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Antioxidant And Antibacterial Activity Against *Staphylococcus Aureus* Of Essential Oil From Fennel Seeds (*Foeniculum Vulgar* Mill.) Application In The Preservation Of Minced Meat

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Abstract

Aromatic plants have traditionally been used to season foods and extend their shelf life. Most of their properties are due to the essential oils produced by their secondary metabolism. The aim of this work is to make a contribution to demonstrating the antioxidant and antimicrobial activity of the essential oil of fennel seeds.Initially, this study is focused on extracting the essential oil from these seeds using the hydrodistillation process and highlighting the antioxidant activity of this oil using the β- carotene bleaching method. In a second phase, the work focused on evaluating the oil's antibacterial activity against a pathogenic bacterium, Staphylococcus aureus, the most frequently incriminated in food poisoning, using the microatmosphere method.The oil in our study showed modest antibacterial activity against the S. aureus strain tested, which was found to be susceptible. The minimum inhibitory concentration (MIC) of the essential oil determined by the solid dilution method was 31.25 µl/ml ethanol.These results suggest a favorable outcome in the field of food preservation. This is why these oils have been used to preserve minced meat at refrigeration temperature.

Keywords*: essential oils, Foeniculum vulgare Mill. antioxidant activity, antibacterial activity, minced meat.*

INTRODUCTION

Meat products are highly vulnerable to lipid oxidation and microbial spoilage. The hygienic quality of meat depends, on the one hand, on microbial contamination and, on the other hand, on lipid oxidation. These two factors are generally due to, and above all encouraged by, poor handling of meat products during chilling, storage, distribution and preparation **(Lebret and Picard, 2015). Indeed,** the slaughterhouse is one of the major critical points for meat hygiene **(Salifou *et al.*, 2010)**. It is considered to be the stage where the greatest opportunities for contamination (80 to 90%) of meat microflora reaching consumers occur **(Jouve, 1990).**

Refrigerated storage is generally the most common method of preserving fresh meat and meat products. To prolong the duration of refrigerated storage, antimicrobial and antioxidant additives, particularly of synthetic origin, are added to the meat. However, consumers are increasingly demanding the use of natural products as alternative preservatives in food products, as the safety of synthetic additives has been questioned in recent years **(Solomakos *et al.* 2008, Khaleque *et al.* 2016, Engel et *al* 2023).**

As a result, many phytochemicals, including essential oils, are beginning to attract a great deal of interest as potential sources of natural bioactive molecules. They are being studied for their possible use as antioxidant, antimicrobial, anti-inflammatory and anticancer agents. Essential oils are substances that occupy a special place through their use in medicine, aromatherapy and agri-food **(Robert *et al.* 2005; Rota *et al.* 2008; Sarkic and Stappen, 2018).**

The aim of our work is to evaluate the antioxidant activity of the essential oil extracted by hydrodistillation from fennel seeds (*Foeniculum vulgare* Mill) and to study the antibacterial effect of this oil '*in vitro'* and also '*in vivo'* by incorporating it into a food matrix of animal origin (minced beef).

1. Materials and methods

1.1. Presentation of biological material

1.1.1. Fennel seeds

The fennel seeds were purchased from a herbalist in June 2021. The seeds came from a private farm in the Ain Ouelman region, Setif (Algeria). The seeds are cylindrical in shape, brown in colour and vary in length from 5 to 8 mm. The seeds were cleaned and placed in clean bags for later use.

1.1.2. Microorganism used

The bacterium chosen was a strain frequently found in human pathology, *Staphylococcus aureus,* which is a food-borne species responsible for food poisoning, thus constituting a public health problem.

This strain was kindly provided by the microbiology laboratory of the Lakhroub Veterinary Institute (Constantine, Algeria) in agar-preserved form.

1.1.3. Minced meat

The beef purchased at the market was obtained from animal muscles 4 days *post- mortem,* stripped of their peripheral layers, made up of connective tissue and fat. The prepared meat was then placed in a mincer to be processed into minced meat. The minced meat was wrapped in sterile paper and transported to the microbiology laboratory in a refrigerated chamber (6±1ºC) for the next 30 minutes.

1.2. Extraction of essential oil from fennel seeds

Extraction was carried out using a Clevenger-type hydrodistiller using intact fennel seeds after grinding. The extracted essential oils were dehydrated with sodium sulphate (Na_2SO_4) and stored at around 4±1ºC in hermetically sealed opaque glass bottles, protected from light and oxygen. The yield of essential oils determined is the ratio between the weight of the extracted oil and the weight of the plant material used **(Akroute, 2001).** This is expressed as a percentage.

1.3. Evaluation of the antioxidant activity of the essential oil

The antioxidant activity of fennel seed essential oil was carried out using the β- carotene decolourisation method described by **Kulisic *et al.* (2004)**.

Ten milligrams of β-carotene were dissolved in 100 ml of acetone. One millilitre (1 ml) of this solution was introduced into a flask containing 20 mg linoleic acid and 100 mg Tween 40. The acetone was removed by rotavapour at 50°C for 5 min and, to the residue, 50 ml of hydrogen peroxide was slowly added to form the emulsion (A).

Two hundred microlitres (200 µl) of fennel seed essential oil, at a concentration of 4 mg/ml ethanol, was mixed with 5 ml of emulsion (A). A negative control, without essential oil, consisting of 200 µl of ethanol and 5 ml of emulsion (A), was prepared. A second emulsion (B) containing 20 mg linoleic acid, 100 mg Tween 40 and 50 ml hydrogen peroxide was also prepared.
A solution consisting of 200 µl of ethanol and 5 ml of emulsion (B) was prepared and used to calibrate the spectrophotometer. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored by a spectrophotometer by measuring the absorbance at 470 nm after 120 min.
Vitamin C was used as a positive control.
The kinetics of emulsion decolorization in the presence and absence of antioxidant (negative control in which the sample is replaced by 200 µl of ethanol) is monitored at 470 nm and at regular time intervals (every 20 min) for 120 min.
Antioxidant activity was expressed as a percentage and calculated using the following equation described by **Amin *et al.* (2004):**

$$AA\ (\%) = [1-[(A_{E0} - A_{ET}) / (A_{C0} - A_{CT})] \times 100$$

AA (%): Antioxidant activity
AE0 : absorbance value of the emulsion in the presence of the essential oil or vitamin C measured at t=0.
A :C0 value of the absorbance of the negative control emulsion measured at t=0.
AET : absorbance value of the emulsion in the presence of the essential oil or vitamin C measured at t=120 min
ACT : absorbance value of the negative control emulsion measured at t=120 min.

1.4. *In vitro* antibacterial activity test

2.4.1. Preparing the inoculum

Antibacterial tests must be carried out on young cultures (18 to 24 h) in the exponential growth phase. For this study, the selected *S. aureus* strain was revitalised by inoculation into a liquid medium, BHIB. After incubation for 24 h at 37ºC, a second subculture was carried out on nutrient agar in Petri dishes, which were incubated for 18 h at 37º

2.4.2. Preparation of the bacterial suspension

From the young cultures obtained, 3 to 5 well-isolated colonies were suspended in 5 ml of sterile physiological water, followed by vortexing for a few seconds. The suspension was standardised to 10^6 CFU/ml using a spectrophotometer set to a wavelength of 620 nm. It is assumed that an optical density of between 0.08 and 0.1 measured at 625 nm is equivalent to a concentration of 10^8 CFU/ml **(Mohammedi, 2006).** The inoculum suspension is diluted 1:100 in sterile physiological water to obtain a concentration of 10^6 CFU/ml.

2.4.3. Testing the sensitivity of the *S. aureus* strain to fennel seed essential oil

Three (03) Petri dishes were half filled with Mueller Hinton liquid agar and left to solidify. The bacterial suspension of *S. aureus* containing 10^6 CFU/ml was inoculated on the surface using sterile swabs.
The technique involves placing sterile 6 mm antibiogram discs soaked in a specific quantity (5 µl) of the essential oil in the middle of Petri dishes in direct contact with the seeded agar. After incubation (37°C for 24 h), the absence of bacterial growth is shown by a translucent zone on the agar with a more or less clear outline, tending to be circular when the disc is well centred. The results are measured with a caliper and expressed as a diameter in mm **(Singh *et al.* 2006).**
According to **Ponce *et al* (2003),** sensitivity to oil was classified by the diameter of the inhibition halos: not sensitive (-) for diameters less than 8 mm; sensitive (+) for diameters from 8 to 14 mm; very sensitive (++) for diameters from 15 to 19 mm and extremely sensitive (+++) for diameters greater than 20 mm.

2.4.4. Determination of the minimum inhibitory concentration (MIC)

The MIC is determined for oils with antibacterial activity on bacterial strains that are more or less sensitive **(Billerbeck, 2003)**. The results showed that the essential oil of fennel seeds was active against *S. aureus.*
Although essential oils are considered to be safe additives **(Lambert *et al.*, 2001),** their use is often limited by the organoleptic criteria of the food. For this reason, it is necessary to determine the MIC (i.e. the lowest

concentration of oil capable of inhibiting all bacterial growth without affecting the sensory quality of the food) **(Caillet and Lacroix, 2007).** The difficulty in using essential oils in water-based culture media is their low solubility. Several substances have been used for this purpose **(Beuchat, 1976; Marino *et al.*, 2001)**. In our case, we used ethanol.
The different concentrations of essential oil prepared in alcohol were incorporated into the supercooled culture medium (45°C) in the following proportions: 1 ml of diluted essential oil and 3 ml of supercooled Mueller Hinton agar, then the tubes were vortexed before pouring into Petri dishes. After solidification, the agar plates were surface inoculated as described above, using the same bacterial load (10^6 CFU/ml). The plates were incubated at 37°C for 24 hours.

1.5. Testing antibacterial activity *in vivo*

1.5.1. Optimising MIC applied to minced meat

It has been well established that the effective doses of essential oils approved in "*in vitro*" antibacterial tests may not be the same when applied to a food matrix. **Burt (2004)** has suggested that MICs should be multiplied by a coefficient that can vary between 2 and 100, if they are to be applied in a food to obtain satisfactory results.

Given that the essential oil of fennel seeds has a bitter taste and in order not to alter the organoleptic quality of the minced meat, we considered it useful to multiply the MIC value by a fairly low coefficient equal to 2.

1.5.2. Preparation of samples

The minced meat was transported to the laboratory in an isothermal cooler and stored at 6±1°C until use.
100 grams (g) of minced meat were weighed into a sterile glass container and then divided into 2 samples of 50 g each. The first sample was mixed with essential oil (10 µl) to obtain a concentration equal to twice the MIC. The second sample represented the control sample, which was mixed with sterile distilled water (10 µl). Both meat samples were then inoculated with pathogenic *S. aureus* bacteria (10 µl). The whole of each sample was homogenised using a sterile homogeniser to distribute the oil and bacteria evenly throughout the meat **(Boubrit and Boussad, 2007).**
The essential oil studied was diluted in ethanol, a solvent that may have antibacterial activity. For this reason, we also added 10 µl of ethanol to the control sample in order to monitor the kinetics of microbial development in the presence and/or absence of the essential oil.
The three samples were then placed in sterile glass containers and stored at refrigeration temperature (6±1°C).

1.6. Microbiological analyses

In order to determine the effect of the essential oil applied to the minced meat inoculated with the bacteria tested, microbiological analyses were carried out to monitor the kinetics of bacterial growth in the presence and/or absence of the essential oil.
The bacterial load was estimated from samples taken on day one, day four and day seven (D1; D4; D7). To do this, 10 g of minced meat was taken from each sample, then introduced into 90 ml of sterile peptone water (0.1%) and ground using a grinder for 1 min. Decimal dilutions in physiological water are prepared from the stock solution. 1 ml of the stock solution and dilutions were inoculated into Petri dishes containing approximately 15 ml of Chapman agar, a selective culture medium for the bacteria under study.
The results are read after incubation at 37°C for 24 h by direct counting of colonies characteristic of the species. The count is expressed in log CFU/g as the average of two determinations.

2. Results and discussion

During our work, the plant part being extracted consisted of dried and crushed fennel seeds. The essential oils are recovered in small quantities, are pale yellow in colour and have a pungent, aromatic odour.
The following table summarises the average yields of essential oils extracted (mean ± standard deviation).

Table 1. Essential oil yield.

Essential oils	Whole seeds	Ground seeds
Yield (%)	0,82 ± 0,05	0,93 ± 0,08

It can be seen that the extraction yield using crushed seeds is higher than that using seeds in the dry state. This could be explained by the increased surface area in contact with the extraction solvent.
From the results quoted in the literature, it is clear that fennel seeds contain few essential oils. The results obtained in this work are inferior to those cited by **Garnéro (1996)**, whose yield varies from 2.5 to 6%, with an average of 3.5%.
This difference in yield is probably due to a loss of oil in the aqueous phase of the distillate and also in the inner wall of the glassware. The yield of essential oils depends on several factors: the plant parts used, the products and reagents used during extraction, the geographical origin and harvesting period of the plant, the degree and conditions of drying (temperature and duration), and the presence of parasites and weeds **(Bajpai *et al.*, 2008).** The rest of the work was continued with the essential oil extracted from crushed fennel seeds.

2.1. Assessment of antioxidant activity using the β-carotene bleaching method

Assessment of the antioxidant activity of fennel seed oil is based on the decolorization property of β-carotene. The absorbance values of the different emulsions are shown in Fig. 1.
Fig. 1 shows that the absorbance values of the negative control emulsions and those of the vitamin C decreased with incubation time. However, the emulsion containing the essential oil of fennel seeds did not show a significant decrease in absorbance.

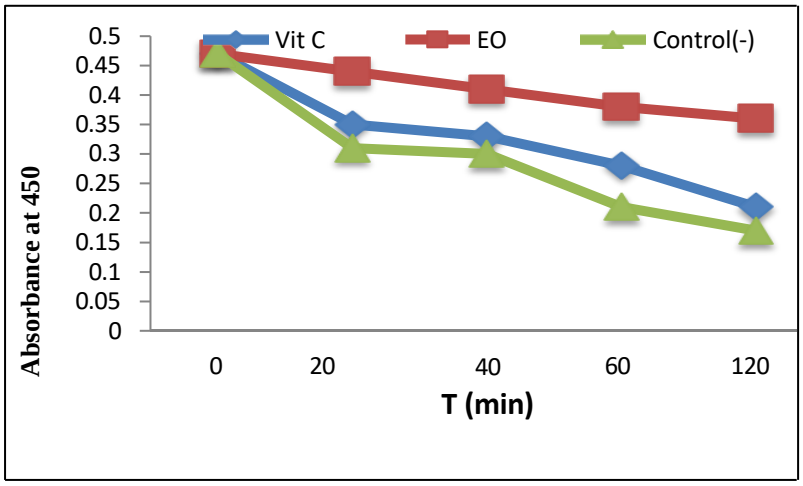

Fig 1: Fig. 1: Absorbance values at 450 nm of the different emulsions.

The essential oil showed a significant inhibition percentage equal to 63.3% compared with vitamin C (13.**3%).** These results are probably due to the high specificity of the β- carotene bleaching assay for lipophilic compounds **(Gachkar *et al.*, 2007)**.
Frankel and Meyer (2000) hypothesised that apolar antioxidants (essential oil from fennel seeds in the present study) exert greater antioxidant properties because they are concentrated within the lipid-water interface, thus preventing the formation of lipid radicals and the oxidation of β-carotene. Polar antioxidants (vitamin C), on the other hand, remain diluted in the aqueous phase and are therefore less effective in protecting lipids.

An extract that delays or inhibits β-carotene bleaching can be described as a free radical scavenger and as a primary antioxidant (**Liyana-Pathirana *et al.*, 2006),** which is defined as a chemical compound that delays or prevents the initiation stage and interrupts the propagation stage of the lipid oxidation reaction **(Miliauskas *et al.*, 2004**), which is the case of our essential oils.
The anti-free radical effect of the essential oil of fennel seeds could be due to the presence of a large proportion of phenolic compounds **(Velioglu *et al.*, 1998)**. According to **Muckenstrum *et al* (1997),** this activity could also be due to major compounds such as anethole: an oxygenated monoterpene known for its antioxidant activity.

Lu and Foo (1995) have already reported that antioxidant compounds work synergistically with each other to produce a wide range of antioxidant activities, creating an effective defence system against free radical attack.

2.2. Sensitivity of the bacterial strain *S. aureus* to the essential oil of fennel seeds.

We tested the antibacterial activity of our oil using the microatmosphere method. By measuring inhibition halos, we were able to determine the degree of sensitivity of the *S.aureus* bacterial strain to our essential oil. The observations made on the effect of fennel seed essential oils on the growth of the bacterial strain tested are shown in Figure 2.

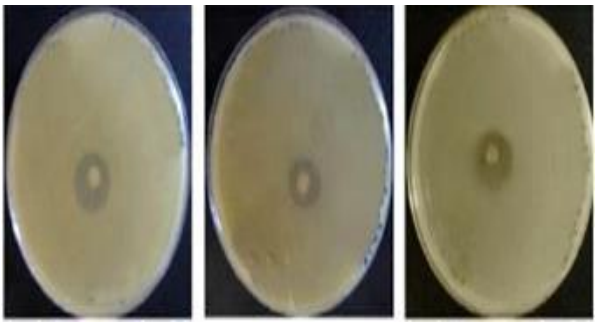

Fig. 2: Photographs showing the antibacterial effect of fennel seed essential oil on *S. aureus* using the microatmosphere method**.**

The above results show that our oil has antibacterial activity against *S. aureus*, with a zone of inhibition of around 11.7 ± 0.624 mm. According to **Ponce *et al.* (2003),** we can say that the bacterial strain studied is sensitive to our oil.

According to the research carried out by **Bouguerra (2011)**, the test of the antibacterial activity of fennel seed essential oils using the disc diffusion method against the same *S. aureus* bacterium shows an inhibition diameter corresponding to 8.67 ± 0.889 mm**.** These results are relatively lower than those obtained using the microatmosphere method. This difference could be explained by the effect of the method used to assess antimicrobial activity and the sensitivity of the strain tested.

According to **Kalemba and Kunicka (2003),** the sensitivity of a microorganism to essential oils depends on the properties of the essential oil and the microorganism itself. It is well known that Gram bacteria^{+}, such as *S. aureus*, are more sensitive to essential oils than Gram bacteria^{-} .

The main active components in essential oils against food-borne pathogens generally contain 1% phenolic compounds such as carvacrol, eugenol and thymol **(Dorman and Deans, 2000**). The antibacterial properties of these compounds are partly linked to their lipophilic nature, which leads to accumulation in bacterial walls, thus disrupting the function and permeability of cell membranes, as well as degradation of the cell wall, causing damage to the membrane **(Helander *et al.*, 1998).**

2.3. Determination of the minimum inhibitory concentration (MIC)

According to the results obtained during the "*in vitro*" antibacterial test, the *S. aureus* strain proved to be sensitive to our essential oil, so we proceeded to determine the MIC.

Before using an antibacterial molecule as a preservative in food, the minimum inhibitory concentration (MIC) must be estimated **(Tiwari *et al.*, 2009)**.

The solid dilution method enabled us to determine the MIC of the essential oil of fennel seeds, which was of the order of 31.25 µl of oil/ml of ethanol (3.12%), characterised by a total absence of bacterial growth. These results show that *S. aureus* is not very sensitive to our oil.

Gram-forming bacteria^{+} are considered to be the most sensitive because they have structural features that are more susceptible to essential oils **(Abdul Rahman *et al.*, 2010)**.

However, according to **Bassole *et al* (2002),** staphylococci are already known for their antibiotic resistance, making *Staphylococcus aureus* less sensitive.

2.4. Test of antibacterial activity in the presence of pathogens inoculated into minced meat

Based on the results of *in vitro* antibacterial tests, we were able to follow the development kinetics of *S. aureus* inoculated into minced meat in the presence of essential oil of fennel seeds.

Fig. 3 shows the antimicrobial activity of fennel seed essential oil on *S. aureus* applied to minced meat kept at refrigeration storage temperature for a period not exceeding 8 days.

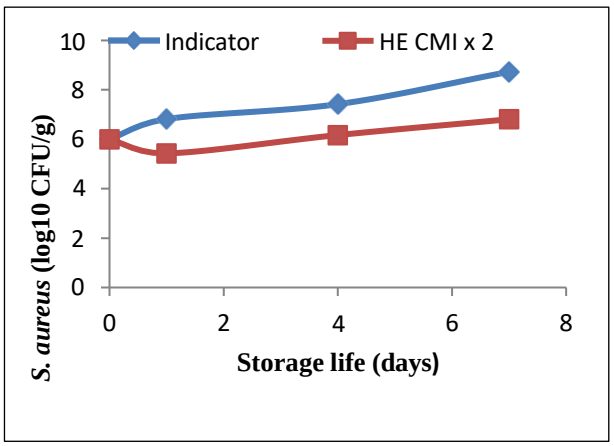

Fig.3: Antimicrobial activity of fennel seed essential oil on *S. aureus* applied to minced meat.

It can be seen from this graph that the maximum number of *S. aureus* during the entire storage period was recorded in the meat samples that did not contain the essential oil (control). These control samples reached a value of 8.73 Log CFU/g at the end of the storage period (on the 7th day).

It turns out that the presence of our oil significantly reduced the microbial load of *S. aureus* throughout the storage period. The fennel seed oil reduced the bacterial count after 24 h of storage, but after that, the oil only slowed down growth compared with the control. The number of *S. aureus* reached a value of 7.21 Log CFU/g on the 7th day. This explains why fennel seed oil has very little antibacterial activity against *S. aureus once* it comes into contact with it.

According to the literature, there aren't many studies on the application of fennel seed essential oils in food matrices. However, according to the references, this oil does have antibacterial activity. Studies by **Bagamboula *et al* (2004)** have shown that various constituents of essential oils such as carvacrol in oregano, fenchone in fennel and eugenol in cloves have antibacterial activity.

It should be noted that an essential oil tested experimentally "*in vitro*" may not have the same effect in a food product. In general, according to **Burt (2004),** to obtain a significant antibacterial effect in a food matrix, higher concentrations must be used. In general, essential oils have fairly strong and powerful odours, which can be a limiting factor for their application in foodstuffs. However, **Caillet and Lacroix (2007)** have pointed out that undesirable organoleptic effects can be limited by carefully selecting the essential oil according to the type of food being considered. On the other hand, several researchers have used combined systems between essential oils and other preservatives.

Djenane *et al* (2002) reported that meat treated with natural antioxidants and packaged in a modified atmosphere showed chemical and microbiological stability over a long period compared with untreated meat. **Caillet and Lacroix (2007)** also reported that the combination of essential oil and moderate heating (55°C for 1 min) totally inhibited *Salmonella* sp. whereas in the absence of oil, heating for more than an hour was required to achieve the same result.

Burt (2004) has suggested that low water content in food can hinder the action of antimicrobial agents on target sites in the bacterial cell. Thus, a high level of water and salt would facilitate the action of essential oils. The same author also found that a high fat content can significantly reduce the action of essential oils in meat products. The formation of a protective layer of fat around the bacteria or the lipid fraction in the food may absorb the antimicrobial agent, reducing its concentration and effectiveness in the aqueous phase.

Some studies have successfully demonstrated the potential applications of essential oils to reduce or control pathogenic flora in food products. The essential oils best suited for application to meat and meat products are eugenol, coriander, clove, oregano and thyme (**Motlagh *et al.*, 1991).**

Oussalah *et al* (2007) reported that the difference in the antibacterial activities of essential oils can be linked to their concentration, nature and content, functional groups, the type of essential oil and the degree of antibacterial activity.

Essential oils extracted from seeds are often complex mixtures of different compounds, some of which have antimicrobial properties. The composition of essential oils from the same species varies according to geographical location, climatic conditions, etc. As a result, their antibacterial properties also vary. It is therefore of paramount importance to separate and identify the active components present in an oil with antimicrobial properties **(Kotzekidou *et al.*, 2008).**

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