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Identification of an antimicrobial peptide from *Chamaemelum nobile*

Identificación de un péptido antimicrobiano de *Chamaemelum nobile*

Diana Daniela Portela Dussán[1], Sandra Mónica Estupiñan Torres[2] and Silvio Alejandro Lopez-Pazos[1]*

[1]*Faculty of Sciences, Universidad Antonio Nariño, Carrera 3 Este 47A-15, Bogotá D.C., Colombia.*
[2]*Faculty of Health Sciences, Universidad Colegio Mayor de Cundinamarca, Calle 28 No. 5B-02 Bogotá D.C., Colombia.*

*Corresponding author: alejandrolopezpazos@uan.edu.co

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Abstract

Chamaemelum nobile, or Roman chamomile, is a plant containing anti-inflammatory and antimicrobial properties. Antimicrobial peptides (AMPs) are part of the plant defense system including lipid transfer peptides (LPTs). Our objective is to identify a LTP-related protein from *C. nobile* (cnLTP). PCR was performed on *C. nobile* DNA for identifying cnLTP gene. Bioinformatics was used for their characterization, and a sensitivity test was carried out on *Rhizoctonia solani*. cnLTP has 99 amino acids, 9.8 kDa, isoelectric point of 9.39, 33 aliphatic residues, aliphatic index of 85, hydropathicity of 0.127, four alpha-helices and four disulfide bridges. An inhibitory activity of apoplastic fluid of *C. nobile* was determined at 1 μg/mL on *R. solani*. This study contributes in the knowledge of a novel and non-characterized LTP using *in silico* and experimental related approaches.

Keywords: Roman chamomile, Antimicrobial peptide, Lipid transfer protein, *Rhizoctonia solani*.

Resumen

Chamaemelum nobile, o manzanilla romana, es una planta que contiene propiedades antiinflamatorias y antimicrobianas. Los péptidos antimicrobianos (AMP) son parte del sistema de defensa de las plantas, que incluye a los péptidos de transferencia de lípidos (LPT). Nuestro objetivo es identificar una proteína relacionada con LTP de *C. nobile* (cnLTP). Se realizó la PCR sobre el ADN de *C. nobile* para la identificación del gen cnLTP; se utilizó bioinformática para su caracterización, y una prueba de sensibilidad contra *Rhizoctonia solani*. cnLTP tiene 99 aminoácidos, 9,8 kDa, punto isoeléctrico de 9,39, 33 residuos alifáticos, índice alifático de 85, hidropaticidad de 0,127, cuatro hélices alfa y cuatro

puentes disulfuro. Se encontró que el fluido apoplásico de *C. nobile* (1 μg/mL) inhibe el desarrollo de *R. solani*. Este estudio contribuye al conocimiento de un LTP novedoso y no caracterizado, utilizando enfoques *in silico* y experimentales.

Palabras clave: Manzanilla romana, péptido antimicrobiano, proteína de transferencia de lípidos, *Rhizoctonia solani*.

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Orcid IDs:

Diana Daniela Portela Dussán: https://orcid.org/0000-0003-2949-7033
Sandra Mónica Estupiñan Torres: https://orcid.org/0000-0002-6937-4567
Silvio Alejandro Lopez-Pazos: https://orcid.org/0000-0002-3682-4162

1 Introduction

Pathogenesis-related proteins (PR) can be detected at the basal level in healthy tissues of plants, but increase their presence under the attack of pathogens. PRs activation may only begin after a phase of plant-pathogen interaction from recognition of molecular patterns associated with microorganisms (PAMPs) by recognition receptors of these patterns (PRR) present on the cell membrane of plants, or through the recognition of elicitors as nucleotide binding domain and leucine-rich repeat (NB-LRR) proteins. The concentration of PRs increases with the transduction of chemical signals as salicylic acid, ethylene and jasmonic acid during the first activation stage of defense mechanisms (Zhou and Zhang, 2020; Boyd et al., 2013). Antimicrobial peptides (AMPs) are one part of PRs, which have a molecular weight of less than 10 kDa and *in vitro* antimicrobial activity. AMPs are produced by microorganisms, animals and plants as defense mechanism against pathogens (Bin Hafeez et al., 2021).

AMPs comprise less than 200 amino acids are expressed constitutively and are inducible in susceptible tissues as leaf primordia, stomata and epidermis, e.g., it was evidenced in *Nicotiana megalosiphon* which after inoculation with *Peronospora hyoscyami* sp. *tabacina* over expressed in leaves a defensin gene, or *Brassica oleracea* inoculated with *Xanthomonas campestris* pv. *campestris* expressed on stem and leaves AMPs genes, and it was also observed that pepper inoculated with *X. campestris* pv. *vesicatory* caused high transcription in a CaAMP1 gene, and their expression generates tolerance to *Phytophthora sojae* (Sharma et al., 2022; Niu et al., 2020; Jiang et al., 2011; Portieles et al., 2010). It has been evidenced that cationic AMPs as alpha/beta thionins, defensins and lipid transfer peptides (LTPs) interact with anionic groups of the phospholipid bilayer in microorganisms. It also forms micelles to allow ion flow to the extracellular space with osmotic decompensation, cell lysis and death (Kovaleva et al., 2020).

Anionic AMPs, e.g., cyclotides, interact with the cytoplasmic membrane of pathogens through electrostatic forces between polar residues of an AMP and hydrophilic heads of phospholipids through recognition of the chiral glycerol backbone. The electrostatic force of this complex junction is determined by an ionic interaction between the ammonium group of phosphatidylethanolamine and the carboxyl group of a conserved glutamate from loop 1 of the AMP (Venkatesan and Roy, 2023; Troeira Henriques and Craik, 2017). The permeabilization suffered by the cytoplasmatic membrane in the microorganism/AMP interaction is not clear. Four possible mechanisms have been described: (i) it comprises peptide helices that form a bundle in the membrane with a central lumen; (ii) AMPs accumulate on the surface of a lipid bilayer interacting with the hydrophilic heads of phospholipids and covering the membrane like a carpet causing disorganization of the lipid bilayer; (iii) AMPs are inserted into the membrane, forming a pore and inducing continuous bending of the lipid monolayer, orienting the hydrophobic surface of the membrane towards the outside and the hydrophilic surface inwards, forming an aqueous channel with loss of polarity; (iv) AMP causes a competitive displacement of Ca2+ and Mg2+ (Boparai and Sharma, 2020; Li et al., 2012).

Roman chamomile *Chamaemelum nobile* (L.) is an aromatic perennial herb. The plant has a branched and elongated stem with a height ranging from 20 to 30 cm, which supports a floral head, formed by white leaves with segmented ligules and well-defined leaflets. It grows at temperate areas, and it is adaptable and used on several types of acidic soils with enough water. *C. nobile* contains some 0.24 - 1.9% volatile oils and 120 secondary metabolites, including flavonoids (quercetin, luteolin, apigenin, patuletine), alpha-bisabolol and bisabolol oxides A and B, azulenes (chamazulene), monoterpenes (alpha-pinene), sesquiterpenes (farnesene), coumarins (herniarina and umbelliferone). Its composition allows recognizing therapeutic properties (anti-inflammatory, spasmolytic, sedative, antioxidant, anticoagulant, antihyperlipidemic, repellent and antimicrobial). World production of *C. nobile* is over 1000 tonnes/year. To produce 0.8 - 1.5% of essential oils, 800 kg of floral stem/ha is used. Phenolic compounds and alkanes can be obtained from seed, fruit, or roots. *C. nobile* oils are effective *in vitro* against *Staphylococcus aureus* (0.1 mg/mL), *Escherichia coli* (0.1 mg/mL), *Bacillus subtilis* (0.05 mg/mL), *Pseudomonas aeruginosa* (12.5 and 25 mg/mL), *P. tolaasii* (300 mg/mL), *Candida albicans* (0.1 mg/mL), and fungi as *Aspergillus candidus*, *Penicillium* sp., *Fusarium culmorum* and *A. niger* (900 mg/mL) (Ghaedi

et al., 2015; Kazemian et al., 2015; Srivastava et al., 2010; Saderi et al., 2005).

The aim of this research is to find a cationic AMP from *C. nobile* (named cnLTP) focused on attempting to identify the DNA sequence of a LTP. The parameters were bioinformatics and molecular tests, and evaluation on *Rhizoctonia solani*, a potato pathogen.

2 Materials and methods

2.1 *C. nobile* culture

Commercial Roman chamomile seeds were used in a propagation medium (Murashige and Skoog Salt and Vitamin Mixture (MS) supplemented with 20 mg/L sucrose, 0.1 mg/L, benzylaminopurine (BAP), 0.5 mg/L naphthaleneacetic Acid (NAA), 0.25 mg/L gibberellic acid (GA3). The seeds were maintained in a condition of constant temperature (20 °C) and humidity (70%).

2.2 PCR for detection of cnLTP gene

For DNA extraction, *C. nobile* leaves from *in vitro* plantlets were dipped in a tube containing lysis buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), precipitation solution (isopropanol) and 1X TE buffer as diluent (Edwards et al., 1991). PCR was carried out using l X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer and 0.2 U Taq DNA polymerase. Thermo cycle condition was a denaturation step at 95 °C for 5 min, 30 cycles consisting of denaturation (95 °C × 1 min), annealing (50 °C × 1 min), extension (72 °C × 1 min), and an final extension (72 °C × 5 min).

The PCR products were determined using 1.2% agarose electrophoresis, and sequenced (Macrogen, USA). The forward and reverse primers for LTP were 5′-GACTGCTCAACGGTTCAGTAAAGTTGA-3′, and 5′-TCAACTTTACTGAACCGTTGAGCAGTC-3′ respectively (Rojas, 2010). The obtained sequence data was deposited in GenBank (ID: MT294300).

2.3 *In silico* characterization of cnLTP

Nucleotide sequence of the cnLTP was employed to find its amino acid sequence, using Translate from Expasy server (http://web.expasy.org/translate/) (Artimo et al., 2012). To predict the feasible role of the AMP, a comparative analysis was completed using BLASTp (http://www.ncbi.nlm.nih.gov/BLASTp/) (Shah et al., 2018). The biochemical annotation of the cnLTP was done using ProtParam to find similar sequences in protein databases (https://web.expasy.org/protparam/) (Artimo et al., 2012). The homology modeling of the cnLTP was performed using I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2009). The best hit obtained on cnLTP annotation was found to LTP from *Nicotiana tabacum* and was taken as a template (PDB ID: 1T12A; 73% identity, 91% of query coverage; E-value of 7e-43). Figures were done by DeepView (https://spdbv.vital-it.ch/) (Johansson et al., 2012).

2.4 Susceptibility test for *R. solani* using *C. nobile* apoplastic fluid

Apoplast washing fluid (AWF) from *C. nobile* was extracted from leaves (Butt et al., 2019; Gentzel et al., 2019). Mature leaves (1 g) were first rinsed using sterile ddH_2O to remove cytoplasmic exudes from damage cells, and the fresh weight was then determined. Leaf segments were infiltrated in a bottle containing 250 mL of sterile ddH_2O at 4 °C and vacuum pressure of 20 kPa, which allows the air to escape and to facilitate the flow of water into the intercellular space, washing the AWF molecules. Once the pressure method was completed, the bottle was shaken slowly until it released air and vacuum to both reduce cell lysis and avoid cytoplasmic contamination.

The vacuum pressure was repeated until the leaves were completely infiltrated, its color turning to dark. The AWF was recovered from leaf segments dried with soft laboratory paper and wrapped in parafilm, and were centrifuged at 4 °C, 10000 rpm for 10 min. The Bradford assay was used to measure protein concentration (Bradford, 1976). Quality assessment for AWF peptides was performed by tricine-SDS-PAGE. The preliminary evaluation on the biological activity of the AWF on *R. solani* already completed by using 10 cm of the mycelium that was grown in PDA medium, and AWF lanes were added to each side and extended beyond the ends of the existing fungal structure on a gradually

decreasing basis, using concentrations of 0.1 μg/mL - 1 μg/mL.

A suspension of *P. aeruginosa* strain PAO1 was used as a positive control (negative control - water instead of AWF as sample, data not shown). *R. solani* was incubated for five days at 30 °C. The effect of AWF proteins on *R. solani* was measured by the displacement of the mycelium situated in the Petri dish.

3 Results

Roman chamomile seeds were cultured in a MS medium supplemented with growth hormones to object of induce germination and growth of seedlings suitable for extracting genomic DNA. PCR identified a 300 pb product that could be determined by sequencing. This sequence was compared with the nucleotides entries recorded in the GenBank database using BLAST program (Shah et al., 2018). The DNA sequence (297 pb) had close linkage with tomato LTPs (identity 96%, E value 4e-57, coverage 96%, NCBI). This knowledge, in turn, enables deduction of the cnLTP sequence (99 residues) that the DNA then encodes. It only contains coding DNA sequences for mature peptide without signal peptide or polyadenylation site. The sequence of amino acids in the cnLTP, and hence protein function, is determined by eight C residues that are linked in four disulfide bonds (Figure 1A).

The theoretical molecular weight of cnLTP is 9.8 kDa, its isoelectric point is 9.39. In addition, the peptide is made up of 33 aliphatic residues (A, V, I, and L), and the aliphatic index of the cnLTP is 85. This index determines the volume occupied by aliphatic side chains, 12 positively charged residues given the cationic conformation of the cnLTP at neutral pH, and three negatively charged residues. The hydropathicity is 0.127, which indicates the presence of a hydrophobic cavity where interaction with lipids also takes place. cnLTP is made up of four alpha-helices. The first helix (P2-Q24) presents a H1A structure (P2-S5) formed by two folds in His3 and Gly4, and H1B (P18-L23) formed by four folds in Q11, G15, C19 and Y22. The second helix H2 (C32-L39) contains three folds in R34, G35, L39. The third helix H3 (P46-A62) comprises seven folds in D48, K50, A52, T54, L56, K57, A59, and N61. The fourth helix H4 (L68-C78) encloses a fold in G69, I74 and S76. The four alpha-helices are connected by three short loops, L1 (G25-G31), L2 (L40-T45) and L3 (I63-N67) (Figure 1B).

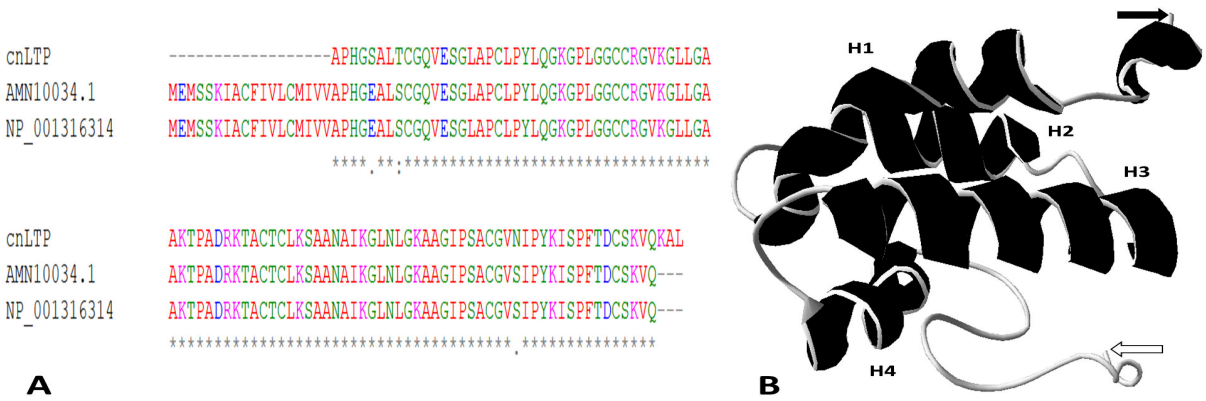

Figure 1. Structure of cnLTP. A. Alignment of two LTPs of tomato against cnLTP associated with physicochemical properties according to Clustal Omega (Sievers and Higgins, 2014): red is small plus hydrophobic (together with aromatic -Y), blue is acidic, magenta is basic – H, green is hydroxyl plus sulfhydryl plus amine plus G, gray corresponding to unusual amino and imino acids. B. 3D model of cnLTP, black arrow indicates the N-terminal, white arrow shows the C-terminal. Helices are indicated and colored in black.

cnLTP is stabilized by four disulfide bridges formed among C9-C33, C19-C55, C32-C78 and C53-C92, which were calculated throughout DISULFIND (http://disulfind.dsi.unifi.it/) (Ceroni et al., 2006). cnLTP has 50 hydrophobic residues: A, V, L, I, P, F and C, most of them are found inside of the

peptide, forming a hydrophobic cavity which is a characteristic of AMPs (Li et al., 2012).

Biological evaluation of *C. nobile* apoplastic fluid was carried out considering the protein concentration (2.37 mg/mL), as well as the intrinsic presence of low molecular weight peptides of approximately 10 kDa, indicating that there are LTPs (Figure 2A). Antagonism tests indicated an inhibitory effect on *R. solani* at a concentration of 1 μg/mL (Figure 2B-2C).

4 Discussion

Peptides allow the bacteria to evade the host defense and proliferate by preventing cell signaling, cell migration and can even kill response cells directly. Peptides have complicated damage mechanisms on cell membranes (rupture), protein synthesis (inhibition), second messengers (activation), or a defense response (activation) (Yang and Yousef, 2018). It has been suggested that a specific biochemical characterization focused on specific biological activity, or a computer system-assisted design are relevant ways for the new biotechnological options discovery (Rondon-Villarreal and Pinzon-Reyes, 2018).

For LTPs, a signal sequence is responsible for directing the peptide towards the cytoplasmic membrane where it is cleaved at the N-terminus by an aminopeptidase, and the mature peptide is exported to the intercellular space where it exerts its biological activity (Pagnussat et al., 2012). The mature cnLTP has a conserved substitution (T-S), plus two semi-conserved substitutions (E-S, N-S). It has been reported that the conservation for S phosphorylation site (which could be exchanged with phosphorylatable T or E) would become one of the key residues to protein folding (Pearlman et al., 2011). The presence of the hydrophobic cavity is essential in the LTPs family, because it allows for binding and transferring lipids, e.g., a LTP from wheat, which had a hydrophobic cavity able to attract prostaglandin B2 and mobilize between microsomal fractions and mitochondria. The LTP hydrophobic cavity can be attached to saturated fatty acids (12–19 carbon atoms), unsaturated FAs of different chain length (16–18 carbon atoms) and geometry of unsaturation, lysolipids (14–16 carbon atoms), and jasmonic acid (Melnikova et al., 2016; Tassin-Moindrot et al., 2000).

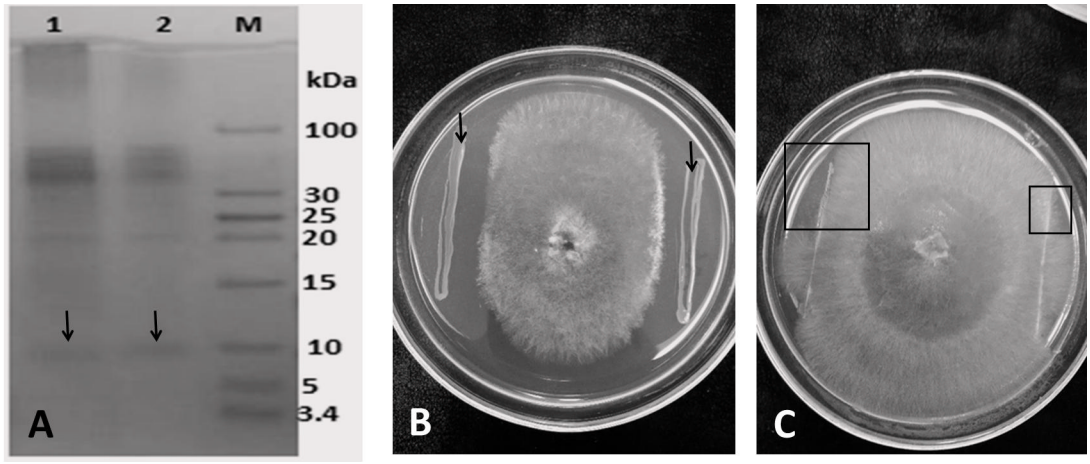

Figure 2. Bioassay of *C. nobile* AWF on *R. solani*. A. Tricine SDS-PAGE showed a band of 10 kDa related to LTPs in *C. nobile* AWF (lines 1 and 2, M is molecular weight marker). B. Positive control contained lanes with suspension of *P. aeruginosa* strain PAO1 (black arrows). C. *R. solani* mycelium with *C. nobile* AWF lanes to each side, which were gradually being extended on the medium; the squares showed inhibition areas.

LTPs are divided into two groups according to their molecular weight and structural conformation: (i) type 1 (9 -10 kDa) are made up of four alpha-helices, characterized by having inhibitory activity on phytopathogens, (ii) type 2 (7.0 kDa) are made up of four alpha-helices, this type of peptides did not show inhibitory activity on plant pathogens (Finkina et al., 2016). cnLTP belongs to the LTP type

1 because of molecular weight (9.8 kDa) and its four alpha-helices. LTPs have biological activity against bacteria and fungi as *Clavibacter michiganensis, P. solanacearum, P. syringae, Alternaria brassicola, Ascochyta pisi, Colletotrichum lindemuthianum, F. solani, F. graminearum, F. culmorum, F. oxysporum, Botrytis cinerea, Sclerotinia sclerotiorum, Verticillium dahliae* (Finkina et al., 2016). Antagonism tests indicated an inhibitory effect of *C. nobile* AWF on *R. solani*, which has been verified and is as objective as possible, e.g., antimicrobial activity of oils of *C. nobile* has been evaluated on *Penicillium* sp., and *Aspergillus* sp. (Sharifzadeh et al., 2016). The location of a LTP in cell wall and their externalization toward the intercellular space by a signal sequence has suggested its presence in apoplastic fluid, as evidenced in *Arabidopsis thaliana*, *B. oleracea*, *Ricinus communis* and *Vigna unguiculata* (Missaoui et al., 2022). Two LTPs (IWFI and IWFI2) were isolated from intercellular washing fluid of *Beta vulgaris* with antifungal activity on *Cercospora beticola*, demonstrating their presence in AWF (Nielsen et al., 1996). I-TASSER can tell us how the cnLTP interact with cellular components, so cnLTP can be chemically attached to palmitic acid since it is also covers 30% of all phospholipids (Carta et al., 2017).

5 Conclusions

This project explored the problem of identifying and characterizing a novel AMPs from *C. nobile*. It was possible to find relevant features of cnLTP (e.g., hydrophobic cavity or disulfide bridges), as well as to assess its activity on *R. solani*. Finally, medicinal plants as *C. nobile* may be a source of interesting AMPs to be used in Biotechnology, because they hold keys to improved synthesis of new or improved molecules by creating conditions for its successful adaptation.

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Author Contribution

DDPD; Conceptualization, investigation, data processing, visualization, writing the original draft. SMET; Project administration. SALP; Conceptualization, investigation, project administration, data processing, visualization, writing the original draft, writing-review and editing.

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