Original Article

# Evaluation of Antimicrobial and Antioxidant Properties of Eucalyptus Extracts in Zein Films to Improve of Minced Sheep Meat Packaging Shelf Life

**Masoud Habibi Najafi [1], Seyed Ebrahim Hoseini [2*], Seyad Ali Yasini Ardakani [3], Zhaleh Khoshkhoo [4], Nargess Mooraki [4]**

[1] PhD student of Food Sciences and Technology, Tehran North Branch, Islamic Azad University, Tehran, Iran. [2] Department of Food Science and Technology, Tehran Science and Research Branch, Islamic Azad University Tehran, Iran. [3] Department of Food Sciences and Technology, Yazd Branch, Islamic Azad University, Yazd, Iran. [4] Department of Food Science and Technology, Tehran North Branch, Islamic Azad University, Tehran, Iran.

## Abstract

Meat products are delicate to quality deterioration due to their rich nutritional composition. The quality deterioration is due to microbial and chemical changes. In order to extend the food shelf-life and maintain product safety, quality and coolness, it is necessary to select sufficient materials and packaging technologies, of which biodegradable films containing antimicrobials. The use of essential oils and plant extracts such as eucalyptus became the main replacements for synthetic additives have rich in phenolic component such as 1,8-cineole. Research data has illustrated that the EO extract exhibited various biological effects, such as antioxidant, anti-hyperglycemic and antibacterial activities. Zein is a renewable polymeric material with potential applications in the plan of food packages. This context evaluates application of Eucalyptus extract in protein corn zein polymer among refrigerated storage of ground sheep meat. The data about the present study were explicit as means ± SD of triplicate. The significance of difference was performed by analysis of variance (ANOVA), and Tukey's test was used for mean comparison. Results showed bioactive film made of zein containing the natural antioxidants, eucalyptus, was effective at delaying the lipid oxidation of raw sheep meat during refrigerated storage. The best treatment was 1.5 percent eucalyptus extract in zein film that provides minced sheep meat shelf life for 6 days (double effect).

**Keywords:** Antimicrobial, Antioxidant, Eucalyptus, Extracts, Zein, Film, Meat

## Introduction

Meat products are delicate to quality deterioration due to their rich nutritional composition. The quality deterioration is due to microbial and chemical changes. Meat is a so perishable product due to its chemical composition delicate to bacterial raid. The microbial growth catalyzes changes in fragrance, color and food texture, resulting in reduced shelf life and increasing the risk of food borne illness. Moreover, the oxidative processes lead to degradation of lipids, proteins, pigments, and constitute major mechanisms of quality deterioration in meat and meat products (Shah, Bosco et al. 2014).

Phenolic compounds are between the dominant components of several essential oils and can found up to 85% of the total composition of some essential oils. In recent years, essential oils have been used widely as natural additives in food, especially combined with other preservation method, which is called hurdle technology (Pateiro, Barba et al. 2018).

Therefore, in recent years, there is an attitude to study the use of new polymers to replace or minimize the use of synthetic ones in the production of packaging. Zein is a renewable polymeric material with potential applications in the plan of food packages. Zein include of a group of alcohol soluble proteins from corn seed, as well as known as prolamins, very low solubility in water and which have thermoplastic properties. In contrast to films derived from other proteins,

**Address for correspondence:** Mr. Seyed Ebrahim Hoseini, Department of Food Science and Technology, Tehran Science and Research Branch, Islamic Azad University Tehran, Iran.
E.mail: ebhoseini@yahoo.com



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films derivate from zein have been reported to possess favorite heat seal characteristics suitable for packaging usage (Dominguez, Barba et al. 2018).

This context evaluates application of Eucalyptus extract in biopolymer corn zein among refrigerated storage of ground sheep meat.

## Materials and Method

### Materials

Zein was supplied by Merck Germany; Glycerol, magnesium nitrate and phosphorus pent oxide were supplied by Merck Germany. Tryptone Soy Broth, Agar bacteriological, Buffered peptone water, Plate Count Agar, Palcam Agar and Violet Red Bile Agar were provided by Merck Germany. Acetic acid glacial and potassium iodide (KI) were supplied by Merck Germany and 1-Decanol by Alfa Aesar, citric acid; 1- diphenyl-2-picryl-hydrazyl (DPPH); 2, 2-azino-bis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS); silica gel; methanol; polysorbate 20 (Merck, Germany).

### Preparation of eucalyptus extract

Fresh leaves of *E.camaldulensis var*. *Myrtaceae* were collected (October 2019) from Yazd (middle of Iran). The leaves were dried for 2 weeks in the shade at ambient temperature (25 – 30 ◦ C) and then crushed to obtain a powder. This product was stored in plastic bags at 4 ◦.The essential oil was extracted from dried leaves by sox let for 4 h in a laboratory. After decantation the oil was dried stored at 4-6 ◦ C and using sodium sulfate anhydrous until utilization.

### Film preparation

The film-forming solution was ready by dissolving 16 g of zein powder in 80% (v/v) ethanol-water solution and stirring for 30 min at 80 °C. Glycerol at 15% (g glycerol/g dry zein powder) was added to the solution and at 30 °C it was stirred for 8 min, then extracts at 0.5 or 1.5% (g /g dry zein) was combined and it was stirred for 8 min. Films were obtained by casting, that the film-forming solution was spread on a hard polyethylene surface using an spread bar and heated at 80 °C for 20 min after complete drying; the films were pared off.

Control films were prepared as described above but without incorporating the extract. The mean thickness of the films was 21 ± 1 mm, measured with a digital micrometer (Kashiri, Cerisuelo et al. 2017).

### Meat Collection and Preparation

Fresh sheep meat was purchased from a local market (Yazd, Iran) and immediately transported to the laboratory. The sheep samples were obtained from topside muscle. Fat containing of minced meat was about 30 g /100 g. The sheep meat was minced three times and packaged with zein film, with film without extract or film with different concentrations of extract. The minced sheep meat were divided into five treatment groups as follows: (1) sample coated with zein film without extract; (3,4) sample coated with zein film combined with 0.5 and 1.5% (g/g) eucalyptus essential oil and (5) sample coated with mix of 0.75% eucalyptus and 075% mint extract. Finally the samples stored at 4-6 °C for 3–6 days.

### Film characterization

- **Film thickness**

The thickness of films, was measured using a micrometer (accuracy of 0.001 mm) and expressed in mm. Measurements were taken at three random sites on each five film. For the evaluation of mechanical properties, the mean and standard deviation were calculated.

- **Solubility of film**

The films were cut into 20 mm radius disc, dried in an oven at 70°C for 2 h and then weighed in order to obtain the initial dry mass. The dried films were immersed into 50 mL capped falcon tubes containing 25 mL of distilled water and placed inside the shaker oven for 24 h at 25°C. Thereafter, the solution was filtered through Whatman filter paper (No. 1) to retrieve the remaining undissolved film. The remaining film pieces were placed in the oven at 70°C for 2 h and then weighed to define the final dry mass of film. The percentage of weight loss was considered as water solubility of the films (Davoodi, Kavoosi et al. 2017).

- **Water vapor permeability**

The water vapor permeability (WVP) of the film samples was measured with a modification of the ASTM E96-95 (ASTM, 1995) gravimetric method (McHugh, Avena-Bustillos, & Krochta, 1993) using Payne permeability cups (Elcometer SPRL, Hermelle/s Argen- teau, Belgium) of 3.5 cm in diameter. Six round samples per formulation were cut, and the thickness was measured in six points per sample. WVP was determined at 25 ◦C and 53–100% RH gradient, which was generated by using an oversaturated Mg (NO3)2 solution and pure water, respectively. The side of the film which was in contact with air during drying was oriented toward the gas phase at 53%RH. The cups were weighed every 1.5 h, for 24 h with an analytical balance. After the steady state was reached, the slope obtained from the weight loss vs. time was used to calculate WVP (Moreno, Atarés et al. 2015).

- **Tensile strength and elongation at break**

Tensile strength and elongation at break were determined in accordance with the ASTM method D 882-02 with a Texture Analyzer Testing Machine (Brookfield, USA). Before testing, the film samples were balanced in a desiccator at 65 ± 5% RH over a saturated solution of magnesium nitrate for two days. The films were cut into 60 mm × 10 mm pieces which the area of one piece was used for each experiment. However, 20 mm of the films were within the mandibles, and so, the initial length of the film was taken as 40 mm.(Davoodi, Kavoosi et al. 2017)

The thickness of the films was measured at different points with a micrometer and the average was taken. The initial cross-sectional area of film cuts was 10 mm × 10 mm average thickness in mm. The tensile strength test was then performed by stretching the film cut at a speed of 50 mm/min. The software (U58) of testing machine directly provided the tensile strength and elongation at break of the films (Davoodi, Kavoosi et al. 2017).

### Texture analyzer

The mechanical behavior of the films was analyzed using a texture analyzer (Brookfield, USA) according to ASTM D882 (2001). Twelve film stripes (25 mm wide and 100 mm long) per formulation were tested. Film ribbons were mounted in the tensile grips and drawled at a rate of 50 mm/min until breaking. The elastic modulus (EM (MPa)), tensile strength at break (TS (MPa)) and percentage of elongation at break (%E) were decided from stress–Hencky strain curves, obtained from force deformation data.

### Optical properties: Transparency, color and gloss

A spectrocolorimeter (UNICCO, USA) was used to obtain the infinite reflectance spectra of the film samples. Measurements were taken on black and white backgrounds. The internal transmittance (Ti) of the films was specified by applying the Kubelka–Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra, following the methodology described by Pastor. Twelve samples per formulation were analyzed, and three measurements per sample were taken. The measurements were performed on the side of the film in contact with air during drying.

CIE-L*a*b* coordinates: lightness (Lab*), chrome (Cab*) and hue (hab*) of the films were obtained from the surface reflectance spectra using D65 illuminant/10◦ observer.

The gloss was measured on the film side in contact with air during drying, at a 60◦ incidence angle, according to the ASTM standard D-523 (ASTM, 1999); using a flat surface gloss meter.Twelve replicates were obtained per formulation. The results were described of gloss units, relative to a black glass standard (value near to 100).

### Micro-structure of film

- FTIR

For preparation of samples, Cutted 50 mg of film and powdered, then mixed with the 0.2 mg KBr salt powder in mortar. The two powders pour into a specific mould and fill it under a partial vacuum (to remove the air) and by a special Jack is established as a small, transparent pill. After the sample was prepared, the disk was placed inside the shimatzu assay and the resulting spectra were obtained at the wavelength range of 4000- 650 cm-1 (2.5-15.4 µm) and Resolution 4 cm-1 and the number of scanning 64. By using the IR Solution software, the obtained spectra were processed.

- SEM

The film microstructure was performed using scanning electron microscopy (SEM). The samples were coated on aluminum tubes using standard method, and were coated with gold (100 A). The micrograph using the aggravated voltage of 25 - 30 kV was measured (Goldstein, Newbury et al. 2017).

### Characterization of active properties

- Antimicrobial ability of the films

The effectiveness of the antimicrobial ability of the films in a real food system was tested in minced sheep meat, which was obtained from a local supermarket and was processed instantly after arriving at the lab. The meat was ground with a mincer and amounts, 10 g in weight, were shaped by using Petri dishes to obtain the test samples. Then, the surface of both sides of the samples was coated with the films with 0, 0.5 and 1.5 percent of eucalyptus extract (Moreno, Atarés et al. 2014).

All the samples were stored at 4 ◦C for 3 and 6 days; however, total count of bacteria and s. aureus counts were obtained. Each sample was homogenized in a Stomacher blender with 90 mL of buffered peptone water for 3 min. Then, serial dilutions were made and plated out. (Moreno, Atarés et al. 2015).

- Antioxidant properties
  - ✓ DPPH radical scavenging activity

Twenty micro liters of each dispersion or standard antioxidant were added to 1 mL of 0.2 mm DPPH in 95% methanol. The mixture was stirred and kept in the dark for 30 min at room temperature. A decrease in absorbance of each sample was measured at 517 nm. The percent inhibition of DPPH was obtained by the following formula:

$$\text{The percentage inhibition of DPPH} = [(A_{DPPH} - A_{test}) / A_{DPPH}] \times 100$$

ADPPH is the absorbance of DPPH solution and Atest is the absorbance of DPPH solution in the presence of the dispersions. IC50 (50% DPPH inhibition) was calculated from the graph plotting the percent inhibition of DPPH against different concentrations of dispersions (Davoodi, Kavoosi et al. 2017).

  - ✓ TBARS value

The evaluation of lipid stability was performed by measuring TBARS at distance of 2 days during storage. For preparation, 10 g of sample were triturated with 25 ml of precooled 20% trichloroacetic acid (TCA) in 2 M orthophosphoric acid solution for 2 min. The content was then quantitatively transferred into a cup by rinsing with 25 ml of chilled distilled water. Then mixed and filtered through What man No. 1 filter paper. Three milliliters of TCA extract were mixed with 3 ml of TBA reagent (0.005 M) in test tubes and placed in a dark room for 16 h. A blank sample was made by mixing 3 ml of

10% TCA and 3 ml of 0.005 M TBA reagent. The absorbance was measured at a fixed wavelength of 532 nm with a scanning range of 532 nm to 533 nm using a UV–vis spectrophotometer. The TBA value was calculated as mg malonaldehyde per kg of sample by multiplying the absorbance value with a factor of 5.2 (Biswas, Chatli et al. 2012).

## Analysis of Extract

### • Total phenolic

The polyphenol content of eucalyptus extracts was quantified by Folin–Ciocalteau's reagent assay and expressed as Gallic acid equivalents (lg GAE/g). Briefly, 100 mm of extract (250 lM concentrations) were mixed with 2 ml of 2.0% Na2CO3 buffer and incubated at room temperature for 2 min. The total volume was made to 2.4 ml by adding distilled water. After addition of 100 mm of 1 N Folin–Ciocalteau's reagents the reactions tube was further incubated for 30 min at room temperature, and the absorbance was read at 720 nm. The amount of total phenolics was determined by a standard calibration curve (y = 0.001x 0.009 and r2 = 0.992; where, y = absorbance, x = Gallic acid concentration, and r2 = correlation coefficient) constructed using standard Gallic acid solutions from 250 to 5000 mg/ g concentrations (Biswas, Chatli et al. 2012).

### • HPLC analysis

HPLC analyses were carried out appendix the method described by Conde et al. The HPLC instrument was a Varian 5000 equipped with a Perkin-Elmer LC-55 UV-VIS detector, set at 280 nm. (I) Analytical scale: The analyses were performed with a Hypersil ODS (5 lm) column (250 mm 64.6 mm ID) from CS-Chromatography Service (Langer-wehe, Germany), the flow rate was 1.2 ml/min. and the injection volume was 20 ml. The mobile phase consisted of 3.5% acetic acid in water (A) and 3.5% acetic acid in methanol (B). A linear fluent gradient was used, starting with 20% B, increasing to 100% B within 40 min. the final conditions were maintained until the end of the run. (ii) Semi-preparative scale: The conditions were corresponding to those of analytical HPLC except for the dimensions of the column (250 mm 610 mm ID), particle size (7 lm), injection volume (100 ml) and the flow rate (3 ml/min). Fractions were collected with the fraction collector Frac 100 (Pharmacia-Biotech) (El-Ghorab, El-Massry et al. 2003).

## Analysis of raw meat

### • Fat and protein contents values of meat

The fat content of the raw sheep meat was determined according to ISIRI742 (Iran National Standard).Firstly, the raw meat ground well with grounder and mix to times, to homogenized the meat.

For evaluate of protein content performed a method according of ISIRI924 (Iran National Standard)

### • Color values of meat

The color of the raw sheep meat was determined using a Color Difference Meter (Tes-135 Colorimeter, Tes Co. Taiwan). Color was characterized in terms of the L* (lightness/white), a*(red/green), and b* (yellow/blue) color space values. Measurements were made vertical to the band surface at five different locations per sample; mean values (L*, a*, and b*) from the samples were analyzed, and triplicate bands were analyzed to achieve an average colorimetric value (Zhang, Wu et al. 2014).

## Statistical analysis

The data about the present study were explicit as means ± SD of triplicate. The significance of difference was performed by analysis of variance (ANOVA), and Tukey's test was used for mean comparison using SPSS version 18. $P < 0.05$ was considered to be significant.

# Results and Discussion

## Identification of compounds with ant oxidative activity

HPLC separation illustrates that the extract obtained by ethanol digestion contains two main compounds with absorbance at 280 nm. For their identification HPLC separation was repeated on a semi preparative scale HPLC column, the corresponding eluent fractions were collected and freeze-dried. GC-MS analysis showed that these compounds are Gallic and Ellagic acid. HPLC co-injection of the authentic compounds confirmed that assignment. Quantitative evaluation of the HPLC chromatograms showed that the extract contained 4.6% of gallic acid and 22.4% ellagic acid. A ferric thiocyanate assay with corresponding quantities of gallic acid and ellagic acid leads to an inhibition effect of 65% after 12 days. Bearing in mind that the complete ethanol digestion extract showed an inhibition effect of 82%, gallic acid and ellagic acid are to be considered as mainly responsible for the ant oxidative effect of the ethanol extract of E. camaldulensis var. brevirostris leaves.

**Table 1.** HPLC Analyses of Eucalyptus Extract

| Sample | Area | Conc. Ppm | ug/g |
|---|---|---|---|
| **Caffeic acid** | 6.2 | 1.51 | 15.1 |
| **Ellagic acid** | 3884 | 511 | 5110 |
| **rutin** | 160 | 58.93 | 589.3 |
| **kaepeferol** | 142 | 5.97 | 59.7 |

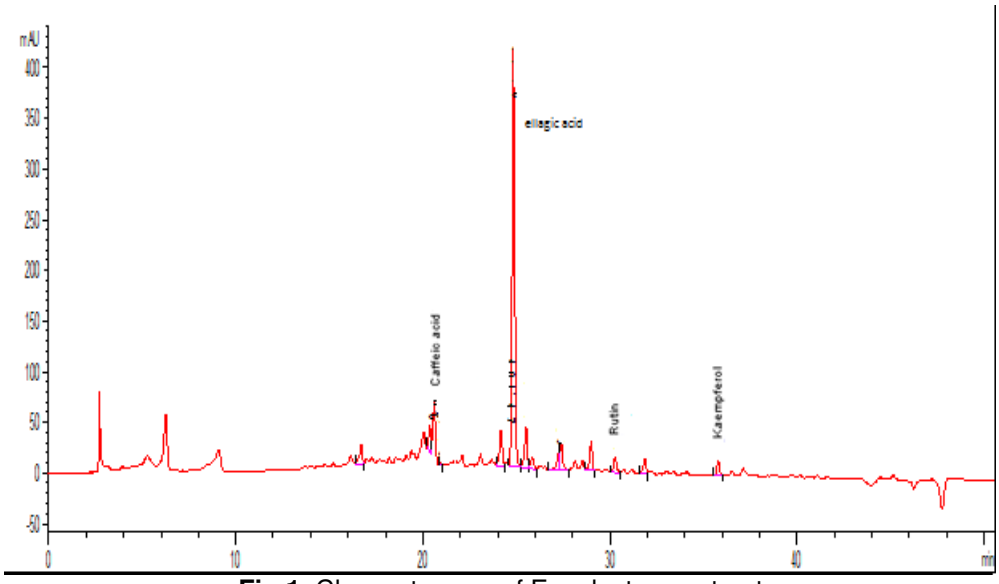


**Fig.1.** Chromatogram of Eucalyptuse extract

## Antimicrobial activities of Eucalyptus Oil

Eucalyptus oil has a high antimicrobial activity (Gilles ET al.2010). The most rich component of eucalyptus oil is 1, 8-cineole, that known for its antimicrobial properties (Pattnaik et al. 1997). Lis-Balchin & Deans (1997) implied that EOs containing high concentrations of 1, 8-cineole.

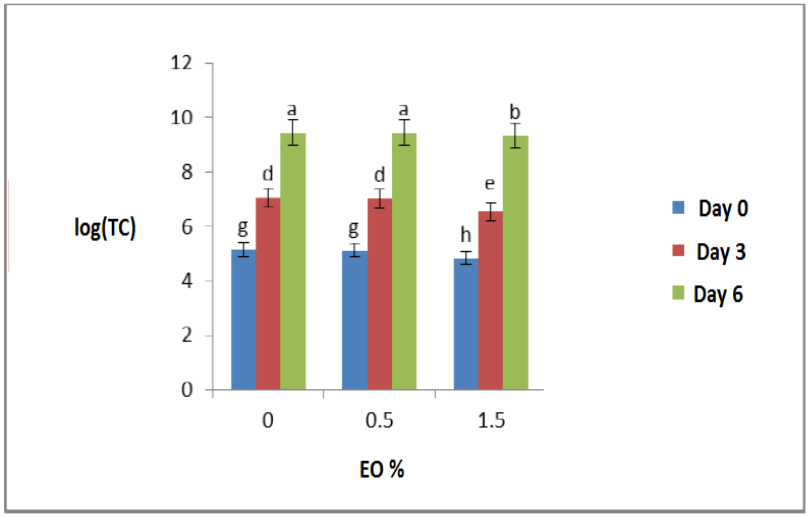


**Fig. 2.** Comparison of log total count in EO% in zein films on 0, 3, 6 days in 4ºC

## Antioxidant activity

- DPPH

DPPH is a free radical compound commonly used to detect the free radical scavenging ability of extracts (Amarowicz ET. Al 2004). DPPH is scavenged and DPPH-H produce, then the colour of the solution transition from purple to yellow, and the detected degree of change by the decrease in absorbance at 517 nm (Isono et al, 2005). The results of the experiments are illustrate the eucalyptus extract has a high DPPH radical scavenging activity. The strong activity of eucalyptus caused by the presence of eugenol, the main factor of eucalyptus, which is known for its antioxidant activity.

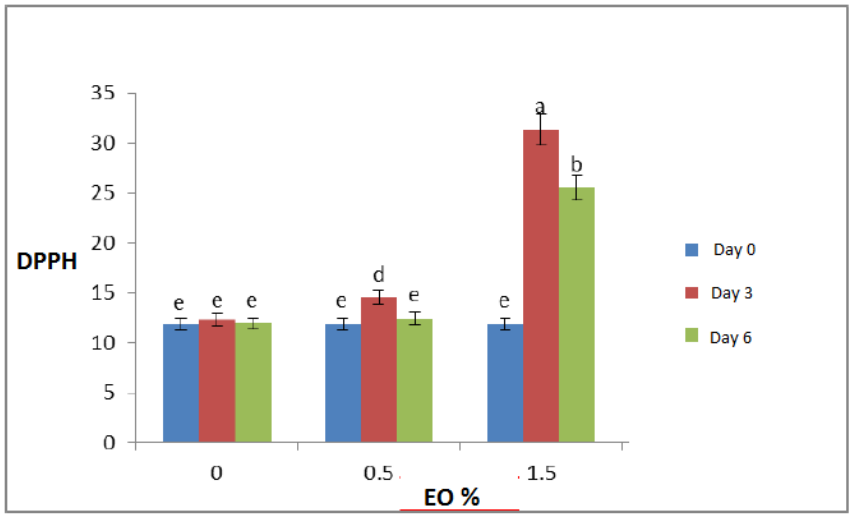


**Fig. 3.** Comparison of DPPH in EO% in zein films on 0, 3, 6 days in 4°C

- TBARS

TBARS analysis showed the creation of secondary products of lipid oxidation, generally malondialdehyde, which may contribute to the off-flavour of oxidized fat. Antioxidant effects of hydro-ethanolic extract on the TBARS values of sheep meat during storage (4 °C) for 6 days analysis. At day 0, the TBARS values ($P < 0.05$) were found to be the same for all meat samples. The TBARS values of all treated samples were significantly lower ($P < 0.05$) than those of the control for each sampling during storage, showing that the extract had highly protective effects against lipid oxidation in meat sheep meat. Somewhat TBARS of samples increased at the storage period.

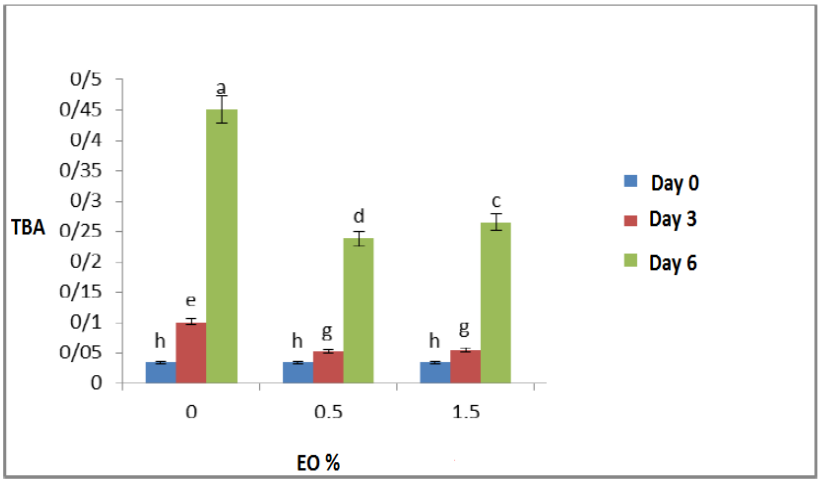


**Fig. 4.** Comparison of TBA in EO% in zein films on 0, 3, 6 days in 4°C

## Colour values

The lightness (L*) values of the sheep meat samples was imperceptibly affected by the addition of extract. However, the control samples showed decreased L* values at the end of the storage period. The L* (lightness) values of films treated with eucalyptus extract were relatively ($P < 0.05$) higher than those of the control over the entire storage period.

As expected, sheep meat samples treated with extract showed an intense red colour, and thereby higher values of a*, than the control sheep meat samples.

**Table 2. Comparison of colour (B*) in EO% in zein films on 0, 3, 6 days in 4°C**

| B*/Day | 0% | 0.5% | 1.5% |
|---|---|---|---|
| **0** | -10±1cd | -10±1cd | -10±1cd |
| **3** | -10.67±0.58d | -7±0.58b | -11±1d |
| **6** | -6±1ac | -5±1a | -8.67±0.58c |

**Table 3. Comparison of colour (A*) in EO% in zein films on 0, 3, 6 days in 4°C**

| A*/Day | 0% | 0.5% | 1.5% |
|---|---|---|---|
| **0** | 25±1a | 25±1a | 25±1a |
| **3** | 19.33±0.58b | 12±1d | 18±0.58b |
| **6** | 16±1c | 9±1e | 12.33±1d |

**Table 4. Comparison of colour (L*) in EO% in zein films on 0, 3, 6 days in 4°C**

| L*/Day | 0% | 0.5% | 1.5% |
|---|---|---|---|
| **0** | 45±1a | 45±1a | 45±1a |
| **3** | 48.67±0.58d | 34±0.58f | 54±0.58a |
| **6** | 50±1c | 34±1f | 52.67±0.58b |

## Physical properties of zein films

After the incorporation of extract at 0.5 or 1.5% no visual phase separation was observed, and the eucalyptus hydro-alcohol extract was homogeneously distributed in the polymer matrix. Zein possesses a high content of hydrophobic amino acids, facilitating compatibility and dispersion of the eucalyptus hydro-alcohol extract in the solid polymer matrix. However, the films lost transparency and acquired a yellowish colour. The average thickness of the films with 0.5 or 1.5% extract was 33 ± 2 µm, similar to the control films.

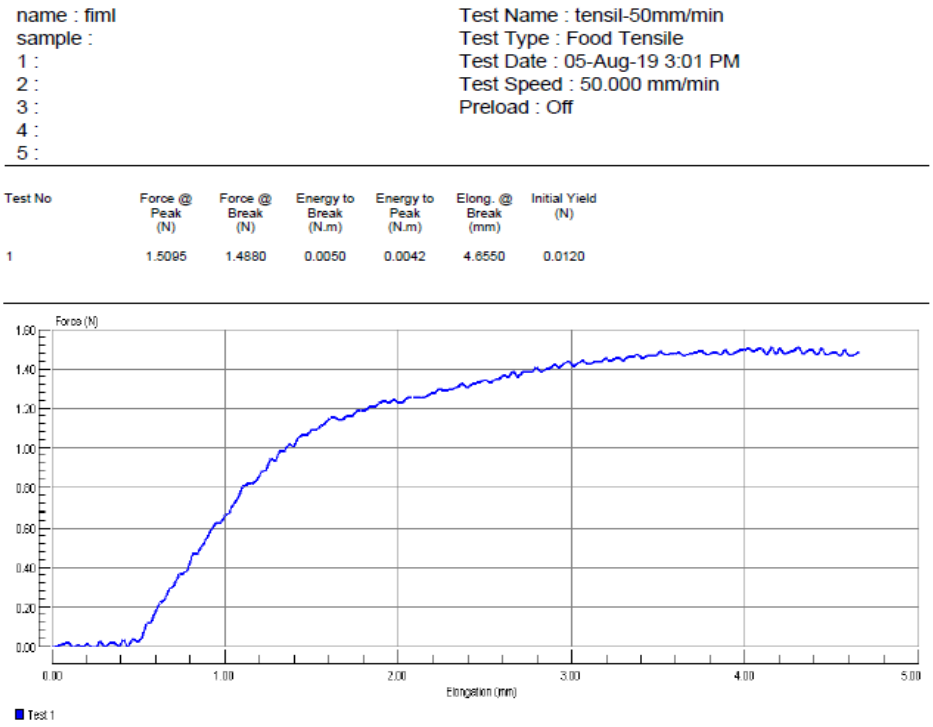


**Fig. 5.** Comparison of film tensile in zein films

## Water vapor permeability (WVP)

Water permeability is a critical parameter for packaging materials. In this work, water flow through the film into the cell was measured at humidity gradients of 70% and 90%. The water flow increases with the gradient, as expected, because the gradient is the driving force of the mass transport phenomena. From the slopes in this representation (dm/ dt) and the film thickness (L) and the vapor pressure gradient ð½DRH $pvÞ, the WVP values were estimated.

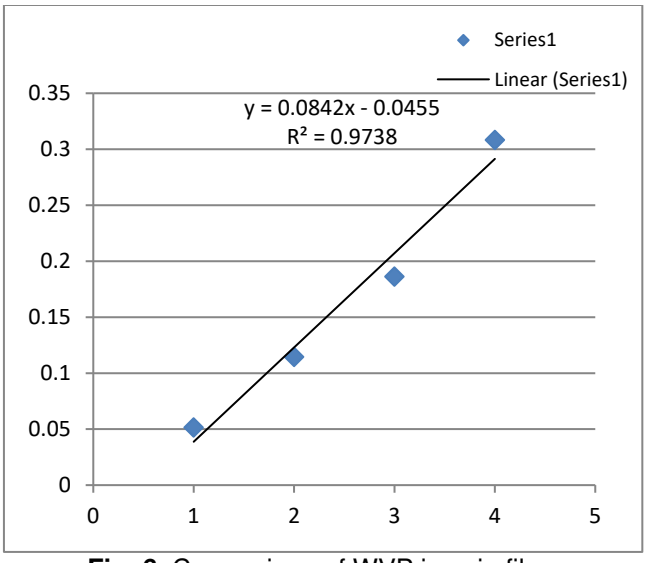


**Fig. 6.** Comparison of WVP in zein films

## Conclusions

The bioactive film made of zein containing the natural antioxidants, eucalyptus, was effective at delaying the lipid oxidation of raw sheep meat during refrigerated storage. The use of these compounds, with several beneficial effects in health (antinflammatory, anticancer, protector cardiovascular, prevention of diabetes, etc.) is a good strategy to replace synthetic preservatives in meat. Also, in previous studies, this film exhibited adequate mechanical and oxygen barrier properties (Bermúdez-Oria et al. 2017).

The results demonstrate the effectiveness of Eucalyptus extracts in inhibiting microbial growth, reducing lipid oxidation, maintaining or improving sensory characteristics and extending the shelf-life of raw sheep meat during storage at 4 °C for 6 days. The antioxidant properties of eucalyptus extract showed that it had good antioxidant activity with higher polyphenol and flavonoid contents.

This study demonstrated that the film formulation improved the oxygen barrier property and enhanced the oxidative stability of sheep relative to the film control, without natural antioxidant, during storage at 4 °C. The best protective effect was obtained for film containing 1.5 percent eucalyptus, which reduced lipid oxidation during 6 days, possibly by the combined effect of the film acting as an oxygen barrier and the antioxidant protection of EO. An edible active film with these antioxidants might have a double positive benefit; one on the oxidation stability of the meat fat itself, and a second health benefit for the consumer. However, very in-depth studies would be required to confirm this secondary benefit as these preliminary results are promising, additional research will be required to improve the formulation to optimize the film's moisture content as well as the sensory properties of meat treated with edible film containing phenolic compounds. Therefore, to get an edible active film with these antioxidants had a double benefits, a positive effect on the oxidation stability of meat fat and beneficial effect in the human organism.

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