

A study of suitability of some conventional chemical preservatives and natural antimicrobial compounds in allelopathic research

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SUMMARY

The impact of three *conventional chemical preservatives* (sodium benzoate, potassium sorbate and salicylic acid) and a natural antimicrobial compound (thymol) on germination, dynamics of growth and accumulation of fresh biomass (g per seedling) of *Lactuca sativa* L., cultivar Great Lakes, was studied under laboratory conditions. The tested conventional chemical preservatives demonstrated strong inhibitory effects (*GI* 27.1-0.0%) on germination and initial development of *L. sativa*, and they cannot be used in allelopathic studies in the laboratory.

An addition of thymol at 0.5-1.0 ‰ concentration showed no inhibitory effect (*GI* varied 81.7-84.6%) on germination and initial development of *L. sativa*. Thymol can therefore be used as a natural antimicrobial compound in allelopathic studies in the laboratory.

Keywords: Allelopathy; Plants; Lettuce; Chemical preservatives; Thymol

INTRODUCTION

The body of research work on allelopathic interrelations between cultivated plants and weed species has grown over the last few decades with a purpose of finding and using environmentally friendly and harmless-to-humans chemical compounds synthesized by some plant species that are fit to be used for regulation of plant pests and diseases, as well as against weed vegetation (Kefeli, 2003; Younesabadi et al., 2014). Most of these studies were performed under laboratory conditions by preparing aqueous extracts from different weed species, and the studies focused on finding their inhibitory and/or stimulatory effects on germination and initial development of other plants (Nasr & Shariati, 2005; Kalinova et al.,

2012; Ravlić et al., 2012; Soltys et al., 2013; Takemura et al., 2013; Yarnia et al., 2013; Soliman & Zatout., 2014; Ayeni & Akinyede, 2014; Baličević et al., 2014; Ravlić et al. 2014, Petrova et al., 2015). The prepared extracts are extremely “unstable”, create suitable conditions for development of various microorganisms that partly change extract pH and show negative osmotic effects on germination and initial development of some plants (Blum et al., 1992; Basile et al., 2000; Botelho et al., 2007; Palaniappan & Holley 2010; Soliman & Zatout, 2014).

Extracts are therefore required to be kept at lower temperatures (Phillips, 2008; Chandra et al., 2012; Hassan et al., 2012; Borghetti et al., 2013) or lyophilized, which requires expensive laboratory equipment or addition of antimicrobial compounds that are classified

as chemical preservatives. According to Russell and Gould (2003), Stopforth et al. (2005), Heydariyinia et al. (2011), Dai et al. (2010) and Levinskaitė (2012), sodium benzoate and potassium sorbate have long been used as conventional chemical preservatives in food and other products to reduce, by over 60%, the colonial growth of different fungi, such as *Aspergillus niger*, *Penicillium notatum*, *Penicillium digitatum*, *Penicillium italicum*, etc.. Reports on addition of conventional chemical preservatives and natural antimicrobial compounds and their influence on seed germination and initial development of seedlings under laboratory conditions are sporadic, extremely limited and contradictory.

According to Peterson and LaRue (1981), sodium benzoate does not disturb the development cycle of *Glycine max* (L.) Merrl., but significantly suppresses pathogenic microorganisms. Karuna et al. (1991) suggested adding sodium benzoate during seed germination of species of the genus *Sorghum* under *in vitro* conditions to prevent pathogenic fungus growth. The research of Usman et al. (2014) showed that conventional chemical preservatives inhibited the growth of seedlings and accelerated the processes of aging and death of plants.

According to Marinov-Serafimov et al. (2007), an addition of thymol to extracts showed no inhibitory effect on germination and initial development of test plants.

The purpose of the present study was to find suitable conventional chemical preservatives or/and natural antimicrobial compounds that do not inhibit germination and initial development of lettuce, *Lactuca sativa* L., and could therefore be used in allelopathic studies conducted under laboratory conditions.

MATERIAL AND METHODS

An experiment was conducted under laboratory conditions at the Institute of Forage Crops – Pleven, Bulgaria, to study the suitability of three conventional chemical preservatives (sodium benzoate, potassium sorbate and salicylic acid) and a natural antimicrobial compound (thymol) in allelopathic studies of germination and early seedling growth of *Lactuca sativa* L. cv. Great Lakes (Davidson et al., 2005). Trial treatments and product characteristics are shown in Table 1.

Chemicals and reagents. Sodium benzoate, potassium sorbate and salicylic acid were purchased from Galen-Pharma Ltd. (Bulgaria), and agar and thymol from FOT Ltd. (Bulgaria).

Bioassays. Suitability of the conventional chemical preservatives and natural antimicrobial compound was examined using an adapted method of Fujii et al. 2003. Petri dishes (diameter 90 mm) were pipetted with 20 ml

Table 1. Trial treatments and product characteristics

Treatment	Chemical name	Substance identification	Type	Concentration, ‰
1.	Control (distilled water)	-	-	-
2.				0.5
3.	Thymol	Phenol, 5-methyl-2-(1-methylethyl)-	natural antimicrobial compound	1.0
4.				1.5
5.				2.0
6.				0.5
7.	Sodium benzoate	Benzoic acid	conventional chemical preservative	1.0
8.				1.5
9.				2.0
10.				0.5
11.	Potassium sorbate	Benzoic acid	conventional chemical preservative	1.0
12.				1.5
13.				2.0
14.				0.5
15.	Salicylic acid	Salicylic acid	conventional chemical preservative	1.0
17.				1.5
18.				2.0

1.0% agar, and four concentrations of three *conventional chemical preservatives* (sodium benzoate, potassium sorbate and salicylic acid) and a natural antimicrobial compound (thymol) were added, i.e. 0.5, 1.0, 1.5 and 2.0%. Distilled water was used as a control. The samples were stored for 72 h at $18 \pm 2^\circ\text{C}$, and then five seeds of *L. sativa*, cv. Great Lakes (Shiraishi et al., 2002; Gilani et al., 2010; Yeasmin, 2013; Qureshi et al., 2014) were added. The prepared samples were placed in an incubator at $22 \pm 2^\circ\text{C}$ in the dark for five days. Each treatment consisted of five replicates, including the control treatment.

Effect assessment. For assessing experimental results, the following parameters were used:

1. Quantitative parameters: the number of germinated seeds in each treatment; percent of germination in each treatment (%); 2. Biometric parameters: root, stem and seedling length, mm; fresh biomass weight per seedling, g. Length was measured using graph paper and weight on an analytical balance; 3. Statistical evaluation and calculated formulas:

The dynamic development index (*DDI*) was determined by the equation (1)

$$DDI = \left\{ \frac{t \log^2}{\log b - \log a} \right\} \quad (1)$$

where *a* and *b* are germinated seeds (%), length (mm) and/or fresh biomass (g) of seedlings, respectively in the control and in each treatment; *t* - duration in days.

Response index (*RI*) was determined by the equation (2) (Williamson & Richardson 1988)

$$RI = \frac{T}{C} - 1 \quad (2)$$

where *C* - characteristic in the control treatment; *T* - characteristics in each treatment.

Growth rate and accumulation of fresh biomass of the root and germ was determined using an adapted formula by Dauta et al. (1990) as the equation (3)

$$\mu = \left\{ \frac{\ln N_t - \ln N_0}{t} \right\} \quad (3)$$

where *N_t* - length (mm) or fresh biomass (g) of seedlings in each treatment; *N₀* - length (cm) or fresh biomass (g) of seedlings in the control treatment; *t* - duration in days.

The rate of emergence (*GR%*) was determined by the equation (4)

$$GR_{\%} = \left(1 - \frac{(N_t - C_n)}{(N_c)} \right) \quad (4)$$

where *N_t* - germinated seeds in each treatment (%); *N_c* - germinated seeds in the control treatment (%); *C_n* - concentration, %.

The index of plant development (*GI*) was determined by the equation (5) (Gariglio et al. 2002)

$$GI = \left[\left(\frac{G}{G_0} \right) \cdot \left(\frac{L}{L_0} \right) \right] \cdot 100 \quad (5)$$

where *G* - germinated seeds in each treatment, %; *G₀* - germinated seeds in the control treatment, %; *L* - average length (cm) of seedlings in treatment transformed into percentage as against the control treatment; *L₀* - average length (cm) of seedlings in the control treatment considered as 100%.

Seedling vigor index (*SVI*) was determined by the equation (6) (Islam et al. 2009)

$$SVI = \left(\frac{SG}{100} \right) \quad (6)$$

where *S* - length (mm) or biomass weight (g) in the seedling treatments and the control; *G* - germinated seeds, %.

Coefficient of allometry (*CA*) was determined by the equation (7) (Isfahan & Shariati 2007)

$$CA = \frac{L_s}{L_r} \quad (7)$$

where *L_s* - length (mm) or fresh biomass weight (g) of the stem, *L_r* - length (mm) or fresh biomass weight (g) of the root for each treatment.

The percentage of seed germination in each treatment was previously transformed by the equation (8) (Hinkelman & Kempthorne 1994)

$$Y = \arcsin \sqrt{(x_{\%}/100)} \quad (9)$$

where *x_%* - germinated seeds for each treatment, %.

The effective concentrations required to induce half-maximal inhibition of growth (*LC₅₀*) and 95% confidence intervals were calculated according to Hamilton et al. (1977). Measurements of pH were performed by a Digitales PH-Meter PH -100 ATC. The collected data were analyzed using the software Statgraphics Plus for Windows Ver. 2.1 and STATISTICA Ver. 10.

RESULTS AND DISCUSSION

The conventional chemical preservatives (sodium benzoate, potassium sorbate and salicylic acid) and natural antimicrobial compound (thymol) inhibited seed germination of *L. sativa* from 3.8 to 100%, compared with the control treatment (Table 2).

Lethal effect on seed germination of *L. sativa* increased with rising concentrations of the conventional chemical preservatives (sodium benzoate, potassium sorbate and salicylic acid).

Thymol had different effects on seed germination as the percentage of germinated seeds in that treatment varied from 3.8 to 16.8%, compared to the control treatment, and the differences were not statistically significant ($p=0.05$) (Table 2).

The chemical preservatives and the natural antimicrobial compound had different effects, corresponding to concentrations on the response index (*RI*), rate of emergence (*GR%*) and dynamic development index (*DDI*) of *L. sativa* (Table 3).

Increasing concentrations of the chemical preservatives and natural antimicrobial compound were found to reduce seed germination of *L. sativa*, and the *DDI* and *GR%* values decreased from 0.31 to 85.0, while the *RI* increased from 9.5 to 300.0% compared with the lowest applied concentration.

The effects of pH, sodium benzoate, potassium sorbate, salicylic acid and thymol on seed germination of *L. sativa* were investigated per applied concentration.

As the pH of the growth medium varied from 1.9 to 8.5, depending on the type of preservative (conventional

Table 2. Effects of sodium benzoate, potassium sorbate, salicylic acid and thymol on seed germination of *L. sativa*

Treatments	Concentration, %	Parameters					pH	*LC ₅₀
		Germination, % ± SE	Mean length, mm			Seedling weight, g ± SE		
			root ± SE	stem ± SE	seedling ± SE			
Control	0	68.4±7.37f	14.25±0.81cd	19.19±1.17ef	33.44±1.68e	0.00763±0.0014d	6.41	
Thymol	0.5	65.5±2.03ef	14.39±0.94cd	20.00±1.05f	34.39±0.94e	0.00791±0.0005d	8.47	>83.8
	1.0	65.8±3.57ef	15.92±1.18d	19.48±1.21f	35.40±2.16ef	0.00944±0.0007e	8.32	
	1.5	60.6±4.47def	15.68±1.02d	17.16±1.35e	32.84±2.03e	0.00867±0.0005de	8.00	
	2.0	56.9±2.59de	13.32±1.16c	14.71±1.21d	28.04±2.26d	0.00780±0.0009d	7.72	
<i>Average</i>		62.2±3.17	14.83±1.08	17.84±1.21	32.67±1.85	0.00846±0.0007	8.13	
Sodium benzoate	0.5	52.6±4.57d	2.71±0.18b	8.67±0.68c	11.39±0.83c	0.0045±0.0010c	6.28	0.69 (0.65-0.74)
	1.0	13.3±7.67b	1.12±1.11ab	1.44±0.18ab	2.56±0.24ab	0.0017±0.0016b	6.71	
	1.5	0.0a	0.0a	0.0a	0.0a	0.0a	6.88	
	2.0	0.0a	0.0a	0.0a	0.0a	0.0a	7.13	
<i>Average</i>		16.48±3.06	0.96±0.32	2.53±0.22	3.49±0.27	0.00155±0.0003	6.75	
Potassium sorbate	0.5	35.5±5.85c	2.29±0.16ab	3.43±0.44ab	5.71±0.46ab	0.0017±0.0009b	7.16	0.51 (0.45-0.58)
	1.0	0.0a	0.0a	0.0a	0.0a	0.0a	6.87	
	1.5	0.0a	0.0a	0.0a	0.0a	0.0a	6.82	
	2.0	0.0a	0.0a	0.0a	0.0a	0.0a	6.79	
<i>Average</i>		8.88±1.46	0.57±0.04	0.86±0.11	1.43±0.12	0.00043±0.0002	6.91	
Salicylic acid	0.5	0.0a	0.0a	0.0a	0.0a	0.0a	2.83	-
	1.0	0.0a	0.0a	0.0a	0.0a	0.0a	2.25	
	1.5	0.0a	0.0a	0.0a	0.0a	0.0a	2.21	
	2.0	0.0a	0.0a	0.0a	0.0a	0.0a	1.90	
<i>Average</i>		0.0	0.0	0.0	0.0	0.0	2.30	

Means marked with different letters differ at $P=0.05$ level of probability in LSD test; ± SE - standard error of mean; *LC₅₀ value unit, per mille (95% confidence interval).

chemical preservatives and/or natural antimicrobial compound), three groups of reaction were discerned: *I.* Alkaline to neutral - thymol, pH 7.72-8.47; *II.* Acidic to neutral - sodium benzoate and potassium sorbate, pH 6.28-7.16, and *III.* - salicylic acid, pH 1.90-2.83 (Table 2).

A correlation was detected between germination and the medium pH for sodium benzoate ($r = -0.901$) and salicylic acid ($r = +0.984$), and a slighter for potassium sorbate ($r = -0.454$), while thymol had $r = -0.286$.

The impact of growth medium pH on seed germination of *L. sativa* ranged from indifferently to actively harmful and can be expressed as a function of their oxidation.

Oxidative processes were the strongest under the influence of salicylic acid: $Y = -100.31 + 66.87 \cdot \sqrt{\text{pH}}$; $r = 0.959$; $R^2 = 0.920$, followed by sodium benzoate:

$Y = -1152.09 - 433.98 \cdot \sqrt{\text{pH}}$; $r = -0.886$; $R^2 = 0.785$, potassium sorbate: $Y = -814.42 - 303.89 \cdot \sqrt{\text{pH}}$; $r = -0.474$; $R^2 = 0.274$ and the slightest under thymol: $Y = -123.11 - 21.26 \cdot \sqrt{\text{pH}}$; $r = 0.323$; $R^2 = 0.105$.

Furthermore, analogous results were obtained when determining the $\text{LC}_{50(\text{p}=0.5)}$ in seed germination of *L. sativa*, depending on the inhibitory effects of the chemical preservatives, and they could be conventionally graded: thymol [$>83.8\%$] \rightarrow potassium sorbate [0.51 (0.45-0.58) %] \rightarrow sodium benzoate [0.69 (0.65-0.74) %] (Table 2).

The data in biometric measurements of seedling length (mm) enabled an objective estimation of differences in the initial development stages of *L. sativa*, depending on the type and concentration of chemical preservatives (Table 2).

Table 3. Indexes of development and coefficients of depression in seed germination and initial development of *L. sativa*

Treatments	Concentration, %	Germination			Seedlings length					Seedling weight			GI	
		DDI	RI	GR%	DDI	RI	$\mu\text{x}10^2$	SVImm	CA mm	DDI	RI	$\mu\text{x}10^2$		SVI $\text{g} \times 10^7$
Thymol	0.5	-12.1	-0.042	95.0	18.7	0.028	0.56	22.5	1.39	14.5	0.037	0.72	6.26	81.7
	1.0	-13.5	-0.038	94.7	4.0	0.139	2.61	25.1	1.05	2.5	0.237	4.26	8.91	84.6
	1.5	-4.3	-0.114	86.4	-29.0	-0.018	-0.36	19.9	1.09	4.1	0.136	2.56	7.52	61.7
	2.0	-2.9	-0.168	80.3	-3.0	-0.161	-3.52	16.0	1.10	23.8	0.022	0.44	6.08	44.3
<i>Average</i>		-5.5	-0.091	89.1	-218.8	-0.002	-0.05	20.7	1.16	4.9	4.85	2.16	7.23	64.2
Sodium benzoate	0.5	-2.0	-0.231	76.2	-0.5	-0.659	-21.54	6.0	3.20	-1.0	-0.410	-0.106	2.03	27.1
	1.0	-0.3	-0.806	18.0	-0.2	-0.923	-51.39	0.3	1.29	-0.4	-0.777	-0.300	0.29	5.7
	1.5	0	0	0	0	0	0	0	0	0	0	0	0	0
	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Average</i>		-0.4	-0.759	22.3	-0.3	-0.873	-41.26	0.7	2.90	-0.3	-0.790	-31.24	0.26	3.8
Potassium sorbate	0.5	-0.8	-0.481	51.2	-0.3	-0.829	-35.35	2.0	1.50	-0.4	-0.777	-0.300	0.29	13.6
	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.5	0	0	0	0	0	0	0	0	0	0	0	0	0
	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Average</i>		-0.5	-0.670	31.2	-0.2	-0.954	-61.56	0.3	1.49	-0.2	-0.948	-58.97	0.02	1.6
Salicylic acid	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.5	0	0	0	0	0	0	0	0	0	0	0	0	0
	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Average</i>		0	0	0	0	0	0	0	0	0	0	0	0	0

Seedling length reduction was the greatest when potassium sorbate was used (92.2-100%), followed by sodium benzoate (65.9-100%), and the least after thymol treatment (1.8-16.2%), but only at higher concentrations, as compared to the control treatment with distilled water, the differences being statistically significantly reduced at $P=0.05$, with an exception of the lowest concentrations applied (Table 2 and 3).

Thymol applied at concentrations of 0.5 and 1.0 ‰ did not inhibit the growth of *L. sativa* seedlings and can therefore be used as a natural antimicrobial compound in allelopathic studies.

The dynamics of accumulation of fresh biomass in early stages of *L. sativa* growth depend on the type of chemical (conventional chemical preservatives - sodium benzoate, potassium sorbate and salicylic acid, and natural antimicrobial compound - thymol) and follow the observed dependence of seedling length (Tables 2 and 3).

Mathematical and statistical analyses of data showed that the tested sodium benzoate, potassium sorbate, salicylic acid and thymol had inhibitory and/or stimulatory effects on the initial development of test plants.

A relatively slight phytotoxic effect was observed under the lowest concentration of 0.5 ‰ and increasing towards 2.0 ‰. *RI* and μ decreased from 0.6 to 6.4 times, but the *DDI* and *SVI* increased from 0.2 to 1.6 times (Table 3).

Therefore, seed germination can be considered as a less sensitive period in individual development of *L. sativa* plants, whereas seedling growth and accumulation of fresh biomass allow to be used as a potential test for determination of toxicity or inhibitory effect of sodium benzoate, potassium sorbate and salicylic acid, as well as thymol in the laboratory.

An analysis of variance for the conventional chemical preservatives (sodium benzoate, potassium sorbate and salicylic acid) and natural antimicrobial compound (thymol) in terms of phytotoxicity evaluation based on germination and initial development of *L. sativa* (Figure 1) showed that the type of chemical compounds had the most dominant influence and significant effect, from $\eta^2_{\text{partial}}=0.78$ to $\eta^2_{\text{partial}}=0.94$. The applied concentrations of sodium benzoate, potassium sorbate, salicylic acid and thymol were factors with significant impact on the variation of phytotoxicity, from $\eta^2_{\text{partial}}=0.08$ to $\eta^2_{\text{partial}}=0.65$. Interaction of the type of chemical compound (conventional chemical preservative and natural antimicrobial compound) and concentration had a relatively small proportion of total variation of $\eta^2_{\text{partial}}=0.04$ to $\eta^2_{\text{partial}}=0.66$.

The obtained results were analogous when determining the index of initial development (*GI*) of the test plants (Table 3).

The tested conventional chemical preservatives (sodium benzoate, potassium sorbate and salicylic acid) provoked an inhibitory effect on germination and initial development (*GI*) of *L. sativa*.

The analyses showed that the addition of sodium benzoate, potassium sorbate and salicylic acid resulted in high degrees of toxicity to *L. sativa* – *GI* varied on average from 27.1 to 0.0%, as compared to the control treatment with distilled water (*GI*=100%) (Table 3).

The addition of thymol at 0.5 and 1.0‰ concentrations showed no inhibitory (*GI* varied 81.7-84.6%) effect on germination and initial development of *L. sativa*. Thymol can therefore be used as a natural antimicrobial compound in allelopathic studies in the laboratory.

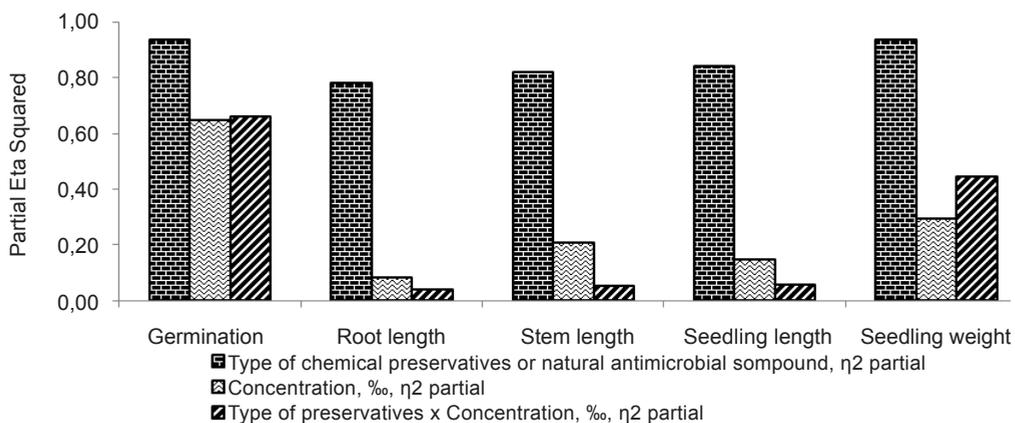


Figure 1. Relative phytotoxic effects (η^2_{partial}) of the conventional chemical preservatives and natural antimicrobial compound on germination and initial development of *L. sativa*

CONCLUSIONS

The tested conventional chemical preservatives (sodium benzoate, potassium sorbate, and salicylic acid) had strong inhibitory effects (GI 27.1- 0.0%) on germination and initial growth of *Lactuca sativa* L. and cannot be used in allelopathic studies in the laboratory.

Thymol added at 0.5 and 1.0‰ concentrations exerted no inhibitory effect (GI varied 81.7-84.6%) on germination and initial development of *L. sativa*. Thymol can therefore be used as a natural antimicrobial compound in allelopathic studies under laboratory conditions.

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Proučavanje pogodnosti nekih konvencionalnih hemijskih konzervansa i jednog prirodnog antimikrobnog jedinjenja u istraživanjima alelopatije

REZIME

U laboratorijskim uslovima je ispitivan uticaj tri konvencionalna hemijska konzervansa (natrijum benzoate, kalijum sorbet i salicilna kiselina) i jednog prirodnog antimikrobnog jedinjenja (timol) na klijanje, dinamiku rasta i akumulaciju sveže biomase (g/izdanku) *Lactuca sativa* L., sorta Great Lakes. Ispitivani konvencionalni hemijski konzervansi pokazali su jako inhibitorno delovanje (GI 27.1-0.0%) na klijanje i početni razvoj *L. sativa* i stoga ne mogu biti korišćeni u istraživanjima alelopatije u laboratorijskim uslovima.

Dodatak timola u koncentracijama 0.5-1.0 ‰ nije pokazao inhibitorno delovanje (GI u rasponu 81.7-84.6%) na klijanje i početni razvoj *L. sativa*. Timol otuda može biti korišćen kao prirodno antimikrobno jedinjenje u istraživanjima alelopatije u laboratorijskim uslovima.

Ključne reči: Alelopatija; Biljke; Salata; Hemijski konzervansi; Timol