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Plant Spices as Sources of Natural Antimicrobials for Food

Guadalupe Virginia Nevárez-Moorillón*

Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua. P.O. Box 1542-C Chihuahua, Chihuahua, Mexico

Correspondence: * **vnevare@uach.mx**

ABSTRACT

Plant spices are described as those parts of the aromatic plants which have special aroma and flavor compounds that can be extracted in aromatic essential oils. The chemical nature of antimicrobials in spices suggests that they act in the natural defence mechanism of plants. Spices have been used in food preparation in warm climate countries, where they are commonly found and used in spicy traditional cuisine. Worldwide concern on the use of toxic preservatives in food has lead to the combination of mild antimicrobial barriers, according to the hurdle technology concept. Spices and their essential oils are one of the most important natural antimicrobials considered in this application. Studies on the antimicrobial effect of spices as natural antimicrobials have been conducted in two areas: growth inhibition in broth or agar cultures, and the addition of spices or essential oils to food systems. When the spice or the essential oil is added to a food system, the study of the antimicrobial effect is complex, since there are interactions of the natural antimicrobials with the food components. Most of the time, the concentration of the natural antimicrobial that is inhibitory or bactericidal under controlled culture conditions, is not efficient when added to a complex food system. Therefore, even though there are multiple references on the antimicrobial effect of spices, there are still many questions to be answered, from the mechanism of action and the best conditions for their use in food systems, to the study of those not-yet analyzed spices around the world.

Keywords: antimicrobial tests, food systems, hurdle technology

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INTRODUCTION

An ancient quest of humanity is to seek the best method for food preservation, so that it can be transported, distributed and consumed safely. Man has developed many food preservation methods, from the antique use of sugar for fruit preservation, to the use of salt or drying of meat. Along with sugar and salt, spices have been used as food additives. Development of food preservation methods includes the use of physical or chemically based methods, and recently biological based methods are important in food industry (Gould 1996).

Currently, the food industry uses a very large amount of chemical antimicrobials, and their use will continue to grow. The most common antimicrobials used include salt, sugar, organic acids and benzoates. Many of those antimicrobials, especially organic acids, have limited spectra of usage. Their antimicrobial action is determined by physicochemical characteristics of food, especially pH. Chemical antimicrobials can be synthesized or can be obtained from natural sources, but excess use of artificially synthesized antimicrobials has generated rejection from the consumer, who is constantly being informed of collateral effects of an abuse in chemical additives in food. Among the problems reported are food allergies and risk analysis data that correlate some food additives such as nitrate, to cancer. The ideal characteristics of a good antimicrobial are: a wide antimicrobial spectrum, not toxic to humans, effective at low concentrations, minimally affected by the food pH and no notorious effects on odor, color or flavor at the concentration required for its antimicrobial action. The antimicrobial will be more valuable if it is presented as a powder, be soluble in water, not corrosive, stable during storage, but above all, not expensive (Doyle and Beuchat 2007).

Worldwide tendencies on minimal food processing and the reduction on the use of synthetic additives, has revitalized the search for new antimicrobials, especially those obtained from natural sources. Efforts have now concentrated on the cultural traditions of ethnic groups, especially in gastronomy and traditional medicine. In food preparation, the importance of spices as food additives has been particularly recognized. Besides the sensorial improvement of food flavor due to the addition of spices, their antimicrobial effects can help to increase the food shelf life (Nielsen and Rios 2000; Ates and Erdogrul 2003). Spicy food plates are common in warm climates, where they also function as food antimicrobials (Sherman and Flaxman 2001).

More than 1,300 plants with recognized antimicrobial

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properties have been documented (Velluti *et al.* 2003), and approximately 80 food products of plant origin have a high concentration of antimicrobials, including basil, cinnamon, clove, garlic, leek, mustard, pepper, thyme, oregano and rosemary (Cowan 1999; Draughon 2004). In recent years, the search for new antimicrobials has increased, especially considering the development of alternative food treatment technologies and the growing market for minimally processed fresh products (Ponce *et al*. 2004; Leite de Souza *et al.* 2005).

The interest in spices has also increased, especially in the characterization of its components and its effects on biological systems for applications in a wide variety of areas, including pharmaceutical applications (Adam *et al.* 1998; Ates and Erdogroul 2003; Lin *et al.* 2005), alternative medicine and natural therapies (Kalemba and Kunicka 2003; Nostro *et al.* 2004), in control of phytopatogens (Bouchra *et al.* 2003; Daferera *et al.* 2003; Jajakumar *et al.* 2007) by the use of plant extracts, or as additives in the food industry (Cutter 2000; Ceylan *et al.* 2004; Dorman and Deans 2000; Leite de Souza *et al.* 2005). In particular in food, the restrictions imposed by regulatory agencies on the abuse of chemical added preservatives (Guynot *et al.* 2003) has lead the way to more natural additives, that can help on the increment in shelf life (Nielsen and Rios 2000; Kong *et al.* 2007). However, the use of spices in food is limited, due to the high concentrations that are needed for their microbicidal activity, and the strong flavor that those concentrations add to food products (Lambert *et al.* 2001; Si *et al*. 2006).

SPICES AND THEIR ESSENTIAL OILS

The word spices derive from the Latin word *species*, meaning "earth fruits". Technically, it refers to the hard parts of aromatic plants, such as seeds, crust, buds or fruits, although the aromatic leaves of some plants (which should be referred as herbs) are also included (Doyle and Beuchat 2007). Spices have always been related to magic, as they are considered as a witch's tool for their enchantments, because they have been related to aphrodisiac, healing and magical properties. In antiquity, spices were considered as treasures, and were even used as money for exchanging merchandise. Each spice has a unique flavor and aroma, which is the result of the presence of different chemical compounds, derived from the plants' secondary metabolism. Pigments, simple phenols, polyphenols, phytoalexines, terpenes, terpenoids, are among the different chemical compounds related to the spices particular properties (Cowan 1999).

The product of hydro-distillation of spices is known as essential oil, and contains many of the compounds related to aroma and flavor. Essential oils are usually soluble in alcohols and other organic solvents, and are widely used in the perfume and beverage industries (Hammer *et al.* 1999; Kalemba and Kunicka 2003). Many of the compounds present in the essential oils are terpenes and terpenoids, derived from isoprene units, including some 12,000 chemical compounds that represent almost 10% of the total substances produced by plants (Cowan 1999; Daferera *et al.* 2000).

The physiological role in plants of the compounds present in essential oils can be related to protection mechanisms of the plant against microbial plant pathogens, as well as against insects. Some other compounds are responsible for aroma; quinones and tannins are plant pigments, and many other compounds are related to flavor. If spices or their compounds can inhibit or destroy predator organisms of plants, they can also protect humans from infections, and can also protect foods from microbial deterioration (Sherman and Flaxman 2001).

CHEMICAL COMPOSITION OF ESSENTIAL OILS

Chemical composition of plants' essential oils depend on a large number of parameters, including environmental and climatic conditions, season of collection, dehydration process and storage conditions (Daferera *et al*. 2000; Baydar *et al*. 2004). As expected, antimicrobial activity of essential oils also varies according to the phenologic stage of the plant at the time of collection and the climatic conditions present during the plant growth. Müeller-Riebau *et al*. (1997) found a different composition in the essential oil of different aromatic plants, depending on their collection dates. It has also been reported variation on the antifungal activity of the lemongrass essential oil during different seasons, being more potent the essential oil obtained from May to November (Mishra and Dubey 1994). European oregano (*Origanum vulgare* sbsp. *Hirtum*) composition has also qualitative and quantitative differences depending on the growth conditions and the time of collection. Fluctuations are so important, that concentrations of the main components, carvacrol, thymol and cymene, vary up to 10 times among samples (Jerkovic *et al.* 2001).

In another report, the concentration of thymol in plants of *Thymus vulgaris* from Brazil varied from 31 to 52% depending on the harvesting time. The percentage of recovered oil also varied from 0.2 to 0.3% (Atti-Santos *et al.* 2004). The biological activity and composition of savory (*Satureja parnassica*, *Satureja thymbra*) essential oil was also affected by harvesting time, as reported by Chorianopoulos *et al.* (2006b). In greenhouse experiments, moisture content did not have a significant effect on the content and composition of essential oil in Mexican oregano (*Lippia berlandieri* Schauer). Plant age, on the other hand, did have an effect on the amount of essential oil recovered; being the youngest plants the ones that contained a higher concentration (Dunford and Silva 2005).

Even though the antimicrobial activity of essential oils is attributed mainly to the components present in larger quantities, it is important to consider the antagonistic or synergistic effect of minor components that affect the overall effect. Each component in the essential oil has its own contribution on the biological effect of the whole mixture; it is well known that the essential oil mixture is more effective than the components added separately (Daferera *et al.* 2003). Although usually one or two components are responsible for most of the antimicrobial activity, the effect of individual components is usually done to identify the main compounds responsible for the biological activity (Bouchra *et al.* 2003).

The analysis of the components present in spices' essential oils can also be used as a tool to characterize and differentiate plant species. In many cases, plants of different genera or even different plant families are recognized as the same or similar spice. The use of chemical analysis for taxonomy purposes have been reported for the differentiation of oregano from marjoram (Baranska *et al.* 2005), as well as for oregano from different geographical locations (Kokkini *et al.* 2004). The variability of oregano species were evaluated by D'Antouno *et al.* (2000) considering their growth areas, and the essential oil was classified into three groups according to its composition: the first group had a high content of compounds derived from the metabolic pathways of carvacrol and thymol; the second group was characterized by a different composition of sesquiterpenes and a large proportion of linalool, and the third group had abundant quantities of sesquiterpenes.

In the search for optimization of essential oil extractions, new methodologies such as supercritical extraction have been studied. Sub-critical and super-critical fluid extraction using CO_2 as dissolvent has been tested on European oregano, since it is not toxic or explosive and is easily removed from the essential oil (Gaspar *et al.* 2000; Arcila-Lozano *et al.* 2004). The use of supercritical CO_2 give higher yields of essential oils, and a total of 24-27 compounds were found in the essential oil of oregano; the distribution of the compounds extracted was a function of the extraction time (Alves Rodríguez *et al.* 2004; Santoyo *et al.* 2005, 2006). When the components that are responsible for the biological activity of a spice are well known, the development of methodologies to increase their concentration in the volatile

fraction can be applied. Castillo-Herrera *et al.* (2007) developed a method for extraction of essential oil from Mexican oregano, enriching the phenolic content, regardless of the geographical origin of the plant used.

ANTIMICROBIAL EFFECTS OF ESSENTIAL OILS

Although the antimicrobial activity of herbs and spices has been identified a long time ago, the scientific literature on their biological activity has intensified in recent years (**Tables 1, 2**). This antimicrobial effect has been studied for either bacteria or fungi and yeast, responsible for food deterioration as well as for food-borne pathogens.

Table 1 includes some examples of antimicrobial analysis of several spice essential oils against a wide variety of bacterial strains related to foods. The effect against the most common food-borne pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. are found in many scientific reports. There is also a wide variety of essential oils tested, but there are common species such as clove, thyme, oregano, anise, basil, coriander, cinnamon, lemongrass and marjoram. The antimicrobial effect is higher for Gram negative bacteria, but *Bacillus cereus* is one of the most sensitive bacterial species (Ultee *et al.* 1999, 2002). Kim *et al.* (1995) reported the antibacterial activity of 11 essential oils, along with thymol and carvacrol against five food-borne bacteria, and demonstrated that carvacrol, citral and geraniol presented strong bactericidal activity.

The approach of antimicrobial analysis is either the evaluation of many essential oils against one or two different microbial species, or the evaluation of a wide variety of bacterial and fungal spices against few essential oils or their main components (**Tables 1, 2**). Spices such as oregano, thyme, rosemary and clove, are the ones that show the better effects. There are also differences on the antimicrobial effect by the strains tested. *Staphylococcus*, *Escherichia*, *Salmonella*, *Listeria* and *Bacillus* are among the most reported bacterial genera, and they show a large variability on their susceptibility to the same essential oil; this effect could be due to differences in response of the isolates to environmental stresses or pathogenic factors. There are not many reports that address this question.

The antimicrobial effect of spices has also been reported for yeasts, and one of the pioneer works was reported by Conner and Beuchat (1984), that demonstrated a strong effect of several essential oils against 13 yeast of importance to foods. The antifungal effect of several essential oils of plants from Greece was also evaluated by inhibition of radial growth, spore germination and conidial growth of *Penicillium digitatum*. According to the results, the essentials oils of oregano, thyme, dictamus, and marjoram completely inhibited fungal development at relatively low concentrations (250-400 mg/L). The authors suggest that the antifungal effect of the essential oils is the result of a synergistic interaction amongst their components (Dafarera *et al.* 2000).

The effect of different essential oils was also studied by Basilico and Basilico (1999) showing that oregano (*Origanum vulgare*), mint (*Menta arvensis*) and basil (*Ocimum basilicum*), had an antifungal effect on *Aspergillus ochraceus* growth, as well as on the inhibition of ochratoxin production; small concentrations of the essential oil, however, presented an increased mycelial growth, but inhibited mycotoxin production. A similar effect was observed by Portillo *et al.* (2005) that evaluated Mexican oregano against fungi and yeasts related to food spoilage. Four different effects were observed when 25 fungal strains were analyzed: no fungal growth inhibition; a concentration-dependent inhibition of fungal growth; an increase in fungal growth by low concentrations of oregano but inhibition at higher concentrations; an increase in fungal growth by high concentrations of oregano, but inhibition by low concentrations.

The antifungal effect of several essential oils has been studied on the growth of specific fungal strains of concern for a particular food group. Guynot *et al.* (2003) evaluated the effect of volatile compounds of 16 different essential oils versus fungi related to bread product contamination, such as *Eurotium herbariorum* and *E. rubrum*. In order to better simulate the effect of the essential oils, the volatile fraction was tested in a medium containing wheat flour, adjusted to different water activities. Suhr and Nielsen (2003) also tested the essential oil against bread-contaminant fungi, but this time, the essential oil was added to the agar plates, and the antifungal effect was evaluated as inhibition of radial growth (**Table 2**).

When the antimicrobial activity of essential oils is studied, the effect is considered as the result of the interaction of all the compounds present; in order to understand the effect of the predominant compounds, some authors have also studied de effect of individual compounds (**Table 1**). Main components such as carvacrol, thymol, p-cymene and γ-terpene were evaluated for antimicrobial activity against *E. coli* O157:H7, carvacrol-thymol combinations showed additive effects (Burt *et al.* 2005). A high concentration of carvacrol in European oregano essential oil was significant in the inhibition of food-borne pathogens such as *E. coli*, *S. typhimurium*, *S. aureus* and *B. subtilis*, among others (Sivropoulou *et al.* 1996).

When carvacrol was mixed with food stabilizers, such as agar or carrageenan, the antimicrobial activity of the phenolic compound was improved; therefore, the components of the essential oils can interact with food components, increasing or possible decreasing their biological activity (Burt *et al.* 2005). Lambert *et al.* (2001) reported that is possible to obtain the same inhibition of *P. aeruginosa* and *S. aureus* with either thymol-carvacrol combinations or with oregano essential oil. The authors suggest that the antimicrobial effect of those compounds is due to damages to the cell membrane, with an effect on the pH and inorganic ions' cell equilibrium.

MECHANISM OF ACTION

Since the essential oils of spices are a complex mixture of compounds with different structures, it is difficult to assess the mechanism of action of the mixture. Therefore, research has focused on the mechanism of action of some of their main components, and as a consequence, the mechanism of action of the essential oil. Still, there are few reports on the effects of the spices' essential oils on microbial cells. It has been suggested that the antimicrobial activity is the result of a damaged enzymatic cell system, especially those associated with energy production and the synthesis of structural compounds (Gill and Holley 2004, 2006). It has also been reported that the effect of phenolic compounds on microbial growth and the production of toxins could be due to the ability of these compounds to modify the microbial cell permeability, allowing the loss of macromolecules from within. Furthermore, they can interact with membrane proteins, causing structural and functional deformity (Rhayour *et al.* 2003).

In Gram positive bacteria, the action of the phenolic compounds involves the leakage of potassium ions from the cell, with a decrease in the internal pH, a collapse on the membrane potential and inhibition of ATP generation; all those events lead to cell lysis and death (Ultee *et al.* 1999; Lambert *et al.* 2001). Under the microscope, those effects are shown as cytoplasm coagulation and breakage of the cell wall (Oussalah *et al.* 2006a). Spices essential oil also inhibits DNA and RNA synthesis, as well as protein and polysaccharide synthesis in bacteria and fungi. In the later, the changes are similar to the effects of medical antibiotics (Kalemba and Kunicka 2003).

Several authors have reported that the degree of microbial inhibition of spices' essential oils can be attributed to the presence of compounds with an aromatic ring in its structure (Velluti *et al.* 2003; Sokmen *et al.* 2004). The high antimicrobial activity of the phenolic compounds can be ex-

Table 1 Antimicrobial analysis of spices against foodborne bacteria.

Spices	Microorganisms	Method used	Reference
Black pepper (*Piper nigrum*), clove (*Syzygium aromaticum*), geranium (*Pelargonium graveolens*), nutmeg (*Myristica fragans*), oregano (*Origanum vulgare*) thyme (*Thymus vulgaris*.)	Twenty five bacterial strains, including foodborne, animal and plant pathogens, food spoiling	Well-diffusion test (4 mm diameter, 15 µL of essential oil added)	Dorman and Deans 2000
European oregano (*Origanum scabrum* and *Origanum microphyllum*)	*S. aureus*, *S. epidermidis*, *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa*	MIC by microplate assay.	Aligiannis *et al.* 2001
Twelve different essential oils, including anise, basil, cardamom, coriander, fennel, parsley, oregano, and rosemary	*L. monocytogenes*, *S. aureus*, *E. coli* O:157:H7, *Y. enterocolitica*, *P. aeruginosa*, *L. plantarum*	Filter paper disk diffusion test (24 mg essential oil in each disk), followed by Microbial Lethal Concentration (MLC) by twofold dilutions	Elgayyar *et al.* 2001
Dill (*Anethum graveolens*), coriander seeds and leaves (*Coriandrum sativum*), Eucalyptus (*Eucalyptus dives*) Essential complete and fractionated oil	*E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, *S. aureus*, *P. fragi*, *S. grimesii*, *E. agglomerans*, *Y. enterocolitica*, *B. cereus*, Group A *Streptococcus*, *Lactobacillus sp*	MIC in microplates. Individual fractions and mixtures were analyzed	Delaquis *et al.* 2002
96 essential oils and 23 oil compounds	*C. jejuni*, *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*	Bactericidal activity (BA50) was defined as the percentage of the sample in the assay mixture that resulted in a 50% decrease in CFU relative to a buffer control.	Friedman *et al.* 2002
Bay (*Pimenta racemosa*), clove bud (*Eugenia caryophyllata*), Oregano (*Origanum vulgare*) and red and light thyme (both *Thymus vulgaris*)	*E. coli*	Disc diffusion and colorimetric determination of MIC using Alamar Blue.	Burt and Reinders 2003
Thymus species from Portugal *Thymus mastichina*, *T. camphorates*, *T. lotocephalus*	*E. coli*, *L. monocytogenes*, *P. mirabilis*, *Salmonella spp.*, *S. aureus.*	Disk agar diffusion test (3 µL in 6 mm paper disks)	Faleiro *et al.* 2003
17 spices, including cloves, thyme, oregano, allspice, basil, rosemary, and marjoram	*Shigella sonnei*, *Shigella flexneri*	Spices added to either Mueller-Hinton broth or agar (1%); visual observation of growth. Determination of MIC by agar dilution method. Effect of other factors such as pH, a_w, NaCl by agar dilution method	Bagamboula *et al.* 2003
Cinnamon bark, Thyme (*Thymus vulgaris*), Perilla, Lavender	*S. aureus E. coli*	Minimal concentration of vapor phase to inhibit growth of the microorganisms; Plate dilution test	Inouye *et al.* 2003
Anise (*Pimpinella anisum*), Coriander (*Coriandrum sativum*), Chinese cinnamon (*Cinnamomum cassia*), juniper (*Juniperus oxycedrus*)	*B. brevis*, *B. cereus*, *B. megaterium*, *B. subtilis*, *E. faecalis*, *K. pneumoniae. L. monocytogenes*, *M. luteus*, *M. smegmatis*, *P. aeruginosa*, *S. aureus*, *Y.enterocolitica*	Alcohol, ethyl acetate, acetone and chloroform extracts tested by disk diffusion method	Ates and Erdogrul 2004
Ginger (*Zingiber officinale*), fingerroot (*Bosenbergia pandurata*) and turmeric (*Curcuma longa*) essential oil extracted with ultrasound assisted solvent-extraction	*L. monocytogenes*, *S.* Typhimurium DT 104	Agar dilution test for determination of MIC and MBC.	Thongson *et al.* 2004
Lemon balm (*Melissa oficinalis*)	*P. aeruginosa*, *E. coli*, *S. enteritids*, *S. typhi*, *S. sonei*, *Sarcina lutea*, *M. flavus*, *S. aureus*, *S. epidermidis*, *B. cereus*	Hole-plate agar diffusion method	Mimica-Dukic *et al.* 2004
Fourteen essential oils (simple or mixed)	*S. aureus and C. albicans*	MIC by OD and metabolic dye test (INT). Disk diffusion and hole-plate diffusion assays	Donaldson *et al.* 2005
Cumin (*Cuminum cyminum*) stored for 30 years	*S. epidermidis*, *S. aureus*, *B. subtilis*, *B. pumilus*, *E. coli*, *P. aeruginosa*, *Salmonella abony. S. cerevisiae*, *C. albicans*, *A. niger*	Agar diffusion cup	Jirovetz *et al.* 2005
Basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*)	Thirteen bacterial strains	Hole-plate agar diffusion test	Bozin *et al.* 2006
Satureja spinosa	*S. aureus*, *E. coli* O157:H7, *L. monocytogenes*, *S. enterica* serovar Enteritidis, *B. cereus*	Conductance method and optical density measures of growth	Chorianopolous *et al.* 2006b
18 different essential oils	*Vibrio parahaemolyticus*	NIC by microplate serial dilution	Yano *et al.* 2006
Basil (*Ocimum basilicum*), Lemon balm (*Melissa officinalis*), Marjoram (*Origanum majorana*) Oregano (*Origanum vulgare*), Rosemary (*Rosmarinus officinalis*), Sage (*Salvia trilobata*), Thyme (*Thymus vulgaris*)	*B. cereus*, *E. coli*, *L. monocytogenes*, *P. aeruginosa.*	Spot-on-agar test, microplate dilution assay for individual essential oils. For combinations of the essential oils tested, a check board method was used, to test for interactions.	Gutierrez *et al.* 2008

plained by the alkyl substitution in the aromatic ring. The importance of the hydroxyl group was confirmed by comparing the efficiency of carvacrol with cymene, which lacks the hydroxyl group in its structure. Carvacrol antimicrobial activity was higher than cymene, suggesting the destabilization of the cytoplasmic membrane, reducing the pH gradient across the cytoplasmic membrane (Ultee *et al.* 2002; Veldhuizen *et al.* 2006). Recently, Xu *et al.* (2008) demonstrated that the antimicrobial effect of thymol and carvacrol against *E. coli* is caused by an increase in cell membrane permea-

Table 2 Antimicrobial analysis of spices against foodborne fungi.

Spices	Microorganisms	Method used	Reference
Black cumin (*Nigella sativa*), coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), dill (*Anethum graveolens*), laurel bay (*Laurus nobilis*), oregano (*Origanum onites*), parsley (*Petroselinum sativum*), spearmint (*Mentha spicata*), sweet basil (*Ocimum basilicum*), white mustard (*Brassica alba*).	*Aspergillus flavus*, *Aspergillus niger*, *Geotrichum candidum*, *Mucor* spp., *Penicillium roqueforti* and 3 *Penicillium* spp.	Spore suspension spread over agar plates containing different concentration of spices. Results were expressed as days until visual fungal growth and spore germination was observed.	Akgül and Kivanc 1988
Oregano (*Origanum vulgare*), mint (*Menta arvensis*), Basil (*Ocimum basilicum*), sage (*Salvia officinalis*) and coriander (*Coriandrum sativum*)	*Aspergillus ochraceus* and ochratoxin production	Growth in YSE broth added with essential oil. After incubation, ochratoxin production and dry mycelium mass was determined.	Basilico and Basilico 1999
Thyme (*Thymus vulgaris*), Oregano (*Origanum vulgare*), Dictamus (*Origanum dictamus*), Marjoram (*Origanum majorana*), Lavander (*Lavandula angustifolia*) Rosemary (*Rosmarinus officinalis*) Salvia (*Salvia fruticosa*)	*Penicillium digitatum*	Inhibition of radial growth, conidial germination.	Daferera *et al.* 2000
Mustard essential oil, cinnamon oleoresin, oregano oleoresin, clove oleoresin, vanilla oleoresin, garlic essential oil	*Aspergillus flavus*, *Endomyces fibuliger*, *Penicillium commune*, *Penicillium corylophilum*, *Penicillium discolor*, *Penicillium palitans*, *Penicillium polonicum*, *Penicillium roqueforti*, *Penicillium solitum*, *Pichia anomala*	Agar plates were inoculated with vegetative fungalmaterial,. Essential oils were placed in filter paper on the lid of the plate and growth was recorded.	Nielsen and Rios 2000
Origanum scabrum and *Origanum microphyllum*.	*Candida albicans*, *Candida tropicalis*, *Torulopsis glabrata*	MIC by microplate assay.	Aligiannis *et al.* 2001
Twelve different essential oils, including anise, basil, cardamom, coriander, fennel, parsley, oregano, and rosemary	*A. niger*, *Geotrichum*, and *Rhodotorula.*	Filter paper disk diffusion test (24 mg essential oil in each disk), followed by Microbial Lethal Concentration (MLC) by twofold dilutions	Elgayyar *et al.* 2001
Melisa (*Melissa officinalis*), lavander (*Lavandula angustifolia*), Spearmint (*Mentha piperita*)	Five food spoilage yeasts, *S. cerevisiae* was used as reference	Disc diffusion test and broth dilution assay, determining deltaOD$_{640}$ by the addition of essential oil to broth cultures	Araújo *et al.* 2003
Cinnamon bark, Thyme (*Thymus vulgaris*), Perilla, Lavender	*T. mentagrophytes A. fumigatus C. albicans*	Minimal concentration of vapor phase to inhibit growth of the microorganisms. Plate dilution test for essential oils.	Inouye *et al.* 2003
Volatile compounds of 16 different essential oils	*Eurotium amstelodami*, *E. herbariorum*, *E. repens*, *E. rubrum*, *Aspergillus flavus*, *A. niger*, *Penicillium corylophilum*	Agar plates added with flour and at different water activities, inoculated with fungal spores. Essential oils were places in filter paper on the lid of the plate.	Guynot *et al.* 2003
Bay, cinnamon leaf, clove, lemongrass, mustard, orange, sage, thyme and rosemary essential oils	*Penicillium roqueforti*, *Penicillium corylophilum*, *Eurotium repens*, *Aspergillus. flavus*	Rye bread-based agar added with different concentrations of essential oils. Inhibition of radial growth was measured. Also, slices of rye bread were inoculated with fungal spores and incubated in an chamber saturated with essential oil volatiles	Suhr and Nielsen 2003
Cumin (*Cuminum cyminum*) stored for 30 years	*S. cerevisiae*, *C. albicans*, *A. niger*	Agar diffusion cup	Jirovetz *et al.* 2005
Basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*)	Six fungal strains	Antifungal: concentrations of essential oil in microplates, inoculated with known concentrations of spores. (microdilution assay).	Bozin *et al.* 2006
Anise (*Pimpinella anisum*), Boldus (*Pëumus boldus*), Peppermint (*Mentha piperita*), Oregano (*Origanum vulgare*), Peperina (M*inthosthachys verticillata*)	*Aspergillus* section *Flavi*	Spore germination and germ-tube elongation of fungal strains in agar plates with different concentrations of essential oils. Fungal growth in plates with essential oil and different water activities	Bluma *et al.* 2008

bility.

Aldehydes, such as citral, are also known to have a strong antimicrobial activity that can be attributed to the double bond in its structure, which gives to the molecule a high electronegative potential. The electronegative compounds can interfere with biological processes that involve electron transfer and can also react with proteins and nucleic acids (Dorman and Deans 2000). Another components commonly found in essential oils are terpenes, which are highly lipophilic, and they are reported to interfere with membrane enzymes, such as those involve in the respiratory chain. Some other components present in essential oils interfere with protein translocation across membranes, and with membrane-bound enzymes (Cowan *et al.* 1999).

Bennis *et al*. (2004) reported the cell wall and cell membrane damage of *Saccharomyces cerevisiae* cells by eugenol and thymol, as demonstrated by electron microscopy and release of organic molecules. The authors placed yeast cells in contact with fungicidal concentrations of eugenol (3 mM) and thymol (1.8 mM) and determined viable cells, as well as the release of organic material from the destroyed cells (260 nm OD). By electron microscopy, the cells showed severe damage in their cell surface, with a morphological damage by the presence of thymol. The effect of oregano essential oil and irradiation on *E. coli* O157:H7 and *L. monocytogenes* were observed by changes in the composition of murein and the internal and external ATP concentration. Those organisms were chosen because of their

Gram reaction, since it is well established that essential oils are more effective against Gram negative bacteria. In both cases, changes in the composition of murein cell wall components were observed; the murein was not disrupted by the treatment of oregano alone, but the composition and relative percentage of several muropeptides were modified, with the consequent effect on cell wall integrity. The effect of irradiation was more evident on the disequilibrium of ATP concentrations inside and outside the cell wall, and the effect of both factors was synergistic (Caillet *et al.* 2005; Caillet and Lacroix 2006).

Oussalah *et al.* (2006a) showed that Spanish oregano (*Corydothymus capitatus*), Chinese cinnamon (*Cinnamomum cassia*), and savory (*Satureja montana*) essential oils create an increase in cell wall permeability in *E. coli* O157:H7 and *L. monocytogenes*, with the consequent release of cell constituents, a decrease in ATP concentration and a decrease in the internal pH. On the other hand, Rasooli *et al*. (2006) demonstrated the deleterious effect of two essential oils of the *Thymus* genus, against *Aspergillus niger*. The authors report a strong antifungal effect, and when cells were observed by electron microscopy, MIC of the essential oil caused severe damage to the cell wall, cell membrane, and cellular organelles. The changes could also be observed visually, with changes in mycelial morphology and spores.

ANTIMICROBIAL COMPOUNDS NOT FOUND IN ESSENTIAL OILS

The essential oil of spices concentrates low molecular weight volatile compounds that are usually responsible for odor and flavor of the aromatic plants. Most of the antimicrobial activity of spices has been related to the compounds found in their essential oil; however, the strong odor and flavor can be an impediment for the use of essential oils as food additives. The study of spices has also focused on the identification of other compounds, different than those found in the essential oils that can also have antimicrobial activity.

Hydrosols, also known as floral water, are the co-products of hydrodistillation of aromatic plants. Hydrosols are complex mixtures of traces of the essential oil components and water-soluble compounds. Sagdic (2003) studied hydrosols from oregano (*Origanum vulgare* L., *Origanum onites* L., *Origanum majorana* L.) and thyme (*Thymus vulgaris* L., *Thymus serpyllum* L.) grown in Turkey and found that they were inhibitory at 10-25 mL/100 mL concentrations, and bactericidal at 50 mL/100 mL when tested against *E. coli* O157:H7, *S. aureus* and *Yersinia enterocolitica.* On the other hand, it has been demonstrated that volatile compounds of European oregano with antimicrobial capacity, include a series of compounds attached to glucosidic residues that are not found in the essential oil (Mastelic *et al.* 2000). Cilantro essential oil was shown to be an effective antilisterial; furthermore, the essential oil was separated by fractional distillation and fractions of cilantro essential oil that were deficient in phenolic compounds have a strong antilisterial activity (Delaquis *et al.* 2002)

The variety of chemical compounds found in plants that have antimicrobial activities is extensive. Although many of the most recognized compounds are present in essential oils, there are many other chemicals from non-aromatic plants that also have antimicrobial properties. Cowan (1999) wrote an excellent review on the nature of chemical antimicrobials found in plants.

THE STUDY OF NATURAL ANTIMICROBIALS

The chemical nature of spices essential oils, especially its high volatility and low water solubility, represent a significant problem when assessing biological activities, including antimicrobial properties. Much has been discussed on the appropriate method for determination of antimicrobial activity of essential oils, on the reproducibility of results, as well as on the correlation between *in situ* and *in vitro* studies. This is especially important, since most of the microbiological methods includes the use of water-based culture media, were essential oils are only partially soluble.

Methods used to test biological activity of essential oil includes qualitative techniques such as disk and well diffusion assays (Elgayyar *et al.* 2001; Ates and Erdogrul 2004; Mimica-Durik *et al.* 2004), as well as quantitative assays based on dilution of the tested substance, such as those used to determine the Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal (or Fungicidal) Concentration (MBC, MFC) (Delaquis *et al.* 2002; Thongson *et al.* 2004; Bozin *et al.* 2006). Although diffusion assay methods are fast and are usually compared to dilution methods, they are considered not reliable, since do not consider factors such as the differential diffusion of oil constituents through agar (Hood *et al.* 2003). On the other hand, dilution methods include agar dilution, broth dilution and microplate-based microdilution, but there are some reports on the carry-over of volatile compounds between the wells in a microplate, when highly volatile compounds are tested (Donaldson *et al.* 2005). Dilution-based methods also have the disadvantage of the difficulty of obtaining homogeneous mixture of oil in the test medium. To overcome this problem, the use of surfactants such as Tween 80 or chemical solvents such as ethanol or dimethyl sulfoxide have been used, but for many microorganisms, the solvent rather than the oil tested, are the ones that exhibits antimicrobial activity (Hood *et al.* 2003; Donaldson *et al.* 2005). Also, the use of dye indicators of microbial activity, such as Alamar blue or fluorescent dyes have been used to help on the determination of MIC, when suspension of antimicrobial compounds are turbid, and determination of bacterial growth is difficult (Burt and Reinders 2003; Donaldson *et al*. 2005; Xu *et al.* 2008).

Lambert and Pearson (2000) first proposed the use of Optical Density (OD) data and application of a mathematical model to obtain MIC and NIC (Non-Inhibitory Concentration) values. The authors used the OD data to generate areas-under-the-curve values and fractional areas that were used in a model derived from the Gompertz equation to determine MIC and NIC. They first reported the use of their model on the dose response of *S. aureus*, *E. coli* and *P. aeruginosa* against several inhibitors. However, OD readings can have several sources of error, including dead or not active cells that still contribute to turbidity. Marino *et al.* (1999) suggested the use of a bioimpedometric method that can detect metabolic activity of microorganisms, based on variations on an electrical signal supplied directly into the culture medium. The time to detect biological activity was used as response variable to evaluate antimicrobial properties of thyme against Gram positive and Gram negative bacteria. The method has also been used to determine MIC by Chorianopoulos *et al.* (2006a), using the time to detection, maximum conductance and slope of the conductance curve to obtain data and adjust it to the model proposed by Lambert and Pearson (2000). In the report, the authors used the method for determination of CMI and NIC of *Satureja spinosa* against several food-borne pathogens.

Determination of antifungal activities of essential oils is an even more complex task, considering the difficulties on assessment of viable fungal cells. Methods such as spreading a spore suspension over a Petri plate and addition of essential oils to wells or paper disks are used as techniques for preliminary detection of antifungal activity (Elgayyar *et al.* 2001; Jirovetz *et al.* 2005). Also, biomass production in the presence of the antimicrobial has proven to be an effective method for filamentous fungi assays (Basilico and Basilico 1999). Among the most reliable methods used in antifungal analysis of essential oils, is the inhibition of radial growth, because data can resemble a bacterial growth curve (Portillo *et al.* 2005). The problem of achieving a homogeneous concentration of the essential oil in the Petri plates is a disadvantage, but it is also shared with most of the methods used to test antifungal activities. Spore germi-

nation and germ-tube elongation of fungal strains placed in contact with the essential oil has recently been reported for analysis of several essential oils against *Aspergillus* strains (Bluma *et al.* 2008). Although it can be time-consuming, the methods provide important information on the effect of essential oil compounds on fungal growth.

Suhr and Nielsen (2003) demonstrated that the antifungal activity of 10 different essential oils was dependent on the method of analysis. They used a culture medium with a composition similar to rye bread, and also tested the inhibition when the essential oil was spread on a slice of rye bread. In the *in vitro* analysis, thyme was more effective, while mustard was more effective when added to the bread slice. It has also been reported that small concentrations (50 µL) of different essential oils (cinnamon, clove, basil, thyme), applied to a bread-based agar, totally inhibited the growth of the most important bakery spoilage molds (*Eurotium*, *Aspergillus* and *Penicillium*), while the same oils were not effective on mold inhibition when those were tested on cake analogues (Guynot *et al.* 2003). Therefore, medium composition, exposure time, as well as environmental factors (temperature, pH and water activity) are all important factors to consider in the selection of the method of evaluation for antimicrobial activity.

As stated before, the most active components of essential oils are volatiles; therefore, several authors have suggested the development of methods to test the volatile fractions of essential oils for antimicrobial activity. Inouye *et al.* (2003) proposed a method to test the minimal concentration of the vapor phase that inhibited the growth of bacterial strains on Petri plates. The authors reported the inhibition of *S. aureus* and *E. coli* as well fungi and yeast (*Candida albicans*) by the volatile fraction of thyme, cinnamon bark, perilla and lavender, and reported the effect as MID (Minimal Inhibitory Dose per unit space). Results correlate to those obtained by MIC, but they demonstrate that very low concentrations of the antimicrobial compounds in the vapor phase are antimicrobial. Antifungal activity has also been tested by placing a filter paper impregnated with a known concentration of essential oil, on the lid of a Petri plate, and then the plate is sealed to achieve an equilibrated atmosphere saturated with volatile compounds. Inhibition of fungal growth by radial growth, or time to detection of micelial growth is used as response variables (Nielsen and Rios 2000; Guynot *et al.* 2003; Suhr and Nielsen 2003).

Lack of agreement between agar-based methods and evaluation of antimicrobial activity with food model systems, shows the importance of the interaction of factors such as fat and protein content in foods, and their role in water activity and chemical reactions with the product that carries the antimicrobial effect. The interaction of those factors, suggests the use of more food analogue products in standard laboratory methods (Suhr and Nielsen 2003).

USE OF SPICES IN FOOD

Since man has used plant extracts, including essential oils for medicinal and pharmaceutical purposes, one of the main interests in spices or their essential oils has also been food preservation. Nowadays, natural antimicrobials are a common alternative to extend the shelf life of different products, by either inhibiting their growth or by killing the microorganisms present (Dadalioğlu and Evrendilek 2004; Draughon 2004). According to reports by the World Health Organization (WHO), it is estimated that up to 80% of human population uses plant extracts or their active compounds for the abovementioned reasons (Arcila-Lozano *et al*. 2004).

On the other hand, the ability of the microorganisms to growth and easily adjust to environmental changes, allows them to develop resistance mechanisms against antimicrobials in a short period of time; therefore, the challenge of developing new food preservation methods, is a continuous task (Dorman and Deans 2004). The challenge is to find natural antimicrobial products that can be easily added to food, without disturbing the nutritional or sensorial quality of the product, and that does not represent a significant increase in formulation, processing or storage costs (Gould 1996; Doyle and Beuchat 2007). The balance between effective dose and consumer acceptability of food products is an actual puzzle for the food industry (Skandamis and Nychas 2000). The incorporation of natural additives to foods must be evaluated in the real capacity of the products to cause a beneficial effect on the microbiological quality and sensorial properties of the foodstuffs, with the expected effect of increasing shelf life. Up to recent years, most of the reports were on antimicrobial effects of natural products that were evaluated under laboratory conditions, without considering the complex food environment where the antimicrobials would be added.

The quantitative evaluation of natural antimicrobials through the development of mathematical models done using data from food model systems can help on the prediction of the efficiency of the antimicrobial additives under real conditions (Baranyi and Tamplin 2003; Kong *et al.* 2007). Predictive microbiology, as the quantitative study of microbial ecology in foods, help on the description of the microbial response to environmental factors, including those present in the food system as a microenvironment: pH, water activity, temperature, etc. Predictive microbiology is based on the assumption that the microbial response of a microorganism studied in the laboratory, under the same environmental conditions that are present in the food system, will have the same behavior as the one expected when the microorganism is present in food. Therefore, the mathematical models developed using data from laboratory studies, can then be used to predict the behavior of the microorganism when present in foods. Models can be confirmed by doing experiments in food systems (Devlieghere *et al.* 1999). Since the food matrix is very complex, predictive models usually consider only the main environmental factors that are the microbial growth barriers; but usually some other factors that are not considered crucial, have synergistic or antagonist effects, and have an important role in the final outcome of microbial growth. For that reason, mathematical models for the prediction of microbial growth in food systems have also been developed (Devlieghere *et al.* 1999; Skandamis and Nychas 2001; Skandamis *et al.* 2002).

The new developments in food preservation have as common objective, minimal food processing, in order to present to the consumer, a food product that most closely resembles the natural one. New preservation techniques such as light pulses, ultrasound, pulsed electric fields and high pressure processing among others, are methods not based on high temperature to reduce the number of microorganisms in food (Butz and Tauscher 2002). The modification of other environmental food factors such as pH, water activity, modified atmospheres or additives, are also used alone or in combination with the abovementioned processing methods (Gould 1996). A common characteristic of all those preservation techniques is that they are considered of moderate antimicrobial action, so that the use of one of the methods alone does not warranty the extension in shelf life of the food product. The concept was proposed by Leistner (Leistner and Gorris 1995; Leistner 2000), as hurdle technology, and has been widely used in the development of new food products. Among the additives that are used in hurdle technologies, the spices or their essential oils are present.

Nowadays, it is common to find in scientific literature, reports that describe the combination of moderate antimicrobials and alternative methods, for the control of foodborne pathogenic bacteria and fungi, as well as the control of spoilage microorganisms for extension of shelf-life. The reports can deal with the effect on the microbial growth of a specific pathogen or microorganism, and few also propose a model that describes the effect of the combined factors on the control of microorganisms in food systems. One of those reports is that of Pol and Smid (1999), describing that the combination of oregano's essential oil and nisin produced a synergistic effect when tested under laboratory con-

ditions against *L. monocytogenes* and *B. cereus*. At low nisin concentrations, the combination with oregano essential oil increased the bactericidal effect of the antibiotic, decreasing 3-log UFC/mL of the pathogens more when both antimicrobials were present than when the nisin was added alone. The effect of oregano has also been tested against *Clostridium botulinum* growth and toxin production. The antitoxigenic activity of the oregano essential oil was high under laboratory conditions, but when tested in a food model (pork meat), the concentration of oregano needed to achieve the antimicrobial effect was higher than the needed in laboratory tests, which made its use impractical. The authors also reported a synergistic effect of oregano essential oil with sodium nitrate (Ismael and Pierson 1990).

The use of spices or essential oils alone, does not guarantee the control of pathogens in food. The combination of spices and organic acids was necessary to control the growth of *C. perfringens* in cooked meat during cooling and storage (Sabah *et al.* 2004). The same research group also demonstrated that carvacrol, cinnamaldehyde, thymol and oregano essential oil, were tested on the control of *C. perfringens* growth during cooked ground turkey chilling process. Cinnamaldehyde was the most effective antimicrobial at low concentrations (Juneja and Friedman 2007). On the other hand, the combination of organic acids and cinnamon has also been reported to control growth of *E. coli* O157:H7 in apple juice (Ceylan *et al.* 2004).

In another study done in meat carcasses, the meat was sprayed with a commercial solution of herbal extracts, and found a reduction in the number of pathogenic bacteria recovered from the surface (Cutter 2000). The effect of the essential oil of oregano against *E. coli* O157:H7 was also tested using as a food model eggplant salad, showing a significant reduction in the number of bacterial cells (Skandamis and Nychas 2000). The same research group determined that the combination of controlled atmospheres or vacuum pack and oregano essential oil, controlled the growth of *Salmonella* Typhimorium in meat pieces (Skandamis *et al.* 2002).

Another developing area in food preservation is the use of biodegradable and/or edible films, used for food packing. Films based on biodegradable compounds such as starch or chitosan, have limited or non-antimicrobial properties; therefore, the addition of essential oils can increase the antimicrobial capacity of the films, and improve the quality of the films for food preservation. Zivanovic *et al.* (2005) reported the addition of essential oils of anise, basil, coriander and oregano into chitosan films, and demonstrated their efficiency on the control of *E. coli* O157:H7 and *L. monocytogenes*, being oregano essential oil the one that better controlled microbial growth. Edible films based on apple puree were added with cinnamon, lemongrass and oregano, and their antimicrobial properties were tested. The films added with antimicrobials were effective in the control of *E. coli* O157:H7, increased oxygen permeability and decreased water vapor permeability (Rojas-Graü *et al*. 2006). In another report, alginate-based films were added with essential oils of oregano (*Corydothymus capitatus*), cinnamon (*Cinnamomum cassia*), and savory (*Satureja montana*), and treated with $CaCl_2$ as well. The films were tested for effective control of *E. coli* O157:H7 and *S.* Typhimorium added to beef fillets, showing an antimicrobial activity during the five days of treatment. The authors placed special attention to the rate of active essential oil compounds released from the films (Oussalah *et al.* 2006b). Lopez *et al.* (2007) evaluated the antimicrobial effect of the vapors released by polypropylene (PP) and polyethylene/ethylene vinyl alcohol copolymer (PE/EVOH) flexible films added with different essential oils, and found that a large proportion of cinnamon and oregano oils were needed to inhibit the growth of fungi (4% w/w), Gram positive bacteria (8-10%) and up to 12% was needed for Gram negative bacteria, but even at that high concentration, *P. aeruginosa* was not inhibited.

Most of the spice essential oils are considered as GRAS (Generally Recognized as Safe), but their use as food additives is limited to their efficiency, since they are mild antimicrobials and large quantities are require to achieve microbicidal effects. It is important to obtain the MIC (Minimal inhibitory concentrations) and MBC (Minimal bactericidal concentration) values, since those concentrations can lead to the determination of optimal use of spices. Furthermore, the complex environment produced by the interaction of the physicochemical properties of a food product, can modify the antimicrobial efficiency of added spices. Therefore, the optimized use of spices can balance the efficiency as food preservative, and the sensorial acceptability of those food products added with spices (Fisher and Phillips 2006).

CONCLUSION

The addition of spices to food has been a common practice in many societies, and the commerce associated with spices has been related to historical events, such as the discovery of America. With today technological advances in food preservation, but especially with the interest on the conservation of natural nutrients in foods, the use of spices as mild antimicrobials has received special attention. Despite the abundant and important research done on the use of natural antimicrobials under laboratory conditions and in food systems, there are still many questions to be addressed. There is a need for standardized methods for determination of antimicrobial effect of spices, so that results can be easily compared. Also, there are many questions to be answered on the production of essential oils by plants, its regulation and the effect of environmental factors on the production of the biological active compounds. The search for new plant antimicrobials or the identification of new active compounds in the already studied essential oils is also a task to be attended. Finally, when considering the addition of spices to foods, the interaction with the other food components is important to determine the best conditions for their efficient use. The study of spices has still many areas for research, to finally apply the results obtained in the preparation of innocuous food.

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