Efficacy of olive leaf extract (Olea europaea L. cv Gentile di Larino) in marinated anchovies (Engraulis encrasicolus, L.) process

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A B S T R A C T

In this study, the antimicrobial activity and the preservative properties of olive leaf extract (OLE) Olea europaea L. “Gentile di Larino” cultivar, were evaluated. The antibacterial activity was performed in vitro against spoilage bacterial strains: Pseudomonas fluorescens (ATCC 13525), Pseudomonas fragi (ATCC 4973), Pseudomonas putida (ATCC 17514), Brochothrix thermosphacta (ATCC 11509), Clostridium sporogenes (ATCC 11437), and Listeria innocua (ATCC 33090). About the preservative properties of OLE, they were evaluated in the marinating process of anchovy fillets. During the process have been determined the change of sensory characteristics and monitored these chemical parameters: pH, aw, salt content (% NaCl), total volatile basic nitrogen (mg/100g), and trimethylamine nitrogen (mg/100g). Moreover, were determined the spoilage bacteria on raw material, after 7 days and at the end of marination process, 22 days. The OLE exhibited an inhibitory effect against the bacteria tested. In marinated anchovy fillets, showed that the extract improves their shelf life without modifying the organoleptic characteristics of the product; this suggests that it could be considered in the food industry as a natural antioxidant and antimicrobial food additive.

1. Introduction

In recent years, the consumer demand for natural foods with no preservatives has increased; in particular the request of olive leaf extract (OLE) and its use as food additive, both for its high phenolic content and for its antimicrobial and antioxidant activity (Lee and Lee, 2010; De Leonards et al., 2008, 2018); like other phenolic compounds naturally occurring in numerous food and known for their beneficial biological and physiological properties, such as anti-inflammatory, antiallergic, anticarcinogenic, anti hypertensive, antiarthritic and antimicrobial activities (Lombardi et al., 2012; Micol et al., 2005; Liu et al., 2017). OLE is a dark brown, bitter-tasting liquid derived from the leaves of the olive tree (Olea europaea L., Oleaceae), contains many different compounds, specifically biophenols, which are thought to give the extract its varied therapeutic properties (Difonzo et al., 2017; Sahin et al., 2017). The most abundant biophenol is oleuropein, a secoiridoid composed from elenolic acid and hydroxytyrosol, which is considered the major bitter constituent in olive fruits with a high concentration especially in green olives up to 1–2 % (De Leonards et al., 2016; Moudache et al., 2016). Other biophenols such as verbascoside, apigenin-7-glucoside, luteolin-7-glucoside and hydroxytyrosol are present in lower quantities (Japón-Luján et al., 2006; Iorizzo et al., 2016). Like many natural products, variation due to differences such as geographical location, plant nutrition, harvesting time, climate and cultivar, can influence the composition of the extracts which could influence the antibacterial and activities of the extracts (Korukluoglu et al., 2010). Phenolic compounds are known to inhibit the growth of Escherichia coli, Klebsiella pneumoniae, and Staphylococcus aureus (Aziz et al., 1998; Paster et al., 1988). Some authors (Tassou and Nychas, 1991; Holley and Patel, 2005) specified that Oleuropein inhibit sporulation of Bacillus cereus. Hydroxytyrosol also reported to be effective against clinical human pathogenic strains of Haemophilus influenzae, Moraxella catarrhalis, Salmonella typhi, Vibrio para-haemolyticus, and Staphylococcus aureus (Bisignano et al., 1999). Several reports have been published on olive leaf, especially its antimicrobial activity against microorganisms such as Helicobacter pylori, Campylobacter jejuni, Staphylococcus aureus, and others bacteria were studied from some authors (Sudjana et al., 2009). The anchovy (Engraulis encrasicolus, L.) represents an important economic resource for the Mediterranean region. Most of the catch is used for human consumption as fresh, salted, marinated, and freezeed. Marinated fish are semi-preserved fish products, ready-to-eat...
with no heat treatment and are a high-value gastronomy product (Fuentes et al., 2010). Organic acid and salt are added to retard the action of bacteria and enzymes, resulting in a preserved product with a limited shelf life (Yeanes and Casales, 2008). The use of 6.5% of salt in combination with acetic acid are important because it makes the bacteria more sensitive in fact their growth is decelerated; moreover, the salt improves the texture and taste (Simat et al., 2011). Some authors found that an increase in the vinegar content of the marinating solution to extend the shelf life may cause defects in the taste and odor of the final product (Yeanes and Casales, 1995; Kilinc and Caiki, 2004; Sallam et al., 2007).

The aim of this research has been to examine the activity of the OLE against a wide range of food spoilage bacteria, and to investigate the use of olive leaf extract as preservative in the marination process of anchovy fillet, in order to extend their shelf life and preserve their quality without the loss of their specific sensory properties.

2. Materials and methods

2.1. OLE preparation and phenolic composition

The olive leaves (Olea europaea L.) were randomly handpicked in mid-October 2014 from olive trees, “Gentile di Larino” cultivar, located in Larino, Molise region (Italy). After the harvest, the olive leaves were transported to the laboratory of the Department of Agriculture, Environment and Food Sciences of the University of Molise, where they were air-dried at room temperature, for 10 days before use. About 100 g of olive leaves were homogenized with an Ultra Turrax homogenizer; for the extraction was used a solution of methanol: water (80:20, v/v); after that solvent was evaporated using a rotary evaporator. Before using the extract, it was lyophilized to a dry powder, re-dissolved in water and then frozen. The total phenol contents were determined by Folin–Ciocalteu's method and the calculation of their content in OLE was carried out using the gallic acid calibration curve. The results were expressed as mg gallic acid per mL of extract (mg GA/mL); whereas the analysis of phenolic compounds was performed by HPLC (Ioriozzi et al., 2014, 2016). The HPLC analysis was performed using a Varian ProStar 230 instrument (Mulgrave, AUS), equipped with a UV–VIS detector and set at a wave-length of 280 nm. The Chromatographic separation was carried out according to the IOC method (The International Olive Oil Council, 2009), using the ternary solvent system constituted by: H3PO4–bistilled water 0.2% v/v (eluent A), methanol (eluent B), acetonitrile (eluent C) and with the following gradients (A/B/C): 0 min 96/2/2%; 24 min 50/25/25%; 27 min 40/30/30%; 36 min 0/50/50%; 49 min 96/2/2%. Identification of oleuropein in OLE was based on retention times in comparison with the corresponding standard.

2.2. Microorganisms and culture conditions

The microorganisms used for this study were six spoilage bacterial strains: Pseudomonas fluorescens (ATCC 13525), Pseudomonas fragi (ATCC 4973), Pseudomonas putida (ATCC 17514), Brochothrix thermosphacta (ATCC 11509), Clostridium sporogenes (ATCC 11437), and Listeria innocua (ATCC 330909). All microorganisms were sub-cultured on Muller-Hinton broth (Oxoid Ltd. CM0405, England) at the appropriate temperature for 24h. The OLE was frozen, re-dissolved in water at a concentration of 25.89 mg/mL, sterilized by filtering through 0.22 μm Millipore filters, and analyzed for their antimicrobial activity.

2.3. Agar Well Diffusion Assay

The antimicrobial activity of OLE was performed by Agar Well Diffusion Assay (Azizollahi Aliabadi et al., 2012), using Muller-Hinton agar (Oxoid Ltd. CM0337, England) and under aseptic conditions. All the bacterial cultures were diluted to obtain a microbial suspension of 10⁶ cfu/mL. The Petri plates containing 20 mL of culture medium were inoculated with 200 μL of microbial suspension and allowed to dry in a sterile chamber. The plates with wells of 8 mm diameter were spotted with 100 μL of OLE. Sterile water was used as negative control and chloramphenicol (Sigma-Aldrich, USA) 100 μg/mL as a positive control. The plates were incubated at the appropriate temperature for 24h. The antimicrobial activity was evaluated by measuring the inhibition zone against the tested microorganisms.

2.4. Determination of minimum inhibitory concentration (MIC)

Determination of MIC was carried out according to EUCAST Definitive Document (The Definitive Document E.DEF 3.1, 2000). Each strain was tested with different concentrations of OLE, that it was serially diluted in water to obtain concentrations ranging from 1.62 mg/mL at 25.89 mg/mL, and before use sterilized by filtrating through 0.22 μm Millipore filters. All the microbial cultures were diluted to obtain a micro-suspension of 10⁶ cfu/mL. The petri plates containing 20 mL of Muller Hinton culture media (MHB, Merck, Germany), were inoculated with 200 μL of microbial suspension. The plates with wells of 8 mm diameter were spotted with 100 μL of various concentrations of OLE, from 1.62 mg/mL at 25.89 mg/mL, and then incubated at appropriate temperature for 24h. Sterile water was used as negative control and chloramphenicol (Sigma-Aldrich, USA) 100 μg/mL as positive control.

2.5. Marinating process

The fresh anchovy fillets were obtained from a local fisherman in Termoli (Molise). The process consisted of gutting, heading and filleting of the anchovies followed by washing to remove blood spots. The fillets were placed in the plastic containers, and they were added with marinade solution. The marinade solution used for the experimental trials consisted of 10% NaCl and 2% acetic acid. The experimentation was divided in two batch A and B; batch A (control) consisted by anchovy fillets and marinade solution and batch B by anchovy fillets, marinade solution and with the addition of OLE (10 mg/mL). The fillets were kept in marinade solutions in a ratio 1:1 (fish: marinade solution) for 22 days and the fish temperature was kept below 5 °C. This ratio according to Capaccioni et al. (2011) decreases the immersion marinating time without damaging their sensorial characteristics. During the entire production process as well as storage, fillets were completely immersed in the marination batch.

2.6. Microbiological assay of anchovy fillets

The microbiological assays they were made on fresh anchovy fillets and on samples taken after 7 days and at the end of the marination process, after 22 days. About 10 g of sample was mixed with 90 mL (0.1 %) of sterile peptone water, in a stomacher for 1 min at room temperature. Decimal dilutions were performed for plating. In addition to aerobic mesophilic bacteria and lactic acid bacteria counts, for fish samples, psychrotrophic bacteria, coliform, yeast, and mold counts were also monitored during storage. For psychrotrophic and mesophilic aerobic bacterial count, sample dilutions were plated in plate count agar (PCA, Oxoid CM325) and incubated at 7 °C for 10 days and 28 °C for 48/72h; for yeast and mold counts, potato dextrose agar (PDA, Oxoid CM139) was acidified to a pH value of 3.5 by tartaric acid, 0.1 mL of sample dilutions were spread on PDA and incubated at 30 °C for 5 days; Violet Red Bile Agar (VRBA, Oxoid CM107) by double layer poured plate method was used for coliform bacterial count, incubated at 37 °C for 24h, and De Man, Rogosa and Sharpe agar (MRS, Oxoid) by poured plate method was used for lactic acid bacterial counts, incubated at 30 °C for 72h.

2.7. Physical-chemical analysis of anchovy fillets

The fillets of each sample were analyzed for activity water (aw), sodium chloride, pH, and acetic acid. Water activity was determined using
The marination process, after 22 days, was carried out by statistical software (SPSS, Inc., Chicago, IL, USA). Significance of differences was determined by one-way ANOVA (Friedman test) using the Duncan post hoc analysis.

3. Results and discussion

3.1. Chemical characteristics of OLE

The HPLC analysis of OLE extract allowed the identification of eight phenolic compounds (Table 1): oleuropein, verbascoside, luteolin-7-glucoside, rutin, vanillin, vanillic acid, catechin, and hydroxytyrosol. The total phenol content of the extract was 25.89 mg GA/mL. The retention times (min), the absolute peak area (%) and the main compounds present in OLE are shown in Table 1. The aqueous extract exhibited a profile in which oleuropein was the compound present in the highest quantity, with other biophenols such as verbascoside and luteolin-7-glucoside present in lower quantities.

3.2. Agar well diffusion assay and MIC

Table 1

| Phenolic compounds | Retention times (min) | Absolute peak area (%) |
|--------------------|-----------------------|------------------------|
| Hydroxytyrosol     | 4.80                  | 1.46                   |
| Catechin           | 8.40                  | 0.04                   |
| Vanillic acid      | 14.08                 | 0.62                   |
| Vanillin           | 14.69                 | 0.04                   |
| Rutin              | 17.20                 | 0.05                   |
| Luteolin-7-glucoside | 18.05            | 1.36                   |
| Verbascoside       | 20.03                 | 1.10                   |
| Oleuropein         | 22.70                 | 24.53                  |

MIC: minimum inhibitory concentration. Each value is expressed as mean ± standard deviation (n = 3) (p < 0.05).

4. Conclusions

The chemical composition of OLE conditioned the antimicrobial effects observed. The high content of oleuropein and the other phenolic compounds identified in the extract might contribute for its antimicrobial properties.

As regards to the use of OLE for the marination process of anchovy fillets, the results of the microbiological analysis were reported in Table 3. After 7 days of the marination process, in batch B that contain OLE, psychrophilic bacteria counts were <10 cfu/g instead in the batch A (control) was 4.5 × 10^2 cfu/g, so this result shows that OLE had inhibited in only seven days these microorganisms. At the end of the marination process, after 22 days, we have found for all microorganisms tested a microbiological count <100 cfu/g both in the sample of batch A (control) and both in batch B (OLE treated fillets), as also found in other studies (Fuselli et al., 1994). So, this suggests that the high concentration of sodium chloride 10% and the presence of acetic acid 2% had an inhibitory effect against many spoilage and pathogen bacteria as reported by several authors (Sen and Temelli, 2003; Gökoglu et al., 2004).
TMA-N is caused by the reduction of trimethylamine oxide by bacterial containing also OLE, was much lower, 11.40 mg/100g. Formation of extract (10 mg/mL).

A-e values in the same column labelled with different letters are significantly different (p < 0.05).

| Micromicroorganisms | Raw material | Marination process | Batch A day 7 | Batch A day 22 | Batch B day 7 | Batch B day 22 |
|---------------------|-------------|--------------------|--------------|---------------|--------------|---------------|
| Viable counts       | 4.5 × 10⁴   | 3.0 × 10²          | <10          | 2.6 × 10²     | <10          | 2.1 × 10²     |
| Lactic bacteria      | 4.3 × 10³   | 2.8 × 10²          | <10          | 2.1 × 10²     | <10          | 2.1 × 10²     |
| Psychrophilic        | 7.0 × 10⁴   | 4.5 × 10²          | <10          | 1.0 × 10²     | <10          | 1.0 × 10²     |
| Yeasts               | 1.0 × 10²   | <10                | <10          | <10           | <10          | <10           |
| Total coliforms      | <1.0 × 10⁶  | <10                | <10          | <10           | <10          | <10           |

Batch A (control): anchovy fillets and marinade solution.
Batch B: anchovy fillets, marinade solution and with the addition of Olive leaf extract (10 mg/mL).

Table 4

Chemical analysis during marination process of anchovy fillets.

| Marination process | days 0 | days 7 | days 22 |
|--------------------|--------|--------|---------|
|                     | Batch A | Batch B | Batch A | Batch B | Batch A | Batch B |
| pH                 | 6.10±   | 6.12±   | 3.81±   | 3.74±   | 3.61±   | 3.55±   |
| aw                 | 0.45    | 0.38    | 0.23    | 0.31    | 0.19    | 0.21    |
| Sodium chloride    | 0.094±  | 0.095±  | 0.056±  | 0.126±  | 0.046±  | 0.083±  |
| Acetic acid (%)    | 0.74±   | 0.69±   | 0.79±   | 0.75±   | 0.74±   | 0.71±   |
| TBA (mg/MA/Kg)     | 1.66±   | 1.62±   | 8.10±   | 4.12±   | 10.42±  | 5.68±   |
| TVB-N (mg/C2)      | 0.22    | 0.02    | 0.90    | 0.51    | 0.98±   | 0.35    |
| TMA-N (mg/C6)      | 1.10±   | 1.15±   | 3.30±   | 2.40±   | 4.5±    | 2.72±   |

Batch A (control): anchovy fillets and marinade solution.
Batch B: anchovy fillets, marinade solution and with the addition of OLE (10 mg/mL).

OLE, the TBA value at the end of marination process, was 5.68 mg MA/Kg, below the consumption level, so a good quality of anchovy fillets. The salt content in anchovy fillets at the beginning of the marination process it was 3.72% and it was increased during the process both into the control (batch A), it was 6.10% after 22 days, and both in OLE treated fillets (batch B) that it was 5.14%, due to diffusion of salt from marinade solution to fillets. The acidity content (% acetic acid) of the control (batch A) and OLE treated anchovy fillets (batch B) at the beginning of the marination process were 0.74% and 0.69% respectively. At the middle stage of the process, after 7 days, it's increased due to the diffusion of acetic acid through tissue. At the end of the marination process, after 22 days, the acidity values of both control and OLE treated anchovy fillets, were almost remained the same as the initial values.

3.5. Sensory analysis of marinated anchovy fillets

The results of sensory analysis of anchovy fillets, after the marination process (22 days), for each batch A (control) and B (with the addition of OLE), were reported in Table 5. The highest result has been obtained for the batch B 16.77 points against 10.61 points of batch A, so this suggests that OLE also has a positive role in preserving texture, appearance, and the organoleptic characteristics of the product.

4. Conclusion

Safety problems related to increasing use of chemical substances in food preservation are receiving growing attention (Neumann et al., 1983). The use of natural products can be a possible and desirable alternative to the use of chemical preservatives in the food industry. In the marination process of anchovy fillets, OLE has delayed the oxidative deterioration, TVB-N and TMA formation and had a positive effect on texture, appearance, and organoleptic characteristics of the fillets. These results show that OLE could be considered in the food industry as a natural preservative and antimicrobial additive (Sudjana et al., 2009).

Like many natural products, variation due to differences such as geographical location, plant nutrition, and cultivar can influence the composition of the extract. In fact, the future prospective will be to evaluate the OLE properties using leaves harvested in different period of the year, not only in autumn, and in different geographical location, to evaluate the different composition and characteristics that the extract can have based on the seasonality, location, and harvest period of the leaves.

Declarations

Author contribution statement

Bruno Testa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Silvia Jane Lombardi, Vincenzo Macciola: Performed the experiments.

Mariantonietta Succi: Contributed reagents, materials, analysis tools or data.
Patrizio Tremonte: Analyzed and interpreted the data.
Massimo Iorizzo: Conceived and designed the experiments; Wrote the paper.

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The authors declare no conflict of interest.

Additional information
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