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Antimicrobial activity of crude extracts of *Pseudocalymma alliaceum* (Lam.) leaves against clinically important microorganisms isolated from clinical specimens

Actividad antimicrobiana de los extractos crudos de las hojas de Pseudocalymma alliaceum (Lam.) contra microorganismos de importancia clínica aislados de pacientes hospitalizados

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Abstract: Introduction. In the last years, antibiotic resistance has been increased worldwide. Nevertheless, complications in antibiotic treatment infection remain a significant cause of morbidity and mortality among hospitalized patients. Medicinal plants have been used as alternativetherapies for human infectious diseases as they contain components of beneficial value. *Pseudocalymma alliaceum* leaves are used to treat different diseases based on traditional medicine for populations in South America. Objective. For this reason, the study aimed to evaluate the antimicrobial activity of aqueous, methanol, and hydroalcoholic extract of dried leaves of *P. alliaceum* on clinically important bacterias (*Escherichia coli*, *Morganella morganii*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Salmonella typhi*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA) and the yeast *Candida albicans* isolated from clinical specimens. Methodology. We used Mueller–Hinton broth in agar well-diffusion method to perform the antibacterial and antifungal activity. Results: The results showed that only aqueous extract exhibited antibacterial effect against *E. coli* [inhibition diameter (23.2±1.0 mm) minimum inhibitory concentration (312 µg mL) and minimum bactericidal concentration (156 µg mL)] and *E. aerogenes* [inhibition diameter (24.4±1.6 mm), minimum inhibitory concentration (119 µg mL) and minimum bactericidal concentration (78 µg mL)]. Conclusion: *P. alliaceum* demonstrated *in-vitro* antibacterial activity against Gram-negative bacteria. Further *in vivo* studies must be improved to identify its action mechanism.

Keywords: Gram-negative bacteria; Pathogenic Bacteria; Medicinal plants; Antibiotic resistance; Alternative treatment.

***Resumen**: Introducción. En los últimos años la resistencia a antibióticos ha ido incrementado en el mundo. No obstante, las complicaciones que se derivan de la farmacoresistencia sigue siendo una causa importante de morbilidad y mortalidad en pacientes hospitalizados. Por otro lado, las plantas medicinales se han utilizado como remedios para las enfermedades infecciosas tanto en animales como en humanos, ya que contienen diversos componentes de valor beneficioso. Las hojas de Pseudocalymma alliaceum (Lam.) se utilizan para tratar diferentes enfermedades basadas en la medicina tradicional en diferentes poblaciones de América del Sur. Objetivo. Por esta razón, el objetivo de este estudio fue evaluar la actividad antimicrobiana de los extractos acuoso, metanólico e hidroalcohólico de hojas de P. alliaceum sobre microorganismos de importancia clinica (Escherichia coli, Morganella morganii, Citrobacter freundii, Klebsiella pneumoniae, Enterobacter aerogenes and Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus resistente a la meticilina (SARM) y la levadura Candida albicans aislados de pacientes hospitalizados. Metodología. Para determinar la actividad antibacteriana y antifúngica se utilizó el método de difusión en agar Resultados. Los resultados mostraron que solo el extracto acuoso exhibió actividad antibacterial contra E. coli [diámetro de inhibición 23.2±1.0 mm) concentración mínima inhibitoria (312 µg mL) y concentración mínima bactericida (1.56 µg mL)] y E. aerogenes [diámetro de inhibición (24.4±1.6 mm), concentración mínima inhibitoria (119 µg mL) concentración mínima bactericida (78 µg mL)]. Conclusión. P. alliaceum demostró mejor*

actividad antimicrobiana contra bacterias Gram-negativas. Por lo tanto, deben realizarse más estudios para poder identificar su mecanismo de acción.

***Palabras clave**: Gram negativas; Bacterias patogénicas; Planta medicinal; Resistencia a antibioticos; Alternativa de tratamiento.*

Introducción

Antibiotic resistance has become an emergent public health problem in hospitals and the community worldwide, causing high mobility and mortality. The inappropriate dosage of antibiotics is the main reason for resistance in bacteria, and the global emergence of multi-drug resistance limits the efficiency of current medications and causes treatment failure (Muteeb *et al*., 2023).

Antibiotic resistance reduces antibiotics' efficacy, making the treatment of patients difficult, costly, or impossible. To address these problems, several actions must be improved, such as the mandatory regulation of antibiotic therapy, the identification of the molecular mechanisms of resistance, and the development of new molecules and alternative therapeutic strategies.

On the other hand, advances in finding new sources of natural products with antibacterial action and expanding antibiotic chemical variety are providing chemical leads for new medications (Barbieri *et al*., 2017; Song *et al*., 2018). Several plants contain secondary metabolites used as popular folk drugs in treating infectious diseases while simultaneously mitigating many of the side effects often associated with synthetic antibiotics (Sharma *et al*., 2017). In recent years, the antibacterial properties of medicinal plants have been increasingly reported from different regions of the world (Mickymaray *et al*., 2016; Al-Snai *et al*., 2018; Panda *et al*., 2020).

Pseudocalymma alliaceum is a member of the family Bignoniaceae, used in traditional medicine for its antiarthritic, anti-inflammatory, analgesic, antipyretic, and antirheumatic properties (Dugasani *et al*., 2019). However, scarce scientific background has been published indicating the microbial activity of *P. alliaceum*. Granados-Echegoyen *et al*. (2014) reported the larvicidal activity of the aqueous, methanol, and ethanol extracts of *P. alliaceum* leaves against the mosquito *Culex quinquefasciatus*. Also, Srivastava (2017) described the antifungal effect of the aqueous extract from *P. alliaceum* leaves against *Fusarium oxysporum* f. sp. *gladioli*. Phytochemical screening of *P. alliaceum* leaves has been shown to contain diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, and 1-octen-3-ol as major compounds (Rao *et al*., 2019).

On the other hand, Granados-Echegoyen *et al*. (2014) analyzed essential oils from leaves, showing that the major components are diallyl disulfide and diallyl sulfide.

Nosocomial infections represent one of the most important and emerging public health problems (Vock *et al*., 2019). According to the Infectious Disease Society of America, the main bacteria linked with these kinds of infections are the extended-spectrum β-lactamase (ESbL)-producing and carbapenem-resistant

Enterobacteriaceae (CRE), metallo- β-lactamase (MbL)-producing *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, *Candida albicans* is the most common yeast that causes fungal infections and is normally found in the skin microbiota and mucous membranes of healthy people. It causes wide spectrum infections that go from superficial to lethal systemic infections, particularly in immunocompromised patients. Nowadays, an increased number of isolated yeasts resistant to antifungal therapy are recognized worldwide (Mayer *et al*., 2013).

The advent of these microorganisms has sparked inquiries regarding the future of therapeutic drugs. In line with the findings mentioned above, searching for alternative methods that use plants is crucial, and these assays should focus on the inhibitory mechanisms of these medically significant microorganisms. For this reason, we designed the present work to investigate the antimicrobial activity of the aqueous, methanol, and hydroalcoholic extracts of *P. alliaceum* leaves against several pathogenic microorganisms isolated from clinical specimens.

1. Material y métodos

1.1. Plant material

Fresh leaves of *Pseudocalymma alliaceum* were collected in different regions of the southern coastal state of Oaxaca (16o 29’ 20.67” N, 95o 09’ 15.70” O). Plant material were identified and compared to voucher specimens at the Herbarium of CIIDIR-OAX for authentication. Leaves were washed with water to remove all unwanted materials, air-dried, pulverized in a mill, and stored until further use.

1.2. Plant extract preparation

Briefly, 200 g of powdered plant material was soaked in 350 mL of solvent for 72 h. The solvent was filtered through Whatman filter paper No. 1 (Whatman, UK), while the residues were used for a second extraction. After the second extraction, the filtrates were concentrated under reduced pressure using a rotary evaporator at 26 °C. The crude extracts were collected and stored at 4 °C (Abubakar & Haque, 2020).

1.3. Microbial strains and culture media

Microorganism strains were granted by the Microbiology Department of the Hospital Regional De Alta Especialidad, Veracruz, Mexico. The panel of test organisms for primary *in vitro* antibacterial screening in this study is summarized in Table 1. Brain heart infusion media (Sigma-Aldrich) was used to culture the activation of the tested bacteria. Colonies of *C. albicans* were maintained on Biggy agar (MCD LAB). Mueller

Hinton Broth (Sigma-Aldrich) was used for antibacterial and antifungal assays. All isolates were identified by biochemical tests.

Table 1

Panel of test microorganism for primary antibacterial and antimycotic in vitro screening

Microorganism	Characteristic	Species	Source	Antibiotic resistance pattern
Bacterias	Gram-positive coccus, MDR, #02	MRSA	Clinical isolation	AM, CF, CTX, CAZ, CXM, DC, E, PEF. PE
	Gram-positive coccus, MDR, #05	MRSA	Clinical isolation	AM, CF, CTX, CAZ, CXM, DC, E, PEF. PE
	Gram-positive coccus	*S. aureus*	ATCC 537064	AM, E, PE
	Non-Enterobacteriaceae	*P. aeruginosa*	Clinical isolation	AM, CF, NF
	Enterobacteriaceae	*C. freundii*	Clinical isolation	Sensible
	Enterobacteriaceae	*E. coli*	Clinical isolation	AM
	Enterobacteriaceae	*M. morganii*	Clinical isolation	CF
	Enterobacteriaceae	*E. aerogenes*	Clinical isolation	A, CF
	Enterobacteriaceae, encapsulated	*S.thypimurium*	Clinical isolation	Sensible
	Enterobacteriaceae, encapsulated	*K. pneumoniae*	Clinical isolation	AM
Yeast	Dimorphic yeast #01-06	*C. albicans*	Clinical isolation	KTZ
	Dimorphic yeast #01-10	*C. albicans*	Clinical isolation	KTZ

Note: Gram-positive antibiotic tested. AM: ampicillin; CF: cefalotin; CTX: cefotaxime; CAZ: ceftazidime; CXM: cefuroxime; DC: dicloxacillin; E: erythromycin; GE: gentamicin; PEF: pefloxacin; PE: penicillin; TE: tetracycline; SXT: sulphamethoxazole/ trimethoprim. Gram-negative antibiotic tested. AK: amikacin; AM: ampicillin; LEV: levofloxacin; CF: cefalotin; CTX: cefotaxime; CRO: ceftriaxone; CL: cephalexin; GE: gentamicin: NET: netilmicin; NF: nafcillin; FEP: cefepime; SXT: sulphamethoxazole/ trimethoprim. Antifungal. KTZ: ketoconazole.

1.4. Assay for antibacterial activity by the agar well diffusion method

The plant extracts were tested for antibacterial activity using the disk diffusion method (Klančnik *et al*., 2010). The tested organisms were cultured on blood agar plates for 24 h at 35 ± 2 °C. The colonies were inoculated in a normal saline solution. The turbidity was then adjusted to equal the turbidity of a 0.5 McFarland standard, providing a final inoculum of 1.5×10^8 CFU/mL. About 100 mL of the test organism inoculum was spread on a Mueller Hinton agar plate. Sterile 6 mm paper with the plant extracts (12.5, 6.25, and 3.12 mg mL), a negative control (distilled water), and a positive control (25 *µg* sulphamethoxazole/trimethoprim discs *for Gram-positive bacteria and* 25 *µg* cefotaxime discs for *P. aeruginosa* and Gram-negative bacteria***)*** were then placed on the inoculated plates. The plates were incubated at 35 ± 2 °C for 24 h. Each extract form

was evaluated with three repetitions. Antibacterial activities were estimated by measuring the diameters of the zones of inhibition in mm against the bacteria tested.

1.5. Assay for antifungal activity by the agar well diffusion method

The plant extracts were tested for antifungal activity using the disk diffusion method. The test yeasts were cultured on Biggy agar plates for 24 h at 37 °C. A suspension containing 10^6 CFU/mL was prepared in 0.9% NaCl using a spectrophotometer (γ = 550 nm, optical density [OD] = 0.380). About 100 mL of the test yeast inoculum was spread on a Mueller Hinton agar plate supplemented with 2 % glucose and 0.5 µg/mL of *methylene-blue, according to* Clinical Laboratory Standard Institute *(CLSI) antifungal susceptibility tests and Barry et al. (2003).* Sterile 6 mm paper with the plant extracts (12.5, 6.25, and 3.12 mg mL), a negative control (distilled water), and a positive control (*amphotericin B disc, 100 µg**)*** were then placed on the inoculated plates. Then, the plates were incubated at 37 °C for 24 h. Each extract form was evaluated in triplicate. Antifungal activities were estimated by measuring the diameters of the zones of inhibition in mm against the yeast tested.

1.6. Minimum inhibitory concentration (MIC)

Twelve vials containing 1 mL of Mueller Hinton broth (MHB) were prepared. One milliliter of the extract solution was added to 11 vials only, and the first vial was used as a negative control. After homogenization, 1 mL of the solution containing the broth and extract solution was transferred to the second vial containing 1 mL of nutrient broth. In the same method, two-fold serial dilutions were prepared up to the tenth vial. Then, 1 mL of content was discarded from the 10th vial, and the 11th vial was used as positive control. All of the vials except the first and last contained equal volumes, i.e., 1 mL, gradually reducing the concentration of the solution. To all of these vials, 20 µL of microorganism suspension (turbidity equal to a 0.5 McFarland standard, equal to 1.5×10^6 CFU/mL) was put into each vial. The vials were then incubated at 37 °C for 24 h. MIC was taken as the lowest concentration that prevented the growth of the microorganism culture.

1.7. Minimal bactericidal concentration (MBC)

The MBC was carried out according to methods described by Irobi & Daramola (1994). Vials with no visible growth in the MIC assays were cultured using a 10 mL inoculating loop onto blood agar plates at (35 ± 2 °C) for a 24 h incubation. The MBC was defined as the lowest extract concentration that did not result in the appearance of a bacterial colony on the solid medium.

1.8. Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to a one-way analysis of variance (ANOVA), and differences between samples were determined using Tukey's test.

2. Results

2.1. Assay for antibacterial activity by the agar well diffusion method

The disk diffusion method for antibacterial susceptibility testing was initially performed to determine the antibacterial activities of the aqueous, methanol, and hydroalcoholic extracts of *P. alliaceum* against enterobacteria (*E. coli*, *M. morganii*, *C. freundii*, *K. pneumoniae*, *E. aerogenes*, and *Salmonella typhi*), *P. aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Of the extracts tested, only the aqueous extract of *P. alliaceum* showed inhibition of bacterial growth against *E. coli* and *E. aerogenes* (Table 2). The plant extracts exhibited inhibition zones with a diameter of 23.2 ± 1.0 mm for *E. coli* and 24.4 ± 1.6 mm for *E. aerogenes* (**Figure 1**).

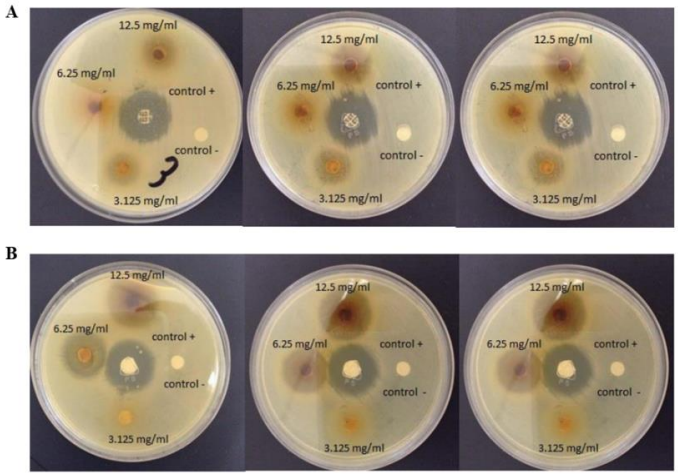
Table 2

Diameters of zones of inhibition of P. alliaceum extracts against clinical isolates of Enterobacteria, S. auereus MRSA, P. aeruginosa and C.albicans strains

Extract	Concentration mg mL	*S. aureus* MRSA #02	*S.aureus* MRSA #05	*S.aureus* ATCC 537064	*P.aeruginosa*	*C.freundii*	*E.coli*	*M.morganii*	*E.aerogenes*	*S.thypimurium*	*K.pneumoniae*	*C.albicans* #01-06	*C.albicans* #01-10
Aqueous	Control	-	-	-	-	-	-	-	-	-	-	-	-
	3.12	.	-	-	-	-	10.4±0.5[a]	-	10.7±0.5[a]	-	-	-	-
	6.25	-	-	-	-	-	15.7±0.5[b]	-	20.4±0.5[b]	-	-	-	-
	12.5	-	-	-	-	-	23.2±1.0[c]	-	24.4±1.6[c]	-	-	-	-
Methanolic	Control	-	-	-	-	-	-	-	-	-	-	-	-
	3.12	-	-	-	-	-	-	-	-	-	-	-	-
	6.25	-	-	-	-	-	-	-	-	-	-	-	-
	12.5	-	-	-	-	-	-	-	-	-	-	-	-
Hydroalcoholic	Control	-	-	-	-	-	-	-	-	-	-	-	-
	3.12	-	-	-	-	-	-	-	-	-	-	-	-
	6.25	-	-	-	-	-	-	-	-	-	-	-	-
	12.5	-	-	-	-	-	-	-	-	-	-	-	-

Note: Different letters in the same column represent a difference between concentrations ($p \leq 0.05$) within the same extract. Results are expressed in mm. -: No zone of inhibition.

Figura 1
Antibacterial effect of P. alliaceum *against Gram-negative bacteria.*



Note: A) Antibacterial effect of aqueous extract of *P.alliaceum* against *E.coli*; B) Antibacterial effect of aqueous extract of *P.alliaceum* against *E. aerogenes.*

2.2. Assay for antifungal activity by the agar well diffusion method

None of the extracts tested showed antifungal activity (**Table 2**).

2.3. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC and MBC assays for *P. alliaceum* are shown in Table 3. Inhibitory activity was exhibited against the clinical strains of *E. aerogens* (119 µg/mL) and *E. coli* (312 µg/mL). The MBC values were 156 µg/mL for *E. aerogens* and 78 µg/mL for *E. coli*.

Table 3
MIC and MBC of aqueous extracts from *P. alliaceum* against clinical isolates of *E. coli* and *E. aerogenes* (µg mL)

Extract	Microorganism	MIC	MBC
Aqueous	*E.coli*	312 ± 0.5 µg mL	156 ± 0.8 µg mL
	E.aerogenes	119 ± 1.1 µg mL	78 ± 0.6 µg mL

MIC: Minimum Inhibitory Concentration; MBC: Minimal bactericidal Concentration

3. Discussion

The presence of such metabolites in the plant extracts studied can provide a preliminary explanation of their antibacterial activities. However, differences were

observed in the activities of the extracts against bacteria and yeast. These could be due to the organospecific production profile (leaves) of the plant or solvent used (water, ethanol, and methanol) and in the mechanism of action of their bioactive elements. It could also be related to the nature of the plant at the time of collection, the storage conditions, and the stability of tested extract metabolites. Phenolic compounds in extracts obtained with polar solvents are sensitive and disintegrated by environmental factors during storing, such as light, temperature, and pH (Pandey *et al*., 2014; Weber *et al*., 2017; Kapcum *et al*., 2018).

This may explain the observed moderate activity. Concerning the extraction method, it was reported that ethanol extracts are more effective against Gram-negative bacteria (Prabakaran *et al*., 2018). Owolabi *et al*. (2007) documented that a concentration of 20 mg/mL of ethanolic extracts from the *Kigelia africana* plant, in the same family of *P. alliaceum*, possessed antibacterial and antimicrobial activity against *S. aureus (*15.0 ± 0.95) and *C. albicans (*0.75 ± 4.6), but not against of *E. coli* and *P. aeruginosa* strains. Moreover, aqueous extracts showed no activity against all of the organisms evaluated. These results are inconsistent with our assay (Owolabi & Omogbai, 2007).

On the other hand, Vadlapudi (2010) reported that methanolic extracts of *P.alliaceum* showed antifungal activity against *Aspergillus flavus* (14.0 mm) and *Fusarium oxysporum* (24 mm). Also, Srivastava *et al*. (2017) reported that aqueous extracts showed activity against *Fusarium* oxysporum, and these results are not similar with those found in our assay, where methanolic and aqueous extracts did not show antifungal activity (Vadlapudi, 2010; Srivastava, 2011). Another assay performed by Arruda *et al*. (2011) reported that the extract of *Jacaranda cuspidifolia* (Bignoniaceae) at a 100 mg/mL concentration was effective against strains of *S. pyogenes* (14.7 ± 0.5 mm), *N. gonorrhoeae* (15.2 ± 0.35 mm), and *S. aureus* (10.7 ± 0.9), with the methanolic extract exhibiting wider zones of inhibition in the agar well diffusion method (Arruda *et al*., 2011). In a more recent study related to the activity of methanol leaf extract of *Newbouldia leavis*, Ugwu *et al*. (2019) found activity against *P. aeruginosa* (16.6 ± 0.3), *E.coli* (17.6 ± 0.6), and *S. aureus* (17.0 ± 0.0) at a 100 mg/mL concentration. The activity of 12.5 mg/mL of the ethanolic extract of *P. alliaceum* against *E.coli* and *E.aerogenes* was found to be similar to that of 25 µg cefotaxime discs.

It has been postulated that the activities of plant extracts against microorganism are focused on the structures and cellular membranes, and due to the presence of a lot of bioactive compounds and diverse chemical profiles, it is likely that the antimicrobial potency is not just produced by one solitary compound; it may be attributed to a synergism effect (Farzaneh *et al*., 2015). Some researchers suggested that compounds of the plant extracts, such as phenolic compounds, alkaloid, and terpenoid, interact with the main proteins and enzymes on the microorganism's membrane. This causes its disruption to disperse a flux of protons to the cell's exterior, which results in cell death or inhibits enzymes essential for amino acid biosynthesis (Piddock, 2006). Other researchers attributed the antimicrobial effect of plant extracts to hydrophobicity characters which enable them to react with the proteins of the microorganism's membrane and mitochondria, distressing their structures and changing their permeability (Silhavy *et al*., 2010).

On the other hand, the capacity of bacteria to avoid the compounds present in extracts may be attributed to the outer membrane found in the Gram-negative wall composed of lipopolysaccharides, which render the bacterial wall impermeable to lipophilic solutes, unlike Gram-positive bacteria which do not have this membrane. This morphologic difference influences their reaction to antimicrobial compounds. In addition, Gram-negative bacteria have overexpressed efflux pumps that prevent the intracellular accumulation of antibiotics (Ugwu *et al*., 2019). Therefore, there is a need to find and develop compounds that are capable of avoiding the efflux pumps.

4. Conclusions

The results of the present study show moderate activity of extracts tested on the Gram-negative bacteria but did not show activity on the Gram-positive bacteria and yeast strains evaluated. On the other hand, this investigation suggests that the aqueous extracts of the studied plant can be used with limitations as potential leads to find compounds to control some bacterial infections, especially for *E. coli* and *E. aerogenes.* Nevertheless, due to the diversity of chemical compounds in plant extracts, the effects of their interaction are equally diverse and may lead to some toxic effects in humans. For this reason, we recommend performing antibiotic, chemical profile, and toxicological tests to ascertain and evade detrimental effects.

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