ARTIGO ORIGINAL
ISSN 1677-5090
DOI: http://dx.doi.org/10.9771/cmbio.v19i2.32895
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Antimicrobial and antibiofilm activities of aqueous extracts of Cucurbita pepo L.

Atividades antimicrobiana e antibiofilme de extratos aquosos de *Cucurbita pepo L.*

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Abstract

Introduction: *Cucurbita pepo L. is an herbaceous plant belonging to the family Cucurbitaceae. The species is popularly used in different countries for the treatment of diabetes and parasitic diseases.* ***Objective:*** *This study evaluated the antimicrobial and antibiofilm potential of aqueous extracts of leaves and seeds of C. pepo.* ***Methodology:*** *the extracts were tested in vitro against strains of Streptococcus pyogenes, Candida albicans and Candida krusei. The antimicrobial activity was performed by the microtiter method and the antibiofilm activity by the violet crystal method.* ***Results:*** *the results demonstrated that the extracts tested showed antibacterial and antibiofilm actions against S. pyogenes, but it was not possible to determine the minimum inhibitory concentration (MIC). The extracts inhibited the growth of C. albicans and C. krusei with MIC of 0.03 mg/mL. The antibiofilm activity of these species did not present either a dose dependence relationship or a synergistic effect when associated with the antifungal Fluconazole®.* ***Conclusion:*** *although there are indications of antimicrobial and inhibitory action in the formation of biofilm, additional studies are necessary to characterize the possible pharmacological effects of the analyzed specie.*

Keywords*: Antimicrobial potential. Antibiofilm. Cucurbitaceae. Cucurbita pepo.*

Resumo

Introdução: *Cucurbita pepo* L. é uma planta herbácea pertencente à família Cucurbitaceae. A espécie é usada popularmente em diferentes países para tratamento de diabetes e parasitoses. **Objetivo:** esse trabalho objetivou avaliar o potencial antimicrobiano e antibiofilme de extratos aquosos de folhas e sementes de *C. pepo*. **Metodologia:** os extratos foram testados *in vitro* contra cepas de *Streptococcus pyogenes*, *Candida albicans* e *Candida krusei*. A atividade antimicrobiana foi realizada pelo método de microtitulação e a atividade antibiofilme pelo método de cristal de violeta. **Resultados:** os resultados demonstraram que os extratos testados apresentaram ação contra *S. pyogenes*, tanto em relação ao controle do crescimento bacteriano como inibição de formação de biofilme, mas não foi possível determinar a concentração inibitória mínima (CIM). Os extratos inibiram o crescimento *C. albicans* e *C. krusei* com CIM de 0.03 mg/mL. A atividade antibiofilme dessas espécies não apresentou relação de dose dependência nem de efeito sinérgico quando associado ao antifúngico Fluconazol®. **Conclusão:** Embora haja indicativos de ação antimicrobiana e inibitória na formação de biofilme, são necessários estudos adicionais para a caracterização dos possíveis efeitos farmacológicos da espécie analisada.

Palavras-chave: Potencial antimicrobiano. Antibiofilme. Cucurbitaceae. *Cucurbita pepo*.

INTRODUCTION

Cucurbita pepo L. is an herbaceous plant belonging to the family Cucurbitaceae, produces branches that can reach 6 m in length. The fruits are popularly known as pumpkin, moranga or jerimum (HEIDEN; BARBIERI; NEITZKE, 2007). The species presents pellet leaves, covered with rough hairs, hollow petioles of up to 50 cm in length. Large flowers of yellow-orange color. The fruits are consumed by man and used in the feeding of domestic animals (LORENZI; MATOS, 2008).

The popular use has awakened the scientific community for research with the species, investigating its potential on treatment of diabetes, hypertension, cancer, infectious and inflammatory processes (CAILI; HUAN; QUANHONG, 2006). The seeds are considered vermifuge, the tea of the flowers is antipyretic. The treaded leaves are used externally for burns and erysipelas (LORENZI; LACERDA; BACHER, 2015).

The excessive and disordered use of antibiotics has favored the emergence of multidrug-resistant organisms (CALDERÓN ROJAS; AGUILAR ULATE, 2017). The drug resistance is a growing threat in infections caused by bacteria and fungi (GEDDES-McALISTER; SHAPIRO, 2019). The mechanisms of fungal resistance involve intrinsic and acquired agents and are associated with a decrease in

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effective drug concentration, altering the drug target or antifungal toxicity by metabolic modification (SANGLARD, 2016). Yeast species of the genus Candida are associated with increased resistance against antifungal agents (PRISTOV; GHANNOUM, 2019).

Biofilm formation is also an important defense mechanism for maintaining the viability of these strains. The bacterial biofilm tolerate antibiotic concentrations 100 to 1,000 times higher compared with their planktonic form (MACIA; ROJO-MOLINERO; OLIVER, 2014). Biofilm is formed through the aggregation of microorganisms that produce and live within an extracellular polymer matrix and are irreversibly bound (SHARMA; SARITA, 2008). The objective of this study was to evaluate the antimicrobial and antibiofilm potentials of leaves and seeds aqueous extracts of C. pepo.

METHODOLOGY

OBTAINING EXTRACTS AND PHYTOCHEMICAL ANALYSIS

Leaves and fruits of C. pepo were collected in Aldeia, Camaragibe, Pernambuco, Brazil. A voucher specimen was deposited in the Herbarium Dárdano de Andrade Lima of the Instituto Agronômico de Pernambuco (IPA), under registration number 91093.

Aqueous extracts were obtained using leaves and seeds of C. pepo. The plant material was oven dried at 40 °C and after 24 h was shredded. Distilled water was used as solvent. The extracts were obtained by decoction, at 100 °C for ten minutes, then frozen and freeze-dried. The phytochemical assay of the extracts was performed by thin layer chromatography (TLC). The analyses were carried out by applying aliquots (15 µL) of the extracts on silica gel chromatographic plates (F254 Macherey-Nagel), using various suitable eluent systems, standards and revelators (RANDAU et al., 2004).

ANTIMICROBIAL ACTIVITY

The antimicrobial activity was carried out with bacterial and yeast strains: Streptococcus pyogenes, Candida albicans URM 5901 and Candida krusei URM 6391. The bacterial strains were obtained from the Collection of Cultures of the Departmento de Antibióticos da Universidade Federal de Pernambuco (UFPE) and the yeast from the Collection of Cultures of the Departamento de Micologia da UFPE.

Bacteria and yeast were grown in Mueller Hinton (MH) and Sabourand Dextrose (SD) agar medium, respectively, overnight at 36 °C (bacteria) and 28 °C (yeast). Subsequently, the colonies were re-suspended in sterile saline solution (NaCl 0.15M) and adjusted to a wavelength of 600 ηm (DO_{600}) to obtain a suspension equivalent to 10^6 colony forming units (CFU) per mL. For the assay, the extract samples were filtered on a syringe filter PVDF sterile 13 mm x 0.22 µm.

The Minimal Inhibitory Concentration (MIC) of the samples was determined by the microtiter test in 96-well plates proposed by the Institute of Laboratory and Clinical Norms (CLSI – Clinical and Laboratory Standards, 2012). The extracts were added (80 µL) in the fourth well from which it was serially diluted in sterile Milli-Q water to the twelfth well of the same row. Posteriorly, 40 µL of the medium Mueller Hinton broth (bacterium) or Sabourand Dextrose (yeast) was added to all wells except the first, which was filled with 200 µL of the culture medium, corresponding to sterility control.

Plates were incubated at 36 °C (bacteria) and 28 °C (yeast) and optical density was measured at time zero and after 24 h of incubation using a microplate reader. MIC_{90} and MIC_{50} corresponded to the lowest concentration of the sample capable of promoting the reduction of ≥ 90% or ≥ 50%, respectively, in optical density, as compared to growth control 100%. For the determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC), aliquots (10 µL) of wells containing sample concentrations ≥MIC_{50} were inoculated into petri dishes containing Mueller Hinton or Sabourand Dextrose agar medium, which were subsequently incubated at 36 °C for 24 h. The MBC and MFC corresponded to the lower concentration of the sample capable of reducing the number of CFUs by 99.9% compared to the initial inoculum. Each assay was performed in triplicate and three independent experiments were performed.

ANTIBIOFILM TEST

The crystal violet method was used to evaluate biofilm formation in flat bottom polystyrene microtiter plates (TRENTIN et al., 2011). In each well 80 µL of water Milli-Q, 40 µL of the Mueller Hinton broth medium and 80 µL of the microorganism suspension (10^8 UFC/mL; in sterile saline solution) were added. The DO_{600} was performed at that time (time zero) using a microplate reader, the plates were incubated at 36 °C (bacteria) and 28 °C (yeast) for 24h. After this time, plaques were read to determine the growth of the microorganism at 600 ηm. The sequential step corresponded to the analysis of the formation of the biofilm that occurs after the removal of planktonic cells from each plate well.

The wells were washed with sterile 0.15M NaCl (three times) and the biofilms were pre-fixed in methanol, followed by setting at 50 °C and labeling with crystal violet 0.4% (w/v) 25 °C. After this step, the wells were washed with water to remove the non-adhered violet crystal and subsequently, the dye adhered to the biofilm was solubilized in absolute ethanol and the absorbance was measured at 570 ηm. All experiments were performed in triplicate. The formation of biofilms was determined by measuring the violet crystal at 570 ηm and the results were expressed as reduction of biofilm biomass percentage compared to control and counting of the CFU. The negative control was done with distilled water. Antibiofilm activity against fungi was performed using the concentrations: 4x MIC; 1x MIC and ¼ MIC, in addition, these concentrations were associated with ½ MIC of Fluconazole®.

The data were expressed as mean ± Standard Deviation (SD) and statistical differences were determined using Dunnett's test. A p-value ≤ 0.05 was considered statistically significant. For statistical analysis was used GraphPad Prim 7.0 software.

RESULTS

The phytochemical profile revealed the presence of monoterpenes, sesquiterpenes and triterpenes in the aqueous extract of seeds, while the leaves extract did not present secondary compounds (Table 1).

Table 1 – *Phytochemical profile of aqueous extracts of leaves and seeds of Cucurbita pepo.*

Secondary metabolite class	Leaves	Seeds
Alkaloids	–	–
Monoterpenes and Sesquiterpenes	–	+
Triterpenes	–	+
Saponins	–	–
Flavonoids	–	–
Coumarins	–	–
Cinnamic acid derivatives	–	–
Tannins	–	–
Anthraquinones	–	–

Positive reaction: +; Negative reaction: –

Source: Own authorship

The antimicrobial activity of the extracts showed that there was no antibacterial action against the strains of S. pyogenes, however there was inhibition of fungal growth (Table 2).

Table 2 – *Minimal Inhibitory Concentration MIC (mg/mL) in vitro of aqueous extracts of leaves and seeds of Cucurbita pepo.*

Extract Microrganism	Leaves	Seeds
Streptococcus pyogenes (A)	---	---
S .pyogenes (B)	---	---
Candida albicans	*0.03*	*0.03*
C. krusei	MIC_{90}: 1 MIC_{50}: 0.5	MIC_{90}: 1 MIC_{50}: 0.25

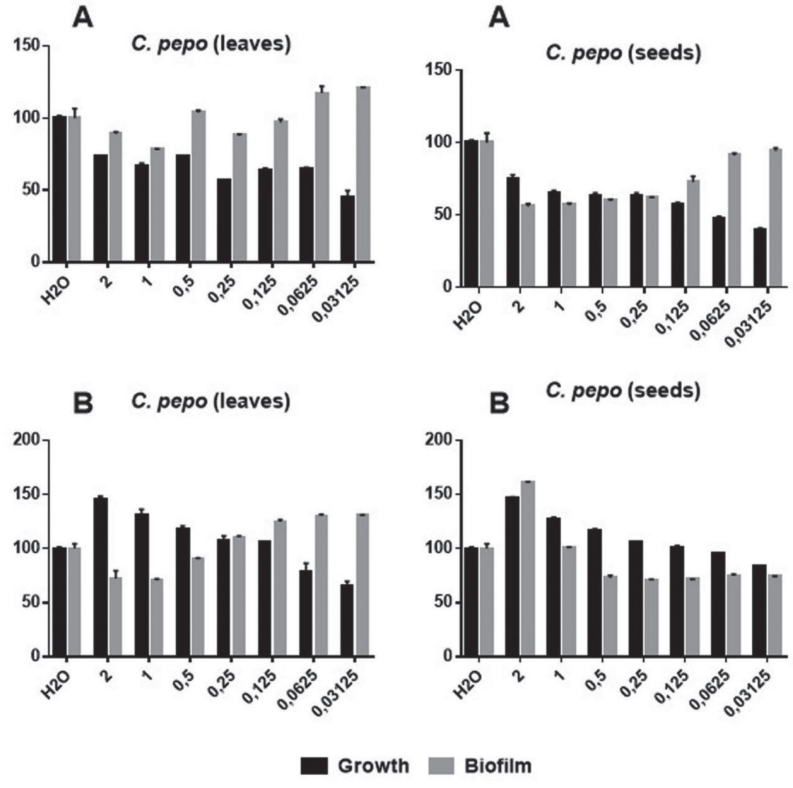
-- – Activity not detected.

Source: Own authorship

(A): Wound isolate; (B): Blood culture isolate

The Figure 1 shows the bacterial growth and biofilm formation capacity of S. pyogenes exposed to C. pepo leaves extracts and seeds. The strains used are clinical isolates of wound secretion and blood cultures. Data are expressed as mean and standard deviation.

Figure 1 – *Bacterial growth and biofilm formation of Streptococcus pyogenes (A and B) against aqueous extracts of Cucurbita pepo (mg/mL).*

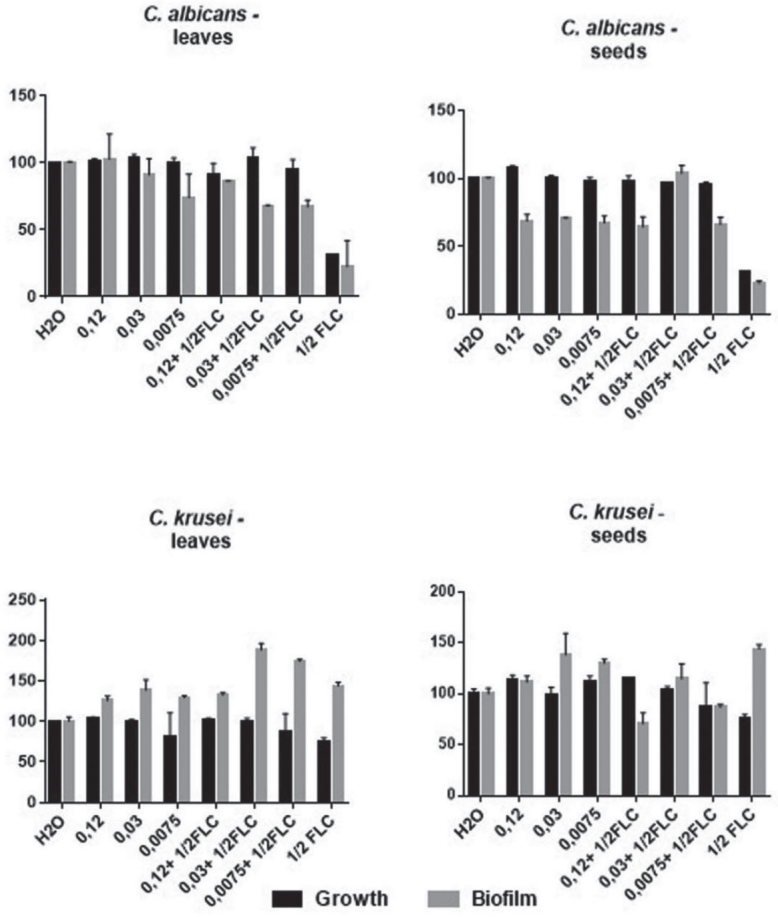


(A): *S. pyogenes wound isolate;* **(B)**: *S. pyogenes blood culture isolate (p≤ 0,05).*

Source: Own authorship

The Figure 2 shows the result of the antibiofilm activity of the extracts against yeast strains. The results show the fungal growth and the biofilm formation capacity of the species. Furthermore, the extracts were combined with ½ MIC of the antifungal fluconazole to evaluate synergistic effect.

Figure 2 – *Evaluation of the growth and biofilm formation of yeasts exposed to aqueous extracts leaves and seeds of Cucurbita pepo (mg/mL).*



Source: Own authorship

DISCUSSION

Triterpenes were also identified in a study with aqueous extract of C. pepo seeds (GONZÁLEZ; GARZA; GUTIÉRREZ, 2010). The presence of alkaloids, steroids and flavonoids has also been demonstrated by González, Garza and Gutiérrez (2010). The methanolic extract of leaves of the species demonstrated the presence of flavonoids, steroids, saponins, diterpenes and phenols (NDERITU et al., 2017). Tetracyclic triterpenoids (cucurbitacins) are common in the family Cucurbitaceae (RAJASREE et al., 2016). Cucurbitacins isolated from seeds of C. pepo exhibited anti-ulcer and antioxidant activity (GILL; BALI, 2011). The divergences of the present work with the literature can be explained by the fact that the extracts were prepared by different methodologies, as well as the previous studies performed the phytochemical prospecting by colorimetric techniques and not by TLC.

The methanolic extract from C. pepo leaves showed MBC below 1024 µg / mL in strains Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, P. stuartii and Enterobacter cloacae (NOUMEDEM et al., 2013).

Biofilm is an organization that is difficult to eradicate using existing antimicrobials, generally associated with high microbial resistance. In clinical practice, this promotes prolonged and costly hospitalizations (BEHLAU; GILMORE, 2008). An alternative to the search for new antibacterial compounds considers the ability to leave bacteria vulnerable, through interruption communication, without inducing stress. Secondary metabolites obtained from natural sources may have this ability (SILVA; SOUZA; ESPINHEIRA, 2019).

Aqueous extract of C. pepo leaves inhibited the growth of S. pyogenes strain isolated from wound, without interfering with biofilm formation. For the S.

pyogenes strain from blood culture, the extract was able to promote inhibition of biofilm formation at the highest concentrations, but did not present inhibitory activity on bacterial growth.

The aqueous extract of C. pepo seeds inhibited growth and biofilm formation for the wound isolate at concentrations greater than 0.25 mg/mL. The lowest concentrations of this extract were not able to inhibit biofilm formation. When tested against blood culture isolate, the seeds extract showed action against biofilm formation up to the concentration of 0.5 mg/mL, without inhibition at the highest concentrations tested. It was also unable to inhibit bacterial growth.

S. pyogenes blood culture isolate showed greater resistance to inhibition of bacterial growth, suggesting that it is a strain with more defense mechanisms. The isolation of multiresistant bacteria from the bloodstream is more frequent. The detection of pathogens in blood cultures is considered an indicator of the spread of an infectious process and is recognized as an important diagnostic resource in cases of infection. The susceptibility profile of these microorganisms should be evaluated to facilitate clinical management (LEÃO et al., 2007).

According to Ogawa et al. (2011), S. pyogenes is a bacterial responsible for superficial infections, such impetigo, or invasive infections, including necrotizing fasciitis, sepsis and streptococcal toxic shock syndrome. The ability to form biofilm is a key virulence mechanism of S. pyogenes. Biofilm guarantees survival and protection of host defensive mechanisms, antibiotics and other environmental changes. Once formed, biofilms are difficult to eradicate (WIJESUNDARA; RUPASINGHE, 2018).

Fungal infections may present superficial involvement, involving mucosal or skin involvement, or manifested systemically through diffusion into the bloodstream, with mortality rates above 40% (LOHSE et al., 2018). The Candida genus acquires resistance during the treatments through mechanisms, such as cellular change to reduce drug absorption and increase the expression of membrane transport proteins, that favors the efflux of antifungals (INIGO; POZO, 2018).

C. krusei has a high incidence in patients with leukemia, responsible for high mortality rates in these patients (HADRICH et al., 2018). The use of prolonged antibiotics, intravenous catheters, chronic diseases and treatment with chemotherapeutic agents are risk factors for the development of fungal infections. Although this species is not very common in wounds, Jud et al. (2017) verified in a skin biopsy the microorganism causing leg ulcer in an elderly patient.

The resistance of microorganisms has generated the need to search for alternative treatments. Fluconazole® may inhibit the formation of biofilms at high concentrations (1024 mg/mL). The search for antifungal agents with anti-biofilm property is necessary (ZHONG et al., 2017).

The results demonstrated that the aqueous extracts of both leaves and seeds showed antifungal activity against the species C. albicans and C. krusei with MIC and 0.25 up to 1 mg/mL. The antibiofilm evaluation showed that the extract of seeds has activity against the C. albicans strain, however the inhibition promoted by the extract was not greater than that obtained with Fluconazole®. The strain C. krusei presented resistance to Fluconazole® and the extracts evaluated.

CONCLUSION

The strains tested showed resistance against the extracts, although the extracts presents action against the growth and formation of biofilm. But, it was not possible to determine the MIC for S. pyogenes. The antifungal evaluation of extracts of leaves and seeds showed MIC 0.03 mg/mL for C. albicans and 1 mg/mL for C. krusei.

The antibiofilm activity of these species showed no dose dependence or synergistic effect when associated with the antifungal standard. It is an edible species, of medicine use, that needs further studies to investigate its pharmacological properties. In addition, its use in combination with antimicrobial agents should be investigated to promote adequate treatment without favoring resistance mechanisms or creating a favorable environment for the growth and/or development of defense mechanisms.

The choice for aqueous extract is justified by popular use, however future research using organic extracts, isolated compounds or seed oil can identify nutritional and medicinal applications of the species. New in vitro and in vivo tests need to be done to confirm the plant's anti-inflammatory, immunomodulatory, antioxidant and healing activities. Scientific works with species are scarce in the literature. Phytotherapics appear as an economical alternative for developing countries that have vast biodiversity. However, it is necessary to use consciously and safely.

ACKNOWLEDGMENTS

The authors grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for financial support.

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Submetido em: 06/08/2019
Aceito em: 08/07/2020