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Molecular Characterization, Heterologous Expression and Antimicrobial Activity of *Phaseolus vulgaris* L. Defensin Peptide (Pv-Def) against various Human MDR Pathogens

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THE ELEVATING worldwide threatening antibiotic resistance, without effective antimicrobials for the prevention and treatment of infections, has put human health at very high risk. Plant defensins are cysteine -rich small cationic peptides, whose tertiary structure is greatly stable due to the presence of disulfide bonds. They exhibit significant antimicrobial activities. The defensin cDNA was successfully obtained from the total RNA extracted from *Phaseolus vulgaris* L. (Cv. Paulista) young green leaves, the CDS was efficiently cloned into pJET1.2/blunt cloning vector, and then subcloned into the prokaryotic expression vector pGEX -4 T -1. To facilitate defensin peptide purification, the sequence fragment was fused in -frame with a GST tag. The pGEX -4 T -1 containing recombinant defensin (pGEX -4 T - 1 -Pv -Def) was transformed into the *E. coli* strain BL21. Glutathione affinity chromatography method of purification yielded 3.7mg/L of recombinant peptide. Western blotting was made using anti -glutathione - S - transferase (GST) antibodies, to detect the production of Pv -Def peptide (of ~ 4kDa) linked to the GST tag (~ 26kDa) of an approximate total molecular weight ~30 KDa. The Pv -Def peptide was successfully cleaved from the GST -tag. The Pv -Def induced growth inhibition of multi -drug resistant (MDR) bacterial culture *Klebsiella* spp , *E. coli* and *Staphylococcus aureus* and fungal culture *Aspergillus flavus* and MDR *Candida albicans*.

Keywords: Alternative antibiotics, Antimicrobial activity, Defensin, Heterologous expression, *Phaseolus vulgaris* L.

Introduction

The worldwide spread of significantly increasing conventional antibiotic resistance due to antibiotic misuse or overuse has prompted the scientific community to expedite the search for non-conventional anti-infective drugs that can benefit global health (El-Shounya et al., 2019; Metwally et al., 2020; Ismail et al., 2022). Since ancient times, natural products have played a significant role in the treatment of human diseases around the world and are a potential source of novel therapeutic compounds due to their specific and vast chemical diversity (Chandra et al., 2017; Afroz et al., 2020). It is well known that plants can evolve multiple constitutive and inducible processes through secondary metabolites, antimicrobial peptides (AMPs), and morphological barriers to protect themselves against pathogenic infections (Mookherjee et al., 2020; Datta & Roy, 2021). All higher organisms possess a barrier defense or an innate immune system, and AMPs are members of numerous protein families that serve these functions. Due to their substantial activity against multidrug-resistant organisms by either stimulating immune responses or their direct action on microorganisms or or, AMPs have recently gained popularity as an alternative to traditional antibiotics. According to

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reports, natural AMPs have minimal to no toxicity for humans. In addition, they are stable in different environments due to their specific characteristics, such as particular structural conformation, overall changes, and disulfide bonds (Afroz et al., 2020). As part of their defense mechanism, plants produce a large number of toxic molecules, including AMPs, which destroy pathogens via phospholipid interaction and membrane permeabilization (da Silva Gebara et al., 2020). Plant defensins are among the most well-known plant AMPs. They are small cationic peptides of (45–54) amino acids and about 5–6 kDa, with a structure of a cysteine-stabilized βαββ pattern. There are eight conserved cysteine residues in most plant defensins (C1 to C8); Disulfide bonds between cysteine residues can be shown by the pattern: C1-C8, C2-C5, C3-C6, and C4-C7 (Kovaleva et al., 2020). Although protein sequence studies on various plant defensins revealed amino acid diversity, only the cysteine peptides and a small number of other residues are clearly conserved (Nawrot et al., 2014; Mahlapuu et al., 2020). This compact structure is resistant to elevated temperatures, high pH, and protease-mediated degradation (Sher Khan et al., 2019) . Plant defensins play a key role in mediating plant abiotic stress, antimicrobial and insecticidal effects, and anticancer effects against several cancer cell lines (Sher Khan et al., 2019; Mahlapuu et al., 2020).

Plant defensin antimicrobial activity is more pronounced against fungi than bacteria. However, inhibition of some Gram-positive bacteria has been reported. The growth of different filamentous fungi and yeasts is inhibited due to incubation with plant defensins (Aerts et al., 2008; Mello et al., 2011; Hegedüs & Marx, 2013). Plant defensins are classified into two groups based on their antifungal properties: The first group (Group I involves plant defensins that exhibit their effect by inhibiting the hyphae's elongation and causing some morphological changes. The second group (Group II involves plant defensins that cause inhibition of fungal growth and does not affect the morphological level (Thomma et al., 2002). Despite the route that plant defensins follow, they need specific targets to bind to the plasma membranes of the fungal cells (Thevissen et al., 2000).

Expression analyses of different plant defensin genes have shown that they respond to various biotic and abiotic stresses by either being constitutively expressed or up-regulated (Nawrot et al., 2014; Goyal & Mattoo, 2014). Numerous defensin recombinant proteins have been reported to be expressed at elevated levels via large-scale production techniques due to their significant positive role as antifungal and anticancer pharmaceutical agents (Thundimadathil, 2012; Domalaon et al., 2016; Parchebafi et al., 2022).

Materials and Methods

Plant seeds and microbial strains

Seeds of *Phaseolus vulgaris* L. (cv. Paulista) were kindly provided by the Horticulture Research Institute (HRI), Agricultural Research Center (ARC), Giza, Egypt.

Bacteria and fungi

Multi-drug resistant (MDR) bacterial culture (*Klebsiella spp* ATCC 700603*, Staphylococcus aureus* ATCC 29213 and, *E. Coli* ATCC 25922). Fungal culture (*Aspergillus flavus* ATCC FBKL3.0167 and MDR *Candida albicans* ATCC JN60639). Both types of cultures were obtained from the American Type Culture Collection (ATCC).

RNA isolation, cDNA synthesis, and molecular cloning

Total RNA extraction from young green leaves of *Phaseolus vulgaris* L. (cv. Paulista) was done using TRIzol Reagent (Invitrogen, Cat. No., 15596026). RT-PCR was performed with LunaScript® RT SuperMix Kit (NEB #E3010). A PCR produced fragment of 250bp length, using *Phaseolus vulgaris* leaves cDNA as a template and the primers Forward: 5' TGCCTTTGGTCTCAACCATCT 3', Reverse: 5' GTTCGGCAAACAGAAGCACA 3'. This fragment was subjected to agarose gel (1.5%) electrophoresis, then the fragment was subjected to purification from the agarose gel and sequencing by Macrogen lab in Seoul, South Korea. The sequence obtained was cleaned and aligned using the Blastn tool provided by the NCBI databases, then it was subjected to the GenBank and offered an accession number (OP009355). A hypothetical protein was deduced from the obtained sequence. The sequence was aligned to other sequences available on the NCBI databases, and a dendrogram was constructed to confirm the close relationship between the obtained sequence and other sequences subjected through previous studies. In addition, SWISS-Model homology analysis was used to predict the deduced amino acid sequence's tertiary structure. The pJET1.2/blunt cloning vector (Thermo Fisher

Scientific, Cat. No. K1232) was used to clone the obtained fragment according to the manufacturer's guidelines. A pair of specific primer forward: 5′-CGC *GGA TCC* GCT CGC TCT GTG TCT TTT GTC TCA ACC-3′, and reverse: 5′- CCG *CTC GAG* -3′ was designed merged to the recognition sites of *BamHI* and *XhoI,* respectively, to be easily subcloned into the prokaryotic expression vector pGEX-4 T-1. The defensin CDS was placed in-frame to be connected to the GST tag protein to aid further purification (Al Kashgry et al., 2020).

Heterologous expression of Phaseolus vulgaris defensin protein (Pv-Def)

The BL21 (DE3) strain of *E. coli* was chosen for transformation by the recombinant vector pGEX-4 T-1-Pv-Def that contains a defensin coding sequence. Positive colonies were selected based on their potential to grow on LB agar plates with 50 g/mL ampicillin. Each selected positive colony was left to grow overnight at 37°C on 5mL ampicillin-containing LB medium. IPTG (Isopropyl β-D-1-thiogalactopyranoside) of 0.1 mM concentration was utilized for the induction of the GST-defensin fusion expression.

Purification of the recombinant protein

After obtaining the bacterial pellet by centrifugation, the expressed recombinant protein was batch purified using Glutathione Sepharose 4B resin (Sigma, GE17-5132-01). The filtered lysate obtained from the bacterial culture was treated with glutathione Sepharose (2mL) in an overhead shaker at 4 °C and left overnight. To eliminate unintendedly bound proteins, the unbound proteins were washed twice with GST binding buffer (10mL) and then twice with GST binding buffer (10mL) that contains Triton X-100 (1%). Subsequently, 1mL of elution buffer (10mM reduced glutathione, 400mM NaCl, and 50mM Tris-HCl pH 8.0) was used to release the bound recombinant GST-defensin peptide. Tris-Tricine gel electrophoresis was used to determine the recombinant protein's purity, whereas the concentration was obtained by applying the Bradford assay after N terminal GST-tag cleavage through overnight digestion with thrombin.

Western blot analysis

Western blot was done to detect the Pv-Def-GST fusion protein in the total protein extracted from the transformed bacterial cells to ensure that the defensin-GST fusion protein was adequately purified as well as to confirm the GST-tag cleavage from defensin. Afterward, 2µg of both the fusion protein and the purified protein were separated by electrophoresis in Tris-Tricine gel (15% (w/v)), The gel staining step was run by placing the gel in 50% acetic acid (v/v), Coomassie R–250 in 10% ethanol (v/v), and for the gel destaining step 12% (v/v) acetic acid and 12.5% (v/v) isopropanol were used.. Polyvinylidene difluoride membrane (PVDF) from Thermo Scientific, US was used for the electro-transfer of the proteins after separation by electrophoresis. It was carried out by utilizing a primary antibody against GST protein, and an anti-mouse-alkaline phosphatase from Sigma-Aldrich was utilized as a secondary antibody. Then, for the detection 5-Bromo-4-chloro-3′-indolyphosphate p-toluidine (BCIP) and Nitro blue tetrazolium chloride (NBT) were employed as the substrates.

Assessing the antimicrobial activity of the purified Pv-Def peptide

Agar well diffusion method

The Agar well diffusion method for bacterial cultures of *Klebsiella* spp., *E. coli,* and *Staphylococcus aureus* and for fungal cultures of *Aspergillus flavus* and *Candida albicans* was performed according to CLSI 2015. Inoculum containing 10^6 CFU/mL for bacterial cultures CLSI 2012 and spore suspensions for fungi were adjusted from $0.1*10^6$ to $5.5*10^6$ spore/ml. Inocula were spread on Muller Hinton agar plates using a sterile swab, then 5mm diameter wells were bunched into the agar media. Finally, 1mL of Pv-Def was placed in each well . As a final step the plates were subjected to incubation for 24-48h at 37°C for bacteria and 28°C for fungi (Erhonyota et al., 2022; Tessema et al., 2023).

Disk diffusion method

A blank sterile disk saturated by Pv-Def purified peptide was placed onto the surfaces of inoculated plates with bacterial and fungal pathogens. Finally, plates were subjected to incubation for 24-48h at 37°C for bacteria and 28°C for fungi (Ngamsurach & Praipipat, 2022; Carvalho et al., 2023).

Results

cDNA sequence analysis and phylogenetic relationships

In the current study, total mRNA was extracted from *Phaseolus vulgaris* L. (Paulista cv.) and was used to obtain first-strand cDNA by Reverse Transcriptase (RT) PCR. *Phaseolus vulgaris* L. defensin cDNA (Pv-Def) was PCR amplified

using defensin-specific primers.

The nucleotide sequence of Pv-Def cDNA was given GenBank Acc. No.: OP009355. It was subjected to the BLASTn tool provided by the NCBI databases, and a phylogenetic tree was constructed between our fragment and the closely related sequences available in the NCBI (Fig. 1 and Table 1).

Homology modeling analysis of the obtained amino acid sequence has shown that Pv-Def possesses the plant defensins' general tertiary structure, which is made up of one α-helix and three antiparallel β-sheets, possessing the βαββ arrangement. The eight cysteine residues are responsible for their stabilization due to the intermolecular disulfide bonds that form among them (Fig. 2 a and b).

Pv-Def's heterologous expression, cloning, and purification

The Pv-Def cDNA was successfully cloned into the pJET1.2/ blunt cloning vector, it was then subcloned in the prokaryotic expression vector pGEX-4 T-1, and the recombinant pGEX-4 T-1-Pv-Def was finally produced. The production of the recombinant Pv-Def fused to the GST tag was successful in the E. coli, BL21 (DE3) pLysS expression system after being transformed with the recombinant pGEX-4 T-1-Pv-Def. The IPTG-induced E. coli BL21 colonies were subjected to total protein extraction. After tricine SDS-PAGE of the total protein extraction from the induced-positive bacterial cells, the Pv-Def protein band fused with the GST protein was observed (Fig. 3). Through the use of glutathione affinity chromatography, the Pv-Def fused with the GST tag was purified. Subsequently, 3.7mg/L of the purified Pv-Def peptide was obtained, and it appears as a clear band of approximately ~30kDa (26kDa GST + ~ 4kDa Pv-Def peptide). Tris-Tricine gel electrophoresis has confirmed an efficient cleavage of the GST tag from the Phaseolus vulgaris peptide (Pv-Def).

Immunodetection of the recombinant Pv-Def peptide

Western blotting was performed to verify the existence and expression of the Pv-Def -GST fusion protein and to detect the Pv-Def peptide after being cleaved with thrombin. Total soluble proteins from positive bacterial cultures incubated for 2, 3, and 4h after IPTG induction, along with both purified Pv-Def-GST fusion protein and purified Pv-Def, were resolved by tricine SDS-PAGE (12%). Electroblotting was followed to transfer the separated proteins onto the PVDF membrane.

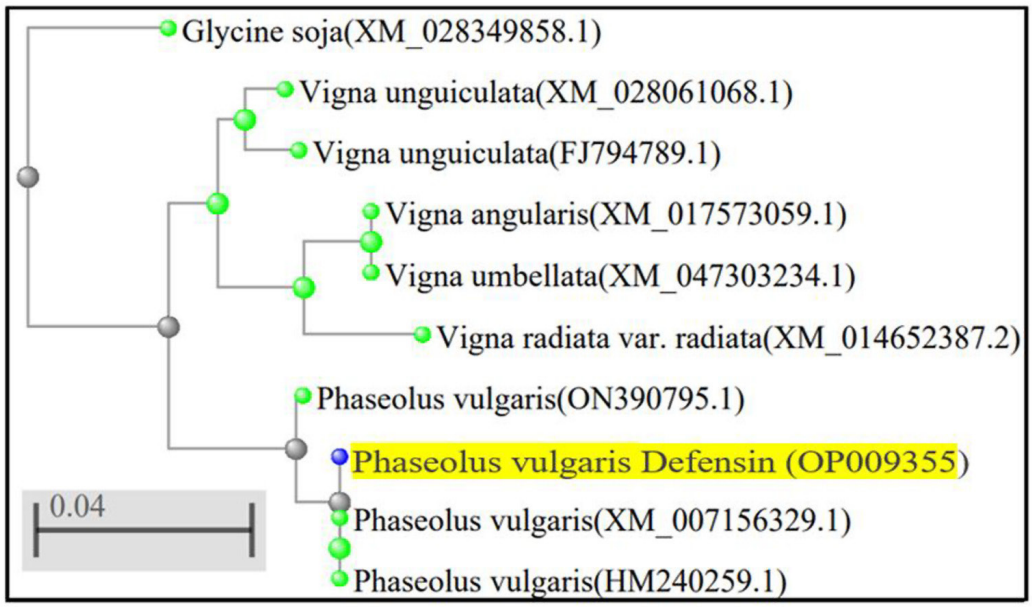

Fig. 1. The phylogenetic tree showing the close relationship between the *Phaseolus vulgaris* L. (cv. Paulista) defensin gene sequence (OP009355) and other closely related sequences available on the NCBI databases. A hypothetical protein was deduced, and its sequence analysis revealed that the obtained sequence contains only 165bp in the open reading frame (ORF), which encodes for a predicted single polypeptide chain of 54 amino acids in length

TABLE 1. BLASTn results for the *Phaseolus vulgaris* L. (cv. Paulista) defensin gene sequence (OP009355) against NCBI databases

Subject species	Subject ID	Query cover (%)	E-value	Identity %
Phaseolus vulgaris	HM240259.1	100	6e-97	100
Phaseolus vulgaris	XM_00715632	100	6e-97	100
Phaseolus vulgaris	ON390795.1	58	5e-53	99.07
Vigna unguiculata	FJ794789.1	95	7e-77	94.83
Vigna unguiculata	XM_028061068.1	98	1e-78	94.44
Vigna umbellate	XM_047303234.1	98	3e-75	93.33
Vigna angularis	XM_017573059.1	98	3e-75	93.33
Vigna radiata	XM_014652387.2	98	9e-71	92.13
Glycine soja	XM_014652387.2	98	2e-67	90.71

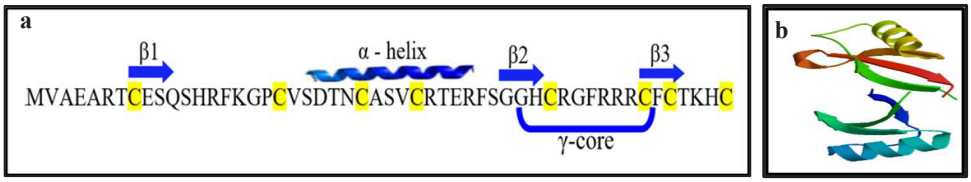

Fig. 2. The hypothetical amino acid sequence and structure for *Phaseolus vulgaris* L. (cv. Paulista) defensin polypeptide chain; (a) The sequence showing the βαββ and the eight cysteine residues characteristic of plant defensins and the γ-core required for their activity, (b) SWISS-Model homology constructed the deduced amino acids' tertiary structure following the common tertiary structure of plant defensin composed βαββ arranged in a homo-dimer state

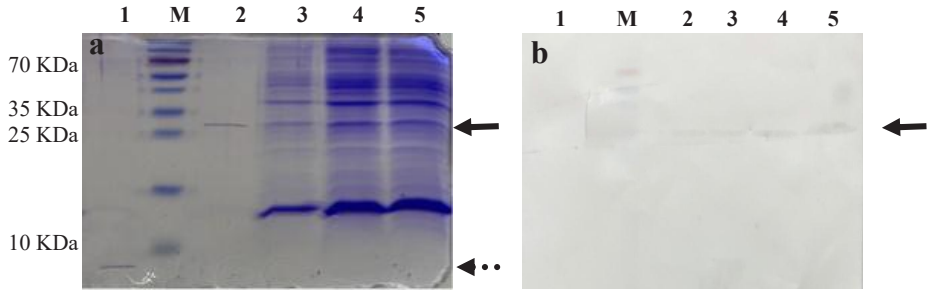

Fig. 3. (a) 14% Tricine SDS-PAGE showing the induction time course for Pv-Def-GST in *E. coli* BL21 plysS (DE3) + RIL, M: marker, 1: purified Pv-Def peptide after cleavage from the GST tag by thrombin treatment (indicated by a dotted arrow), 2: Purified GST-tagged Pv-Def (indicated by a solid arrow) and 3,4, and 5 are the total proteins extracted from Bl21 *E. coli* after 2h, 3h, and 4h induction. (b) The corresponding western blotting using anti-GST antibody showing positive signals in 2-5, while no signal is shown in 5 (1-5 are the same samples shown in Tricine SDS-PAGE in (a)

The primary antibody used was that against GST protein, while alkaline phosphatase-conjugated universal anti-mouse antibodies were employed as secondary antibodies. BCIP and NBT were utilized as detection procedure substrates. Results obtained from the tricine SDS-PAGE have shown the presence of Pv-Def -GST in the soluble proteins of the positive bacterial cultures after 2, 4, and 6h of incubation from IPTG induction. Moreover, the purified fusion Pv-Def -GST and the cleaved protein Pv-Def appear at the expected band size (Fig. 3 a). In addition, western blot analysis revealed specific signals for the expressed recombinant Pv-Def-GST protein in the total soluble proteins and for the purified Pv-Def-GST protein, but none for the purified cleaved Pv-Def peptide (Fig. 3 b).

Assessing the purified Pv-Def peptide's antimicrobial activity

The antimicrobial activity of Pv-Def was tested against several MDR bacterial pathogens, including *klebsiella* spp., *E. coli* and *Staphylococcus aureus*, and fungal pathogens, including *Aspergillus flavus* and MDR *Candida albicans* showing inhibition of pathogen by agar well diffusion method and disk diffusion method estimated by defensin (Fig. 4).

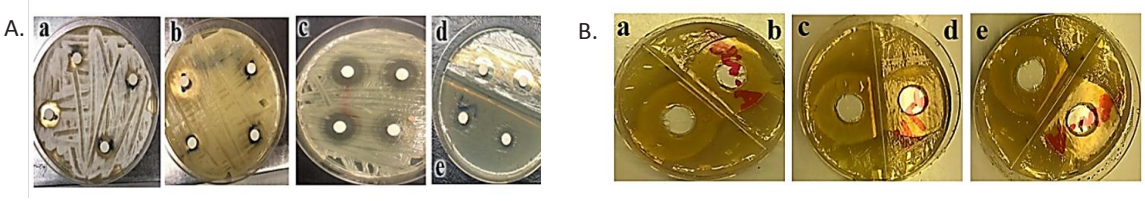

Fig. 4. The antimicrobial activity of the purified Pv-Def peptide's against MDR bacterial and fungal species using two different methods. A. Disk diffusion method; B. Agar well diffusion method. a. *Aspergillus flavus;* b. *Candida albicans;* c. *Klebsiella* spp.*;* d. *E. coli; e. Staphylococcus aureus*

Discussion

Heterologous expression of Pv-Def and sequence analysis

Plant Defensins are ubiquitous partners of the plant's innate immune system as well as in different organisms (Thomma et al., 2002; Stotz et al., 2009). Although they are different organisms, they act similarly to defend themselves against several microbial invaders that usually follow the same infection strategies despite infecting different hosts. Consequently, plant defensins have emerged as a potential alternative offering broad-spectrum resistance against various pathogenic microbes that can attack host cells (Thomma et al., 2003; Van Baarlen et al., 2007a, b; Stotz et al., 2009; Dowd & Johnson, 2018). Defensins are stable, small, cationic, cysteine-rich host defense peptides (45-54 amino acids) having a low molecular weight of almost 4-5 KDa. Numerous confer antibacterial, antifungal, and anticancer activities (García-Olmedo et al., 1998; Guzmán et al., 2016; Guillén et al., 2017; Perez-Rodriguez et al., 2022).

Sequence and structure analysis

The multiple sequence alignment (MSA) performed for pv-Def with other sequences available at the GenBank databases shows a similarity percentage of 99.07-100% with other *Phaseolus vulgaris* defensins (ON390795.1, XM_007156329.1, and HM240259.1) and an average similarity percentage of 90.71-94.83% with other defensins of closely related species in the same family (Table 1). Additionally, the phylogenetic tree (Fig. 1) has grouped the *Phaseolus vulgaris* defensins along with Pv-Def obtained in this study in one cluster. The predicted polypeptide chain (Fig. 2 a) possesses one -helix, three antiparallel β-sheets (βαββ), and is stabilized by eight cysteine residues that confer resistance to pH and temperature changes (Fig. 2 b) (Poon et al., 2014; Shafee et al., 2016). Moreover, the Pv-Def hypothetical protein sequence contains the γ-core structural motif, which possesses the conserved sequence GXC3-9C and is composed of two antiparallel beta-strands β2-β3 and a loop region called the β2-β3 loop (Sonderegger et al., 2018; Kovaleva et al., 2020).

Heterologous expression and Immunodetection of Pv-def

Total soluble proteins were extracted from different positive bacterial cultures after IPTG induction for 2, 3, and 4h. Tricine SDS-PAGE electrophoresis has shown that the Pv-Def-GST fusion protein was found in total soluble protein samples and the purified sample as well at the expected band size (~29kDa) 24kDa for Glutathione Transferase Tag and 5kDa for Pv-Def peptide as shown in Fig. 3 a (Al Kashgry et al., 2020; dos Santos & Franco, 2023). Furthermore, the Pv-Def polypeptide chain cleaved from the GST-tag using thrombin has been shown to be in the expected band size of 5kDa, as demonstrated in Fig. 3 b (Di Somma et al., 2021; Deo et al., 2022).

Antimicrobial activity of Pv-Def

In this study, isolation of the defensin coding sequence from *Phaseolus vlgaris* L was performed. It was subsequently cloned in pGEX-4T-1, which resulted in the expression of the Pv-Def peptide. The existence of inhibitory zones determined the purified Pv-Def's antimicrobial activity against the bacterial species *Klebsiella* spp*., E.Coli,* and *Staphylococcus* aureus and the fungi species *Aspergillus flavus* and *Candida albicans.* Purified *Pv*D1 and *Pv*D1r plant defensins isolated from *Phaseolus vulgaris* have been shown to exhibit inhibitory activity against the growth of the pathogenic yeast strain *Candida albicans* and different yeast cells and filamentous fungi (de O Mello et al., 2014). This finding aligns with results obtained from the application of Maize defensin MzDef that has shown potent antibacterial activities against *Bacillus cereus* and *E. coli* as well as moderate activity against *Staphylococcus aureus* and *Staphylococcus*

enterica (Al Kashgry et al., 2020). However, VrD1, mungbean recombinant defensin, has been shown to possess an antibacterial effect against *Staphylococcus epidermidis* and *Salmonella imurium* (Chen et al., 2005).

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Competing interests The authors report no conflicts of interest regarding this work.

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Ethics approval: Not applicable.

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التوصيف الجزيئي والتعبير الجيني غير المتجانس والنشاط المضاد لبروتين الدفنسين (Defensin Peptide) من نبات الفاصوليا ضد العديد من الميكروبات متعددة المقاومة للمضادات الحيوية

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يؤدي ارتفاع مقاومة العديد من الميكروبات للمضادات الحيوية بدون مضادات فعالة للوقاية والعلاج من العدوى إلى تعريض صحة الإنسان لخطر كبير. بروتتين الدفنسين (ببتيد) (Defensin Peptide) النباتي عبارة عن ببتيدات كاتيونية صغيرة غنية بالسيستين وهيكله الرباعي الأبعاد ذو درجة ثبات عالية لاحتوائه على روابط كبريتية ثنائية. ومن المعروف أن لهذا البروتين نشاطا مضادا للعديد من الميكروبات. تم في هذا البحث استخلاص جين الدفنسين من أوراق نبات الفاصوليا في صورة الرنا المراسل (mRNA) وتم تحويله إلى الدنا المكمل (cDNA) بإنزيم النسخ العكسي حيث تم تحميله على أحد ناقلات التعبير الجيني، وتم إدخال الناقل في بكتريا القولون (*E.coli*) ليسهل فصل وتنقية الببتيد المكمل (Recombinant peptide) الناتج من الدنا معاد الإتحاد (Recombinant DNA). تلى ذلك فصل وتنقية الببتيد النباتي بطريقة الفصل الكروماتوجرافي. تم إجراء تقنية الطبعة الخاصة بالبروتينات (Western Blot) في وجود أجسام مضادة تخصصية للكشف عن البتيد النباتي المرتبط. بعد الحصول على الببتيد النباتي في صورة نقية (وزنه الجزيئي حوالي 4 كيلو دالتون)، تم إجراء تجربة للكشف عن نشاطه ضد العديد من الميكروبات البكتيرية (*Klebsiella* spp. , *E. coli* and *Staphylococcus aureus*) والفطرية (*Aspergillus flavus* and MDR *Candida albicans*) التي تصيب الإنسان والممقاومة للعديد من المضادات الحيوية. وقد أظهر الببتيد النباتي كفاءة ملحوظة في نشاطه المثبط للميكروبات المستخدمة في الدراسة.