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 30,000 are hospitalized and 500,000 die as a result of foodborne illness (Satcher, 2000). In 2009 there were 13,161 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 Varas, 2006). 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; Urquiga 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 anti-pruritic, 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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EXTRATO ETANÓLICO DE HOJAS DE Bixa orellana L.: UN POTENCIAL CONSERVANTE NATURAL DE ALIMENTOS

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RESUMEN

Este trabajo estableció la concentración mínima inhibidora (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 Bysschlamys 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 EC50 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 inhibidora (CMI) do extrato etanólico de folhas de Bixa orellana L. contra bactérias e fungos de interesse alimentar, tais como Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium, Shigella sonnei, Listeria monocytogenes, Candida albicans, Saccharomyces cerevisiae, Aspergillus niger, Penicillium chrysogenum e Bysschlamys fulvas. Além disso, se determinou a atividade antioxidante do extrato etanólico avaliando sua capacidade seques- tradora 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 inhibidos. O extrato etanólico de folhas de B. orellana mostrou boa capacidade sequestradora do radical DPPH com uma EC50 de 7710 ±0,6318ppm.
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 1024 ppm. The dilution method proposed by Muller-Hinton 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 24 h 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.

In order to determine total phenolic compounds, 100μl of the EELB solution (5 mg 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 5 min. The sonicated solution and 1250μl of 20% Na₂CO₃ (Merck®, Germany) were mixed and left to rest for 2 h in the dark. The solution absorbance at 725 nm was measured and was expressed as mg tannic acid/g extract. The procedure was repeated three times for each sample (Singleton and Rossi, 1965).

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 2000 mg·ml⁻¹ and then mixed in proportion 10:990 by volume with 25 μl of a solution of DPPH in methanol (A517, control = 0.300). This mixture was incubated in the dark at room temperature for 30 min. The absorbance of the sample was measured in a spectrophotometer (Spectronic 20, Genesys²) at 517 nm 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.

**Results and Discussion**

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 512 ppm for L. monocytogenes and 256 ppm for both S. aureus and the B. cereus. As comparison, the MIC of nisin was found to be 2, 64 and 256 ppm for L. monocytogenes, S. aureus and B. cereus respectively. The results were consistent with the MIC value of 62.5 ppm 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 16000 ppm) 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 512 ppm for E. coli, S. sonnei and S. typhimurium and 1024 ppm for P. aeruginosa. In comparison, MICs for nisin were 1024 ppm for E. coli, S. sonnei and S. typhimurium and 32 ppm for P. aeruginosa. The EELB was found to have a higher antimicrobial activity than nisin and, therefore, it could become a good alternative 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 differences 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 (Bourgad 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 bactericidal and fungicidal 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 (Tsuihya et al., 1996; Cushnie and Lamb, 2005). On the other hand, Raga et al. (2011) isolated and identified a bioactive sesquiterpene (ishwarrano) 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.

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 (DPH₃) 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 DPH₃ (<10% DPH₃) 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 (EC₅₀). For ascorbic and tannic acid the EC₅₀ values obtained were 1.73 ±0.02 and 1.06 ±0.01ppm, respectively, while for EELB the EC₅₀ was found to be 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 EC₅₀ (7.71
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