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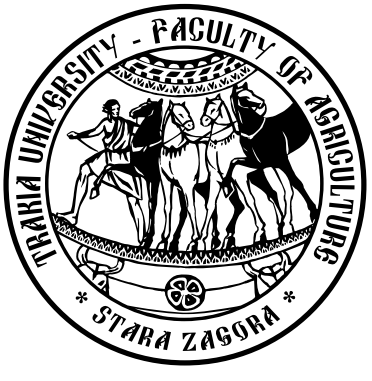

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Antibacterial activity of ethanol extracts of fifteen Bulgarian plants

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Abstract. *Analyses were performed of the antimicrobial activity of 15 herbs and spices (lemongrass, sour cherry, horseradish, ginger, St. John's wort, common centaury, fig, clove, rose geranium, dill, rosemary, oregano, savory, smoketree and wild thyme) widely spread and used in Bulgaria and of some combinations thereof by the agar disk diffusion method. Total phenol content was the highest in the smoketree extract (43.80±1.50 GAE/ml), followed by rosemary (27.80±1.20 GAE/ml), clove (25.17±0.26 GAE/ml), wild thyme (24.83±1.20 GAE/ml), and oregano (23.50±2.00 GAE/ml) extracts. It was established that ethanol extracts of many tested plants showed inhibitory action against S. aureus and E. coli. The most potent effect was observed with extracts of St. John's wort, smoketree and clove. With combination of plant extracts, very good results were demonstrated in mixtures of St. John's wort with wild thyme, with savory and with clove. The said extracts may be used as active constituents in biopolymer matrices for development of functional antimicrobial films needed for food and pharmaceutical industries.*

Keywords: antimicrobial activity, natural antimicrobials, plant extracts, total phenols

Introduction

Contamination of food products with spoilage and pathogenic microorganisms increases the risk of food disorders and poisoning and constitutes a significant social issue affecting consumers' health status. One traditional method of preventing food contamination is the use of chemical preservatives, which act as antimicrobial compounds inhibiting the growth of undesirable microorganisms. Available data in literature about the negative effects of synthetic additives due to demonstrated carcinogenic properties or presence of residual toxicity (Rangan and Barceloux, 2009) and the growing concerns about microbial resistance to conventional preservatives have boosted the search for alternative solutions. One such solution is the use of natural antimicrobial compounds (Bowles and Juneja, 1998; Baljeet et al., 2015). Of particular interest in this regard are spices and herbs, which are considered safe on account of their traditional application without documented harmful impact and/or toxicological analyses.

It is known that, as a result of secondary metabolism, plants synthesize substances such as alkaloids, flavonoids, glycosides, tannins, terpenes, etc., responsible for their antimicrobial properties (Zaika, 1988; Cowan, 1999; Negi, 2012). Many authors believe that antimicrobial activity lies in the foundations of multiple applications of plant essential oils and extracts not only in the food industry but also in pharmaceutical products, alternative medicine and natural therapies (Hammer et al., 1999).

A number of published studies are available in literature on the antimicrobial activity of spice and herbal extracts against microorganisms, including food pathogens. Tested herbs and spices with strong antimicrobial activity against food pathogens include extracts and essential oils of cinnamon, mint, peppermint, rosemary, oregano, clove, black pepper, ginger, cumin, thyme, etc. (Smith-Palmer et al., 1998; Hara-Kudo et al., 2004; Ertürk, 2006; Al-Turki, 2007; Sofia, 2007; Ababutain, 2011; Keskin and Toroglu, 2011; Kumar et al., 2014; Shehadi et al., 2014; Baljeet et al., 2015; Mostafa et al., 2018).

This study was aimed at determining the content of phenolic substances and the antibacterial activity of ethanol extracts of fifteen herbs and spices and combinations thereof against *S. aureus* and *E. coli*.

Material and methods

Plant material. Lemongrass, sour cherry, horseradish, ginger, St. John's wort, common centaury, fig, clove, rose geranium, dill, rosemary, oregano, savory, smoketree and wild thyme were obtained from licensed local suppliers of herbs (Bioprograma) and spices (Rigana). The common name, scientific name and part used, are presented in Table 1.

Reagents and chemicals. All reagents and solvents used in the experiments were of adequate analytical grade and were obtained from Merck Co., Fluka and Marvin (Bulgaria).

Plant material humidity. The humidity of the plant raw material was determined by „Sartorius-thermo control YTC 01L".

Plant extract preparation. For the extraction process, ethanol in concentration of 70% v/v was used. Raw plant materials were dried, except for horseradish and ginger, which were used fresh (Table 1). All samples were chopped to 0.5-1mm with Fritsch cutting mill. Extraction proceeded at room temperature, as a stationary 7-day process with raw material: solvent ratios of 1:3 (horseradish and ginger) and 1:10 (for the remaining plants). The extracts samples were centrifuged for 10min at 3000 x g (Beckman J2-21M). Supernatant was collected and stored at 4°C for 24h and filtered through a sin-

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Table 1. Plant parts used and raw material-to-solvent ratio

No	Common name	Scientific name	Used part	Preliminary preparation	Raw material: solvent ratio
1.	Lemongrass	*Cymbopogon citratus* L.	Leaves	Dried and chopped	1:10
2.	Sour cherry	*Prunus cerasus* L.	Stems	Dried and chopped	1:10
3.	Horseradish	*Armoracia rusticana* L.	Root	Chopped	1:3
4.	Ginger	*Zingiber officinale* Rosc.	Root	Chopped	1:3
5.	St. John's wort	*Hypericum perforatum* L.	Aerial part	Dried and chopped	1:10
6.	Common centaury	*Centanrium erythreaea* Rafn.	Aerial part	Dried and chopped	1:10
7.	Fig	*Ficus carica* L.	Leaves	Dried and chopped	1:10
8.	Clove	*Syzygium aromaticum* L.	Flower buds	Dried and chopped	1:10
9.	Rose geranium	*Pelargonium roseum* auct.	Leaves	Dried and chopped	1:10
10.	Dill	*Anethum graveolens* L.	Seeds	Dried and chopped	1:10
11.	Rosemary	*Rosmarinus officinalis* L.	Aerial part	Dried and chopped	10
12.	Oregano	*Origanum vulgare* L.	Aerial part	Dried and chopped	1:10
13.	Savory	*Satureja hortensis* L.	Aerial part	Dried and chopped	1:10
14.	Smoketree	*Cotinus coggygria* Scop.	Leaves	Dried and chopped	1:10
15.	Wild thyme	*Thymus serpyllum* L.	Aerial part	Dried and chopped	1:10

tered glass filter (pore size 40μm). The extracts were stored in a dark place at temperature under 10°C for further analysis.

Determination of dry matter content in plant extracts. Dry matter in plant extracts was quantified according to the Bulgarian State Standard (BSS EN 12145:2000). Quantity of extractive substances is expressed in mg/ml.

Total phenolic content in plant extracts. Total phenolic contents in plant extracts were estimated by Folin-Ciocalto reagent method (Singleton and Rossi, 1965). Briefly, 0.5ml of extract was mixed with 3.0ml distilled water, 0.25ml Folin–Ciocalteu reagent and 0.75ml of 20% sodium carbonate. The final volume was made up to 5ml with distilled water. The absorbance of the reaction mixtures was measured at 725nm after 2h with spectrophotometer „Specol-11". The total phenolic content of extracts was expressed as milligram equivalent of Gallic acid/ml extract (mg GAE/ml).

Evaluation of antibacterial activity. Tests for antimicrobial effect of extracts against *S. aureus* ATCC 43300 and *E. coli* ATCC 25922 were carried out by the agar disk diffusion method. For that purpose, sterile paper discs (6mm) were soaked with 20μl of the relevant plant extract and placed on the surface of Muller-Hinton agar, pre-inoculated with 0.5ml bacterial suspension (10^6 CFU/ml). The plates were left for 30min at room temperature to allow the diffusion of extract and evaporation of solvent, and then were incubated at 37°C for 22h. Inhibition zone (IZ) of diameter exceeding 8mm was considered a positive result. The paper disc impregnated with a solvent was used as a control. Three parallel experiments were carried out.

Determination of minimum inhibitory concentration. Method of serial dilution: Used strains: *S. aureus* ATCC 43300, *E.coli* ATTC 25922, obtained from NBIMCC (National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria). The analysis was performed in compliance with NCCLS guidelines (1990). 0.400ml of 10^6 cells/ml suspension of the respective bacterial strain were inoculated in test-tubes with 4ml liquid nutritional medium containing various extract concentrations. Extract concentrations ranging from 0.10 to 5.41 mg/ml were tested.

Samples were incubated at 37°C for 24 hours. Optical density at 600nm was measured, against non-inoculated nutritional medium. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test sample causing over 90% reduction of light absorption by control sample (inoculated medium without extract).

Statistical analysis. The results were analyzed using the Microsoft Excel program. The significance between different data ($p<0.05$) was evaluated by analysis of variance (ANOVA). Results were presented as mean ± SD.

Results and discussion

Obtaining extracts from Bulgarian spices and medicinal plants

In this work, fifteen different herbs and spices, widely spread and used in Bulgaria, were analyzed. Of all plant material, only horseradish and ginger were in fresh state, with moisture content of 75.00% and 82.24%, respectively. All other herbs and spices were dried to residual moisture content from 3.49 to 9.50% (Table 2). These plant materials were used for obtaining ethanol extracts with the parameters described in Material and methods section.

Table 3 presents data of extractive substance contents in ethanol extracts. Depending on the type of plant material, the quantity of extractive substances contained in 1.00ml liquid extract varied from 0.79 mg/ml (horseradish) to 50.00 mg/ml (smoketree).

Table 2. Residual moisture content of dried and fresh plant materials

Plant	Lemongrass	Sour cherry	Horseradish	Ginger	St. John's wort
Residual moisture content, %±SD	7.10±0.11	9.40±0.14	75.00±1.02	82.24±1.85	8.51±0.12
Plant	Common centaury	Fig	Clove	Rose geranium	Dill
Residual moisture content, %±SD	7.75±0.10	9.05±0.17	3.49±0.04	9.02±0.13	9.10±0.10
Plant	Rosemary	Oregano	Savory	Smoketree	Wild thyme
Residual moisture content, %±SD	9.50±0.12	8.20±0.11	7.60±0.14	7.90±0.10	8.06±0.13

Table 3. Extractive substance contents in ethanol plant extracts

Plant	Extractive substance contents (mg/ml)±SD	Plant	Extractive substance contents (mg/ml)±SD	Plant	Extractive substance contents (mg/ml)±SD
Lemongrass	0.99±0.01	Common centaury	21.0±0.40	Oregano	23.00±0.60
Sour cherry	15.00±0.50	Fig	1.51±0.01	Rosemary	23.10±0.34
Horseradish	0.79±0.01	Clove	37.00±0.98	Savory	23.80±0.50
Ginger	8.53±0.22	Rose geranium	14.78±0.41	Smoketree	50.00±0.70
St. John's wort	33.00±0.90	Dill	7. 90±0.50	Wild thyme	23.20±0.30

Table 4. Total phenol content in plant extracts. Data presented as Gallic acid equivalent ± SD.

Plant	Ethanolic extract (mg GAE/ml)	Plant	Ethanolic extract (mg GAE/ml)
Lemongrass	0.80±0.01	Rose geranium	8.30±0.10
Sour cherry	1.90±0.01	Dill	1.00±0.01
Horseradish	1.90±0.04	Rosemary	27.80±1.20
Ginger	0.50±0.01	Oregano	23.50±2.00
St. John's wort	10.85±0.04	Savory	20.90±1.30
Common centaury	1.28±0.03	Smoketree	43.80±1.50
Fig	0.90±0.05	Wild thyme	24.83±1.20
Clove	25.17±0.26		

Total phenol content in plant extracts

Table 4 displays the total phenolic content of the test extracts presented as equivalents of gallic acid (mg GAE/ml extract). The obtained values were within a broad range of 0.50 to 43.80 mg GAE/ml. The highest phenol content was observed in the smoketree extract, while the lowest - in ginger, lemongrass and fig. Of the other studied extracts, high concentration of phenolic compounds was also observed in rosemary, clove, wild thyme, and oregano extracts, but compared to smoketree, the values were lower by 36.5 to 52.3%. Another extract with relatively high phenol content was St. John's wort with 10.85mg GAE/ml.

Phenolic substances are synthesized as a result of secondary metabolism and are a commonly found compound class in plants. It is well-known that secondary metabolites can be protective (against viruses, microbes, parasites) and/or signaling compounds as well as protectors against UV radiation and oxidative stress. In this regard, they provide massive reserve of biologically active substances of diverse biological impact. High content of phenolic substances in the tested extracts is a prerequisite for expected antibacterial activity. Moreover, multiple studies report that phenolic substances have significant contribution to antiradical and antimicrobial properties of medicinal plants and spices (Moreno et al., 2006; Cetin-Karaca and Newmann, 2015).

Phenolic content in plants may vary due to differences in plant physiology, seasonal variations, extract production. We assume this to be the reason for different values of total phenol content in our test extracts compared to the results reported by other researchers (Georgieva and Mihaylova, 2015; Turgay and Esen, 2015).

Microbiological analyses of obtained plant extracts

Tests of antibacterial activity by the agar disk diffusion method showed that most tested extracts exhibit inhibitory actions against *S. aureus* and *E. coli* (Figures 1 and 2).

The largest inhibition zones (IZ) were observed in St. John's wort extracts (40mm for *S. aureus* and 12mm for *E. coli*), smoketree (15mm for *S. aureus* and 13mm for *E. coli*) and clove (14.3mm for *S. aureus* and 11.3mm for *E. coli*). The main constituent of the clove extract is the phenolic compound eugenol, which has significant antimicrobial effect (Pavesi et al., 2018). With smoketree, the high content of tannins determines the strong antibacterial activity which can be explained by blocking the protoplasm of microorganisms (Negi, 2012). Unlike the other tested herbs

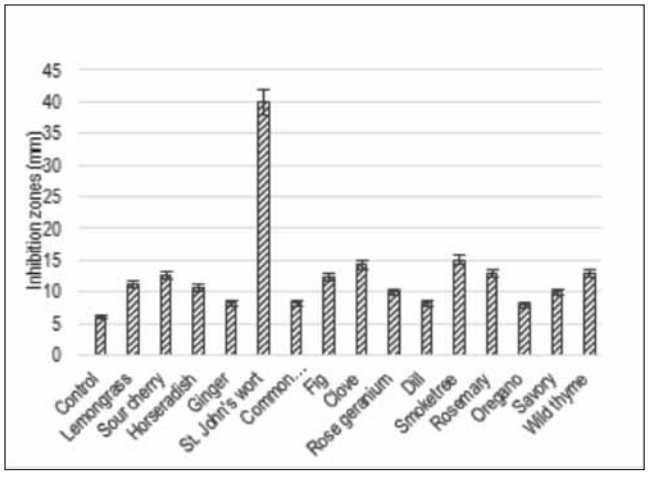

Figure 1. Antimicrobial activity of ethanol extracts of fifteen plants against *S.aureus* determined by the agar diffusion method

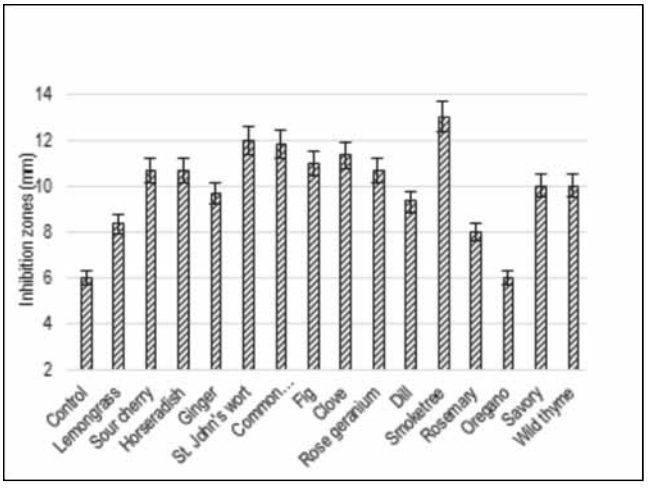

Figure 2. Antimicrobial activity of ethanol extracts of fifteen plants against *E.coli* determined by the agar diffusion method

and spices, smoketree is not suitable for use in foods but may be included in biopolymer films and coatings for achieving antimicrobial effect. St. John's wort extract inhibits the growth of both test strains, with particularly potent effect on *S. aureus*. Supposedly, its antibacterial action is mainly due to its hypericin and hyperforin constituents (Reichling et al., 2001). Previous studies with St. John's wort extract reported strong antibacterial effect on Gram-positive bacteria (such as *S. aureus*), which was confirmed by our study (Males et al., 2006; Radulovic, 2007; Okmen and Balpınar, 2017). Unlike our research, however, other studies show an absence of antibacterial effect on Gram-negative microorganisms (Males et al., 2006; Hodžić et al., 2010). Such difference may be due to seasonal changes in plant composition, different extraction procedures, concentration of crude extracts and strains of test bacteria. Savory, rosemary and thyme are spices commonly used in Bulgarian and Balkan cuisine. The main components of thyme and savory essential oils are the phenolic compounds thymol and carvacrol, in respect whereof significant antimicrobial effect has been reported in literature (Lambert et al., 2001; Adiguzel et al., 2007; Negi, 2012). The rosemary extract contains 1.8-cineol, borneol, camphora, camphene, phenolic acids (caffeic, ferulic, and rosmarinic) which are responsible for the broad range of biological effects including antibacterial activity. The inhibitory action of rosemary and thyme is also more potent in *S. aureus* (13mm), compared to *E.coli* (IZ 8mm and IZ 10mm, respectively). Sour cherry extract exhibits antibacterial activity on both test strains, with inhibition zones to *S.aureus* and *E. coli* of 13mm and 11mm, respectively. The observed extract activity is probably due to the terpenes contained therein (Piccirillo et al., 2013). The other extracts exhibit mild inhibitory effect to both strains. Control samples showed negative results.

Antibacterial activity of mixed plant extracts was also tested. The selection of combinations was based on the results of antibacterial tests of individual extracts and their compatibility in terms of taste and flavor (when mixed). Combinations include: sour cherry + horseradish; lemongrass + rose geranium; horseradish + dill; ginger + St. John's wort; St. John's wort + fig; fig + clove; St. John's wort + clove; clove + oregano; lemongrass + clove; sour cherry + clove; savory + oregano; wild thyme + St. John's wort, and savory + St. John's wort in ratio1:1.

Figures 3 and 4 show the results for antimicrobial activity of combination plant extracts against *S. aureus* and *E.coli*.

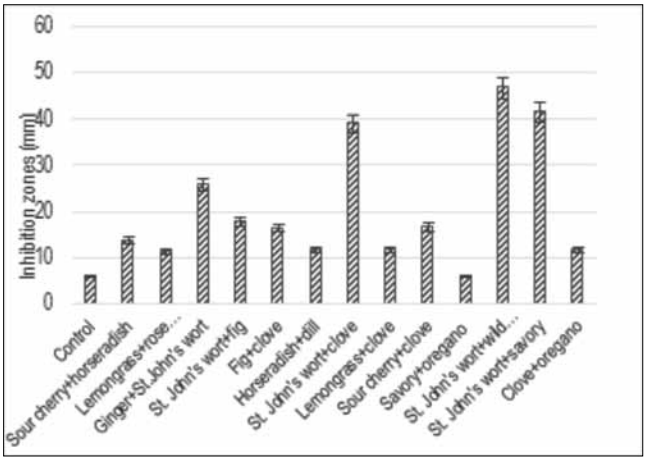

Figure 3. Antimicrobial activity of mixed plant extracts against *S.aureus* determined by the agar diffusion method

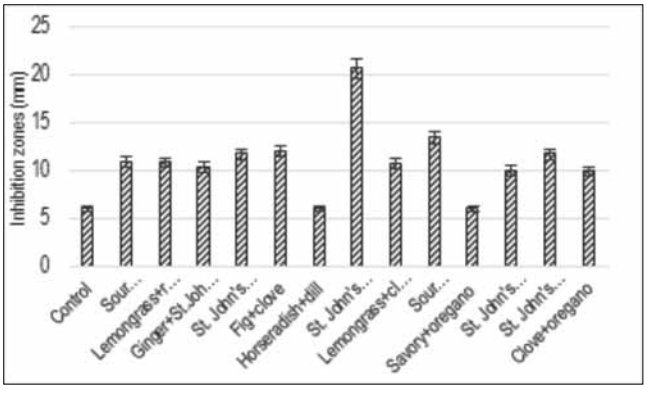

Figure 4. Antimicrobial activity of mixed plant extracts against *E. coli* determined by the agar diffusion method

It can be seen from the figures that the combinations of St. John's wort with wild thyme, savory and clove show very good results. Sour cherry + clove combination also showed good inhibitory effect on both strains. These results are representative of synergy between active ingredients in combination extracts, which individually exhibit less potent inhibitory action. The synergic effect of mixing different extracts or essential oils has been reported by other authors, too (Rivera et al., 2014).

Table 5 presents data of minimum inhibitory concentration of liquid plant extracts. MIC to *S. aureus* varies from 0.04 mg/ml for clove and 0.11 mg/ml for smoketree, to 0.33 mg/ml for St. John's wort and 2.90 mg/ml for wild thyme. In *E. coli*, growth inhibition was observed at higher concentrations - from 0.55 mg/ml (for smoketree) to 4.50 mg/ml (for oregano and wild thyme). The results show that *S.aureus* is more sensitive to test extracts compared to *E.coli*. Results are consistent with published reports about stronger effect of plant extracts on gram-positive bacteria compared to gram-negative ones (Smith-Palmer et al., 1998; Males et al., 2006).

Table 5. Minimum inhibitory concentration (MIC) of plant ethanol extract against *S.aureus* and *E.coli*

Plant ethanolic extract	MIC, mg/ml		Plant ethanolic extract	MIC, mg/ml	
	S.aureus ATCC 43300	*E.coli* ATTC 25922		*S.aureus* ATCC 43300	*E.coli* ATTC 25922
Lemongrass	4.50	5.41	Rose geranium	ND*	ND*
Sour cherry	0.60	0.55	Dill	4.80	5.41
Horseradish	ND*	ND*	Rosemary	0.21	3.12
Ginger	ND*	ND*	Oregano	1.20	4.50
St. John's wort	0.33	3.30	Savory	1.08	2.16
Common centaury	0.84	ND*	Smoketree	0.11	0.55
Fig	ND*	ND*	Wild thyme	1.90	4.50
Clove	0.04	1.11			

*ND – not detected due to solution opacity or strong coloration

Conclusion

The results from microbiological analyses of extracts from fifteen different herbs and spices (lemongrass, sour cherry, horseradish, ginger, St. John's wort, common centaury, fig, clove, rose geranium, dill, rosemary, oregano, savory, smoketree and wild thyme) define the extracts with the highest inhibitory effect on both tested strains (*S. aureus* and *E. coli*). These are Saint John's wort, clove, savory, smoketree and wild thyme. With regard to combination extracts, the best effects on both strains exhibit the following combinations: Saint John's wort + clove, Saint John's wort + wild thyme and Saint John's wort + savory. The synergic antibacterial effect observed in combination extracts was more potent than the one seen in individual separated extracts. These eight extract variants may be determined as suitable for incorporation in biopolymer matrices and production of functional antimicrobial films for use in food and pharmaceutical industries.

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