

Estudos em Biociências e Biotecnologia:

Desafios, Avanços
e Possibilidades

Manuel Simões
(organizador)

 EDITORA
ARTEMIS
2021

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**Dados Internacionais de Catalogação na Publicação (CIP)
(eDOC BRASIL, Belo Horizonte/MG)**

E82 Estudos em biociências e biotecnologia [livro eletrônico] : desafios, avanços e possibilidades / Organizador Manuel Simões. – Curitiba, PR: Artemis, 2021.

Formato: PDF

Requisitos de sistema: Adobe Acrobat Reader

Modo de acesso: World Wide Web

Inclui bibliografia

Edição bilíngue

ISBN 978-65-87396-50-7

DOI 10.37572/EdArt_211221507

1. Biociência. 2. Biotecnologia. 3. Biomedicina. 4. Bioética.
I. Simões, Manuel.

CDD 574

Elaborado por Maurício Amormino Júnior – CRB6/2422

PREFÁCIO

A biotecnologia baseia-se em conhecimentos multidisciplinares fortemente associados às ciências naturais e exatas, e às ciências aplicadas. As ciências biológicas e o seu enquadramento na biotecnologia têm aplicações em grandes áreas de importância socioeconómica, principalmente na medicina humana e animal, ambiente, agronomia e na indústria. Os processos biotecnológicos são caracterizados por usarem células procariotas ou eucariotas, partes das mesmas ou análogos moleculares - com o objetivo de se obterem produtos e serviços. Avanços significativos na biotecnologia surgiram das sinergias estabelecidas entre engenheiros, cientistas e reguladores para transformar descobertas científicas em novos processos e produtos, com impacto socioeconómico. A elevada dinâmica académica e industrial no desenvolvimento de conhecimento em ciências biológicas e biotecnologia é revelador da sua importância. Contudo, a necessidade de atualização dos avanços científicos, em conjugação com a transformação desse novo conhecimento em conteúdo curricular técnico-científico relevante são desafios para um eficaz processo formativo de recursos humanos altamente qualificados. O enquadramento ético e regulamentar de novos processos e produtos é igualmente desafiante.

Este livro foi dividido em quatro partes: a primeira parte reúne capítulos (1 a 6) relacionados com as biociências e a biotecnologia na área biomédica. A segunda parte concentra capítulos (7 a 11) na área do ambiente. A terceira parte é composta pelos capítulos 12 a 14 que se enquadram em aspetos da bioprospeção. A quarta parte contém os capítulos 15 e 16 que abordam aspetos do ensino/aprendizagem em biotecnologia e da bioética, respetivamente. Neste contexto, pretende com este livro contribuir para que estudantes e professores do ensino superior, ligados às biociências e à biotecnologia, quer a nível de graduação quer de pós-graduação, possam ter uma perspetiva de avanços na área. Este livro pode ser também útil a profissionais ligados a setores nos quais as biociências e a biotecnologia têm um papel de relevo, bem como para professores do ensino pré-académico.

Manuel Simões

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CAPÍTULO 4

EFFECT OF *Zinnia peruviana* ROOT EXTRACT ON THE PRODUCTION OF MICROBIAL BIOFILMS¹

Data de submissão: 09/09/2021

Data de aceite: 28/09/2021

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ABSTRACT: The increase of antimicrobial resistance is a growing global public health. One mechanism by which microorganisms acquire resistance to antibiotics is the biofilms. Approximately 80% of chronic and recurrent microbial infections are due to this resistance strategy produced by a diverse group of microorganisms. This context has motivated the search for new antimicrobial substances with anti-biofilm activity. The extensive and varied flora of the Central West Region of Argentina offers an important resource for the study of natural products in the search for antimicrobials with potential therapeutic use in clinical infections. Within these species, *Zinnia peruviana* (L.), is an herb with proven antimicrobial properties in its aerial part. The objective of this work was to evaluate the effect of the addition of acetonic extract from the roots of *Z. peruviana* at different concentrations on the biofilm production of *L. monocytogenes*, *E. coli* and *C. albicans*. Biofilm production was evaluated by determining adherence to 96-well microplates U-bottom. The tested extract inhibited 29% and 50% of the biofilm production of *L. monocytogenes* with 0.625 mg ml⁻¹ and 5 mg ml⁻¹ respectively. For *E. coli* the reduction was 33% (0.625 mg ml⁻¹) and 51% (0.078 mg ml⁻¹) while for *C. albicans* a significant reduction in biofilm formation was observed with 3 concentrations of extract: 59% (5 mg ml⁻¹), 42% (0.625 mg ml⁻¹) and 44% (0.078 mg ml⁻¹). All the biofilm reduction values showed significant differences ($p < 0.05$). The

¹ Trabajo presentado anteriormente como Abstract en BIOCELL, 45(3):62, 2021. ISSN 0327- 9545 / ISSN 1667-5746 (versión en línea). Este estudio fue financiado por Ciencia y Técnica de la Universidad Nacional de San Luis (PROICO 02-0218 y 02-2516). Los autores declaran no tener ningún conflicto de interés.

high biological activity of the roots of *Z. peruviana* represents an alternative potential for the treatment and control of microbial infections. Further studies on toxicity and bioapplicability are needed.

KEYWORDS: *Zinnia peruviana*. Acetonic root extract. Microbial biofilms.

EFEECTO DE EXTRACTO DE RAÍZ DE *Zinnia peruviana* SOBRE LA PRODUCCIÓN DE BIOPELÍCULAS MICROBIANAS

RESUMEN: El aumento de la resistencia a los antimicrobianos constituye una creciente preocupación de la salud pública mundial. Un mecanismo por el cual los microorganismos adquieren resistencia a los antibióticos es la formación de biopelículas. Aproximadamente el 80% de las infecciones microbianas crónicas y recurrentes se deben a esta estrategia de resistencia producida por un grupo diverso de microorganismos. Este contexto ha motivado la búsqueda de nuevas sustancias antimicrobianas con actividad anti-biofilm. La extensa y variada flora de la Región Centro-Oeste de Argentina ofrece un importante recurso para el estudio de productos naturales en la búsqueda de antimicrobianos con potencial uso terapéutico en infecciones clínicas. Dentro de estas especies, *Zinnia peruviana* (L.), es una hierba con propiedades antimicrobianas demostradas en su parte aérea. El objetivo de este trabajo fue evaluar el efecto de la adición de extracto acetónico de raíces de *Z. peruviana* a distintas concentraciones sobre la producción de biofilm de *L. monocytogenes*, *E. coli* y *C. albicans*. La producción de biofilm se evaluó por determinación de la adherencia a microplacas de 96 pocillos fondo en U. El extracto ensayado inhibió el 29% y 50% de la producción del biofilm de *L. monocytogenes* con 0,625 mg ml⁻¹ y 5 mg ml⁻¹ respectivamente. Para *E. coli* la reducción fue de 33 % (0,625 mg ml⁻¹) y 51% (0,078 mg ml⁻¹) mientras que para *C. albicans* se observó una significativa reducción en la formación de biofilm con 3 concentraciones de extracto: 59 % (5 mg ml⁻¹), 42 % (0,625 mg ml⁻¹) y 44 % (0,078 mg ml⁻¹). Todos los valores de reducción de la producción del biofilm presentaron diferencias estadísticamente significativas ($p < 0,05$). La elevada actividad biológica de las raíces de *Z. peruviana* representa un potencial alternativo y alentador para el tratamiento y control de infecciones microbianas. Se necesitan más estudios sobre toxicidad y bioaplicación.

PALABRAS CLAVE: *Zinnia peruviana*. Extracto acetónico de raíz. Biopelículas microbianas.

1 INTRODUCTION

Worldwide, antibiotics are used to treat infections in humans and animals. In addition to therapeutic use, antibiotics are commonly added to animal feed in small amounts as prophylaxis and for growth promotion purposes, promoting a steady increase in antimicrobial resistance of microbes and a decrease in the ability of available antimicrobials to treat common infections. Antimicrobial resistance (AMR) represents a health threat and an emerging risk to the global population of entering a post-antibiotic era in which existing antibiotics are gradually rendered ineffective due to resistance. This increased resistance is mainly associated with the overuse and misuse of antibiotics, the appearance of

resistant strains and the slowdown in the discovery of antimicrobial drugs (Lofa *et al.*, 2019; Asmerom *et al.*, 2020). One important mechanism whereby microorganisms acquire resistance to antibiotics and evade the immune system is by forming biofilms. Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces (Vasudevan, 2014). Biofilm-associated cells have the ability to adhere irreversibly on a wide variety of surfaces, including living tissues and indwelling medical devices as catheters, valves, prosthesis, and so forth (Parsek and Singh, 2003). They are considered an important virulence factor that causes persistent chronic and recurrent infections; they are highly resistant to antibiotics and host immune defenses (Grant and Hung, 2013).

Bacteria protected within biofilm exopolysaccharides are up to 1,000 times more resistant to antibiotics than free-floating (planktonic cells) (Sharma *et al.*, 2019), which generates serious consequences for therapy and severely complicates treatment options (Sun *et al.*, 2013). Biofilm resistance is due to several reasons, like restricted diffusion of antibiotics into biofilm matrix, expression of multidrug efflux pumps, type IV secretion systems, decreased permeability, and the action of antibiotic-modifying enzymes (Alekhshun and Levy, 2007). The increased biofilm resistance to conventional treatments enhances the need to develop new control strategies (Simoes *et al.*, 2007). Biofilm inhibition is considered a major drug target for the treatment of various bacterial and fungal infections, and pharmacological development of these drugs is now extensively studied (Namasivayam *et al.*, 2013).

The microorganisms producing biofilm comprise a diverse group of organisms, including both Gram-negative and Gram-positive bacteria, as well as fungal species. Gram-positive specie involved in infections associated with the formation of biofilm is *Listeria monocytogenes* that produces listeriosis due to food contamination and coinfections with other bacterial species. Among Gram-negative infections, *Escherichia coli* is responsible for hemolytic uremic syndrome, acute diarrhea, and urinary tract infections. *Candida* species have emerged as a significant cause of morbidity and mortality and account for approximately 72% of all nosocomial fungal infections. *C. albicans* is a very common opportunistic pathogen producing oral, vaginal and or systemic candidiasis. Furthermore, biofilm formation by *Candida* has been documented on a variety of medical devices such as catheters, dialysis and joint devices. While fungal implant infections are less common than bacterial infections, they tend to be more serious or troublesome. Antifungal drugs, mainly polyenes and azoles, are commonly used to treat *Candida* infections. However, efficacy of these drugs is limited in many cases due

to development of resistance, poor penetration power in biofilm and undesirable side effects (Jafri and Ahmad 2019; Ahmad *et al.*, 2020). Given the alarming incidence of antimicrobial resistance in microorganisms of clinical importance, there is a continuing need for new and effective therapeutic agents. This context has led to the investigation of new antimicrobial substances from plant species popularly used in folk medicine. Therefore, the study of new agents of natural origin with antimicrobial and antibiofilm activity constitutes an important clinical challenge.

The field of natural products of plant origin currently links two axes of study. From a structural point of view, natural products pose significant challenges for the application of strategies that allow their isolation, purification and structural elucidation. However, the axis of greatest importance is undoubtedly their bioapplicability, since the bioactivity reported in the literature for many secondary or chemically derived metabolites makes them potentially interesting in the search and development of molecular models in the design of new drugs. In this framework, the extensive and varied flora of the Central-West Region of Argentina is an important source of renewable resources in the study of natural products and their applications in the search for antimicrobials with potential therapeutic use in clinical infections. *Zinnia* genus contains annual and perennial plants belonging to the family Asteraceae and comprises about 20 species native to South America (Goma *et al.*, 2019). *Zinnia peruviana* (L.) L., commonly known as “Chinita del Campo”, is a native plant that grows in the center and north of Argentina (Del Vitto *et al.*, 1997). The aerial parts of this species present diverse ethnopharmacological uses and have been evaluated for different biological properties (Barboza *et al.*, 2009; Satorres *et al.*, 2012; Mohamed *et al.*, 2017).

On the other hand, the genus *Zinnia* has shown the presence of a diversity of secondary metabolites of different classes (Bastos *et al.* 2020). Among them, the sesquiterpene lactones are one of the main constituents that have been isolated and they are responsible of various biological activities such as anticancer, anti-inflammatory and immunomodulatory effects, anti-ulcer, antifungal and antiviral activities (Lent *et al.*, 1977; Subramaniam *et al.*, 2014).

Previous works of our research group (Mattana *et al.*, 2016; Satorres *et al.*, 2012; Mohamed *et al.*, 2017; Mohamed *et al.*, 2018) motivate the progress and deepening of the study of this promising plant as a possible source of new antimicrobial agents with direct application in clinical microbiology and, as part of our studies of antimicrobial herbal constituents, was studied the *in vitro* the inhibitory effect of *Z. peruviana* roots against biofilm of *L. monocytogenes*, *E. coli* y *C. albicans*.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Zinnia peruviana (L.) L. were collected in El Trapiche and La Florida, San Luis, Argentina (latitude: 33° 7' 0" S; longitude: 65° 5' 0" W), in March 2018/February 2019. A voucher specimen was deposited at the Herbarium of the National University of San Luis (L.A. del Vitto N° 8841).

2.2 PREPARATION OF ACETONIC EXTRACT

Z. peruviana roots collected were air-dried and ground in knife mills to fine grain size. Powdered air-dried material of *Z. peruviana* (200 g) was macerated in 500 ml of acetone at room temperature for one week (Figure 1). The crude acetonetic extract was filtered through Whatman filter paper N° 1, and the filtrates were concentrated under reduced pressure at 40 °C. The extract was dried, weighed (3.1 g) and stored at 4 °C in storage vials for preliminary antimicrobial screening.

Figure 1. **A:** *Zinnia peruviana* plant. **B:** *Z. peruviana* roots collected. **C:** Maceration of *Z. peruviana* roots in acetone.



Photos: own authorship.

2.3 MICROBIAL STRAINS

The microorganisms tested included *Listeria monocytogenes* CLIP 74904, *Escherichia coli* ATCC 35218 and *Candida albicans* ATCC 36801.

2.4 ANTIBIOFILM ACTIVITY. INHIBITION OF THE BIOFILM FORMATION

The potential of the acetonetic extracts from *Z. peruviana* roots to prevent initial cell attachment was investigated through the biofilm inhibition assay on microplate U-bottoms

(Microplate method). In brief, 50 µl of culture medium (supplemented with 1% glucose), 50 µl of bacterial inoculum (10^8 CFU ml⁻¹) and 100 µl of extract at different concentrations were added to each well and incubated at 37 °C for 48 h. Three concentrations were tested: 5 mg ml⁻¹ (C1), 0.625 mg ml⁻¹ (C2) and 0.078 mg ml⁻¹ (C3). The culture was discarded and the adhered content (sessile cells) was washed with sterile physiological solution, fixed with methanol and stained with crystal violet. The optical density reading was performed at 550 nm in ELISA reader (Microplate Reader Benchmark, BIO-RAD). The experiment was carried out in quadruplicate and was repeated 2 times.

Percentages of inhibition for each sample were determined by comparing the mean optical density (OD) of control wells (without extracts added) with the bacterial suspension (with the addition of extracts). Thus the percentage of biofilm inhibition was determined by the formula:

$$\text{Biofilm reduction \%} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

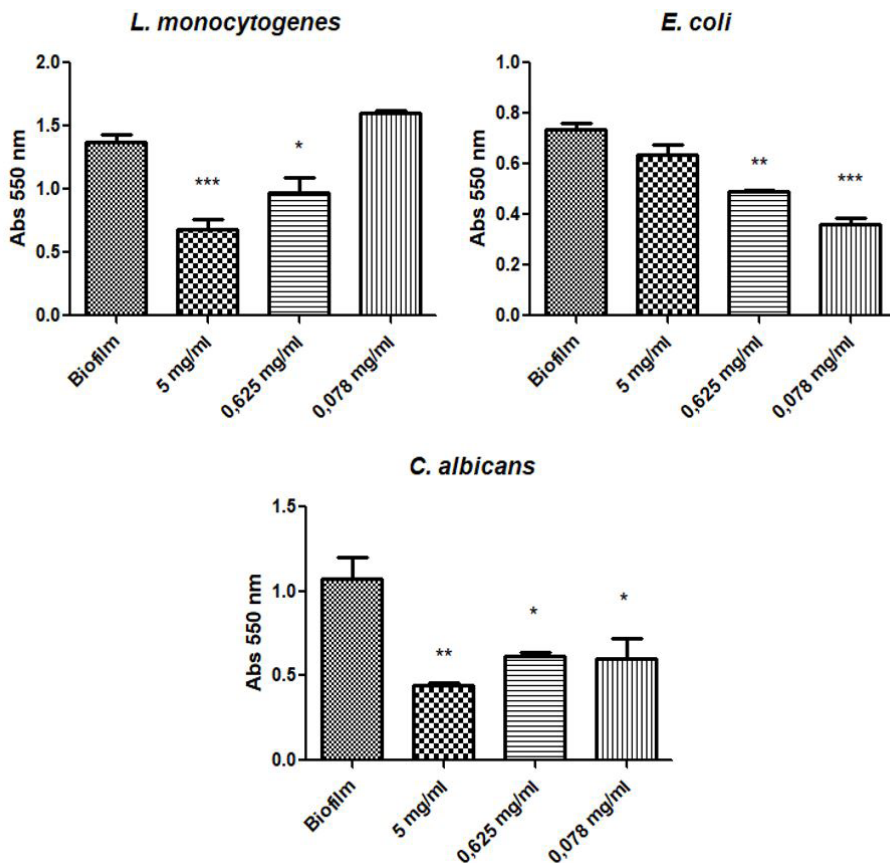
2.5 STATISTICAL ANALYSIS

GraphPad Prism 5 program, One-way analysis of variance (ANOVA), Bonferroni test were used to analyze the result and p values less than 0.05 were considered statistically significant. All experimental data were derived from at least 2 independent experiments.

3 RESULTS AND DISCUSSION

There is recently an increased interest in using green antimicrobials as an alternative to prevent and destroy biofilms, such as plant extracts and phytochemicals. Natural products derived from *Z. peruviana* present biological activity against Gram-positive and Gram-negative bacteria and yeasts. This study assessed the ability of acetic extract obtained from the roots of *Z. peruviana* to prevent further formation of biofilms at 48 h. This extract showed significant inhibition of the biofilm production *L. monocytogenes*, *E. coli* and *C. albicans* ($p < 0.05$) (Figure 2).

Figure 2. Reduction of biofilm production with the addition of *Zinnia peruviana* root extract at three concentrations. * $p < 0.05$ compared with control. ** $p < 0.01$ compared with control. *** $p < 0.001$ compared with control (biofilm). Each value represents means \pm SD.



The acetonic extract inhibited 29% and 50% of the biofilm production of *L. monocytogenes* with 0.625 mg ml⁻¹ and 5 mg ml⁻¹ respectively, following a dose-dependent effect. Concentrations lower than 0.625 mg ml⁻¹ did not show antibiofilm effect for this bacterium. Against *E. coli*, the reduction in biofilm production was 33% (0.625 mg ml⁻¹) and 51% (0.078 mg ml⁻¹) however, the reduction was not dose-dependent. In contrast, *C. albicans* reduced biofilm production with the three concentrations tested with dose-dependent effect with values of 59% (5 mg ml⁻¹) and 44% (0.625 mg ml⁻¹ and 0.078 mg ml⁻¹).

The secondary metabolites of plants present a wide chemical diversity and important pharmaceutical properties. Among them, several classes of phytochemicals have been shown to present antimicrobial properties, either when used alone or together with other antimicrobials products, against clinically important pathogens, interfering

with some of the main factors involved in biofilm development, such as motility, adhesion, intercellular aggregation, and communication (Sakarikou *et al.* 2019). In this case, regarding the antibiofilm activity of the *Zinnia* root extract, the ziniolide compound identified as the major secondary metabolite, would be responsible for this activity by a mechanism that has not yet been elucidated.

It is generally more difficult to eradicate pre-existing biofilms by the extracts. Some reports have also noted that it is less difficult to inhibit cell attachment than to get rid of established biofilm (Cerca *et al.*, 2005; Sandasi *et al.*, 2011). We have carried out studies of the antibiofilm activity of *Zinnia* root extract on the already formed biofilm (data not shown in this work) with less active results to eradicate the biofilm. This confirms that pathogens are able to resist the action of antimicrobials more when they exist in biofilms and their infections are able to persist on different biotic and abiotic surfaces (De La Fuente-Núñez *et al.*, 2013). Factors which cause resistance in biofilms include presence of an extracellular polymeric matrix which causes strong attachment of microbes to surfaces and low antibiotic penetration or increased activity of efflux pumps which expel antimicrobial agents from cells (Jamal *et al.*, 2018) and too interfering with intercellular communication strategies (quorum sensing) of the bacteria, thereby reducing biofilm formation (Merghni *et al.*, 2018). The plant extracts may have interfered with any of these factors. Specifically, the excellent ability of the plant extracts to interfere with the initial stage of biofilms formation may be attributed to interference with forces, such as Brownian, sedimentation, Lifshitz–Van der Waals and electrostatic interaction forces, that favour the deposition and adherence of bacteria to surfaces (Tiwari *et al.*, 2018). Also, since certain organic and inorganic molecules and other nutrients are important for cell growth and hence cell adhesion (Sandasi *et al.*, 2010), it is possible that the plant extracts may inhibit the availability of nutrients.

The strong biological activity of the acetonic extract of the root of *Z. peruviana* demonstrated in this work represents an alternative potential for the treatment of microbial infections. We are conducting other studies to elucidate the antibiofilm action mechanism, as well as cytotoxicity and genotoxicity studies to determine the safety of the acetonic extract of the root of *Z. peruviana*.

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