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EVALUATION OF ANTIBACTERIAL PROPERTIES OF THREE PANAMANIAN PLANTS AGAINST MULTI-DRUG RESISTANT BACTERIA

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ABSTRACT
The increased resistance of pathogenic bacteria to traditional therapy has forced the implementation of different therapies. The consequent use of more than one antibacterial drug can increase toxicity problems, an undesirable situation, especially when it comes to therapy for immunosuppressed patients. Bacterial resistance to different drugs is a war between "humans and bacteria". For this reason, it is important to keep looking for new alternatives for the treatment of pathogenic bacteria. In this study, we determined the antimicrobial activity of methanolic extracts *of Lippia graveolens, Sphagneticola trilobata* and *Gliricidia sepium* against four pathogenic bacteria: *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. *Pseudomonas aeruginosa was* the strain most affected by antagonistic activity of all the extracts evaluated, followed by *E. coli* and *S. aureus*. We found that *S. trilobata* was the plant with the highest activity. We carried out tests mixing extracts (1:1) from different parts of each plant, where there was antagonistic and synergistic activity. These results are very promising, since these bacteria are currently being studied for their high resistance mechanisms to a wide variety of antibiotics.

KEYWORDS
antimicrobial, *Gliricidia sepium, Sphagneticola trilobata, Lippia graveolens*, inhibitory effect.

EVALUACIÓN DE LAS PROPIEDADES ANTIBACTERIANAS DE TRES PLANTAS PANAMEÑAS CONTRA BACTERIAS MULTIRRESISTENTES

RESUMEN

El aumento de la resistencia de las bacterias patógenas a la terapia tradicional ha forzado la implementación de diferentes terapias. El consiguiente uso de más de un antibacteriano puede aumentar los problemas de toxicidad, y esta situación no se desea, especialmente cuando se trata de terapia para pacientes inmunosuprimidos. La resistencia a diferentes drogas por parte de las bacterias es una guerra entre "humanos y bacterias". Por esta razón, es importante seguir buscando nuevas alternativas para el tratamiento de bacterias patógenas. En este estudio, determinamos la actividad antimicrobiana de extractos metanólicos de *Lippia graveolens, Sphagneticola trilobata* y *Gliricidia sepium* contra cuatros bacterias patogénicas: *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* y *Staphylococcus aureus.* Donde *Pseudomonas aeruginosa* fue la cepa más afectada por la actividad antagónica de todos los extractos evaluados, seguido por *E. coli* y *S. aureus*. Encontramos que *S. trilobata* fue la planta con mayor actividad. Realizamos pruebas mezclando extractos (1:1) de diferentes partes de cada planta donde hubo actividad antagonista y sinergista. Estos resultados son muy prometedores, ya que estas bacterias son muy estudiadas en la actualidad por sus mecanismos de alta resistencia a una amplia variedad de antibióticos.

PALABRAS CLAVES

antimicrobiano, *Gliricidia sepium, Sphagneticola trilobata, Lippia graveolens*, efecto inhibitorio.

INTRODUCTION

Infections caused by microorganisms represent a major public health problem with great economic repercussions. Despite advances in medicine, microorganisms have found different strategies to evade antibiotic drugs, such as the development of resistance, which limits useful therapeutic options on the market. Currently, we do not have enough pharmaceutical products to meet the therapeutic needs of the population (Prestinaci *et al.* 2015). Resistance of microbial pathogens to antimicrobial drugs is rapidly increasing worldwide, this has spread the use of combination therapy, consisting of the use of more than one antibiotic per treatment that increases toxicity associated with drug side effects. This situation could be more dangerous when it comes to immune suppressed patients (Fair & Tor, 2014).

Unfortunately, the discovery of new compounds for the treatment of human diseases and their development is a long and expensive process. This makes difficult to study all the plants used in traditional medicine, but many of them contain compounds that could become useful drugs for therapeutic and for this reason, scientists are still interested in medicinal plant study. The plants chosen for the present study were selected based on their wide use in traditional medicine. *Sphagneticola trilobata* is used to treat hyperglycemia, dysmenorrhea, cough, allopathic skin diseases and abdominal pain, some of these biological effects have been supported by scientific studies (Kade *et al*. 2010; Flores & Quinlan, 2014; Rahman, 2015). Additionally, in other countries it has also been determined their antimicrobial activity (Taddei and Rosas Romero, 1999; Utrakoon *et al*. 2009; Govindappa *et al*. 2011; Chethan *et al*. 2012; Toppo *et al*. 2013). *Gliricidia sepium* is used to control fever, muscle pain, and gonorrhea. In Mexico, it is employed as an antipyretic, expectorant and diuretic (Survase & Raut, 2011; Nadhi *et al*. 2016). The extracts of this plant have also displayed potent antimicrobial activity (Nazli *et al*. 2008; Abulude & Adebote, 2009; Jose & Reddy, 2010; Nazli *et al*. 2011; Sukumar & Aparna, 2012; Akharaiyi, Boboye & Adetuyi, 2012; S Kumar & Simon, 2016; Oladunmoye et al., 2018). Finally, *Lippia graveolens* is widely used to treat diarrhea, vomiting, indigestion, dysentery and as antiviral, antiparasitic, nematicidal and antimicrobial (Argueta et al., 1994; Monroy & Castillo 2000; Salgueiro, 2003; López-Aroche *et al*. 2008; Pilau *et al*. 2011; Molina-Garza, 2014; Miller *et al*. 2015).

This research aims to determine the antimicrobial activity of *L. graveolens, S. trilobat*a and *G. sepium*, highlighting its antimicrobial properties against multiresistant bacteria. *Escherichia coli* is one of the most common bacteria provoking intestinal tract infections and neonatal meningitis (Pouillot *et al*. 2012). *Klebsiella pneumoniae* produces very severe pneumonia (Jondle *et al*. 2018). *Pseudomonas aureginosa* causes urinary tract infections that could become systemic in many cases (Mittal *et al*. 2009). *Staphylococcus aureus* infects wounds and can cause septicemia, endocarditis, and toxic shock syndrome (Lin *et al*. 2010). These bacterial strains can present early resistance to all antibiotics used to date, including B-lactams and carbapenems. According to the World Health Organization (WHO), among the multi-resistant strains with priority for the search and development of new antibiotics, the species *P. aeruginosa, K. pneumoniae* and *E. coli* are in the Critical Category and *S. aureus* is in the High-priority Category (Tacconelli *et al*. 2018).

Infinity of tests are carried out in search of the compound that contains an efficient antimicrobial activity. The synergy of the extract mixture could be an alternative strategy, to add to the different experiments, and therapeutics that exist. Toxicity assessment of such mixtures can be useful, and these interactions could be beneficial for the control of diseases produced by microorganisms. (Enke *et al*. 2011; Caesar & Cech, 2019). In this context of constant "arms race between bacteria and man", new drugs are urgently needed that could come from compounds found in plants that live in tropical forests.

METHODS

Collection of plants and extracts preparation.

The plants used in this study (*Lippia graveolens, Sphagneticola trilobata* and *Gliricidia sepium*) were collected in Altos de María, Panama Oeste, Panama and identified in the Herbarium of the University of Panama. The harvested plants were washed with water, disinfected, rinsed with distilled water, and finally dried in the shade at room temperature. The aerial parts (leaves and stems) of the plants were separated and washed again and then dried for seven days at 25 ° C. Once dried, the material was triturated with a blender and placed in methanol for 48 hours, the solvent was decanted and filtered, then evaporated on a rotary evaporator, then on a vacuum pump to obtain the crude extract.

Biological evaluation

Bacterial Strains

Eschericia coli CECT434, *Klebsiella pneuminae* ATCC11296, *Pseudomona aeruginosa* CECT907, and *Sthaphylococcus aureus* ATCC25923 were used to test the antibacterial activity of the methanolic extracts.

Antibacterial Bioassays

Two mg of each extract were dissolved in DMSO and antimicrobial activity was tested using the Bauer & Kirby agar diffusion assay (Woods & Washington, 1995). Bacterial inoculum was prepared at a concentration equal to 0.5 McFarland turbidity standard, 100 ml of inoculum was applied to the surface of a trypticase and soy agar plate at a pH between 7.2 and 7.4. Then, the disks impregnated with the extract of each plant [concentration (0.04 mg / uL)] were placed on the surface of the culture medium. Petri dishes with a diameter of 145 mm were used and a maximum of eight discs were placed in each plate. Three replications were made for each microorganism. Subsequently, the plates were incubated at 37 °C for 24 hours. Each Petri dish was observed with indirect light and each inhibition zone was measured using a previously correctly graduated caliper. The mean of the halo of four replications is expressed in Table 1.

Combination of Extracts

One mg of each crude extract from different parts of the plants, were mixed (1:1) and resuspended in 100 uL of DMSO to homogenize it and create an homogeneous solution in the order mentioned in Table 2. Bioassays were performed using the methodology explained above.

	Extracts							
	Leaves			Stems			Controls	
Bacteria	**LG**	**ST**	**GS**	**LG**	**ST**	**GS**	**CP**	**CN**
S. aureus	-	14.6 ± 0.6	-	-	12.5 ± 0.9	13.5 ± 0.9	46 ± 2.6	-
P. aeruginosa	14.5 ± 1.3	14.5 ± 0.6	14.5 ± 0.5	16 ± 1	16.8 ± 0.3	16 ± 1	21 ± 1	-
E. coli	18 ± 1.7	18.5 ± 0.5	-	15.5 ± 0.9	18 ± 1	-	28 ± 2.6	-
K. pneumoniae	-	-	-	-	-	-	31 ± 1.7	-

Table 1: Antibacterial activity (diameter of the inhibition zone in mm) of the methanol extracts (0.04 mg / µLin DMSO) from stems and leaves of evaluated plants against gram negative and gram-positive bacteria using agar diffusion discs assay. Plants: LG: *Lippia graveolens*, ST: *Sphagneticola trilobata* and GS: *Gliricidia sepium*. CP: Positive control (Trimethoprim with sulfa 10 µg/mL), CN: Negative control (DMSO).

	Extract															Control	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Bacteria	**Lgl+Stl**	**Lgl+Gsl**	**Lgl+Lgs**	**Lgl+Sts**	**Lgl+Gss**	**Stl+Gsl**	**Stl+Lgs**	**Stl+Sts**	**Stl+Gss**	**Gsl+Lgs**	**Gsl+Sts**	**Gsl+Gss**	**Lgs+Sts**	**Lgs+Gss**	**Sts+Gss**	**CP**	**CN**
S. aureus	-	-	-	-	-	-	-	21 ± 0.7	-	-	17 ± 0.7	21 ± 0.7	-	-	19 ± 0.7	40 ± 1.0	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	18 ± 0.0	-	-	-	-	15 ± 1.0	-
E. coli	16 ± 0.0	17 ± 0.7	15 ± 0.7	17 ± 0.7	-	-	-	-	-	-	-	-	-	-	-	22 ± 0.5	-
K. pneumoniae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25 ±0.5	-

Table 2. Antimicrobial effect for the **c**ombination of different methanol extracts (all values in mm). Extract mix: (1) Leaves of *L. graveolens* and *S. trilobata*; (2) Leaves of *L. graveolens* and *G. sepium*; (3) Leave and stem of *L. graveolens*; (4) Leave of *L. graveolens* and stem *S. trilobata*; (5) Leave of *L. graveolens* and stem *G. sepium*; (6) Leaves of *S. trilobata* and *G. sepium*; (7) Leave of *S. trilobata* and stem *L. graveolens*; (8) Leave and stem of *S. trilobata*; (9) Leave of *S. trilobata* and stem of *G. sepium*; (10) Leave of *G. sepium* and stem of *L. graveolens*; (11) Leave of *G. sepium* and stems of *S. trilobata*; (12) Leaves and stems of *G. sepium*; (13) Stems of *L. graveolens* and *S. trilobata*; (14) Stems of *L. graveolens* and *G. sepium*; (15) Stems of *S. trilobata* and *G. sepium.*

RESULTS AND DISCUSSION

The antibacterial activity of leaves and stems extracts of each plant are summarized in Table 1. Methanolic extracts from leaves and stems of *L. graveolens* were positive against *P. aeruginosa* and *E. coli*. Essential oils of *L. graveolens* have been tested against *Streptococcus faecalis*, *P. vulgaris*, *S. aureus*; *S. epidermidis*, *B. subtilis*, *Sarcina lutea*, *S. boydii*, *Vibrio cholerae*, *E. coli*, *Micrococcus luteus*, *Enterobacter agglomerans*, *E. aerogenes*; *Y. enterocolitica*, *S. typhi* and *Enterococcus sp* (Salgueiro *et al*. 2003; Hernandez *et al*. 2009; Hernández-Hernández *et al*. 2014; Castellanos-Hernández *et al*. 2020). Essential oils of *L. graveolens* have phenolic monoterpenoids (e.g., carvacrol, α-terpinyl acetate, *m*-cymene, thymol and β-pinene) with antiparasitic, antifungal, antiviral and antimicrobial activity (Kintzios 2002; Salgueiro *et al*. 2003; Hernandez *et al*. 2009; Quintanilla-Licea *et al*., 2020; Salgueiro *et al*. 2003; Hernández *et al*. 2008; Pilau *et al*. 2011). However, this is the first time that methanolic extracts of leaves and stems of *L. graveolens* are tested against the gram-negative bacteria *P. aeruginosa* and *E. coli*. Methanol extracts of leaves and stems of *S. trilobata* has antimicrobial activity against *S. aureus*, *P. aeruginosa* and *E. coli* with a mean inhibition zone of 14.6 ± 0.6 and 18.5 ± 0.5 respectively. Studies by Toppo *et al*. (2013) found antibacterial activity in methanolic extracts of *S. trilobata* leaves against *S. aureus*, *S. typhi* and *P. aeruginosa* and the methanolic extracts from stems has antimicrobial activity against *S. aureus*. Apparently, this is the first report of an antibacterial activity of leaves and stems of *S. trilobata* against *P. aeruginosa* and *E. coli*. Similar antibacterial activity with methanolic extracts of *S. trilobata* have been found against *Bacillus subtilis, Mycobacterium smegmatis, S. aureus*, *S. epidermidis*, *E. coli*, *Proteus vulgaris*, *P. aeruginosa, Salmonella paratyphi* and *Shigella sonnei* (Taddi *et al*. 1999). Ethanolic extracts of leaves and stems of *S. trilobata* have shown antimicrobial activity against *P. aeruginosa*, *S. aureus, K. pneumoniae, Xanthomonas oryzae and X. axanopodis* (Govindappa *et al*. 2011). *S. trilobata* shows the most promising results since extracts from its leaves and stems show activity against three of the four multi-drug resistant bacteria evaluated in this study.

In the same way, leaves methanolic extracts of *G. sepium* showed a mean zone of inhibition against *P. aeruginosa* of 14.5 mm and methanolic extracts of stems had activity against *S. aureus* and *P. aeruginosa* with inhibition zones of 13.5 mm and 16 mm, respectively.

In this study *K. pneumoniae* presented resistance to all methanolic extracts tested as was found by *Jhon et al*. (2006), Abulude and Adebote (2009) and Nazli *et al*. (2011). A positive antimicrobial activity result was also found with leaves of *G. sepium* against *S. aureus* and *E. coli* as was reported by Akharaiyi *et al.* (2012) and Oladunmoye (2018). However, information about antimicrobial activity of stems is limited. These results showed that *G. sepium* have a good antimicrobial activity against two of the resistant bacteria tested here.

Regarding the susceptibility of the pathogenic bacteria analyzed, *P. aureginosa* was the most inhibited strain by most of the evaluated extracts, followed by *E. coli* and *S. aureus*. This suggests that our results are promising, as these bacteria are currently studied for their extended multidrug resistance.

When comparing the activity of extracts from leaves versus extract from stems to analyze if there is a significant difference between the antimicrobial activity of the organs that integrate the aerial parts of each plant, we found that stem extracts displayed a greater inhibition halo than leaf extracts, this fact was similar for the three evaluated plant species. In fact, the inhibition halos showed by the evaluated extracts were greater than 10 mm, which makes them very promising since all extracts displayed an inhibition zone equal to or greater than 50% of the inhibition presented by the positive control, which is a pure compound.

Table 2 shows the synergism of the extract mixture 1, 2, 3, 4 against *E. coli*; 8, 11, 12 and 15 against *S. aureus*; and 11 against *P. aeruginosa*. It can be noted that not all the extracts were synergistic. Many mixtures of the extracts were antagonistic. It cannot be demonstrated why antagonism or synergism occurs due to the number of compounds in the samples (Enke *et al*. 2011). These findings are interesting for future studies, since their metabolites vary in plants, according to the soil where they are cultivated, the region of origin, temperatures, and other environmental factors (Khan, 2006).

The results obtained corroborate the antimicrobial potential that the three plants evaluated have shown in previous works. *S. trilobata* is the species with the greatest potential against three of the four evaluated bacterial strains. Furthermore, we found that stem extracts are more active than leave extracts, except for extracts evaluated against *E. coli*, where the leave extracts showed greater activity. Regarding efficacy, our study provides additional scientific evidence that the evaluated plants can still be considered by the human population as an alternative remedy to combat bacterial infections. It is also important to note that studies related toxicity are necessary to promote the safe use of these three plants.

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