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Synergistic effects of ethanolic plant extract mixtures against food-borne pathogen bacteria

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Plant extracts are an important part in agroecology, as they benefit environment in combating pathogenic organisms, without resorting to synthetic chemicals. The objective of this study was to evaluate the antibacterial activity of mixtures of ethanol extracts from semi-desert plants [creosote bush (*Larrea tridentata*), tarbush (*Flourensia cernua*) and paddle cactus (*Opuntia ficus-indica*)] against *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. The maximum antimicrobial activity was achieved with the creosote bush–tarbush-paddle cactus mix (1:1:1) v/v, followed by paddle cactus-tarbush (1:1) v/v. *E. coli* was the bacterial strain that showed the highest growth inhibition as consequence of the concentration of plant extracts (4000 and 5000 ppm of tarbush). While with the creosote bush plant extracts, the highest inhibition halos were observed. Synergistic effects were observed when mixtures of ethanolic plant extract against food-borne pathogen bacteria were used, so this may be a better way to design alternative pathogen control methodologies for food-borne pathogen bacteria.

Key words: *Larrea tridentate*, *Flourensia cernua*, *Opuntia ficus-indica*, ethanolic plant extract mixtures, food-borne pathogen.

INTRODUCTION

Food-borne pathogen bacteria are one of the major public concerns worldwide (Tayel and El-Tras, 2010). A variety of microorganisms also lead to food spoilage that is encountered as one of the most important matter concerning the food industry. So far, many pathogens microorganisms have been reported as causal agents of food-borne diseases and/ food spoilage (Natta et al., 2008). Prevention of pathogenic and spoilage microorganisms in food is usually achieved by using synthetic chemical preservatives but some of them are responsible for many carcinogenic and teratogenic attributes as well as residual toxicity, and with growing concern of microbial resistance toward conventional synthetic preservatives (Pundir et al., 2010). In addition, the continuous spreads of multi-drug resistant pathogens have become a serious threat to public health and a major concern for infection control practitioners (Iwalokun et al., 2004). All of the above mentioned are concerns that have put pressure on the food industry for progressive removal of synthetic chemical preservatives and adoption of natural alternatives to obtain its goals concerning safe food with long shelf lives (Agatemor, 2009). In addition, at the present time, there

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Abbreviation: CT, Condensed Tannins; **HT,** hydrolysable tannins; **C,** creosote bush; **T,** tarbush; **P,** paddle cactus.

is a tendency to create environmentally friendly products (Lira-Saldivar et al., 2003).

Many plant derived products such as spices, fruit preparations, vegetal preparations or plant extracts have been used for centuries for preservation and extension of shelf life of foods (Pundir et al., 2010). Today, scientific research reveals that not only chemicals from plants have an effect against a particular disease. But also the antioxidant property from plant extracts gives a beneficial effect to human health (Puangpronpitag and Sittiwet, 2009). Different extracts of plants used for treatment of some gastrointestinal disorders, may be successfully applied to elimination of food borne bacteria (Tayel and El-Tras, 2010). On the other hand, the perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals and the reduced cost of plant preparations (Cock, 2008), suggests the idea that some plant extracts may be used as natural antimicrobial additives to reclaim the shelf –life of foods (Pundir et al., 2010). Considerable research on antimicrobial plant extracts have been reported (Sharma and Hashinaga, 2004; Dupont et al., 2006; Sutherland et al., 2009). But rarely the combined effect of two or three plant extracts on growth of food-borne pathogen bacteria has been reported. Mexican flora is one of the most diverse worldwide due to number of species, biological variability levels and climatic diversity. In this country, more than 200 plant species with antifungal and antibacterial activities, mainly against plant pathogens have been reported (Montes et al., 2000).

The state of Coahuila is a region with a high biodiversity (endemic flora and fauna); some representative plants of the flora region are creosote bush (*Larrea tridentata)*, tarbush (*Flourensia cernua*) and paddle cactus (*Opuntia ficus-indica*). These plants are characterized by a large number and variety of polyphenols. Among the phenolic compounds present are: gallotannins, ellagitannins and condensed tannins, which have protective functions against microorganisms (Osorio et al., 2010). Plant extract consist of a combination of active products of plant metabolism. The large number of chemical compounds that extracts have may interact synergistically, so the resulting effects are higher than those using each component separately (Viveros and Castaño, 2006). Currently, agriculture and food industry uses various synthetic chemicals, but, some of them may promote environmental and health risks, so, there is a tendency to create natural and environmentally friendly products. The use of different solvents like hexane, chloroform and methanol is not allowed in the context of organic production systems (Lira-Saldivar et al., 2003). For this reason, it is importance to study the use of plant extracts obtained organically and identify their applications on inhibition of food-borne pathogens.

The aim of this study was to evaluate the effect of ethanol extract mixtures from *L. tridentata*, *F. cernua* and *O. ficus-indica*, on inhibition of food-borne pathogen bacteria.

MATERIALS AND METHODS

Vegetal material

Leaves and stems of creosote bush (*L. tridentata*), tarbush (*F. cernua)* and paddle cactus (*O. ficus-indica*) were collected from areas nearby to Saltillo, Coahuila Mexico (samples were collected by Diana Morales, and were identified with the numbers CB-03-2010, TB-24-2010 and PC-09-2010 for creosote bush, tarbush and paddle cactus, respectively). Each vegetal tissue was dehydrated at 60°C for two days. The dried samples were grinded in a miller and the powder was sieved at 1 mm. The fine powder obtained was stored in amber bottles or dark plastic bags at room temperature until phytochemical compounds extraction was performed.

Extraction of phytochemical compounds

Each fine powder sample (100 g) was mixed in an Erlenmeyer flask in a 1:4 (w/v) ratio with 70% ethanol. The flask was covered with aluminium foil to avoid light exposition. The mixture was refluxed at 60°C for 7 h. After this, extracts were filtered using Whatman filter paper No. 4. The solvent was removed using a rotary evaporator (Yamato RE540) using a temperature below 60°C, the sample was stored at 5°C in containers covered with aluminum foil until phytochemical analysis were performed.

Analysis of phytochemical compounds

Assay for quantification of tannins of the ethanol extracts was performed using the method reported by Waterman and Mole (1994).

Tannins concentration

Concentration of Condensed Tannins (CT) was spectrophotometrically determined using the method proposed by Swain and Hillis (1959). For condensed tannins determination, an aliquot of 0.5 ml of plant extract was placed in a tube, with 3 ml of HCl/butanol (1:9) and 0.1 ml of ferric reagent.

Analytical standard

On the other hand, it was added to a tube assay serie, [Catechin standard, (+)-Catechin 43412 Fluka from Sigma-Aldrich] in distilled water at different concentrations (0, 200, 400, 600, 800 and 1000 ppm) to obtain a reference curve. The tubes were plugged tightly and heated for 1 h in a water bath at 100°C. After that, they were left to cool and absorbances were read at 460 nm. The concentration of hydrolysable tannins (HT) was determined by the traditional method of Folin-Ciocalteu according to the protocol reported by Makkar (1999), a reference curve was done placing gallic acid to different concentrations (0, 200, 400, 600 and 800 ppm) in assay tubes. The solution of stock gallic acid was to a concentration of 500 ppm and prepared using distilled water. Each one of plant extracts was diluted in a test tube then immediately a 400 µL of commercial Folin-Ciocalteu reagent was added to each tube and the samples were vortexed and left for 5 min. Then, 400 µL of Na_2CO_3 (0.01 M) and 2.5 ml of distilled water was added. Finally, absorbances were read at 725 nm in an UV/visible spectrophotometer.

Antibacterial activity evaluation

For this study, we used four food-borne pathogenic bacteria, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* which were supplied by the Coahuila Public

Table 1. Effect of plant extracts and concentrations on growth inhibition of four different food-borne pathogen bacteria.

Source of variation	Degree of freedom	Mean squares	Pr>F
Replication	2	0.04	0.2603
Concentration (c)	4	10.62	<0.0001
Extracts (e)	12	18.75	<0.0001
Bacteria (b)	3	130.47	<0.0001
c*e	48	0.29	<0.0001
c*b	12	3.53	<0.0001
e*b	36	9.58	<0.0001
c*e*b	144	0.31	<0.0001
Error	518	0.03	<0.0001
Total	779		

Table 2. Area (cm^2) of inhibition halo of four food-borne pathogen bacteria, as an effect of different plant extract mixtures and concentrations.

Bacteria	Inhibition halo (cm^2)
Enterobacter aerogenes	2.71[a]
Salmonella typhi	1.94[b]
Escherichia coli	1.01[c]
Staphylococcus aureus	1.01[c]

Means with the same letter in the same column are not significantly different ($p \leq 0.05$) according to Tukey's multiple range test.

Health State Laboratory (Saltillo, Coahuila Mexico). Each bacterial strain was grown in brain-heart-infusion broth (BHIB). Antibacterial activity of plant extracts against four food-borne pathogens was evaluated in test tubes (16 × 150 cm) with screw cap. Each test tube was filled with 5 ml of sterile BHIB medium, and bacteria were added with a sterile inoculating loop [approximately 1.5 × 10(8) bacterial cells/ml]. Inoculated test tubes were incubated at 37°C for 24 h.

Extracts preparation

Pure extracts of creosote bush (C), tarbush (T) and paddle cactus (P) were obtained at five different concentrations (1000, 2000, 3000, 4000 and 5000 ppm). Also, the extract mixtures (C-P, C-T, P-T) in 1:1, 1:3 and 3:1 relations and the mixture (C-T-P) in a relation 1:1:1 were also included. All these mixtures were used in the same five concentrations too. As control we used a tablet of trimethoprim with sulfamethoxazole with a concentration of 4 and 0.8 mg, respectively. Three Petri plates were used for each treatment, in addition two control treatments (sterile water and BHIB medium without extract) were also included in this study. In each Petri plate we placed disks of paper filters (0.7 cm in diameter) soaked in the extract solution corresponding to a concentration.

Experimental design and data analysis

The experiment to determine the effect of different extract mixtures and concentrations on growth inhibition of four food-borne pathogen bacteria was established under a randomized complete block design with three replications under a factorial arrangement of treatments; where the considered factors were: treatments with thirteen levels (all extract combinations), concentrations with five levels (1000, 2000, 3000, 4000 and 5000 ppm) and bacterial species with four levels (*E. aerogenes*, *E. coli*, *S. typhi* and *S. aureus*). The response variable was the area (cm^2) of the bacterial growth inhibition halo. The data were analyzed using ANOVA. When it was needed, Tukey's multiple range procedure was used for treatments mean separation. In this case, statistical analyses were performed using InfoStat software. In order to determine if the extract combinations had an effect on bacterial growth inhibition, an analysis of mixture experiments design under a simplex centroide design was performed using Statgraphics software.

RESULTS

ANOVA results for the effect of three different plant extracts at different concentration on growth inhibition of four food-borne pathogens are showed in Table 1. There was no observed significant differences among replications, but significant differences (P<0.001) were observed for extract concentrations, extracts, bacteria and all interactions among these sources of variation. In addition, it was observed that as extract concentration increased so does bacteria growth inhibition. *E. aerogenes* and *S. typhi* were the food-borne pathogens where the growth was most inhibited as consequence of the plant extract mixtures and their concentrations. While S*. aureus* was the bacteria less inhibited (Table 2). Figure 1 shows that combination of three extracts in a 1:1:1 proportion was the best treatment for inhibition of bacterial growth, suggesting synergical effects among the extracts, with an inhibition halo of 3.13 cm. The pure extract of tarbush and its combinations with creosote bush or paddle cactus have the same inhibition halo that the pure extract of creosote bush (non-significant differences). *E. coli* and *S. aureus* were the lowest inhibited by the extracts and their combinations. Different combinations of extracts were tested, the lowest 1000 ppm; however, *E. coli* was only inhibited with the highest concentrations of tarbush extracts (4000 or 5000 ppm). On the other hand, *S. aureus* growth was inhibited only with the highest concentrations (5000 ppm) of the C: T (25-75) and C: P (25-75) extract

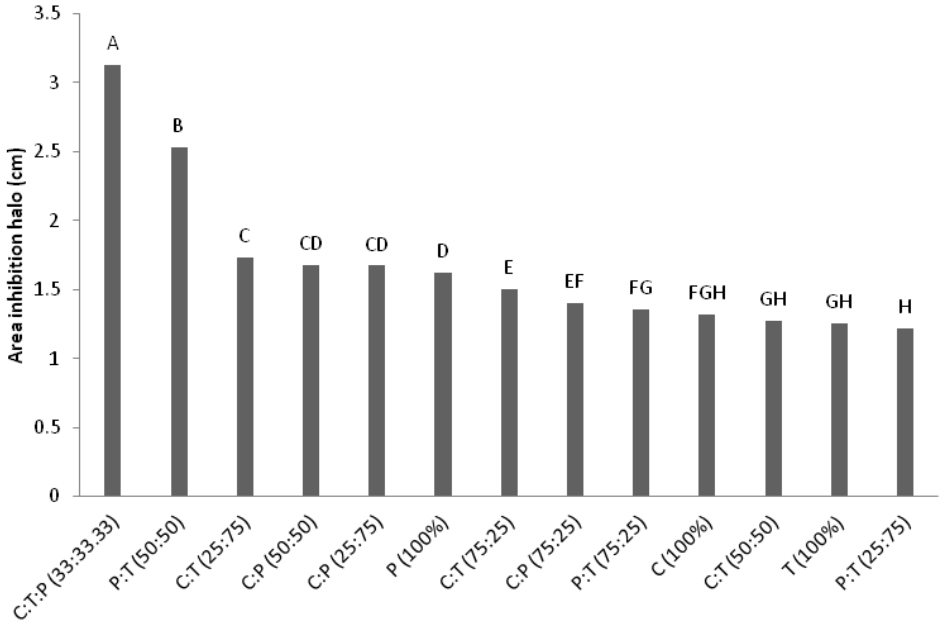

Figure 1. Evaluation of plant extracts as inhibitors, creosote bush (C), paddle cactus (P) and tarbush (T).

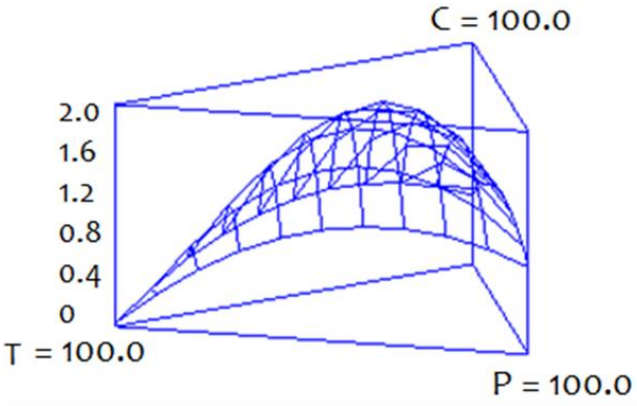

Figure 2. Growth inhibition of *Enterobacter aerogenes* by different plant extracts combinations.

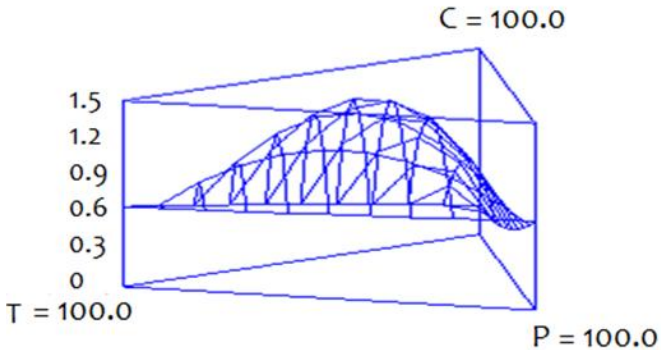

Figure 3. Growth inhibition of *Salmonella typhi* by different extract combinations.

combinations. Most of extract combinations had an inhibitor effect on *E. aerogenes* and *S. typhi*; therefore a mixture analysis was performed under a cubic model, and this analysis was performed using Statgraphics software.

The graphic of response surface showed that the combination of the three extracts (1:1:1) provoked the highest level of growth inhibition of *E. aerogenes* and *S. typhi* (Figures 2 and 3).

DISCUSSION

The significant differences observed among bacteria suggest that extract mixtures affect in a different way each of the tested food-borne pathogen bacteria; while differences among extract mixtures suggest that at least one extract mixture affect in a different way the bacteria growth. In addition, extract concentration has an effect on bacteria growth. A significant difference for the extract concentration-bacteria interaction indicates that a specific extract concentration promotes the highest growth inhibition of a specific bacterium. In same way, the extract-bacteria interaction showed significant differences, showing that a specific extract promotes the highest growth inhibition of a specific bacterium. In addition, the significant differences for the concentration-extract interactions suggest that a specific concentration in each extract is the best for growth inhibition of food-borne pathogen bacteria. Food-borne pathogenic bacteria were more inhibited by the highest extract concentration (Figure 4). It has been reported that extract concentration is very important for microbial control. Alvarez (1999) studied the effect of

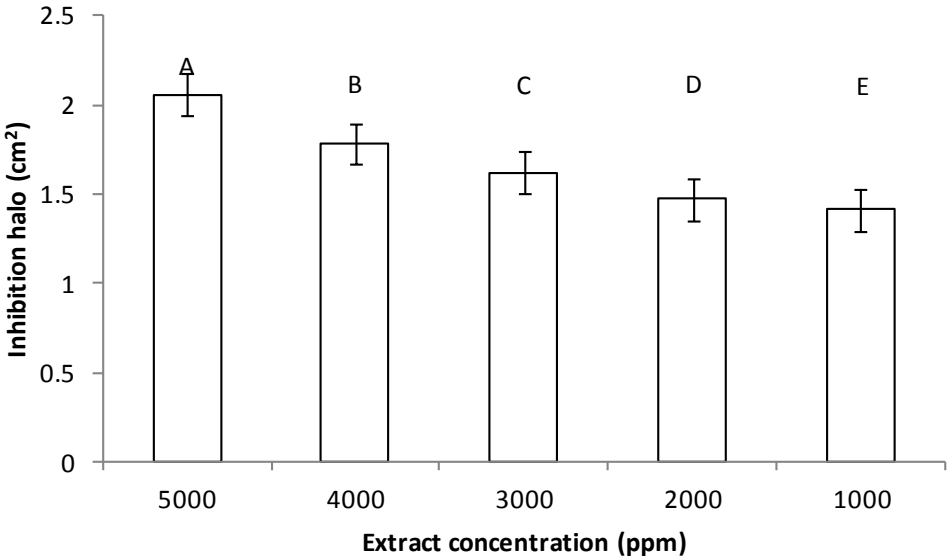

Figure 4. Influence of the extract concentration on bacterial inhibition halo area. Means with the same latter are not different, according to the Tukey multiple range test $p<0.05$. cm^2.

oregano powder and essential oil on growth inhibition of *S. aureus*, *E. coli* and *S. thyphimurium* finding better inhibitory effects with 150 to 200 ppm of essential oil and 1500 ppm of oregano powder for these three bacteria. On the other hand, Guerrero-Rodríguez et al. (2007) evaluated the effect of fresh leaves extract from *F. cernua* on mycelium inhibition and sporulation of three plant pathogens (*Alternaria alternata*, *Collectotrichum gloesporioides* and *Penicillium digitatum*). These extracts were obtained with a mixture of solvents [methanol:chloroform (1:1v/v)], and by sequential extractions with hexane, diethyl ether and ethanol. The highest *A. alternate* mycelial inhibition occurred when the extract was obtained using hexane and methanol:chloroform at 4000 ppm. *E. aerogenes* and *S. typhi* growth was most inhibited by the plant extracts (Table 2). Castillo-Godina (2008) reported that creosote bush, tarbush, pecan nut shells, damiana and leatherstem extracts promoted *E. aerogenes* growth inhibition as high as 60%. These results are similar to those obtained in this study. The different percentages of bacterial growth inhibition can be attributed mainly to the different chemical composition of these plant extracts. That may contain compounds such as diterpenes and flavones that may cause breakdown of bacterial cell membranes by action of terpenes and the bacterial specie membrane composition, which may be a barrier against the chemical extract (Urzua et al., 2006; Cowan, 1999). Figure 1 shows that combination of three extracts in a 1:1:1 proportion was the best treatment for inhibition of bacterial growth, suggesting synergical effects among the extracts, with an inhibition halo of 3.13 cm.

It has been reported that alcoholic extract of creosote bush has antifungal activity against species of *Aspergillus*, *Penicillium* and *Fusarium* (Tequida et al., 2002). López-Guerra et al. (2007) reported that creosote bush is a good source of condensed and hydrolysable tannins which represent 61.12% of the total phenolic content of this plant species. The amount of phytochemicals present in different extracts may be the explanation for the differences of antimicrobial activity among different plant extracts. Scalbert and Williamson (2000) mentioned that the antimicrobial properties of tannins may be because: tannins can form complexes with microbial enzymes and some proteins inhibiting their functions, tannins also inhibit electron transport through membranes and can alter ions like iron and copper thus inhibiting activity of some enzymes which may be essential for microbial life. *E. coli* was only inhibited with the highest concentrations of tarbush extracts; these extracts were those that had the highest polyphenols content and promoted the highest bacterial growth inhibition. Susceptibility of *E. coli* to plant extract has been reported previously; Mounchid et al. (2005) determined that *E. coli* is susceptible to essential oils of *Rosmarinus officinalis* L. and *Eucalyptus globules*. *S. aureus* growth was inhibited only with the highest concentrations (5000 ppm) of the C: T (25-75) and C: P (25-75) extract combinations. High concentration of plant extract for *S. aureus* inhibition had been reported before. Castaño et al. (2010) evaluated the bacterial activity of ethanolic extract and essential oil from rosemary *R. officinalis* L. leaves on microorganisms of interest in food industry: *E. coli*, *S. aureus*, *S. typhimurium*, *Shigella sonnei*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Lactobacillus plantarum*. The growth inhibition of *E. coli* was observed with a high concentration (4092 ppm) of rosemary essential oils.

On the other hand, Burt (2004) determined the minimum inhibitory concentration (MIC) of the rosemary essential oil against *S. typhimurium*, *B. cereus* and *S. aureus*, obtaining values of 20000, 2000 and 8000 ppm, respec-

tively. Synergic effect among creosote bush, paddle cactus and tarbush extracts at a specific concentration (1:1:1) is a good alternative for growth inhibition of *E. aerogenes* and *S. typhi* while that of *S. aureus* and *E. coli* showed the lowest growth inhibition. The highest bacterial growth inhibition was observed with the creosote bush and tarbush extracts and their combinations; this may be attributed to higher concentration of polyphenolic compounds in these plants.

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