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Antibacterial activities of the crude ethanol extracts of medicinal plants against *Listeria monocytogenes* and some other pathogenic strains

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Searches for substances with antimicrobial activity are frequent and medicinal plants have been considered interesting by some researchers since they are frequently used in popular medicine as remedies for many infectious diseases. The aim of this study was to verify the antibacterial effect of ethanol extracts of 13 plants (*Artemisia Herba Alba*, *Lavandula officinalis* L., *Matricaria Chamomilla*, *Eugenia caryophyllata*, *Cistus salvifolius*, *Mentha suaveolens* subsp. *Timija*, *Thymus serpyllum* L., *Lippia citriodora*, *Cinnamomum Zeylanicum*, *Rosa centifolia*, *Thymus vulgaris* L., *Rosmarinus officinalis* and *Pelargonium graveolens*) against *Listeria monocytogenes* and other pathogenic strains. These plants are used more for their therapeutic effects in the aromatization of the traditionally fermented dairy products. For this purpose, the agar well diffusion method was the antimicrobial susceptibility performed test. The major components of extracts tested were identified by gas chromatography coupled with mass spectrometry (GC/MS) analysis. The obtained results revealed *in vitro* anti-*Listeria monocytogenes* activities of all the extracts. Also, the extracts of clove, mint timija, cinnamon, cistus, rose, thyme, wild thyme, artemisia, rosemary, geranium and camomile presented in this order promises inhibitory capacity with MIC value between 0.25 mg/mL for clove extract and 6.75 mg/mL for camomile extract. On the other hand, the antimicrobial activity was mainly a function of their chemical composition, in particular in the nature of their major volatile compounds. This study thus confirmed the possibility of using these plants or some of their components in food systems to prevent the growth of foodborne bacteria and to extend the shelf-life of processed foods.

Key words: Medicinal plants, ethanol extract, *Listeria monocytogenes*, antimicrobial activity.

INTRODUCTION

A variety of microorganisms are capable of inducing food spoilage which is one of the most important concerns of the food industry. So far, many pathogenic microorganisms, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Campylobacter jejuni* have been reported as the

causal agents of foodborne diseases and/or food spoilage (Norajit et al., 2007).

In recent years, food safety concerns have been focused on pathogens, such as *Listeria* which is recognized as one of the leading causes of foodborne bacterial diseases. The problem of human listeriosis following consumption of contaminated foods has increased worldwide (Bortolussi, 2008).

Listeria monocytogenes emerged as an important foodborne pathogen in the latter part of the 20th century.

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Clinical syndromes caused by this microorganism included sepsis in the immunocompromised patients, meningococcal meningitis in infants and adults and febrile gastroenteritis. *Listeria* species are commonly found in raw and unprocessed food products. Major outbreaks of listeriosis, with high morbidity and mortality, have been caused by a variety of foods, including soft cheeses, delicatessen meats and vegetable products (Schlech III, 2000).

L. monocytogenes is a small, gram positive bacillus that can grow in anaerobic or aerobic conditions. It is found widely in the environment, in soil, decaying vegetation and water and may be part of the fecal flora of many mammals, including healthy adult humans. *L. monocytogenes* presents a particular concern with respect to food handling because it can grow at refrigerator temperatures (4 to 10°C), temperatures commonly used to control pathogens in foods. Freezing also has little detrimental effect on the microbe (Schlech III et al., 2005).

Besides, the presence of chemical residues in foods and labelling of preservatives on food packages are major concerns to consumers these days. Therefore, the need for naturally derived compounds and other natural products with antimicrobial properties has been explored (Gould, 1996; Mau et al., 2001).

The growing concern about safety of foods has recently led to the development of natural antimicrobials to control foodborne pathogens. Medicinal plants are some of the most commonly used natural antimicrobial agents in foods. Addition of aromatic plants in foods not only imparts flavour but also provides antimicrobial property (Nevas et al., 2004).

Natural antimicrobial compounds in plants were found to possess antimicrobial activity (Kim et al., 1995). In addition, the antimicrobial property of medicinal plants may differ depending on the forms of added plants, such as fresh, dried, or extracted forms. In order to use plants to control *Listeria* in dairy products, it is essential that antibacterial effects of crude ethanolic extracts of plants against *Listeria* be investigated (Nanasombat and Lohasupthawee, 2005).

In the last years it has been reported that some essential oils are capable of inhibiting foodborne bacteria and extending the shelf-life of processed food (Cosentino et al., 1999).

Essential oils contain phenolic compounds known to possess antimicrobial activity and some are classified as generally recognized as safe (GRAS) substances and therefore could be used to prevent post harvest growth of native and contaminant bacteria (Ponce et al., 2003; Kabara, 1991 In: Rusell and Gould 1991; Singh et al., 2001).

In this study, a screening of medicinal aromatic plants selected for their use in the aromatization of the dairy products was made. Therefore, the aim of this study is to evaluate the minimal inhibitory concentrations and the main components of the extracts by GC/MS, thus to determine antibacterial properties of these plants extracts

used in traditional medicine for future application as natural anti-*Listeria* agents in the dairy products.

MATERIALS AND METHODS

Bacterial strains and culture preparation

The bacterial strains used in this study were *Listeria monocytogenes* ATCC 19117, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Acinetobacter baumannii*. All of them were obtained from the culture collection of the Department of Microbiology in the Hospital Ibn Rochd. Bacterial cultures were maintained on nutrient Agar (Difco Laboratories). They were subcultured monthly and subsequently stored at 4°C. The strains were inoculated in the Brain Heart Infusion (BHI) broth (pH 7.6, Difco) and incubated at 37°C for 24 h.

Preparation of crude ethanol extracts

Thirteen different plants were used in this study (Table 1). These spices were extracted by ethanolic extraction by soaking 20 g of plant part in 100 mL of 90% ethanol for 4 days at ambient temperature. The mixtures were then filtered. The filtrate was concentrated on a rotary evaporator at 45°C for ethanol elimination and the extracts were kept in sterile bottles under refrigerated conditions until use.

Antibacterial assay

The screening of the ethanolic extracts of these plants for antibacterial activity was performed using two methods, the agar well diffusion method and the disk diffusion method, to compare their effectiveness against antimicrobial activity.

Agar well diffusion method

In order to determine the antibacterial spectrum, the antibacterial activity was performed by the agar-well diffusion method as described by Schillinger and Lucke (1989). A volume of 10 mL of agar medium (0.7% w/v) was inoculated with 0.1 ml of fresh overnight culture of the indicator strain (approximately 10^7 CFU/mL) and poured into a Petri dish containing layer of the plate-count agar (PCA). Wells of 6 mm in diameter were punched in the agar and filled with 50 µL of the ethanol extract. After holding the plates at room temperature for 2 h to allow diffusion of the extract into the agar, the plates were incubated at 37°C for 24 h. Then, they were examined for inhibition of the bacterial lawn and the diameters of the inhibition zones were measured.

Disk diffusion method

The disk diffusion test was performed using the standard procedure (NCCLS). The inoculum suspension of each bacterial strain was swabbed on the entire surface of Mueller-Hinton agar (MHA, Biokar-diagnostics). Sterile 6-mm filter paper discs were aseptically placed on MHA surfaces and crude ethanol extracts were immediately added to discs in volumes of 10 µL. The plates were left at ambient temperature for 15 min to allow excess prediffusion of extracts prior to incubation at 37°C for 24 h. Diameters of inhibition zones were measured. Each experiment was done in duplicate.

A microbial susceptibility control test was performed with some

Table 1. List of plants used and their properties.

Common name	Botanical name (Family)	Plant parts	Properties
Artemisia	<i>Artemisia Herba Alba</i> (Asteraceae)	leaves	Tonic, Stimulant, Antispasmodic, emmenagogue.
Lavender	<i>Lavandula officinalis</i> L. (Labiatae)	flowers	Disinfectant, antispasmodic, carminative, cholagogue, healing, diuretic, stimulant and sudorific and flavouring agent
Camomile	<i>Matricaria chamomilla</i> (Asteraceae)	flowers	Antispasmodic, stomachic, anti-inflammatory drug, healing, calming, anti-pains, anti-stress.
Clove	<i>Eugenia caryophyllata</i> (Myrtaceae)	Flower buds	Dental analgesic, carminative, stimulant and antiseptic.
Mint timija	<i>Mentha suaveolens</i> subsp. <i>Timija</i> (Lamiaceae)	leaves	Flavouring agent, anti-nausea and stimulant.
Thyme	<i>Thymus serpyllum</i> L. (Lamiaceae)	leaves	Disinfectant, antispasmodic, carminative, diuretic, expectorant and vermifuge.
Wild thyme	<i>Thymus vulgaris</i> L. (Lamiaceae)	leaves	Disinfectant, antispasmodic, cholaretic, diuretic, healing,deodorizer, revulsive, stomachic, tonic and vermifuge.
Verbena	<i>Lippia citriodora</i> (Verbenaceae)	leaves	Stomachic, antispasmodic with a sedative effect.
Cinnamon	<i>Cinnamomum zeylanicum</i> (Lauraceae)	Barks	Astringent, stimulant, stomachic, aromatic agent, carminative and antiseptic.
Rose	<i>Rosa centifolia</i> (Rosaceae)	Flowers	Aromatic, Perfumery, astringent, disinfectant, depurative and bechic.
Cistus	<i>Cistus salvifolius</i> (Cistaceae)	leaves	Contain tannins antifongic and antimicrobial.
Rosemary	<i>Rosmarinus officinalis</i> (Labiatae)	leaves	Disinfectant, cholagogue, diuretic, stomachic, tonic, carminative, stimulant and flavouring agent.
Scented Geranium	<i>Pelargonium graveolens</i> (Geraniaceae)	leaves	Flavouring agent and stimulant.

antibiotic discs with different targets: Penicillin G (10 units), Nalidixic acid (30 µg), Vancomycin (30 µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Novobiocin (5 µg), Ampicillin (5 and 30 µg). There were used as experimental positive control and ethanol as negative control. The tests were performed in duplicate for the evaluated microorganism.

Minimal inhibitory concentration assay

Based on the previous screening of plant extracts, Artemisia, lavender, camomile, clove, mint timija, thyme, wild thyme, verbena, cinnamon, rose, cistus, rosemary, geranium were identified to have potent antibacterial activity and their minimum inhibitory concentrations (MIC) were determined for *Listeria monocytogenes*. The micro-dilution method using serially diluted (2-fold) plant extracts recommended by the National Committee for Clinical Laboratory Standards (NCCLS) was performed and the MICs were determined by agar well diffusion method, inoculated plates were incubated at 37°C for 18 h. The MICs were determined as the lowest concentration of ethanol extracts inhibiting visible growth of each organism on the agar plate.

GC/MS analysis

The ethanol extracts were analyzed using the Thermo Scientific TRACE GC Ultra™ gas chromatograph. It was fitted with a splitless injector and connected to an MS PolarisQ-Quadrupole Ion

Trap (Thermo Electron) fused silica column VB5 (5% phenyl, 95% methylpolysiloxane, 30 m with 0.25 mm i.d. film thickness 0.25 µm) (J & W Scientific Fisons, Folsom, CA). The injector and interface were operated at 250 and 300 °C, respectively. The oven temperature was programmed as follows: 50°C raised to 250°C (4°C/min) and held for 3 min. Helium was the carrier gas at 1 ml/min. The sample (1 µl) was injected in the split mode (1:20). MS conditions were as follows: ionization voltage EI of 70 eV, mass range 10 – 350 amu. The ethanol extracts components were identified by comparing their relative retention times and mass spectra with those of authentic samples (analytical standards from data base).

RESULTS

Preliminary screening of plant extracts

The anti-bacterial activity of thirteen selected ethanol plant extracts against *L. monocytogenes* is summarized in Tables 2 and 3. The results revealed that the selected ethanol plant extracts showed antibacterial activity with varying magnitudes. The susceptibility of the strain *L. monocytogenes* tested to mint timija, clove, cinnamon, cistus, rose, thyme, wild thyme, artemisia, rosemary, geranium, camomile, lavender and verbena was appreciable.

Table 2. Diameters of inhibition zone of ethanol extracts tested against *L. monocytogenes*.

Ethanol extracts	Diameter (mm)
Mint timija	26 ± 0
Clove	25 ± 1
Cinnamon	22 ± 0
Cistus	20 ± 1
Rose	20 ± 1
Thyme	19 ± 1
Wild thyme	18 ± 1
Artemisia	16 ± 1
Rosemary	16 ± 0
Geranium	16 ± 0
Camomile	15 ± 1
Lavender	15 ± 1
Verbena	14 ± 1

Data are mean of two replications. Includes diameter of well (6 mm).

Minimal inhibitory concentration (MIC)

The MIC values of all the extracts tested against *L. monocytogenes* is shown in Table 4. MIC for selected ethanol extracts ranged from 0.25 to 11.75 mg/mL. It is apparent from the results that the MIC values are high for lavender and verbena, explaining the extent of resistance offered by *L. monocytogenes*, against these ethanol extracts. This study revealed that clove extract showed maximum activity against *L. monocytogenes* with MIC value 0.25 mg/mL followed by mint timija extract with MIC value of 0.315 mg/mL, indicating that clove and mint timija showed excellent antimicrobial activity against *L. monocytogenes*. *L. monocytogenes* is fairly sensitive to all ethanol extracts except lavender and verbena and was showing moderate MIC values against rosemary, geranium and camomile.

Antimicrobial activity

Out of the many ethanol extracts tested, thirteen showed antibacterial activity against one or more bacteria (Table 5). Generally most of the tested organisms were sensitive to many of the ethanol extracts. Mint timija, clove, cinnamon, cistus and rose extracts showed maximum activity against all the bacterial species tested.

The sensitivity of pathogens to ethanol extracts, as determined by agar well diffusion method, showed that the gram-negative bacteria were less sensitive than the gram-positive bacteria to the potent ethanol extracts. *K. pneumoniae* was inhibited by clove, cistus, rose and cinnamon extracts; no inhibition zone was detected for mint timija, thyme, wild thyme, artemisia, rosemary, geranium, camomile, lavender and verbena, indicating that these ethanol extracts do not have any apparent effect on

Table 3. Diameter of inhibition zones of antibiotics against *L. monocytogenes*.

Antibiotic target	Antibiotic	Diameter of inhibition zone (mm)
Protein synthesis	TET 30	33
	CLO 30	25
Cell wall synthesis	PEN G10	25
	AMP30	25
	AMP 5	20
	VAN 30	20
Nucleic acids (DNA replication)	NAL 30	-
	NOV 5	18
Negative control	Ethanol	-

TET= tetracycline (30 µg); CLO = chloramphenicol (30 µg); VAN = vancomycin (30 µg); PEN= penicillin G (10 unit); AMP = ampicillin (5 and 30 µg); NOV = novobiocin (5 µg); NAL= nalidixic acid (30 µg).

Table 4. Values of MIC for different ethanol extracts against *L. monocytogenes*.

Ethanol extracts	MIC (mg/ml)
Clove	0.25
Mint timija	0.315
Cinnamon	0.4
Cistus	0.515
Rose	0.9
Thyme	1.56
Wild thyme	2.15
Artemisia	3.75
Rosemary	5.25
Geranium	6.15
Camomile	6.75
Lavender	11.5
Verbena	11.75

K. pneumoniae.

No inhibition zone of *E. faecalis* growth was observed except for wild thyme, showing that this organism tolerate and survived in the presence of these ethanol extracts. Of all the ethanol extracts tested, clove and wild thyme extracts showed the highest antibacterial activity against majority of tested strains.

Gas chromatography coupled with mass spectrometry (GC/MS)

The major components and their retention times are summarized in Table 6. Among the identified compounds, some of them are known for their interesting biological capacity; mint timija (Piperidine deriv. 40.20%, Piperitenone oxide 38.90%), clove (Eugenol 76.91%), cinnamon (Trans-cinnamaldehyde 12.85%, Germacrene 11.87%), cistus (Aromadendrene 67.94%), rose (Isoamyl

Table 5. Sensibility of other pathogens to ethanol extracts.

Ethanol extracts	<i>S. aureus</i> ATCC 25923	<i>E. cloacae</i>	<i>K. pneumoniae</i>	<i>E. coli</i> ATCC 25922	<i>A. baumannii</i>	<i>E. faecalis</i>
Clove	±	+	+	+	+	-
Artemisia	+	+	-	-	-	-
Cistus	+	+	+	-	±	-
Verbena	+	+	-	-	±	-
Mint timija	+	+	-	±	+	-
Cinnamon	+	+	-	-	±	-
Lavender	±	+	-	-	-	-
Wild thyme	+	+	+	+	+	+
Geranium	+	+	-	+	+	-
Rosemary	±	±	-	-	-	-
Thyme	+	+	-	+	+	-
Rose	+	+	+	-	+	-
Camomile	+	+	+	-	-	-
Negative control (ethanol)	-	-	-	-	-	-

+ = Inhibition; - = no inhibition was observed; ± = weak inhibition.

alcohol 74.40%), thyme (Borneol 69.18%), wild thyme (α-thujene 10.91%, Carvacrol 10.79%, Thymol 8.91%), artemisia (Camphor 41.87%), rosemary (Phthalazine deriv. 30.80%, Verbenone 5.70%), geranium (Cis-Geranyl acetate 42.37%, Geraniol 36.79%), camomile (Phenylindolizine 32.82%), lavender (Linalyl anthranilate 46.29%) and verbena (Allethrolon 17.74%, Borneol 17.06%).

DISCUSSION

It was noted that disk diffusion assay and agar well diffusion method exhibited similar results, but the agar well diffusion revealed a low activity of ethanolic extracts (Olila et al., 2001). The inhibitory effect of thirteen plant extracts on *L. monocytogenes* was investigated *in vitro*. The results revealed the antibacterial potential of all these extracts especially the ethanol extract of clove. Among all ethanol extracts analyzed in this study, the extract of clove was the most effective as an antibacterial agent. The antibacterial activity has been attributed to the presence of some active constituents in the ethanol extracts. Our GC-MS study revealed eugenol (76.91%) to be the major constituent of clove extract.

Antimicrobial properties of ethanol extracts are desirable tools in the control of undesirable microorganisms especially in treatment of infections and in food spoilage. Gram-negative strains are generally more resistant than gram positive strains in solid diffusion tests and this trait has been attributed to the external lipopolysaccharide wall that surrounds the peptidoglycan cell wall of the former (Loã pez et al., 2005). In the present study, clove and wild thyme extracts were found to be equally effective against both gram-positive and gram-negative

organisms.

The degree of antibacterial activity was considered from the MIC values against *L. monocytogenes*. The MIC values were used as guide for the treatment and battle against undesirable microorganisms. The results obtained showed that the MIC values varied according to the extracts and indicated that clove exhibited the strongest antibacterial activity, followed by mint timija. Similar result has been reported by Nanasombat and Lohasupthawee (2005).

The antibacterial activity of clove is attributed to eugenol (2-methoxy-4-allyl phenol). Clove bud oil contains high eugenol (70 - 90%) content (de Guzman and Siemonsma, 1999). This compound is an antimicrobial compound having wide spectra of antimicrobial effect (Kim et al., 1995; Beuchat and Golden, 1989) which may contribute to growth inhibition of enterobacteria. High tannin content (10 - 19%) in clove provided additional antimicrobial activity (Shelef, 1983). Similar findings have been reported by Farag et al. (1989).

Analysis by GC/MS of the mint timija extract showed the presence of piperitenone oxide which is an oxygenated monoterpene with very interesting biological effects including antibacterial and antifungal activities (Brada et al., 2007). Aromadendrene is a sesquiterpene that belongs to the class of Aromadendranes.

Most of the identified components with antimicrobial activity extracted from plants are aromatic or saturated organic compounds and they are soluble in methanol and ethanol (Cowan, 1999). It seemed that the antimicrobial activity of wild thyme can be related to oxygenated monoterpenes as thymol and carvacrol are well known compounds with pronounced antimicrobial potentials (Didry et al., 1993, 1994). Most of the antimicrobial constituents such monoterpenes (pinene, limonene and

Table 6. Major chemical compounds in ethanol extracts tested identified by GC-MS.

Ethanol Extracts	Major compounds	RT	%
Artemisia	Dibromo-4-methoxybiphenyl	6.61	3.55
	Camphor	16.98	41.87
	Glycocholic acid	36.99	1.80
	Bornane	51.55	5.33
	Enopyranoside, deriv.	54.28	36.80
Lavender	β -Ocimene	19.13	14.85
	Camphor	20.86	9.27
	Linalyl anthranilate	21.17	46.29
	Borneol	21.46	10.12
	Terpendiol I	21.81	10.98
	δ -3-Carene	23.72	27.04
	Phthalazine, deriv.	23.87	20.80
	Hotrienol	26.02	12.00
	Trans-Linalool oxide	30.66	3.13
	Caryophyllene oxide	31.52	16.80
Isolongifolene	34.01	4.28	
Camomile	Benzeneacetic acid	17.83	9.53
	Phenylindolizine deriv.	23.87	32.82
Clove	Eugenol	24.54	76.91
	Caryophyllene	26.60	9.33
	Eugenyl acetate	29.84	8.88
	Caryophyllene oxide	31.51	2.45
	Grevillin A	54.29	44.07
	Triazolopyrimidine, deriv.	54.82	10.05
Cistus	Phthalazine, deriv.	23.87	7.34
	Manoyl oxide	42.20	5.56
	Aromadendrene	50.85	67.94
	Methylpentenol	53.15	5.41
Mint timija	Trimethoxy QUERCETIN	5.04	14.75
	Benzeneacetic acid	17.83	10.48
	Phenylindolizine	23.87	31.08
	Piperitenone oxide	27.10	38.90
	Phenoxazone deriv.	29.31	23.33
	Piperidine deriv.	32.23	40.20
	Triazole	32.56	20.90
Wild thyme	α -thujene	8.80	10.91
	2-bornanone	20.86	1.40
	Carvacrol	23.50	10.79
	3-methylresacetophenone	23.80	2.32
	phenylindolizine	23.87	42.16
	Thymol	24.21	8.91
	Benzothiazine driv.	29.31	19.11
Vervain	1,1-Dimethylcyclopentane	7.18	8.27
	Heptane	7.57	3.68
	Isoamyl alcohol	8.27	8.59
	2-methyl 1-butanol	8.36	4.02
	Camphene	9.36	10.86
	2-(Triethylsilylethynyl) Bromobenzene	17.83	10.69
	Borneol	21.46	17.06
	Phenylindolizine	23.87	34.06
	Thymol	25.07	5.17

cineole) contributed to the antimicrobial effect particularly against *L. monocytogenes* (Mourey and Canillac, 2002).

The medicinal aromatic plants and their extracts are generally recognized as containing active antimicrobial compounds. Allicin is a component in garlic oil that inhibits the growth of both gram-negative and gram-positive bacteria. Sulfur-containing compounds found in onions, leeks and chives are also antimicrobial components. Eugenol, carvacrol and thymol are phenolic compounds in cinnamon, cloves, sage and oregano that present antimicrobial activity (Ponce et al., 2003). Prindle and Wright (1997) reported that the antimicrobial activity of phenolic compounds was concentration dependent, affecting enzymatic activity related to energy production at low concentrations and causing protein precipitation at high concentrations.

Many plants contain non toxic glycosides which can get hydrolyzed to release phenolics which are toxic to microbial pathogens (Aboaba and Efuwape, 2001). An important characteristic of essential oils and their components is their hydrophobicity, which enabled them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Sikkema et al., 1994).

Conclusion

In conclusion, mint timija, clove, cinnamon, cistus, rose, thyme, wild thyme, artemisia, rosemary, geranium, camomile, lavender and verbena present promising antimicrobial activity that indicated that these ethanolic extracts have the potential to become technologically useful products as sanitizing agents. Further research will be needed to establish the technical possibility of their use as natural sanitizing agents in dairy products. The plant extracts are promising a big potential for incorporation into various food products for which a natural antimicrobial additive is desired.

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