LWT - Food Science and Technology xxx (2014) 1–5

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Antioxidant and antimicrobial active paper based on Zataria (*Zataria multiflora*) and two cumin cultivars (*Cuminum cyminum*)

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ARTICLE INFO

Article history:
Received 30 December 2013
Received in revised form
26 March 2014
Accepted 20 September 2014
Available online xxx

Keywords:
Active packaging
Antioxidant
Antimicrobial
Zataria multiflora
Cuminum cyminum

ABSTRACT

The antioxidant and antimicrobial properties of *Zataria multiflora* and Iranian and European Cumin were evaluated, first as pure extracts and later as active agents incorporated in paper, manufactured at laboratory scale as an active packaging material. Two different procedures were used for quantifying the antioxidant properties and microbiological studies versus *Staphylococcus aureus*, *Listeria innocua, Pseudomonas* sp., *Salmonella enterica* subsp. *enterica and Escherichia coli* were carried out. All bacteria were inhibited when exposed to the atmosphere generated from 4% to 6% (w/w) of Zataria essential oil in the active coating. Zataria showed the best antioxidant properties. The compounds responsible for the antimicrobial and antioxidant properties are analyzed and discussed.

1. Introduction

In recent years, the major driving forces for innovation in food packaging technology have been the increase in consumer demand for minimally processed foods, the change in retail and distribution practices associated to globalization, new consumer product logistics, new distribution trends (such as Internet shopping), automatic handling systems at distribution centers, and stricter requirements regarding consumer health and safety. Active Packaging (AP) technologies are being developed as a result of these driving forces. Active Packaging is an innovative concept that can be regarded as a mode of packaging in which the package contains compounds specifically added to protect the product against deterioration processes, to extend the shelf life or to enhance safety and/or sensory properties, while maintaining the quality of the product (Rodriguez-Lafuente, Batlle, & Nerin, 2007). This concept is very attractive, especially for fresh food, where the addition of protective agents is not allowed for being considered as “fresh food”. Antimicrobial and antioxidant actions are the main target properties searched by active packaging. Active packaging approaches have been studied *in vitro* and *in vivo* by many researchers, although there are many more papers dealing with *in vitro* test than with real foodstuffs.

The antioxidant packaging may be useful in retarding or inhibiting oxidative deterioration of foodstuffs, especially those with high lipid content. The natural antimicrobial agents inhibit or delay the growth of microorganisms, resulting in the prevention of various diseases (Abdoul-Latif et al., 2010). Several plastics (Lopez, Sanchez, Batlle, & Nerin, 2007) as well as paper (Rodriguez-Lafuente et al., 2007), board and biopolymers have been studied and proposed as active packaging materials and a lot of them contain essential oils (EOs) as active agents (Lopez-de-Dicastillo et al., 2011; Lopez-de-Dicastillo et al., 2012; Nerin, 2012; Rodriguez-Lafuente, Nerin, & Batlle, 2010), either as antioxidants or as antimicrobials. This is not surprising, because EOs are rich sources of terpenes and phenols, with strong antioxidant properties and some of them also show antimicrobial properties. Another interesting feature is that these natural compounds do not have any significant toxicity or environmental impact so they constitute efficient alternatives to conventional antimicrobial agents (Gutierrez, Sanchez, Batlle, & Nerin, 2009). The antimicrobial resistances are also much lower than those found for common synthetic antibiotics (Becerril, Gomez-Lus, & Nerin, 2011; Becerril, Nerin, & Gomez-Lus, 2012; Rodriguez-Lafuente, Nerin, & Batlle, 2008), which constitutes an additional advantage. Most of these EOs are traditionally used in food preparation and are included in

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http://dx.doi.org/10.1016/j.lwt.2014.09.051
0023-6438/

the food additives list, either as flavoring, spices, antioxidants and or antimicrobials. Although this old knowledge about essential oils, their composition and the relationship between the individual components and their properties is unknown in most of the cases.

EOs composition vary with climate, geographic location, collection of plants, drying system and extraction procedure. For this reason, their properties also change with these parameters. Mediterranean countries have a strong tradition of using essential oils and aromatic plants to preserve food, but the specific properties and the real strength as antioxidants and/or antimicrobials are not well known. The main purpose of this research work was to study in depth the antioxidant and antimicrobial behavior of cumin and Zataria EOs, commonly used in Mediterranean countries, as well as the performance of an active paper containing them.

Zataria multiflora is a thyme-like plant that grows wild in central and southern Iran. It is a member of the *Latiatae* family to which mint, rosemary and several other useful medicinal plants also belong. In Iran, *Zataria multiflora* is used in traditional folk remedies for its antiseptic, analgesic (pain-relieving) and carminative (antiflatulence and intestine-soothing) properties. However, its composition has not been published yet.

Cumin (*Cuminum cyminum*) is one of the most commonly used spice condiment in Asia. Cumin seeds are used as popular aromatic herbs and culinary spices. All the cumin varieties are used in traditional and veterinary medicine as stimulant, carminative, astringent and as a remedy against indigestion, flatulence and diarrhea (Amin, Kalantar, Saeid, & Ahsan, 2010) All these properties make cumin a good candidate for being used as protective agent in food packaging, mainly to protect those foodstuffs that cannot be spiked or produced with additives, such as fresh products.

The present study deals with the chemical composition, antibacterial and antioxidant behavior of *Cuminum cyminum*, *Zataria multiflora* and *European cum*in obtained by steam distillation. Additional tests have been done after incorporating these EOs in active paper packaging. The results obtained are shown and discussed.

2. Materials and methods

2.1. Apparatus

A Clevenger apparatus was used for obtaining the essential oils from the plants. A Varian CP-3400 Saturn 2000 gas chromatograph with a mass spectrometer as detector (GC/MS), a microbial culture media (Scharlau), a UV–VIS Spectrophotometer and an Alliance 2795 Separations Module high performance liquid chromatography (HPLC) with UV detector were used. Active materials were prepared at laboratory scale by coating with a K101 Control Coater RK Print-Coat Instruments Ltd. Chemicals.

2.2. Plant materials

The plants (*Cuminum cyminum* and *Zataria multiflora*) were collected from Kerman and Fars provinces of Iran, respectively. *European cumin* EO was supplied by the company Argolide (Barcelona, Spain).

2.3. Essential oil extraction

The aerial parts of plants (*Cuminum cyminum* and *Zataria multiflora*) were dried in an oven equipped with hot air circulation. They were then ground. The essential oil was obtained by steam distillation of ground material with boiling water up to 2 h utilizing a clevenger-type system. The extracted oils were dried over anhydrous sodium sulfate followed by filtering and they were stored at 4 °C in sealed glass vials for further use.

2.4. Essential oil analysis

The GC/MS analyses were executed on a Varian CP-3400 gas chromatograph equipped with a column BPX5 30 m × 0.25 mm × 0.25 um, SGE (Scientific Instrument Service, NJ, USA) coupled to Saturn 2000 mass spectrometer. The column temperature was programmed at 50 °C as an initial temperature, holding for 2 min, with 2.5 °C increases per minute to 265 °C. Injection port temperature was 250 °C and helium was used as carrier gas at a flow rate of 1 mL/min. Ionization voltage of mass spectrometer in the electron impact mode was equal to 70 eV and ionization trap temperature was 170 °C. The mass spectrometer was scanned from m/z 40 to 250. The individual compounds were identified and confirmed thereafter using Kovats retention indices. Pure standards of the compounds were also used to confirm the identification of the compounds.

2.5. Active paper manufacture

The active paper was manufactured as follows: a US Food and Drug Administration (FDA) quality emulsion of paraffin formula supplied by Repsol (Madrid, Spain) was homogeneously mixed with the appropriate amount of the selected EO. This active coating was then applied to 70 g/m^2 paper provided by Antalis (Zaragoza, Spain) using the coating machine above mentioned. Once coated, the properties of this active paper were evaluated.

2.6. Microbial cultures

The antimicrobial activity of three essential oils were studied using the following bacteria: two gram positive bacteria, namely: *Staphylococcus aureus* (American Type Culture Collection, ATCC 29213), *Listeria innocua* (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,* DSM 20643),three gram negative bacteria, namely *Pseudomonas* sp. (Colección Española de Cultivos Tipo, CECT 4335), *Salmonella enterica* subsp. *enterica* (Colección Española de Cultivos Tipo, CECT 556), *Escherichia coli* (American Type Culture Collection, ATCC 25922).

2.7. Antimicrobial testing

2.7.1. Antimicrobial activity tests

2.7.1.1. Solid diffusion assays. A plastic Petri dish (90 mm diameter) containing the appropriate solidified medium was inoculated with 100 μL of a physiological saline solution containing 10^4 colony-forming units (CFU)/mL of the microorganism under study. Five microliters of the undiluted EO were added to a 5 mm diameter sterile blank filter disk, placed on top of the cultured media. After incubation under optimal conditions (temperature and time), the diameter of inhibition zones was measured. All analyses were carried out in triplicate.

2.7.1.2. Vapor diffusion assays. In each test of the antimicrobial activity of the active packaging materials, a Petri dish with the appropriate solidified agar culture medium was inoculated with 100 uL of a 10^4 colony forming unit (CFU)/mL solution of the microorganism under study. Then, the active paper was placed over the Petri dish and non-hermetically tied using a plastic strip. Controls with paper coated by the paraffin formulation but without active ingredients were also prepared for each set of samples. The inhibition of each microorganism under test was calculated by the rate between the number of viable colonies in the Petri dish

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covered by the active paper divided by the number of colonies in the control dish. All tests were performed in triplicate.

2.8. Antioxidant activity

2.8.1. ORAC assay

The Determination of the antioxidant capacity of essential oil was performed according to the ORAC assay previously adapted (Bentayeb, Vera, Rubio, & Nerin, 2009).

The ORAC reaction was undergone in the thermostated autosampler of a chromatographic system Alliance 2795 Separations Module (Waters, Milford, MA, USA) at 32 °C. Reagent solutions were preincubated for 15 min in the autosampler before mixing them to carry out the reaction. First, 800 μL of fluorescein solution were mixed with 100 μL of diluted extract of the sample. After that, 600 μL of the AAPH solution were added to make the reaction starts. Then, 20 μL of the reaction mixture were injected every minute and carried, by a 0.5 mL/min water flow, to the online detector 474 Scanning Fluorescence Detector (Waters, Milford, MA, USA). Excitation and emission wavelengths were set up at 540 and 565 nm, respectively. A total of 50 injections were made for each assay, describing the fluorescence decay. The area under the curve (AUC) was calculated as:

$$\text{AUC (area under curve)} = \left(\frac{f_1}{f_0} + \frac{f_2}{f_0}\cdots + \frac{f_i}{f_0} + \cdots\right) \times \Delta t$$

where f_0 is the area of the first peak observed; f_i is the area of the peak i; and Δt is the time interval between consecutive peaks. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. In order to obtain the calibration curve, Trolox solutions of a concentration 0, 50, 100, 150, 200 and 250 μg/g were analyzed by the same procedure as the samples. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net AUC. The results were expressed as Trolox equivalents as microgram per square meter of active paper. Finally, five consecutive injections of the fluorescein solution were performed at the beginning of the day, in order to check the stability of the fluidic system. The system was considered suitable when the relative standard deviation of the five areas obtained was lower than 2%.

2.8.2. DPPH assay

The DPPH assay was performed from the adaptation of Brand-Williams, Cuvelier, and Berset (1995). For each antioxidant, different concentrations were tested. Antioxidant solution in methanol (0.1 mL) was added to 3.7 mL of a 30 μg/g methanol DPPH solution. The decrease in absorbance was determined at 515 nm for 120 min. For each antioxidant concentration tested, the reaction kinetics was plotted. From these graphs, the percentage of DPPH remaining at the steady state was determined and the values transferred onto another graph showing the percentage of residual DPPH at the steady state as a function of the molar ratio of antioxidant to DPPH. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% expressed as percentage of essential oil in the solution. In this way, the lower the EC50 value, the more antioxidant capacity the extract shows.

2.8.3. Antioxidant activity in paper packaging

The procedure to evaluate the antioxidant capacity of packaging materials, developed by Pezo, Salafranca, and Nerin (2006, 2007 and 2008) is described in the articles (Pezo et al., 2006, 2007, 2008) This procedure is based on the hydroxylation of sodium salicylate by •OH radicals generated by hydrogen peroxide photoreaction. The generated atmosphere, rich in free radicals, passes through the active material contained in a plastic bag. Those materials with radical scavenger properties trap the free radicals while those without antioxidant properties leave the radicals arrive to the final impinger, where a salicylic acid solution is placed. The final reaction products in the impinger are 2,3 dihydroxybenzoic (2,3-DHB) and 2,5 dihydroxybenzoic (2,5-DHB) acids. The quantitative values of both DHB derivatives are related to the number of free radicals which cross the system and arrive at the impinger. When the concentration of 2,5-DHB increases the material is not acting as antioxidant, as the free radicals are not scavenged. This way, the antioxidant capacity can be measured in an objective and quantitative manner. The values of the hydroxylation are shown in percentages, which is the comparison of active packaging to the target (100% hydroxylation means that there are not antioxidant properties involved). The antioxidant capacity of the active papers in the time trials of 24 and 48 h were measured.

2.9. Statistical analysis

All tests were done by triplicate and data represent the average values. The intervals, with a 95% confidence level, are given in the Tables. Calibration plot for chemical analysis was adjusted by least squares.

3. Results and discussion

3.1. Chemical composition of the essential oils

The results obtained by GC–MS analyses of *C. cyminum*, *Z. multiflora* and European cumin are shown in Table 1. As can be seen there are many differences between the European and Iranian cumins. Both have cumic aldehyde as major component, but the European cumin contains β-pinene and much higher concentration of terpinene and carvacrol, while the Iranian cumin is very concentrated in cumic aldehyde. The ratio between the likely active compounds, such as carvacrol, thymol and *p*-cymene and the cumic aldehyde is more favorable for the European than for the Iranian one. This composition will affect the antimicrobial and antioxidant properties.

The differences found could be attributed to the variety of the plant, but also to the manufacture of the plant to obtain the final extract. The European extract was commercially available, whereas the Iranian one was homemade at laboratory scale.

Table 1
Composition of EOs under study expressed as ug/g.

Compound	Iranian cumin	European cumin	Zataria
n-butyl ether	1.721	–	–
p-cymene	30.991	44.324	–
terpinene	5.949	52.956	205.844
terpinolene	1.140	0.574	11.936
linalool	3.026	–	–
iso-borneol	1.227	–	–
tepinen-4-ol	1.488	–	–
α-terpineol	2.142	–	5.666
methyl thymol ether	1.737	–	14.159
cumic aldehyde	468.800	81.194	–
thymol	49.120	52.227	113.535
carvacrol	17.030	35.841	311.615
caryophyllene	3.061	–	26.801
caryophyllene oxide	1.915	–	22.728
a-pinene	–	–	2.431
b-pinene	–	38.981	24.314
camphene	–	–	1.121

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Table 2
Antimicrobial properties of EOs under study, expressed as diameter of inhibition halo (in cm, total Petri dish in the culture 9 cm) versus several strains.

	Salmonella enterica	*Escherichia coli*	*Pseudomonas* sp.	*Staphylococcus aureus*	*Listeria innocula*
Z multiflora	6.8 ± 1.1	6.4 ± 1.6	3.2 ± 0.2	6 0.9 ± 1.1	5.8 ± 1.3
C cyminum (Iranian)	0	2.7 ± 0.6	2.9 ± 0.06	1.2 ± 1	0
C cyminum (European)	0	2.2 ± 0.6	2.2 ± 0.3	0	0

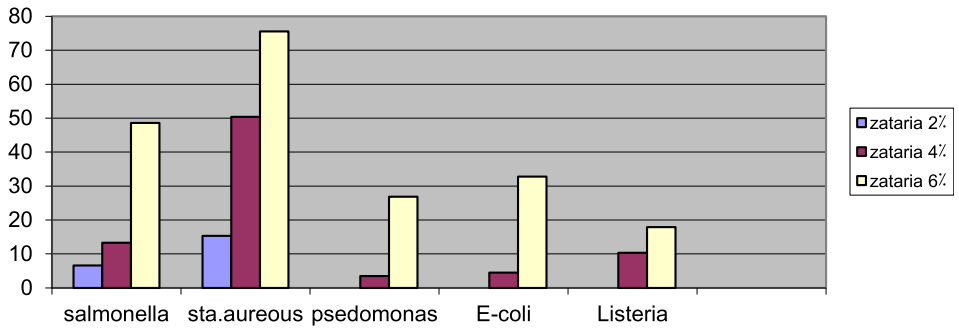

Fig. 1. Antimicrobial performance of active paper containing different concentrations of Zataria.

Zataria composition could be compared to that of oregano, which is essentially made up of carvacrol and thymol, which together range to more than 57% of the total chromatographic area. However, the major compound in Zataria is terpinene, also present at quite high concentration in European cumin. From these results a mixed behavior between European cumin and oregano could be expected for Zataria.

3.2. Antimicrobial activity

The results of preliminary tests on antimicrobial activities against various Gram-positive and Gram-negative food-borne bacteria using solid diffusion assays are shown in Table 2. As can be seen in Table 2 and Fig. 1, the best antimicrobial performance was obtained for *Zataria multiflora*. Both cumins showed little antimicrobial behavior versus *Pseudomonas* and *E. coli* but not enough to get the total inhibition and in any case lower than that of Zataria. The main components of Zataria are carvacrol, terpinene, and thymol and the three compounds have been found to be good antimicrobial agents in previous work (Lopez, Sanchez, Batlle, & Nerin, 2005; Lopez et al., 2007) It is interesting to point out that Zataria partially inhibited *Listeria*, while the cumins did not. Again, this behavior is based on the concentration of carvacrol and thymol, which are the main responsible for the antimicrobial performance.

The three EOs showed activity against both Gram-positive as well as Gram-negative bacteria. *S. aureus*, *S. enterica* and *E. coli* were the most sensitive food-borne bacteria to Zataria, while *Cuminum cyminum* from IRAN showed better activity compared to *the E. cumin*. A separate study on antimicrobial effect of Zataria conducted by Fazeli et al. (2007) reports also higher sensitivity of *S. aureus*, *Salmonella typhi* and *E. coli* to Zataria (Fazeli et al., 2007). Amin et al. (2010) reported antibacterial activity of essential oil of *Zataria multiflora* and it was assessed by agar disc diffusion (Amin et al., 2010). Minimal inhibitory concentration (MIC) was studied and they showed that essential oil of *Zataria multiflora* was effective on pathogenic bacteria, particularly *S. aureus* and *E. coli*. Sagdıc et al. (Sagdic, Karahan, Ozcan, & Ozkan, 2003) studied antibacterial properties of Turkish spice hydrosols and demonstrated that *Cuminu cyminum* had no activity against *S. aureus*, *Salmonella enteritidis* and *E. coli*.

The antimicrobial activity of the manufactured active paper against Gram-positive and Gram-negative bacteria with nominal concentration 2%, 4%, 6% (w/w) of the active agent incorporated into the coating was evaluated ($n = 3$). All bacteria were inhibited when exposed to the atmosphere generated from 4% to 6% (w/w) of Zataria essential oil in the active coating. However, when using 2% (w/w) of the active components, only *Staphylococcus aureus* and *Salmonella enterica* were inhibited. Rodríguez et al. (2007) used cinnamon essential oil as antimicrobial agent at concentrations from 1 to 8% (w/w) in paper packaging and no inhibition of the tested Gram-positive bacteria (*Bacillus cereus*, *Listeria monocytogenes*, *Enterococcus faecalis*, and *Staphylococcus aureus*) was observed. The only active coating that showed inhibitory activity against Gram-negative bacteria was the enriched cinnamon EO (which significantly inhibited the highly relevant organisms *Salmonella cholerasuis*, *Escherichia coli*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*). The minimum amount that demonstrated inhibitory activity against Gram-negative bacteria was 4% (w/w) EO (Rodriguez-Lafuente et al., 2007).

3.3. Antioxidant properties

The values of antioxidant capacity of the extracts are listed in Table 3. As can be seen, Zataria is the most antioxidant species

Table 3
Antioxidant performance of the EOs under study by applying ORAC and DPPH methods.

Spice	Zataria	Cumin	European cumin
ORAC Assay (mg Trolox/g spicy extract)	1.35 ± 0.13	0.72 ± 0.13	0.40 ± 0.07
DPPH Assay (EC50)	0.23% (w/w)	–	–

Table 4
Hydroxylation rate after 24 and 48 h, % RSD, $n = 3$.

% Hidroxylation	24 h	48 h	%RSD 24 h	%RSD 48 h
Blanks BK	100.0%	100.0%	10.8%	15.7%
Paper coated with active paraffin containing tym 2% T1	22.5%	37.4%	22.6%	24.3%
Paper coated with active paraffin containing tym 4% T2	19.5%	38.2%	40.2%	27.9%

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according to the ORAC assay, followed by Iranian cumin and European cumin. The values obtained are lower than those measured by Bentayeb et al. (2009) for the essential oils of oregano or cinnamon (2–2.5 mg Trolox/g EO), but Zataria resulted as antioxidant as rosemary EO (1.5 mg Trolox/g essential oil), which is a widespread used antioxidant essential oil.

When measuring the antioxidant capacity of the extracts by the DPPH method, only Zataria extract showed DPPH response significantly different from that of the blank (Table 3).

The antioxidant capacity of active paper with different characteristics have has been determined following the method of the Pezo et al. (2006, 2007 and 2008). The values obtained from the active paper were compared to the blank paper, i.e. coated paper without active agent. The antioxidant capacity can be seen in Table 4. It can be seen that the active paper containing 4% (w/w) of thyme was the most antioxidant. It should be pointed out that both the antimicrobial and antioxidant behavior of the active paper was demonstrated in vapor phase, without direct contact between the paper and the product, what represents a great advantage for further use in the food packaging frame.

4. Conclusions

Iranian Zataria and cumin and European cumin have been studied as antioxidant and/or antimicrobial agents in active paper for food packaging. Among them the most efficient as both antioxidant and antimicrobial is Zataria. Cumin showed lower antioxidant properties and not enough antimicrobial performance to inhibit *in vitro* the common pathogens.

Acknowledgments

Thanks are given to Gobierno de Aragón and Fondo Social Europeo for financing the GUIA group T-10.

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