The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology 35(2) 16-26

ORIGINAL RESEARCH PAPER

PRELIMINARY STUDIES ON THE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST FOOD BORNE BACTERIA AND TOXIGENIC FUNGI

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Received 2 June 2011
Revised 17 August 2011

The aim of this research was to evaluate the *in vitro* antimicrobial activity of seven essential oils against four different bacterial and five fungal strains that are involved in food poisoning and/or food decay: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella enteritidis*, *Fusarium graminearum, Fusarium culmorum, Aspergillus flavus, Aspergillus oryzae* and *Aspergillus brasiliensis*, using two methods: agar disc diffusion method and disc volatilization method.
The majority of the selected essential oils presented inhibitory activity against all the microorganisms tested but essential oils of oregano, thyme and clove proved to develop the best antibacterial and antifungal activity both in direct contact and volatilization method and could be used for further investigation in active packaging of food.

Keywords: essential oils, antibacterial activity, antifungal activity, agar disc diffusion method, vapour phase method

Introduction

Food safety is a known problem worldwide, affecting hundreds of millions of people that suffer from contaminated food. World Health Organization (WHO) defines this issue as "one of the most widespread health problems and a major cause of the reduction in economic productivity".
Many food products are perishable and need protection against spoilage during preparation, storage and distribution to achieve the desired shelf life. Consumption of foods contaminated with pathogenic microorganisms is a threat to human health,

the percentage of people that falls ill from food borne diseases each year has been reported to reach rates of up to 30% (WHO, 2007).

The development of the food preservation process started from the need to extend storage life. Food conservation is a constant struggle against microorganisms that spoil food and make unsure their consumption. Some preservation systems such as heating, cooling and adding antimicrobial compounds can be used to reduce the risk of food contamination, although these conservation techniques show changes of organoleptic characteristics and loss of nutrients.

In the variety of techniques available for conservation, food industry is investigating other techniques to replace traditional methods of conservation due to the high demand by consumers for nutritious tasty and natural food. In recent years, consumers have become concerned about the processed foods they consume. Synthetic preservatives that are used in foods for decades, can lead to negative health consequences. Thus, case it shows a growing interest in the replacement of synthetic preservatives with natural, efficient and non-toxic ones. They are extracts and essential oils (EOs) of spices and herbs.

Essential oils are mixtures of natural volatile compounds deriving from the plant secondary metabolism, mainly monoterpenes, sesquiterpenes, and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides). They can be obtained by expression, fermentation or extraction but the method of steam distilation is the most commonly used for commercial production.

Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant proprieties. (Burt, 2004; Kordali, 2005). Currently, about 300 essential oils, out of approximately 3,000 are commercially important, especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Burt, 2004; Delamare *et al*., 2007).

Essential oils and their components, naturally occurring, are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, their safety for environment and low risk for pathogens to develop resistance to chemical components mixing, due to the diversity of mechanisms of action. The most interesting area of application for essential oils consist of their incorporation directly into the packaging material, coated onto polymer surface, or immobilized to polymers (P. Appendini, 2002), resulting an antimicrobial packaging which inhibits increasing or decreasing numbers of the more serious food-borne pathogens.

The purpose of this research was to create directly comparable, qualitative data on the capacity of seven essential oils to prevent a diverse range of foodborne and spoilage microorganisms strains (bacteria and fungi) based on two test methods: agar disc diffusion method and disc volatilization method. These two methods are used to screen essential oils for antimicrobial activity, they generate the preliminary, qualitative data only, and permit the selection of the most active essential oils for further evaluation.

Materials and methods

Essential oils

Within this research were used seven high purity essential oils, cinnamon leaf oil (*Cinnamomum zeylanicum*), garlic oil *(Allium sativum),* onion oil *(Allium cepa),* white thyme oil (*Thymus vulgaris),* oregano oil (*Thymus capitatus),* basil oil *(Ocimum basilicum)* and clove bud oil (*Eugenia caryophyllata*) obtained by steam distilation, purchased from Sigma Aldrich, Germany.
The essential oils are placed in brown bottles and their quality parameters (appearance, color, purity, odor, density at -20°C and refraction index at -20°C) were described in an accompanying technical report. These oils were selected based on the literature survey with documented antimicrobial activity.
The oils were dissolved in DMSO (Dimethyl sulfoxide) 1:2 (v/v) to give stock solution after which they were mixed for total solubilization at 180 rpm for 10 minutes. For the bioassay, the stock solutions of essential oils were sterilized by filtration using sterile membrane filters (Millex – GP, pore size 0.22 µm). Until subsequent use, stock solutions of essential oils were stored in a refrigerator at +4°C.

Test organisms

In this research were used pure references strains of food-borne microorganisms involved in food toxi-infections.
The selected test organisms used to evaluate the antimicrobial activity of the essential oils were as follows:

- Pathogen bacteria: Gram positive (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923) and Gram negative (*Salmonella enteritidis* ATCC 13076, *Escherichia coli* ATCC 25922) all purchased from MicroBioLogics, SUA. Source of bacteria strains: ATCC, American Type Culture Collection.
- Toxigenic fungi: *Fusarium culmorum* 46 and *Fusarium graminearum* 96 from I.C.D.A Fundulea, *Aspergillus flavus* and *Aspergillus oryzae* from C.B.A.B Biotehnol, *Aspergillus brasiliensis* ATCC 16404 from MicroBioLogics, SUA.

All the bacterial and fungal strains used in this research were a part of the collection of reference strains of Microbiology – ELISA Laboratory from National Institute of Research & Development for Food Bioresources – Institute of Food Bioresources, Bucharest.

Preparation of test organisms

The cultures of test organisms were maintained in agar slants at +4°C (Plate Count Agar – PCA for bacterial strains and Potato Dextrose Agar – PDA for fungal strains; Biokar Diagnostics, France) and used as stock cultures.
Bacterial inoculums were obtained from reference stock culture inoculated in TSB medium (10 ml), which was incubated at 37°C for 18-24 hours. From the freshly grown cultures were made decimal dilutions in sterile Peptone Physiological Serum, up to the concentration of 10^6 CFU/ml, used for testing essential oils.

Spore suspension (spores were picked using 10 ml of NaCl 1% (w/v) containing 5% Tween 80 (w/v) solution from a fresh stock culture grown on PDA Petri dish for 7 days at 25°C), obtained from fungal culture stock, brought to room temperature, mixed and decimal dilutions prepared in sterile Peptone Physiological Serum to obtain concentrations of 10^5 spores/ml, was used to assess the antifungal activity of the selected essential oils.

In vitro antimicrobial activity testing

Because it appears that no standardized test has been developed for evaluating the antimicrobial activity of possible preservatives against food-related microorganisms, the CLSI (Clinical and Laboratory Standards Institute) method for antimicrobial susceptibility testing has been modified for testing essential oils. We used two preliminary methods: agar disc diffusion method and disc volatilization method (vapour phase activity) for detecting the most efficient essential oils against test organisms.

Agar disc diffusion method

The screening of selected essential oils for antimicrobial activity was done by the disc diffusion method, which is normally used as a preliminary check and to select between efficient essential oils (Lopez et al. 2005, Benkeblia, 2004, Kordaly et al., 2005). The appropiate solidified medium (PCA for bacterial strains and PDA for fungal strains) was inoculated with 100 µl of bacterial inoculum (10^6 CFU/ml) and spore suspension (10^5 spores/ml) and spred over the plates using a sterile rod display in order to get a uniform microbial growth on both control and test plates. After inoculum absorbtion by agar, sterile filter discs (Whatman no 1, England, 6 mm diameter) were impregnated with 10 µl of stock solutions of essential oils and placed on the agar surface using forceps dipped in ethanol and flamed. Filter disc moistened with DMSO solution was placed on the seeded Petri dish as a negative control. Filter discs impregnated with streptomycin solution (50 mg/ml) were used as a reference control.
All Petri dishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the essential oils. The dishes were left for 30 min at room temperature to allow the diffusion of oil, and then were incubated at 37°C for 24h for bacteria and at 25°C, for 48–120h for fungi. After the incubation period, the mean diameter of inhibition halo where test microorganism did not grow (clearly visible inhibition zone) was measured in millimetres, for each disc and evaluated for susceptibility or resistance using the comparative standard method.

Disc volatilization method (Lopez *et al*., 2005)

This method evaluates the activity of essential oils vapours on the same strains, technically near to disc diffusion method.
Solidified medium was inoculated with 100 µl of bacterial inoculums and spore suspension containing 10^6 CFU/ml respectivly 10^5 spores/ml of the microorganism under study. Then, 10 µl of each stock solution of essential oil were added to 6 mm diameter sterile blank filter discs and placed in the center of the cover of the Petri

dish in which was previosly cast a thin layer of medium to avoid the adsorbtion of essential oils onto the plastic material of the cover. The dishes were then sealed using sterile laboratory parafilm to avoid eventual evaporation of the essential oils followed by incubation at 37°C for 24h for bacteria and 25°C for 48–120h for fungi.
Blanks were prepared by adding 10 µl of DMSO solution to the filter discs. The effectiveness of the essential oils was calculated by measuring the diameter (in mm) of the zone of microorganism growth inhibition above the disc.
Each assay in these experiments was repeated three times and the results (mm of zone of inhibition) were expressed as average values (± standard deviation).

Results and discussion

Antibacterial activity

Direct contact versus vapour phase method

The antimicrobial activity of the selected essential oils, both by direct contact and vapour phase, against four bacterial species was qualitatively assessed by the presence or absence of the inhibition zone. The antibacterial activity is summarized in Table 1.

Table 1. Antibacterial activity of tested essential oils

EOs	Mean inhibition zone diameter (mm)* after 24 h of incubation							
	Direct contact				Vapour phase			
	Gram positive		Gram negative		Gram positive		Gram negative	
	B. cereus	*S. aureus*	*S. enteritidis*	*E. coli*	*B. cereus*	*S. aureus*	*S. enteritidis*	*E. coli*
White thyme	35.5	37.7 ±0.31	46 ±0.35	39.3 ±0.1	32 ±0.1	37 ±0.34	48.3 ±0.15	46.3 ±0.23
Clove bud	28 ±0.07	22 ±0.21	25.2 ±0.24	31.2 ±0.17	-	-	-	-
Oregano	45.2 ±0.67	43.5 ±0.42	42.5 ±0.21	42 ±0.28	41.6 ±0.05	26.3 ±0.11	43 ±0.26	27 ±0.1
Cinnamon leaf	20	21.2 ±0.03	19.2 ±0.31	18.7 ±0.17	12.3 ±0.15	-	-	-
Onion	38.2 ±1.09	-	9.5 ±0.21	9.5 ±0.10	-	-	-	-
Garlic	25 ±0.21	-	12.7 ±0.10	13 ±0.21	33 ±0.15	-	-	-
Basil	10 ±0.35	12	18.7 ±0.95	24.5 ±0.07	-	-	-	-

* The diameter of the filter paper disc (6 mm) is included. No inhibition (< 6 mm diameter)

The results revealed that the selected essential oils showed antibacterial activity with a higher activity in direct contact method. On the other hand, only two essential oils presented significant antibacterial effect through the volatilization

method against test bacteria. Among the essential oils, oregano, clove bud and white thyme oil exhibited the most effective antibacterial activity in direct contact method in particular against *E. coli* and *B. cereus*, with inhibition zones of 42 mm, 31.2 mm and 39.3 mm and 45.2 mm, 28 mm and 35.5 for *B. cereus*, having a greater inhibition diameter than the control sample (streptomycin 50 mg/ml). Such an activity could be strictly related to their chemical composition: in fact, carvacrol, thymol and eugenol found in these oils, act on the cell membrane increasing its permeability (Burt S., 2004).
For the reference control, results are shown in Table 2. DMSO solvent as negative control presented no inhibition against the tested strains in any method used proving that it is a suitable solvent for testing oils.

Table 2. Diameter of inhibition zones of streptomycin for the tested bacteria by the direct contact method

Reference control	Mean inhibition zone diameter (mm)* after 24 h of incubation			
	Gram positive		Gram negative	
	B. cereus	*S. aureus*	*S. enteritidis*	*E. coli*
Streptomycin 50 mg/ml	28 ± 0.1	33 ± 0.1	26 ± 0.28	25 ± 0.17

* The diameter of the filter paper disc (6 mm) is included.

The essential oil with the widest spectrum of activity was found to be oregano oil followed by white thyme oil > clove bud oil > cinnamon oil > garlic oil > onion oil > basil oil, in that order. White thyme essential oil presented a higher activity against gram-negative bacteria like *Salmonella enteritidis* ATCC 13076 and *Escherichia coli* ATCC 25922, both in direct contact and vapour phase method, while oregano essential oil was the most efficient against gram-positive bacteria (*Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 25923) (Figure 1-A).

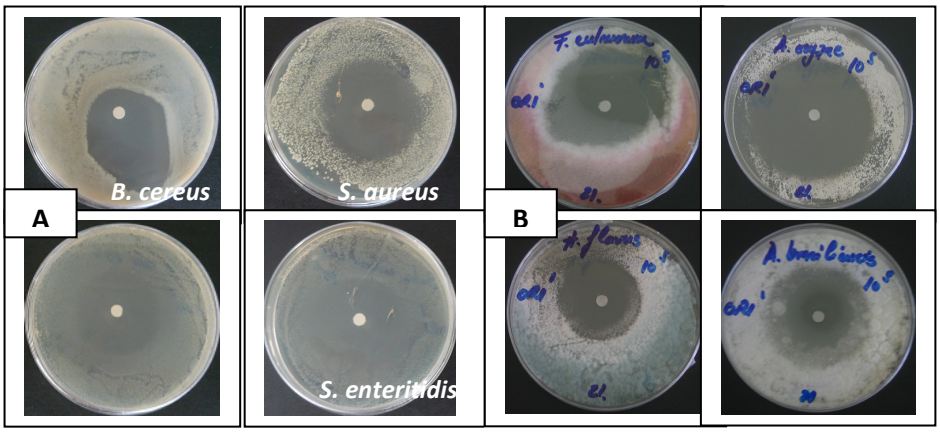

Figure 1. Antimicrobial activity of oregano (ORI) essential oil in direct contact method: A - Inhibition of tested bacteria; B - Inhibition of tested fungi

Activity was also shown by cinnamon leaf oil and basil oil against the same bacterial strains and with the same amount (10 µl/paper disc), with inhibition zones fewer than 30 mm as those presented by white thyme and oregano essential oils. Onion and garlic essential oils exhibit different inhibition levels against *S. enteritidis* and *E. coli,* as shown in Table 1. In the direct contact method, onion essential oil inhibited weakly the development of gram-negative bacteria; both bacteria tested expressed the same sensitivity against onion oil. On the tested gram-positive bacteria, *B. cereus* was strongly inhibited by onion and garlic essential oils, with a diameter of the inhibition zone of 38.2 mm and 25 mm, respectively. The vapour phase of onion and garlic essential oils presented no inhibition against the test strains, except for garlic essential oil that was active against *B. cereus* with a growth diameter inhibition larger than that in direct contact.

Cinnamon leaf oil and basil oil expressed a moderate action against tested strains in direct contact method while in vapour phase method only cinnamon leaf oil showed antibacterial activity against *B. cereus* with a growth inhibition diameter of 12.3 mm. Cinnamon leaf oil presented a greater antibacterial activity against gram-positive bacteria than basil oil. Basil oil was more active against gram-negative bacteria *E.coli* (diameter of inhibition of 24.5 mm).

The probable mechanisms of action of the essential oils on bacteria are given by their hidrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, action that cause the structural damage or complete rupture of the cellular membranes, losses of nutrients and increased permeability (Burt, 2004).

Antifungal activity

Direct contact versus vapour phase method

The growth of commonly occurring filamentous fungi in foods may result in production of toxins known as mycotoxins, which can cause a variety of ill effects in humans, from allergic responses to immunosuppression and cancer. Spoilage and poisoning of food by fungi is a major problem, especially in developing countries. *Aspergillus, Fusarium* and *Penicillium* species are the most important fungi causing spoilage of foodstuff.

Each essential oil showed notable antifungal inhibition zones against fungal strains tested (Table 3 and 4). Fungi susceptibility to these essential oils, as determined by the direct contact method, showed that oregano oil produced a 57.6 mm in diameter inhibition zone against *Aspergillus oryzae* thus presenting the highest inhibitory effects (Figure 1–B).

Clove bud oil and oregano oil presented the most effective antifungal activity in direct contact method, against all the fungal strains tested, but volatile vapours of white thyme oil had the highest antifungal activity against *Aspergillus spp.* tested, with a range of inhibition from 55 mm (*A. oryzae*) to 70 mm (*A. brasiliensis*) respectively. Results have shown that *A. oryza*e was the most susceptible fungal strain to most of the selected essential oils both by the direct method and the vapour phase method.

The inhibiting effects of oregano and cinnamon essential oil in vapour phase were generally higher than those in liquid state (direct contact method). According to both methods, clove bud, oregano and cinnamon leaf were found to be the oils with the widest spectrum of activity against all fungi tested.
The three essential oils, basil oil, onion and garlic oil showed a minor inhibitory effect in both methods, relative to the two most effective essential oils, clove bud and oregano. Cinnamon leaf oil is the most active against *A. oryzae* in the direct contact method but in vapour phase technique, the largest inhibition diameter is present against *F. graminearum* (47.5 mm).
Although white thyme oil proved to be very active in vapour phase against *Aspergillus spp*, in direct contact method the susceptibility of the tested fungi to this oil was weak.

Table 3. Antifungal activity of the tested essential oils by direct contact method

EOs	Mean inhibition zone diameter (mm)* after 120 h of incubation				
	A. o	*A. f*	*A. b*	F. c	*F. g*
White thyme	24.6 ± 0.15	12	15.6 ± 0.11	24.3 ± 0.05	-
Clove bud	48 ± 0.26	28.6 ± 0.05	32 ± 0.17	30 ± 0.25	40.6 ± 0.37
Oregano	57.6 ± 0.05	31.6 ± 0.45	34 ± 0.1	49.6 ± 0.64	-
Cinnamon leaf	39.3 ± 0.15	28 ± 0.17	25.6 ± 0.05	16 ± 0.1	34.6 ± 0.32
Onion	-	17.5 ± 0.35	-	6.0	-
Garlic	-	-	-	6.0	-
Basil	9.6 ± 0.11	8.3 ± 0.05	6.0	6.0	6.0

*The diameter of the filter paper disc (6 mm) is included. No inhibition (< 6 mm diameter)
A.o – *Aspergillus oryzae*, A. f – *Aspergillus flavus*, A. b – *Aspergillus brasiliensis*,
F.c – *Fusarium culmorum*, F. g – *Fusarium graminearum*

Table 4. Antifungal activity of the tested essential oils by the vapour phase method

EOs	Mean inhibition zone diameter (mm)* after 120 h of incubation				
	A. o	*A. f*	*A. b*	*F. c*	*F. g*
White thyme	55 ± 0.32	62.6 ± 0.25	70	-	-
Clove bud	35.3 ± 0.05	22.6 ± 0.05	21 ± 0.1	35.3 ± 0.05	22.3 ± 0.05
Oregano	60 ± 0.2	54.5 ± 0.07	55	57 ± 0.14	56.3 ± 0.11
Cinnamon leaf	42.3 ± 0.25	35.6 ± 0.05	31 ± 0.26	31.6 ± 0.15	47.5 ± 0.35
Onion	-	-	-	-	-
Garlic	-	-	-	-	-
Basil	-	-	-	-	-

*The diameter of the filter paper disc (6 mm) is included. No inhibition (< 6 mm diameter)
A.o – *Aspergillus oryzae*, A. f – *Aspergillus flavus*, A. b – *Aspergillus brasiliensis*,
F.c – *Fusarium culmorum*, F. g – *Fusarium graminearum*

Our data demonstrated that volatile vapours of the majority of essential oils had inhibitory activity against the germination of spores and mycelial growth of the tested fungi. In the paper disc method, the direct contact of all the selected essential oils inhibited the growth of the fungal strains tested (in many cases), but in the vapour phase, the oils were more active giving a higher inhibition zones (as seen in Table 4). The exact mechanism of action of essential oil on fungi is unclear but the

majority of reports agree that oil volatiles result in morphological changes to the hyphae (Cavanagh, 2007). Fungi grow mainly on the surface of the agar medium and might be more susceptible to direct vapour contact while the antimicrobial effect against bacteria might be more dependant on the vapour accumulation into the agar. This could explain why no zones of inhibition were observed in the case of disc volatilization method against bacterial strains.
In this study, volatile vapours of essential oils such as oregano, cinnamon leaf and white thyme exhibited potential antifungal activity with fungicidal inhibition mode. The fungicidal activity was evaluated by measurement of the inhibition diameter after ten days of incubation, when it was observed that the diameter of inhibition was lower than on the fifth day (data not shown). This indicated that the volatile vapours of essential oils could be vanished by evaporation after prolonged exposure time, allowing the fungus to resume growth.

Conclusions

The qualitative assays confirmed the good antimicrobial potential of the tested essential oils. The results suggested that these evaluation techniques could be used as a preliminary, qualitative step, which can determine the sensitivity of many microorganisms to essential oils and select the oils with the best antimicrobial activity, in order to use them for quantitative measurements. In addition, disc volatilization method proved to be a useful method for simple screening of the antimicrobial activity of the vapour phase of essential oils.
In our study, oregano, clove bud, white thyme, cinnamon leaf, basil, onion and garlic essential oils exhibit inhibitory effects against the selected bacterial and fungal strains, both in liquid form and volatile vapours. The result of the direct contact method showed that white thyme, oregano and clove bud oil were the most active against foodborne bacteria. Gram-positive bacteria, *B. cereus* and *S. aureus*, as a mean sensitivity against all essential oils tested, were more sensitive than the Gram-negative bacteria *E. coli* and *S. enteritidis*, but the difference in susceptibility was not soobvious. By disc volatilization method only two essential oils represented by white thyme and oregano oil were found highly effective. The preliminary testing of the antifungal effect of the selected essential oils, showed that oregano, clove bud, cinnamon leaf and white thyme oils were the most active against the fungal strains tested, both in the direct contact method and the disc volatilisation method, with a higher inhibition by volatile vapours. In our study, best results were obtained with oregano, clove bud and white thyme essential oils for bacteria and fungal strains. The antimicrobial activity of these essential oils was more pronounced in the direct contact method but the volatile vapours of these essential oils gave the best results against fungal strains. The importance of these preliminary results is that pathogenic bacteria and some important spoilage and toxigenic fungi can be controlled using plant essential oils. Further investigations include quantitative tests in order to determine the concentration of essential oils (minimum inhibitory concentration) needed to exhibit antimicrobial activity against

food related microorganims in order to use them as natural antimicrobial agents to extend the shelf life and to increase the safety of the processed food.

Acknowledgements

This research was supported by the Sectorial Operational Programme Human Resources Development 2007-2013 under the project number POS-DRU/88/1.5/S/52614 „PhD scholarships to increase the quality of training young researchers in the field of agronomy and veterinary medicine".

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