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PLANT SCIENCE

Antibacterial and cytotoxic activities of *Acacia aroma* extracts

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Abstract

Acacia aroma, native plant from San Luis, Argentina, is used commonly like antiseptic and healing of wounds. The present study was conducted to investigate the *in vitro* antibacterial activity of extracts of *A. aroma* and its toxicity on Vero cell. The bacterial strains used were *Staphylococcus aureus* ATCC 43300, *S. aureus* ATCC 35556, *Listeria monocytogenes* CLIP 74910 and *L. monocytogenes* CLIP 74904. The minimal inhibitory concentration (MICs) and minimal bactericidal concentration (CBMs) was determined and tested at concentration ranges from 5000 to 78µg/mL. On the other hand, the cytotoxic activity on Vero cells was assayed by MTT method. The MIC values of EE and HAE against *S. aureus* was 156µg/mL and 625µg/mL respectively. MBC values were one or two fold higher than the corresponding MIC values in both extracts. *Listeria* strains studied gave MICs and MBCs values of 78µg/mL and 312µg/mLl respectively for EE, while HAE showed less antibacterial activity against this strain (MIC=1250µg/mL and MBC=5000µg/mL). The CC50 was 658µg/mL for EE and 1020µg/mL for HAE. The extracts of *A. aroma* tested *in vitro* showed inhibitory activity against *Staphylococcus* and *Listeria*. These results allow validating the external use of this plant.

Key words: *Acacia aroma,* Antibacterial activity, Cytotoxicity

Introduction

Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries. Bacterial species presents the genetic ability to acquire and transmit resistance against currently available antibacterials. These antimicrobial-resistant bacteria are the causes of numerous clinical problems worldwide and the development and increase of resistance among pathogens causing nosocomial and community-acquired infections and are associated with the widespread utilization of antibiotics (Harvey and Gilmour, 2001; Ruiz-Bolivar et al., 2008; Sakoulas and Moellering, 2008; Howden et al., 2010). Due to the constant emergence of microorganisms resistant to conventional antimicrobials, the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections, is very important to develop new antimicrobial drugs especially from natural products.

In developing countries, a large proportion of the population utilizes medicinal plants for the treatment of infectious diseases. In many places in Argentina is a rich tradition of using herbal medicine for the treatment of various infectious diseases, inflammations, injuries (Zampini et al., 2007; Mattana et al., 2010). Considering the vast potentiality of plants as sources for antimicrobial drugs several authors have investiged the antimicrobial activity of medicinal plants (Srinivasan et al., 2001; Kumarasamy et al., 2002; Masika and Afolayan, 2002; Hamill et al., 2003).

The genus *Acacia*, one of the important genera of the family *Fabaceae* includes aproximately 1350 species and is abundant in Australia, Africa, India and America. Number biological activities have been reported from various species of *Acacia* (Akhtar and Khan, 1985; Li et al., 2003; Solomón and Shittu, 2010; Ramli et al., 2011; Lakshmit et al., 2011). *Acacia aroma* Gill. Ex Hook et Arn, which common name is tusca, is a native plant of Argentina, widely distributed in central and northwest region (Burkat, 1952). This plant is used

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in Argentina folkloric medicine as wound healing, antiseptic and for the treatment of gastrointestinal disorders.

Phytochemistry studies on *Acacia aroma* indicated that the main components of the steam volatile flower oils were methyl salicylate and eugenol. Furthermore, fatty acid profiles of *A. aroma* seed were examined (Lamarque et al., 2000). Mattana et al. (2010) in TLC analysis revealed the presence of flavonoids and saponins. Antimicrobial activity of flavonoids and saponines has also been reported against methicillin-resistant *Staphylococcus aureus* (Alcaráz et al., 2000; Li et al., 2002; Soetan et al., 2006; Tanaka et al., 2007; Kannabiran et al., 2008).

There is little scientific information concerning the antimicrobial activity of *A. aroma.* In Argentina, have been reported only studies on the antimicrobial activity of this plant in Tucuman and San Luis (Arias et al., 2004; Mattana et al., 2010).

The purpose of the present study was to investigate antibacterial activity of hot aqueous and ethanolic extracts of leaves *A. aroma* against *Staphylococcus aureus* and *Listeria monocytogenes* and to determine the degree of toxicity of these extracts in Vero cells.

Materials and Methods

Plant material

Aerial parts of *A. aroma* were collected in January-March of 2010, in the Northwestern region of the province of San Luis, Argentina. Voucher specimens under the number 487, were deposited in the herbarium of the Botany Department, San Luis National University (UNSL). Leaves were used for the study.

Preparation of Acacia aroma extracts

Crude ethanol extracts (EE)

The *A. aroma* leaf powder was macerated in ethanol 95% (V/V) in a 1:3 proportion at room temperature, undergoing mechanical shaking for 4 h, followed by filtration. The extract obtained was concentrated in a rotavapor at 40°C and the vegetable residue was extracted twice again analogously, thereby obtaining the crude ethanol extract.

Hot aqueous extract (HAE)

The *A. aroma* dried and powdered leaves (30 g) were macerated in water (1,400 mL) at 70°C for 120 min. This process was repeated twice. The extract obtained was filtered and lyophilized.

To perform the assays *in vitro* the extracts were solubilised in distilled water and sterilized by filtration through a 0.2µ membrane filter (Microclar).

Microorganisms

The microorganisms used in this study were as followed: *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 35556, *Listeria monocytogenes* CLIP 74910 and *Listeria monocytogenes* CLIP 74904. All organisms were maintained in brain-heart infusion (BHI medium) containing 20% (v/v) glycerol at −20°C. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 Mc Farland.

Antibacterial activity

Determination of minimal inhibitory concentration (MIC)

The MICs of *A. aroma* hot aqueous extract (HAE) and ethanolic extract (EE) were determined by micro-well dilution in tripticase soy broth supplemented with 0.01% (W/V) of 2,3,5-triphenyltetrazolium chloride as visual indicator of bacterial growth (CLSI, 2011). The inicial inocula were diluted 10 times (10^7 CFU/mL). The extracts were dissolved in distilled water to the highest concentration to be tested (5000µg/mL), and then serial two-fold dilutions were made in concentration ranges from 5000 to 78 µg/mL. The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µL of the inoculum. One hundred microlitre aliquot from the stock solutions of the extracts and their serial dilutions initially prepared was transferred into seven consecutive wells. The final volume in each well was 200 µL. The plates were incubated at 37°C for 24 h. Controls were included. MIC was defined as the lowest concentration of the extracts in the medium in which there was no visible growth. The assays were performed in duplicate and then replicate at least twice.

Determination of minimal bactericidal concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bacterial growth. MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37°C.

Cytotoxicity assay

Cell culture

Cytotoxic assays were performed in Vero cells (*Cercopithecus aethiops* green monkey kidney epithelial cell line; ATCC CCL-81) grown in Eagle's minimal essential medium (EMEM) (Gibco, USA),

supplemented with 10% (v/v) heat-inactivated fetal calf serum (Natocor, Argentina), glutamine (30 mg/mL) and gentamicin (50 mg/mL) (Sigma–Aldrich, Italy). Cell cultures were maintained at 37ºC in a 5% (v/v) CO_2 humidified atmosphere.

Determination of 50% cytotoxic concentration

For cytotoxicity assay, the cells were cultured in 96-well culture plates (Cellstar, Greiner Bio-One, Germany). After incubation for 24 h at 37ºC, cells were exposed to increasing concentrations of the extracts. Assays were carried out in triplicate. Monolayers incubated only with EMEM were used as cellular controls. The concentration of the extracts which reduced the viable cell number to 50% (CC_{50}) was determined by MTT method. This colorimetric assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The CC_{50} was measured by the MTT method (Mosmann, 1983). Briefly, monolayers treated with extracts for 48 h at 37ºC were incubated with MTT solution for 4 h at 37ºC. Subsequently, the supernatant was removed and acid-isopropanol (0.04N HCl in isopropanol) was added. After gently shaking for 15 min, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, ELx800) at 570 nm. The Optical Density (OD) was measured at 560nm using a microplate ELISA reader (Labsystems Multiskan MS, Finland). The Survival Fraction % (SF%) in the treated cultures was calculated from the OD, in relation to cultures controls, that represent the 100% viability:

$$SF\ \% = \frac{\text{OD treated cells}}{\text{OD control}} \times 100$$

Statistical analysis

The CC50 values were calculated from concentration-effect curves after nonlinear regression analysis based on Boltzmann sigmoideal curve by the software *GraphPadPrism 5.0*. The results represent the mean ± standard error of the mean values of three different experiments.

Results and discussion

Antibacterial activity

The antibacterial activity of aqueous and ethanolic extracts of leaves of *A. aroma* was assayed against *S. aureus* and *L. monocytogenes* by the broth microdilution method. The ethanolic extract of *A. aroma* was the most active against the microorganisms studied. The Table 1 shows MICs and MBCs values against *S. aureus* and *L. monocytogenes.* This extract inhibited the growth of both microorganism at concentration of 156 µg/mL and 78 µg/mL respectively. The MBC values were one or two fold higher than the corresponding MIC values (625 µg/mL and 312 µg/mL respectively). In order to elucidate whether the observed antibacterial effects were bactericide or bacteriostatic, MBC/MIC ratios were calculated. Extracts with ratios greater than 1 were considered as bacteriostatic, while the other extracts were bactericide. These data allow concluding that all MIC values were bacteriostatic. Higher concentrations of ethanol extract were required to have for bactericidal effect. The hot aqueous extract (HAE) of *A. aroma*, showed a lower degree of antibacterial activity as compared with ethanolic extract. The HAE showed good biological activity against *S. aureus* (MIC=625µg/mL) however, against both strains of *Listeria* the activity was lower (MIC=1250µg/mL) (Table 1).

Some authors established that results would be considered significant if MIC or CBM ≤ 200 µg/mL (Suffredini et al., 2006). Our results with EE are in accordance with those studies. Ethanolic extract exhibited a higher degree of antibacterial activity, compared to aqueous extract. This observation confirmed the evidence from a previous study which reported that alcohol is a better solvent for extraction of antimicrobial substances from medicinal plants than water (Rojas et al., 2006).

Table 1. Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) obtained for ethanolic (EE) and hot aqueous extracts (HAE) from *Acacia aroma.*

Microorganisms	*MIC (µg/ml)*		*MBC (µg/ml)*	
	EE	*HAE*	*EE*	*HAE*
S. aureus ATCC 43300	625	156	625	1250
S. aureus ATCC 35556	625	156	625	1250
L. monocytogenes CLIP 74910	78	1250	312	5000
L. monocytogenes CLIP 74904	78	1250	312	5000

The biological activities of any medicinal plant are direct reflections of the effect and nature of the phytochemicals it contains. Previous phytochemical determination in ethanol and ethylacetate extracts from *A. aroma* done in our laboratory showed flavonoids and sapogenines compounds, and the bioautography assay demonstrated well-defined inhibition zones against *S. aureus* in correspondence with those flavonoids and sapogenines bands (Mattana et al., 2010). These phytochemicals are known to have various pharmacological activities including antimicrobial activity. Thus the presence of these compounds supports the traditional use of *A. aroma* in the treatment of wounds and skin infections.

Cytotoxic activity

The cytotoxic activity was carried out by using MTT assay. The results are graphically represented in Figures 1 and 2. Figure 1 shows the percentage of viability of Vero Cells, incubed 48 h in presence of ethanolic extract of *A. aroma* (EE) employed different concentrations. In this study, it was found that the 50% cytotoxic concentration (CC50) value was 658µg/mL for EE. This extract was not cytotoxic to Vero cells at bacteriostatics and bactericidal concentrations for all microorganisms tested.

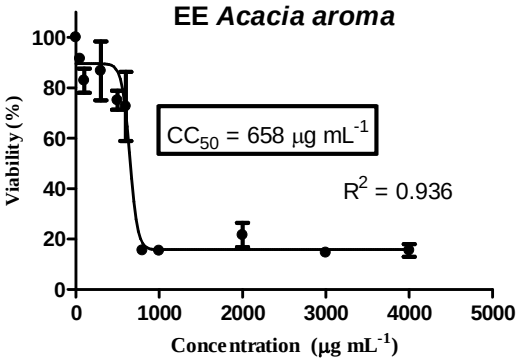

Figure 1. Percentage of viability of cultured Vero cells, incubated 48 h in the presence of ethanolic extract of *Acacia aroma* (EE) employed at different concentrations determined by MTT assay.
Each point represents the mean of four independent trials; CC50 was 658 µg/mL.

The CC50 of hot aqueous extract of *A. aroma* was 1020 µg/mL (Figure 2). This concentration showed not inhibitory activity against *L. monocytogenes,* however inhibited the growth of *S. aureus.*

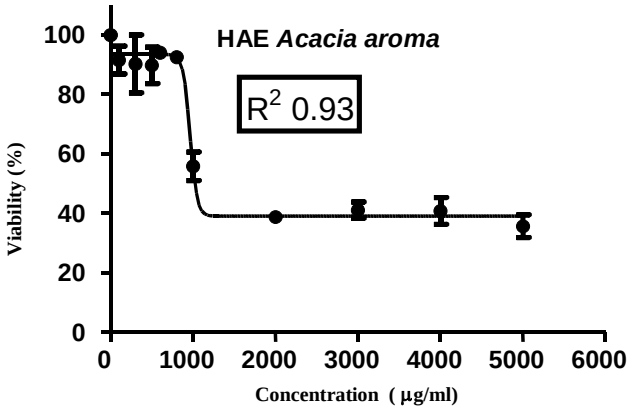

Figure 2. Percentage of viability of cultured Vero cells, incubated 48 h in the presence of hot aqueous extract (HAE) of *Acacia aroma* employed at different concentrations determined by MTT assay.
Each point represents the mean of four independent trials; CC50 was 1020 µg/mL.

Concentrations below 1000 µg/mL are considered non-toxic (Santos et al., 2003). Our results suggest that extracts tested exhibited selective toxicity, that means it present bactericidal or bacteriostatic effect without affect the eukaryotic cells. From the above results, it is concluded that these studies validate the external use of *A. aroma* extracts as complementary or alternative drugs to combat pathogenic microorganisms. Further study of separation and identification of bioactive principles and their evaluation *in vivo* models could clarify the properties of *A. aroma.*

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