ETHANOLIC EXTRACT FROM LEAVES OF *Bixa orellana* L.: A POTENTIAL NATURAL FOOD PRESERVATIVE

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SUMMARY

This paper reports the minimum inhibitory concentration (MIC) of ethanolic extract from leaves of Bixa orellana *L. against bacteria and fungi of interest in the food industry, such as* Escherichia coli*,* Bacillus cereus*,* Pseudomonas aeruginosa*,* Staphylococcus aureus*,* Salmonella typhimurium*,* Shigella sonnei*,* Listeria monocytogenes*,* Candida albicans*,* Saccharomyces cerevisiae*,* Aspergillus niger*,* Penicillium chrysogenum *and* Byssochlamys fulva*. In addition, the antioxidant activity of the ethanolic extract was determined by evaluating their scavenging of the DPPH radical. The ethanolic extract exhibited a broad spectrum of antimicrobial activity for both Gram positive and Gram negative bacteria with MICs of 256-1024ppm. For MICs between 1 and 512ppm, fungi showed greater sensitivity to extract than bacteria. Nisin used as positive control caused growth inhibition of all bacteria tested, with MICs between 2 and 1024ppm. In contrast, fungi were not inhibited by nisin. Results also indicated that the ethanolic extract had good scavenging of DPPH radical with an* EC_{50} *of 7710 ±0.6318ppm.*

Introduction

Foodborne diseases, according to World Health Organization (WHO), is one of the most common health problems in the contemporary world and an important cause of loss in productivity (SIVIGILA, 2001). The Disease Control and Prevention Center in the USA estimates that each year 76 million people get sick, more than 300000 are hospitalized and 5000 die as a result of foodborne illness (Satcher, 2000). In 2009 there were 13161 cases of foodborne diseases reported in Colombia. Among the microorganisms isolated from food and implicated in those outbreaks are *Escherichia coli*, mold, yeast, *Salmonella* spp., mesophylls and coagulase-positive *staphylococci*. Some of the reported symptoms included vomiting, abdominal cramps, fever, dizziness, headache and diarrhea (SIVIGILA, 2009).

Traditionally, among the methods to prevent food contamination are (Kabak *et al*., 2006; Leistner, 2000) heating, reduced water activity, fermentation and the addition of antimicrobial agents (preservatives). The addition of preservatives has been an effective method to control microbial contamination, although in recent years popular demand has shown a marked aversion to synthetic chemical preservatives (Rojas and Vargas, 2008). This has resulted in a growing demand for natural products (plant extracts) which are presumably safer, functional (antimicrobial and antioxidant) and provide nutritional and health benefits. This demand has increased the importance of studies of alternative sources of natural preservatives rich in phenolic compounds (Cowan, 1999; Urquiaga and Leighton, 2000).

The medicinal plant *Bixa orellana* L. (achiote, orlean, roucou or annatto) is an ornamental shrub 3-5m tall with red flowers, native to Central and South America. Its leaves have been used in traditional medicine as an anti-emetic, for the treatment of gonorrhea, as a gargle to cure sore throats, as laxative, as antipruritic, as antipyretic agent, for the treatment of oral tumors, dysentery, jaundice and hepatic diseases (Cáceres *et al*., 1990; Shilpi *et al*., 2006). The mentioned applications are evidence of the antimicrobial activity of the plant, which has also been proven by various authors (Cáceres *et al*., 1990; Irobi *et al*., 1996; Castello *et al*., 2002; Fleischer *et al*., 2003). Coelho *et al*. (2003) conducted a study of antimicrobial activity of extracts of *B. orellana* by the agar diffusion method. They evaluated activity on the fungi *Cryptococcus neoformans* and *Candida albicans* obtained from clinical samples; their results showed that both fungi were resistant to all extracts used (leaf, root, stem and fruit). Irobi *et al*. (1996) reported similar results with ethanol extracts from leaves on *Candida subtilis* and *Aspergillus niger*. Navarro *et al*. (2003) studied and identified antimicrobial activity from leaf and stem extracts of *B. orellana* on the fungi *Trychophyton mentagrophytes* and *T. rubrum* and determined minimum inhibitory concentrations of 2mg/ml of hexane extracts. Cáceres *et al*. (1995) investigated 46

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EXTRACTO ETANÓLICO DE HOJAS DE *Bixa orellana* L.: UN POTENCIAL CONSERVANTE NATURAL DE ALIMENTOS

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RESUMEN

Este trabajo estableció la concentración mínima inhibitoria (CMI) del extracto etanólico de hojas de Bixa orellana *L. contra bacterias y hongos de interés alimentario, tales como* Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium, Shigella sonnei, Listeria monocytogenes, Candida albicans, Saccharomyces cerevisiae, Aspergillus niger, Penicillium chrysogenum *y* Byssochlamys fulvas*. Además, se determinó la actividad antioxidante del extracto etanólico evaluando su capacidad atrapadora del radical DPPH. El extracto exhibió un amplio espectro de acción antimicrobiana tanto para bacterias Gram positivas como Gram negativas, con CMI entre 256 y 1024ppm. Los hongos mostraron mayor sensibilidad al extracto que las bacterias con CMI entre 1 y 512ppm. La nisina, utilizada como control positivo, ocasionó una inhibición del crecimiento de todas las bacterias evaluadas con CMI entre 2 y 1024ppm, mientras que los hongos no fueron inhibidos. El extracto etanólico de hojas de B. orellana mostró buena capacidad atrapadora del radical DPPH con una EC_{50} de 7710 ±0,6318ppm.*

EXTRATO ETANÓLICO DE FOLHAS DE *Bixa orellana* L.: UM POTENCIAL CONSERVANTE NATURAL DE ALIMENTOS

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RESUMO

Este trabalho estabeleceu a concentração mínima inibitória (CMI) do extrato etanólico de folhas de Bixa orellana *L. contra bactérias e fungos de interesse alimentário, tais como* Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium, Shigella sonnei, Listeria monocytogenes, Candida albicans, Saccharomyces cerevisiae, Aspergillus niger, Penicillium chrysogenum *y* Byssochlamys fulvas*. Além disso, se determinou a atividade antioxidante do extrato etanólico avaliando sua capacidade sequestradora do radical DPPH. O extrato exibiu um amplo espectro de ação antimicrobiana tanto para bactérias Gram positivas como Gram negativas, com CMI entre 256 e 1024ppm. Os fungos mostraram maior sensibilidade ao extrato que as bactérias com CMI entre 1 e 512ppm. A nisina, utilizada como controle positivo, ocasionou uma inibição do crescimento de todas as bactérias avaliadas com CMI entre 2 e 1024ppm, enquanto que os fungos não foram inibidos. O extrato etanólico de folhas de B. orellana mostrou boa capacidade sequestradora do radical DPPH com uma EC_{50} de 7710 ±0,6318ppm.*

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plants including *B. orellana*; they measured the antimicrobial activity from leaves and roots against five strains of *Neisseria gonorrhoeae* and found activity only for the leaves extract.

The *B. orellana* pharmacological properties are directly related to the chemical composition of the leaves. Some of the chemical compounds isolated from the leaves include flavonoids, heterosides, sulphated derivates, diterpenes, gallic acid, pyrogallol and essential oils (Schneider *et al*., 1965; Chaco *et al*., 1969; Raga *et al*., 2011). In addition to the antimicrobial properties, polyphenols have also shown other biological properties such as that of antioxidant agents. It has been reported) (Ugartondo *et al*., 2007) that polyphenols inhibit the oxidation of low density lipoprotein LDL related to coronary heart disease and protect DNA from oxidative damage (age-related cancers. For their excellent antioxidant activity and biological functions, some authors have suggested the polyphenols as substitutes for existing synthetic antioxidants, as they can provide technological, scientific, nutritional and medicinal benefits (Harborne and Williams, 2000; Martínez-Valverde *et al*., 2000).

Antimicrobial activity studies have been limited to the evaluation of microorganisms that cause diseases unrelated to food, and the controls have been antibiotics commonly used in the treatment of these diseases (Cáceres *et al*., 1995; Coelho *et al.,* 2003; Fleischer *et al.* 2003; Shilpi *et al*., 2006; Metta *et al*., 2009). The present work presents the potential applications of the ethanolic extract from *Bixa orellana* leaves (EELB) as a food preserver (antimicrobial and antioxidant agent). The antimicrobial activity against bacteria and fungi involved in food contamination was evaluated and compared to other preservatives commonly used in the food industry.

Materials and Methods

Preparation of ethanolic extract from Bixa orellana leaves

Leaves were collected in the municipality of San Luis, Antioquia, Colombia, located at 06°02'N 74°59'O, 1050masl, and were identified as *Bixa orellana* L. red variety at the Universidad de Antioquia Herbarium. The leaves were dried in a conventional oven at 37 ±0.2ºC during 48h. The dry leaves were subjected to an extraction process with 95% ethanol (Merck®, Germany) during 48h. The resulting extract (EELB) was concentrated in a rotary evaporator (Büchi R-124), followed by lyophilization and storage at a temperature of 4 ±0.2°C

Microoganisms used

The food microorganisms or interest used for testing were *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 8739, *Pseudomonas*

aeruginosa ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Shigella sonnei* ATCC 29930, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 2601, *Aspergillus niger* ATCC 16404, *Penicillium chrysogenum* ATCC 10106 and *Byssochlamys fulvas* ATCC 9406. All these species were supplied by ATTC (American Type Culture Collection).

Growth curves were constructed for the bacteria and yeasts to establish the time of exponential growth phase of each microorganism. However, no growth curves were traced for *A. niger*, *P. chrysogenum* and *B. fulvas*, since the determination of minimum inhibitory concentration (MIC) requires that the microorganism be in its spore form; in these cases, crops of molds were prepared in slanted tubes (37 ±0.2°C) until sporulation, spores were scraped with sterile water and this suspension at 0.1 absorbance (600nm) was used in the broth microdilution.

Minimum inhibitory concentration of the extract

The colorimetric broth microdilution method proposed by Abate *et al*. (1998) was used, with some modifications, in the evaluation of the minimum inhibitory concentration (MIC) of the EELB. Diluted solutions of the extract were prepared. The concentration of EELB ranged from 1 to 1024ppm. The diluted extracts were placed in 96-well microplates (Becton Dickinson Labware®, USA) and then culture medium and the microorganism in the exponential growth phase (~1.5×10^8 CFU/ml) were added in each well. After incubation at 37 ±0.2°C for 5h, a 0.8mg·ml^{-1} tetrazolium salt solution, also known as MTT (3-{4.5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide; Alfa Aesar®, Germany), was added into each well and subjected to further incubation at 37 ±0.2°C for 1h, in order to allow the viable microorganisms to metabolize the yellow dye MTT into formazan (purple crystals) (Foongladda *et al*., 2002). The MIC value was considered as the concentration at the first well that did not undergo color change (from yellow to purple). This procedure was successfully applied to all bacteria. In the case of fungi, the incubation time was increased to 24h. The complete procedure was repeated three times for each microorganism.

Culture media used were Mueller-Hinton broth for bacteria and Sabouraud dextrose broth (both from Merck®, Germany) for fungi. After determining the MIC, the wells whose microorganism did not experience any growth were transplanted to a solid medium. After 24h of incubation, the concentration at which there was no microorganism growth was considered as the minimum bactericidal concentration against bacteria and the minimum fungicidal concentration against fungi.

Nisin, which is the only bacteriocin widely accepted as natural food preserver (Abee *et al*., 1995) was employed as a control for bactericidal effects.

Total phenolic compounds in the extract

In order to determine total phenolic compounds, 100µl of the EELB solution (5mg extract/ml methanol) was brought to 500µl using distilled water. The solution was then mixed (1:1) with 250µl of Folin-Ciocalteu reagent (Merck®, Germany) and subjected to sonication for 5min. The sonicated solution and 1250µl of 20% Na_2CO_3 (Merck®, Germany) were mixed and left to rest for 2h in the dark. The solution absorbance at 725nm was measured and was expressed as mg tannic acid/g extract). The procedure was repeated three times for each sample (Singleton and Rossi, 1965).

In vitro antioxidant activity of the extract

Antioxidant activity was measured *in vitro* using the DPPH free radical. The EELB was diluted in methanol (Merck®, Germany) at concentrations ranging from 0.039 to 2000mg·ml^{-1} and then mixed in proportion 10:990 by volume with 25µl of a solution of DPPH in methanol (A517, control= 0.300). This mixture was incubated in the dark at room temperature for 30min. The absorbance of the sample was measured in a spectrophotometer (Spectronic 20, Genesys®) at 517nm against a blank. The antiradical activity was defined as the amount of antioxidant necessary to decrease to 50% the initial concentration of DPPH. Ascorbic acid (Mol Labs®, Colombia) and tannic acid (Carlo Erba®, Italy) were used as references. (Brand-Williams *et al*., 1995). The experimental values are reported as mean ± SD (standard deviation) of three samples.

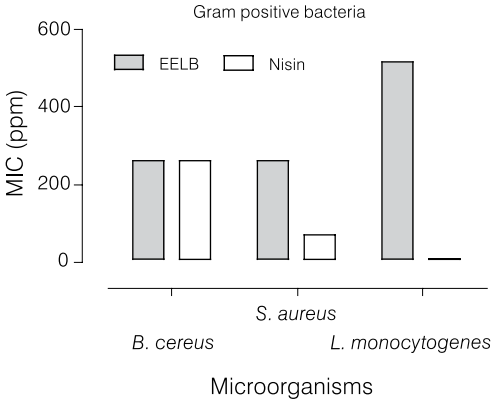

Figure 1. Minimun inhibitory concentration (MIC) of ethanolic extract from leaves of *B. orellana* (EELB) and nisin against Gram positive bacteria.

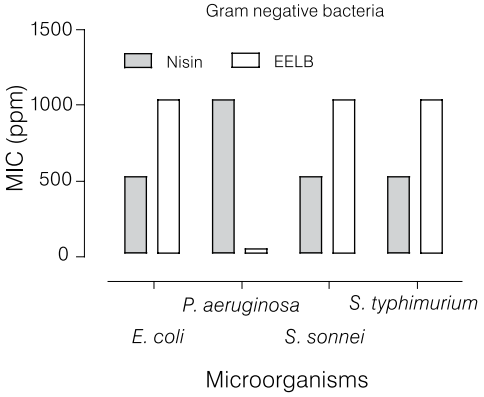

Figure 2. Minimun inhibitory concentration (MIC) of ethanolic extract from leaves of *B. orellana* (EELB) and nisin against Gram negative bacteria.

Results and Discussion

Minimum inhibitory concentration

The antimicrobial activity of EELB and nisin against the Gram positive bacteria is represented in Figure 1. The MIC of EELB was found to be 512ppm for *L. monocytogenes* and 256ppm for both *S. aureus* and the *B. cereus*. As comparison, the MIC of nisin was found to be 2, 64 and 256ppm for *L. monocytogenes, S. aureus and B. cereus* respectively. The results were consistent with the MIC value of 62.5ppm for EELB against *S. aureus* as reported by Metta *et al*. (2009), while Irobi *et al*. (1996) reported MIC of ethanol extract of the leaves of *B. orellana* at higher doses (4000 to 16000ppm) against Gram positive bacteria.

The antimicrobial activity of EELB and nisin against the Gram negative bacteria is shown in Figure 2. MIC for EELB was found to be 512ppm for *E. coli*, *S. sonnei* and *S. typhimurium* and 1024ppm for *P. aeruginosa*. In comparison, MICs for nisin were 1024ppm for *E. coli*, *S. sonnei* and *S. typhimurium* and 32ppm for *P. aeruginosa*. The EELB was found to have a higher antimicrobial activity than nisin and, therefore, it could become a good alternatives as natural preserver in the food industry. These results are consistent with those reported by Fleischer *et al*. (2003), who evaluated the antimicrobial activity of ethanolic extract from leaves of *B. orellana* L. against: *P. aeruginosa*, *E. coli* and *S. typhimurium*, obtaining diameter of inhibition zone

of 19, 22.5 and 17mm in diameter, respectively, while Shilpi *et al*., (2006) found that the methanolic extract from *B. orellana* leaves showed an inhibition zone against *E. coli* but not against *S. sonnei.*

The results of the antibacterial activity test using disk diffusion technique showed minute average inhibition zones that may be caused by limited diffusion of the extracts through agar. However, in broth dilution techniques, the results showed better antibacterial activity as the extracts were directly exposed to the microbes (Metta *et al*., 2009).

Results for the MIC of EELB and nisin against fungi are shown in Figure 3. At a MIC of 512ppm EELB was able to inhibit the growth of *C. albicans* and *S. cerevisiae.* A MIC of 256ppm of EELB was required for *A. niger* and *B. fulva;* while, *P. crysogenum* was inhibited with a MIC of 1ppm. Nisin showed no antifungal effect at the concentrations tested (1-1024ppm) against *C. albicans, P. crysogenum*, *A. niger* and *B. fulva.* For *S. cerevisiae* the MIC of nisin was found to be 256ppm.

The present antifungal activity results are in contrast with those of Navarro *et al*., (2003), who found that the methanol extract from leaves of *B. orellana* was not active against *C. albicans* and *A. niger*. On the other hand, Tamil *et al*. (2011) demonstrated that a leaf extract of *B. orellana* at 1000ppm showed significant inhibition against *C. albicans, A. niger* and the dermatophytes *Trichophyton mentagrophytes* and *T. rubrum*. The differencess in these results may be related to factors that affect directly the quality and quantity of secondary metabolites present in the final extract, such as environmental conditions, rainfall, soil nutrient concentration or time of collection, among others (Bourgaud *et al*., 2001).

The bactericidal and fungicidal activities of EELB were further studied by transplanting the wells that showed no color change to a solid medium, evaluating the bactericidal and fungicidal activity of EELB against all microorganisms tested at the MIC values found in this work. This study has proven the broad spectrum bactericide and fungicide of EELB against microorganisms of interest in the food industry, in contrast with the results of Irobi *et al*. (1996), who found minimal bactericidal concentrations of 95% hydroalcoholic extract from leaves of *B. orellana* only against Gram positive bacteria, while Coelho *et al.,* (2003) showed that this extract has antimicrobial activity against some bacteria of clinical interest, both Gram positive and Gram negative.

The total phenols in EELB were quantified as 99.86 ±0.78mg tannic acid/g extract. Several studies have shown that the alcoholic extracts from leaves of *B. orellana* consist of chemical compounds such as flavonoids, sesquiterpenes, saponins, tannins, alkaloids and steroids (Chaco *et al*., 1969; Lawrence and Hogg, 1973; Harborne and Williams, 2000).

Several studies have been conducted to determine the compounds responsible for the antimicrobial activities of some plants, and found that flavonoids and tannins have antibacterial properties (Coelho *et al*., 2003). The inhibitory activity of leaf and seed extracts of *B. orellana* could be attributed to the presence of flavonoids (Tamil *et al*., 2011). The activity of flavonoids seems to be related to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Tsuchiya *et al*., 1996; Cushnie and Lamb, 2005). On the other hand, Raga *et al*. (2011) isolated and identified a bioactive sesquiterpene (ishwarano) from a dichloromethane extract from leaves of *B. orellana*. They demonstrated the antimicrobial activity of the compound against *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus*, but no activity against *B. subtilis* and *A. niger*. This result could be due to the antimicrobial power of a crude extract being related to different fractions that are compounds of different polarity, and in many cases the presence of all the compounds is required to reach a maximum antimicrobial activity, given the strong synergy among them (Dubey and Kishore, 1987).

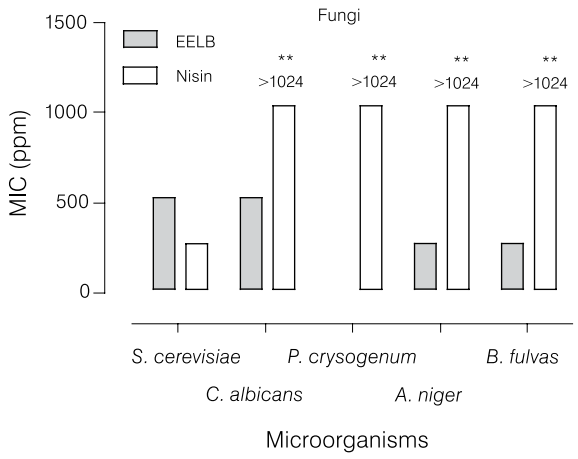

Figure 3. Minimun inhibitory concentration (MIC) of ethanolic extract from leaves of *B. orellana* (EELB) and nisin against fungi.
**Nisin did not inhibit the growth of *C. albicans, P. crysogenum, A. niger* and *B. fulvas* in the range of concentrations tested (1-1024ppm).

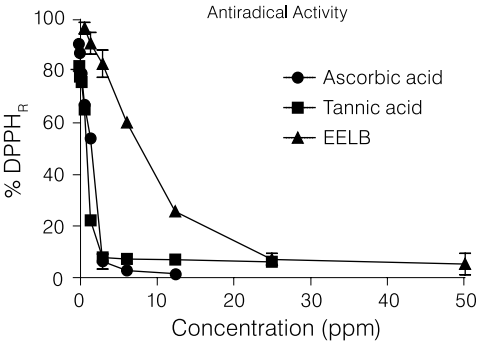

Figure 4. Antiradical activity of ascorbic acid, tannic acid and ehanolic extract from leaves of *B. orellana* (EELB).

In vitro *antioxidant activity*

Antioxidants are compounds that prevent the formation of colors and bad flavors in foods by delaying or inhibiting oxidative degradation of various molecules. It has been reported that phenolic compounds such as tocopherols, tocotrienols and flavonoids have a high capacity to capture free radicals (Urquiaga and Leighton, 2000). Figure 4 presents the percentage of remaining DPPH (DPPH$_R$) as a function of the concentrations of EELB, ascorbic acid and tannic acid. Ascorbic acid was selected for its common use in the food industry, and tannic acid for being reported as a metabolite present in the EELB. According to the results, EELB presents less antiradical activity than ascorbic and tannic acids. The lowest concentration of DPPH$_R$ (<10% DPPH$_R$) requires about 25ppm of EELB and only 3.1ppm of either ascorbic or tannic acid.

The concentration at which the DPPH decreases to 50% of the initial concentration is also known as the effective concentration (EC_{50}). For ascorbic and tannic acid the EC_{50} values obtained were 1.73 ±0.02 and 1.06 ±0.01ppm, respectively, while for EELB the EC_{50} found was 7.71 ±0.63ppm. The statistical analysis demonstrates that there is a statistically significant difference between controls and EELB. Enciso Gutiérrez *et al.* (2010) showed that the hydroalcoholic extract from *B. orellana* leaves has the highest polyphenol content of four medicinal plants studied, as well as the highest concentration of flavonoids, a result that kept the high correlation with the antioxidant capacity tested.

The results of antiradical activity of EELB are positive because the EC_{50} (7.71

±0.63ppm) of the extract is below 1024ppm, which is the highest MIC for antimicrobial activity used in this study. The antiradical properties combined with the bactericidal and fungicidal activities increases the potential application of EELB as a natural food preserver.

Another advantage offered by the EELB is its low toxicity. Shilpi *et al.* (1995) performed acute toxicity tests in mice with a methanol extract from *B. orellana* leaves and concluded that the extract had no toxic effects during the evaluation period at doses up to 4000mg·kg^{-1}. Similary, Dha-Wan *et al.* (1977) reported that the plant has not cytotoxic effects.

Conclusions

The ethanolic extract from *Bixa orellana* leaves (EELB) presents a broad spectrum of inhibition against Gram positive and negative bacteria, and fungi. The antimicrobial activity of EELB is greater on the Gram positive bacteria *B. cereus* and *S. aureus*. The more sensitive Gram negative bacteria were *E. coli*, followed by *S. sonnei* and *S. typhimurium*. Among the fungi, molds were more sensitive to EELB that yeasts.

The minimum inhibitory concentrations (MIC) of EELB for most of the microorganisms used in this study were much lower than those found for nisin, which makes EELB a valid alternative for use as natural preservative in food matrices, due to its broad spectrum of antimicrobial action against bacteria and fungi associated with contaminated meat, dairy products and vegetables. Moreover, considering its good *in vitro* antiradical capacity, the possible replacement of synthetic antioxidants by EELB should be considered.

The understanding of inhibition mechanisms must direct the research on development of natural plant preservatives and its potential application in food matrices.

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