agar-well diffusion method

In this method same as the previous assays the culture suspension 10^6 CFU was pipetted into the petri dishes adding TGE agar. Using special cork metal sterilized hole of 8 mm in diameter were made and filled with 80 µl of the appropriate dilution of the active components. Half-dilution of the liquid component was made from the undiluted component. For the non-liquid components, a stock solution of 30 mg thymol / 600 µl ethanol was prepared and diluted in half with distilled water. For the other component, a stock solution of 200 mg camphor / 200 µl ethanol was prepared and further diluted (in half) with distilled water. Ethanol and sterile distilled water were pipetted into the holes in case of control. Inhibition zones around the holes were measured (Balouiri et al., 2016)

2.2.4. Micro-dilution method

Measurements were made on a 96 well-plate, 50 µl of TGE was pipetted into each well, and 50 µl of appropriately diluted antimicrobial material was placed in the well of the first column. The stock solution of carvacrol became 200 µl/ml ethanol and thymol 30 mg / 600 µl ethanol. Micro dilution and mixing done via a pipette 2-3 times for homogenization, then pipetted 50 µl into the right well and continued to the last well of the plate. Then pipetted 50 µl of the cell bacterial suspensions into the appropriate well. Ethanol was also used as a control. After the incubation 10 µl of resazurin mixture was pipetted into each well, microbial growth were indicated with a pink color while if it remains blue growth inhibition. Based on these, the MIC value were determined. Resazurin solution was made by dissolving 0.025 g of resazurin in 1 ml of water. This was then added to a pre-weighed, 8 ml medium in a sterile test tube, and this solution was weighed at 900 µl into eppendorf tubes. Meanwhile, 0.014 g of menadione was dissolved in 1 ml of DMSO and added to the previously made resazurin solutions.

2.2.5. Gradient-plate method

In this method, culture suspension of 10^6 CFU was pipetted into the petri dishes adding 15 ml TGE agar. This suspension was added to TGE agar and mixed. In a first layer, to 15 ml of TGE agar was added to the petri dishes and left to solidify at an angle of 45 degrees. 15 ml of agar-antimicrobial mixture poured onto the first solid agar layer and allowed to solidify in the normal position. The wrapped dishes were placed in a refrigerator for 48 hours and then 4 strips parallel to the agar surface were drawn on the agar surface with a sterile microfoam swab soaked in microbial suspension. The plates were incubated at 30 and 37 °C for 24 and 48 hours, respectively, and the length of the growth strips was measured with a ruler then the MIC values were determined.

3.0 RESULTS AND DISCUSSION

3.1. Agar diffusion

Based on the 24-hour results obtained (Table 1), it can be observed that γ-terpinene, p-cymene and limonene did not show antimicrobial activity against the studied bacteria. α -pinene partially inhibited the growth of *P. lundensis* but no inhibition zone was observed in *L. monocytogenes*. For *L. monocytogenes* α-pinene and limonene also partially inhibited its growth. Carvacrol showed the highest activity with thymol and eugenol against *P. lundensis*.

Table 1: Inhibition zones (mm ± standard deviation) of *P. lundensis* and *L. monocytogenes* after 24- and 48-hours using agar diffusion method

Active components	**24 hrs**		**48 hrs**	
	P. lundensis	*L. monocytogenes*	*P. lundensis*	*L. monocytogenes*
Thymol	10,287 ±0,459	8,44±1,145	10,01±0,592	8,953±0,457
Eugenol	9,497±0,656	10,32±1,531	9,247±0,487	11,902±0,99
α-pinene	9,14±0,556	-	9,65±0,765	7,453±1,13
γ-terpinene	-	-	-	-
P-cymene	-	-	-	-
Limonene	-	-	-	9,177±0,396
Carvacrol	17,382±4,936	-	17,533±4,053	7,12±0,22
Camphor	9,345±0,735	8,47±0,556	7,59±0,444	8,25±0,03

3.2. Micro-atmospheric diffusion

In this method, active components were not in direct contact with the inoculated medium, thus using larger discs and higher amounts of antimicrobial component were applied. Because the, they were only able to exert inhibition by their volatility. In this method used only the five antimicrobial components (thymol, eugenol, α-pinene, carvacrol, camphor) that were active against the microbes in the previous experiment. Table 2 shows that *P. lundensis* was inhibited by thymol, eugenol, limonene and carvacrol, but none of the components showed activity against *L. monocytogenes*. This may be due to the fact that 24-hour incubation was not sufficient to reproduce. Thymol, eugenol, carvacrol and camphor also showed significant activity against both bacteria, while α-pinene and limonene showed only partial Inhibition. Eugenol and camphor gave the highest inhibition zones.

Table 2: Inhibition zones (mm ± standard deviation) of *P. lundensis* and *L. monocytogenes* after 24 and 48 hours using micro-atmosphere diffusion method.

	24 hrs	**48 hrs**

Active components	*P. lundensis*	*L. monocytogenes*	*P. lundensis*	*L. monocytogenes*
Thymol	23,725±3,479	-	23,567±1,087	23,46±0,05
Eugenol	20,16±3,33	-	23,95±0,02	25,46±0,165
α-pinene	-	-	23,527±0,912	24,82±0,7709
Limonene	23,795±1,336	-	22,57±0,7907	22,77± 0,238
Carvacrol	20,424±0,772	-	24,60±0,06	22,84±0,00
Camphor	-	-	25,955±0,9405	25,89±0,52

3.3. Agar-well diffusion

An agar-well diffusion method was used to determine the MIC value. Here, the components diffuse from the holes into the agar, where they inhibit microbial growth. Based on the results of previous experiments, four antimicrobial components, were applied eugenol, carvacrol, camphor, and thymol. Tables 3 show inhibition zones for *P. lundensis* after 24 and 48 hours of incubation. From the results shown, the second dilution of eugenol resulted in a MIC of 62.5 µl / ml. In the case of carvacrol, MIC of 15.3 µl / ml was observed. The first dilution of camphor had complete inhibition corresponding to a concentration of 500 mg / ml. For thymol the MIC value was 1.563 mg / ml. In summary, the lowest MIC values for *P. lundensis* were observed for carvacrol and thymol after both 24 and 48 h incubation. In case of *L. monocytogenes* after 24 and 48 hours of incubation (Table 3). Eugenol had complete inhibition up to the third limb, which is 31.25 µl/ml MIC, and carvacrol showed MIC 7.813 µl/ml. Thymol had MIC at 1.563 mg/ml. After 48 hours camphor remained at the MIC value of 500 mg/ml. However, in the case of carvacrol, after another 24 h incubation MIC was 3.9 µl/ml. In conclusion, thymol and carvacrol proved as the most active components against *L. monocytogenes* this result were in accordance to the result by Gutierrez et al. (2009).

3.4. Micro-dilution method

For the micro-dilution method to determine the MIC value. The experiment performed with both bacteria repeatedly with thymol and carvacrol. None of the experiments yielded appreciable results, nor did the control group (ethanol) show any growth. Therefore, another gradient plate method was used to determine MIC values.

Table 3: Inhibition zones of active components against *P. lundensis* and *L. monocytogenes* after 24 and 48 hours using agar-well diffusion method

Concentration [µl/ml]	***P. lundensis***								***L. monocytogenes***							
	Eugenol		Carvacrol		Camphor		Thymol		Eugenol		Carvacrol		Camphor		Thymol	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
500	NA	NA	NA	NA	8,54± 0,38	8,81± 1,71	NA	NA	NA	NA	NA	NA	10,885 ±1,237	10,90 ±1,21	NA	NA
250	12,78± 0,38	11,87 ±0,17	23,77 ±0,36	20,41± 0,65	-	-	NA	NA	22,75 ±0,71	22,3± 2,40	19,85 ±0,98	19,5± 0,63	8,26±0, 33	8,26± 0,33	NA	NA
125	12,00± 1,053	11,99 ±1,32	21,45 ±1,69	18,17± 0,672	-	-	NA	NA	19,19 ±1,15	19,26 ±0,33	17,56 ±0,44	17,35 ±0,42	-	-	NA	NA
62,5	8,19 ±0,83	9,25± 0,53	15,86 ±0,26	15,15± 0,56	-	-	NA	NA	16,76 ±1,74	18,17 ±0,95	15,81 ±0,06	15,55 ±0,14			NA	NA
31,25	-	-	12,78 ±0,40	13,96± 0,12	-	-	NA	NA	10,54 ±0,05	10,37 ±0,88	14,20 ±1,06	14,00 ±1,05			NA	NA
15,63	-	-	12,41 ±0,74	12,77± 0,49	-	-	NA	NA	-	-	11,80 ±2,33	11,57 ±2,09			NA	NA
7,813	-	-	-	10,15± 0,14	-	-	NA	NA			8,9±0, 91	8,07± 0,07			NA	NA
3,9											-	7,75± 0,00			NA	NA
25							13,55± 1,414	14,08 ±1,38							16,54± 0,48	16,3± 0,35
12,5							12,61± 0,89	13,22 ±0,38				-			15,07± 0,81	14,95 ±0,70
6,25							10,6±0 ,21	11,77 ±1,02							12,96± 0,11	12,80 ±0,21
3,125							9,42±0 ,6	11,31 ±1,07							12,25± 0,424	11,92 ±0,60
1,563							8,93±0 ,78	9,71± 0,98							8,39±0 ,43	8,15± 0,09

NA: not available

3.5. Gradient plate method

In this method the two most active substances were selected, thymol and carvacrol, based on previous experiments. These were investigated by the gradient plate method, whereby the antimicrobial material in the upper agar layer diffuses into the lower TGE agar layer containing only agar. The components were used in three different concentrations to determine the MIC value. Tables 4 show that there was no proliferation of *L. monocytogenes*, which may indicate that the component was inhibited at the MIC of both thymol and carvacrol such antimicrobial activity also confirmed by Lambert et al. (2001). In *P. lundensis*, did not seen the growth in case of carvacrol. Thymol, on the other hand, inhibited growth at both 24- and 48-hours incubation, resulting in MIC values of 1.887 mg / ml. Comparing the two experiments, the previous agar-hole diffusion experiment showed that the MIC of thymol was 3.125 mg / ml, but at 1.887 mg / ml it had inhibited the growth of *P. lundensis.*

Table 4: Gradient plate experiment result (mm ± standard deviation) after 24 and 48 hours

Dilution ratio	**Concentration**	***P. lundensis***		***L. monocytogenes***	
		24 hrs	48 hrs	24 hrs	48 hrs
Thymol 1.	0,943 mg/ml	26,67±4,726	33,333±3,215	-	-
Thymol 2.	1,887 mg/ml	8±3,605	15,333±2,516	-	-
Thymol 3.	2,830 mg/ml	-	-	-	-
Carvacrol 1.	100 µl/ml	-	-	-	-
Carvacrol 2.	200 µl/ml	-	-	-	-
Carvacrol 3.	300 µl/ml	-	-	-	-

CONCLUSION

Current findings indicate the greater sensitivity of *P. lundensis* to the selected active components. This is explained by the thinner peptidoglycan cell wall, whereas *L. monocytogenes* has a simpler but thicker cell wall with a larger peptidoglycan layer. As a result, it is likely to be more resistant to external influences and antimicrobial components used. further studies required to determine concentration of the combined amicrobial activity of active components.

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