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Potential Antibacterial, Wound Healing and Anti-inflammatory Activities of *Penicillium rubens*, an Endophytic Fungus Isolated from the Leaves of *Cucumis sativus* L.

Mahmoud H. Farghali[a,*], Duaa Eliwa[b], Maisra M. El-Bouseary[a]

[a] Department of Microbiology and Immunology, Faculty of Pharmacy, Tanta University, Tanta, Egypt 31527

[b] Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta, Egypt 31527

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ABSTRACT

This study is the first to report the isolation and bioactivity of *Penicillium rubens* from *Cucumis sativus* L. leaves. *Penicillium rubens* was tested for its potential antibacterial, anti-inflammatory, and wound healing properties. The dried ethyl acetate extract of *P. rubens* (EPR) demonstrated antibacterial activity against *Pseudomonas aeruginosa* (ATCC 27853), showing a 24 mm inhibition zone in the agar disc diffusion method, where the disc was saturated with 1000 µg/mL of EPR in ethyl acetate. The minimum inhibitory concentration (MIC) of EPR against 20 clinical isolates of *Pseudomonas aeruginosa* ranged from 128 to 1024 µg/mL, as determined by the broth microdilution assay. Stimulation of WI38 cells with 20 µg/mL lipopolysaccharide (LPS) led to ~3.08-fold increase± 0.03 in the gene expression of the proinflammatory cytokine, TNF-α, as determined by qRT-PCR, while EPR treatment of LPS-stimulated cells led to a significant reduction in the expression of TNF-α to only ~1.09-fold increase± 0.02 (p<0.0001), providing insights into possible anti-inflammatory activity. The EPR effect on wound healing process was investigated *in vitro* where EPR treatment significantly increased the wound closure percentage (66.64% ± 5.61 and 99.94% ± 0.05) compared to the control cells (13.79% ± 3.98 and 83.37% ± 0.05) as indicated at 24 and 48 hours post-wound induction, respectively. Further studies may be needed to characterize the bioactive compounds responsible for the above-mentioned biological activities, which may help explore alternative therapeutically active compounds.

1. Introduction

The current study is the first to document the isolation and bioactivity of *Penicillium rubens* from *Cucumis sativus* L. leaves. *Cucumis* is a genus of tendril-bearing twining plants that include gerkins, melons, and cucumbers [1]. Cucumber (*Cucumis sativus* L.) is a member of the family *Cucurbitaceae* and, traditionally, has been utilized as a cooling agent, to heal skin issues, and to eliminate overall debility in both rural and urban regions [2]. This plant has been shown to have several pharmacological effects such as anti-inflammatory, anti-wrinkle, antibacterial, antidiabetic, and hypolipidemic effects [3].

Endophytic fungi are a wide group of microorganisms that colonize several plant parts, such as leaves, stems, and roots, without posing any threat to the host plant [4]. Fungal endophytes have recently been the subject of numerous studies as alternate sources of substances with therapeutic potential [5].

Diverse therapeutic advantages, such as antibacterial, antifungal, antioxidant, antitumor, enzymatic, and anticancer abilities, have been demonstrated by the bioactive compounds produced by endophytes [6]. Endophyte-produced secondary metabolites are structurally and functionally similar to those produced by their associated host medicinal plants [7-9]. Previous research reported an anti-inflammatory effect of some fungal bioactive compounds [10], such as the metabolites from *Penicillium bialowiezense* [11]. Another study reported the anti-inflammatory activity of indole-terpenoids from *Penicillium* sp. HFF16 [12]. In addition, different fungal-based derivatives have shown enhancement in the wound healing process [13-15].

Natural products, including the bioactive compounds extracted from endophytic fungi, may be a possible alternative to be used as antibacterial agents. A previous study reported an anti-*Pseudomonas aeruginosa* effect of a dilactone terpenoid from the fungal endophyte *Neofusicoccum luteum* [16]. Another study recorded antibacterial and anti-quorum sensing activities of tannic acid isolated from *Penicillium oxalicum*, an endophytic fungus from *Opuntia ficus-indica* [17]. Also, 1H-pyrrole-2,5-dicarboxylic acid, isolated from *Perenniporia tephropora* FF2 which is an endophytic fungus from *Areca*

* Corresponding author at Tanta University

E-mail addresses: mahmoud.farghali@pharm.tanta.edu.eg (Mahmoud H. Farghali)

catechu L., showed a quorum sensing inhibitory effect on *Pseudomonas aeruginosa*, and represented an antibiotic accelerant when combined with piperacillin or gentamycin [18].

Pseudomonas aeruginosa poses a serious threat to medicine because of its antibiotic resistance and capacity to cause serious infections, especially in immunocompromised people [19]. This bacterium may grow in various conditions, making it a constant threat in hospital settings [20]. Treatment is complicated by its resistance mechanisms, which calls for constant research into novel therapeutic approaches. Reducing *Pseudomonas aeruginosa* influence on public health requires an understanding and commitment to control [21].

The endophytic fungus *Penicillium rubens* has demonstrated significant antimicrobial activity due to its ability to produce bioactive secondary metabolites, particularly β-lactam antibiotics like penicillin. Research has shown that crude extracts from *P. rubens* exhibit strong antibacterial effects against Gram-positive pathogens such as *Staphylococcus aureus* and *Bacillus subtilis*, likely due to the inhibition of bacterial cell wall synthesis [22-25]. Additionally, some studies indicate moderate effectiveness against Gram-negative bacteria, though their outer membrane may limit antibiotic penetration [26]. Furthermore, bioactive compounds such as alkaloids and peptides found in *P. rubens* extracts contribute to their broad-spectrum activity [22,27,28]. These bioactive metabolites could have applications in medicine for combating multidrug-resistant (MDR) infections, or in agriculture as biocontrol agents against plant pathogens [29-32]. Beyond antibacterial activity, *P. rubens* extracts have also exhibited antifungal properties, potentially inhibiting pathogens like *Candida albicans*, *Aspergillus niger*, and *Fusarium oxysporum* [29]. Given the global rise of antibiotic resistance, exploring *P. rubens* and its secondary metabolites may provide new therapeutic options for infectious diseases and sustainable alternatives to synthetic antimicrobial agents.

The present study aimed to investigate the potential wound healing and anti-inflammatory activities of the extract of *P. rubens* isolated from the leaves of *Cucumis sativus*, and its antibacterial activity against *Pseudomonas aeruginosa*.

2. Materials and Methods

2.1. Plant source of the endophytic *Penicillium rubens*

Penicillium rubens was isolated from healthy leaf segments of the medicinal plant *C. sativus* obtained from a nearby farm in Tanta City, Al-Gharbia Governorate, Egypt. It was identified by staff member of the Botany Department, Faculty of Science, Tanta University, Tanta. A voucher specimen (PD-7-22-D6) has been preserved at Pharmacognosy Department at Tanta University, Tanta.

2.2. Isolation of the endophyte *Penicillium rubens* from cucumber leaves

For isolation, the cucumber leaf segments were rinsed with tap water until clean, surface sterilized using a solution of 70% ethanol for 1 min, rinsed 3-4 times using sterile water for 2 min, and dried. The segments were inoculated into sterilized potato dextrose agar medium supplemented with 250 mg/L amoxycillin to prevent bacterial growth following surface sterilization, and then they were cultured for a week at 25± 2 °C. Growing fungus on agar plates was repeatedly subcultured with fresh PDA media resulting in pure strains of *Penicillium rubens*.

2.3. Morphological characterization of *Penicillium rubens*

Penicillium rubens was inoculated into Potato Dextrose Agar (PDA) and the colony morphology was evaluated.

2.4. Fermentation and extraction of fungal culture filtrate

The ethyl acetate extract of *Penicillium rubens* (EPR) was prepared for subsequent characterization and biological testing. The cultures under investigation were grown on PDA plates at 25 ± 2 °C for 7 days and a small block was transferred to pre-autoclaved Erlenmeyer flasks (1 L) containing wheat (100 g) in sterile water under sterile conditions. The fungus was grown under static conditions for four weeks at room temperature away from light. The fungal broth was extracted with ethyl acetate three times and then subjected to filtration over Whatman filter paper. The ethyl acetate fraction was collected and dried out using a rotary evaporator, at 45 °C and the crude extract (230 gm of dried extract, 20% yield, brown color) was stored at 4 °C for further studies.

2.5. *In vitro* antibacterial activity

In this study, twenty clinical isolates of *Pseudomonas aeruginosa* were sourced from the culture collection of the Microbiology and Immunology Department, Faculty of Pharmacy, Tanta University, to evaluate the antibacterial properties of EPR. The agar disc diffusion method was used to assess the activity of EPR against the reference strain *Pseudomonas aeruginosa* (ATCC 27853). For this, one disc was loaded with EPR (1000 µg/mL in ethyl acetate), while discs saturated with gentamicin (40 µg/mL) and ethyl acetate (undiluted) served as the positive and negative controls, respectively [33]. Furthermore, the minimum inhibitory concentration (MIC) of EPR for the twenty isolates was determined using the broth microdilution method in a 96-well microtiter plate [34]. EPR concentrations were prepared in a serial two-fold dilution, ranging from 1024 to 32 µg/mL**.** The agar disc diffusion and MIC determination were conducted in triplicates.

2.6. *In vitro* anti-inflammatory activity

Using DMSO, different concentrations of EPR dried extract, in addition to the anti-inflammatory drug, piroxicam, were prepared and suspended in serum-free RPMI medium. WI38 human fibroblast cells were employed to assess the cytotoxicity of EPR. In a 96-well plate, WI38 cells were plated at a density of $3x10^3$ cells/ well, and incubated with different EPR concentrations and10 µg/mL piroxicam for 48 h. MTT assay was employed to assess the viability of cells

Using lipopolysaccharide (LPS)-stimulated WI38 cells, the EPR effect on the gene expression of the pro-inflammatory cytokine, TNF-α, was investigated [35, 36]. The cells were plated at a density of $5x10^4$ cells/ well in a 12-well plate, with a complete medium of RPMI, LPS was added at a concentration of 20 µg/mL. Then, the cells were incubated for 24 hours. After that, the supernatant was discarded after centrifuging the plate for 5 min at 1650 rpm. Then, 10 µg/mL piroxicam or 1/10 of IC_{50} of EPR was added. The cells were then incubated for 48 hours. After centrifugation, RNA isolation kit (iNtRON Biotechnology, Korea) was used to isolate RNA, following the manufacturer's instruction. One µg RNA was converted into cDNA using SensiFAST cDNA synthesis kit (Bioline, London, UK). The following steps were conducted for quantitative PCR: 1 µL of cDNA, 0.5 µl of 10 pmoles/mL forward primer, 0.5 µl of 10 pmoles/mL reverse primer and 10 µL SensiFAST SYBR (Bioline, London) were mixed. The reaction mixture was completed to 20 µL using nuclease-free water. The following program was employed using a CFX96™ Real-Time System (BIO-RAD, USA): heating at 95 ºC for 10 min, then 40 cycles of 95 ºC for 15 sec, 60 ºC for 30 sec and 72 ºC for 30 sec. The cycle threshold (Ct) of TNF-α gene was normalized with Ct of the housekeeping gene, beta-actin. Sequences of the primers used for TNF-α amplification were 5'-CTCTTCTGCCTGCTGCACTTTG-3' for the forward primer and 5'-ATGGGCTACAGGCTTGTCACTC-3' for the reverse one, while 5'-CACCATTGGCAATGAGCGGTTC-3' and 5'-AGGTCTTTGCGGATGTCCACGT-3' were the sequences of the forward and reverse primers of beta-actin, respectively [37].

2.7. *In vitro* wound healing assay

Assessment of wound healing process was performed as previously described [38], with modifications. WI38 cells were plated in a 24-well plate at a density of 10^4 cells/ well. The plate was incubated for 24 hours. Then, serum-free RPMI was used to wash the cells. Using a sterile 200 µL-pipette tip, the cell monolayer was scratched (wounded), then washed with PBS. The cells were then incubated with or without 1/10 of IC_{50} of EPR for 48 hours. Using phase contrast microscopy, the migration of the cells in the denuded zone was photographed. To quantify the relative wound size at 0, 24, and 48 hours after wound induction, the Image J version 1.49 software was used.

2.8. Statistical analysis

An ANOVA test was performed using Graphpad Prism 10 to investigate the anti-inflammatory activity. An unpaired *t*-test was used for the wound healing assay, the data represented the mean ± standard deviation. The data represented the mean ± standard deviation

3. Results

3.1. Morphological characterization of *Penicillium rubens*

The color of the colony was greenish white with a slightly woolly texture. The reverse is buff in color. It has filiform edges and a slightly uneven surface **(Figure 1).**

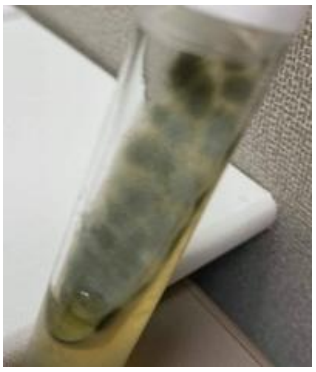

Figure 1. Colony morphology of *Penicillium rubens* isolated from the leaves of *C. sativus* on PDA medium.

3.2. *In vitro* antibacterial activity

EPR exhibited antibacterial activity against the reference strain *Pseudomonas aeruginosa* (ATCC 27853) as determined by the agar disc diffusion method with an inhibition zone diameter (IZD) of 24 mm compared to IZDs of gentamicin and ethyl acetate (22 and 12 mm, respectively). The minimum inhibitory concentration (MIC) of EPR against twenty clinical isolates of *Pseudomonas aeruginosa* was assessed using the broth microdilution assay, with MIC values ranging from 128 to 1024 µg/mL (**Table 1**).

Table 1. MIC values of EPR against the tested *Pseudomonas aeruginosa* isolates (n=20).

MIC (µg/mL)	Number of isolates (%)
128	1 (5)
256	8 (40)
512	9 (45)
1024	2 (10)

3.3. The *In vitro* anti-inflammatory effect of EPR

On WI38 human fibroblast cells, the IC_{50} of EPR was 59.82 µg/mL. One tenth of IC_{50} of EPR was tested for the anti-inflammatory activity on LPS-stimulated WI38 cells. The anti-inflammatory drug, piroxicam, at a concentration of 10 µg/mL was used for comparison. Stimulation with LPS led to ~3.08-fold increase± 0.03 in TNF-α gene expression compared to the non-stimulated cells. EPR treatment of LPS-stimulated cells, showing more than 90% viability of the cells, led to only ~1.09-fold change ± 0.02 compared to the non-stimulated cells, which represents a significant reduction in the expression of TNF-α compared to the LPS-stimulated control cells (p<0.0001). LPS-stimulated cells treated with piroxicam showed a significant reduction in TNF-α gene expression compared to LPS-stimulated control cells (p<0.0001) **(Figure 2)**.

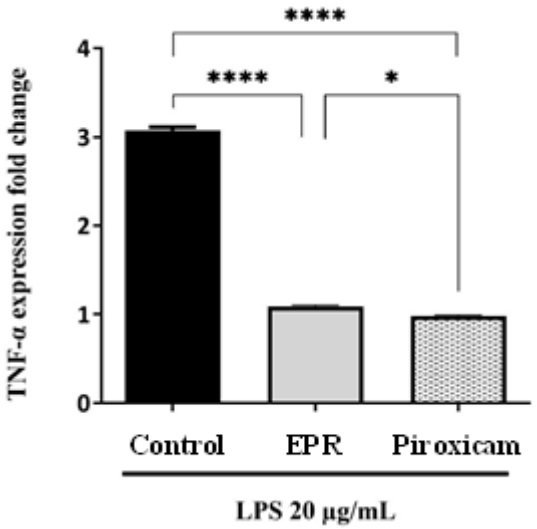

Figure 2. EPR decreases the gene expression of TNF-α. Data represented mean ± standard deviation (n=2), ANOVA test.

3.4. EPR improves the *in vitro* wound healing

Treatment with EPR promoted cell migration and wound closure in WI38 cells (Supplementary Figure S1). As shown in **Figure 3,** Treatment with EPR significantly increased the wound closure percentage (66.64% ± 5.61) compared to the control cells (13.79% ± 3.98) as indicated at 24 hours post-wound induction (P = 0.012). Also, the wound closure percentage significantly increased in EPR-treated cells (99.94% ± 0.05) compared to the control ones (83.37% ± 0.05) at 48 hours post-wound induction (p<0.0001).

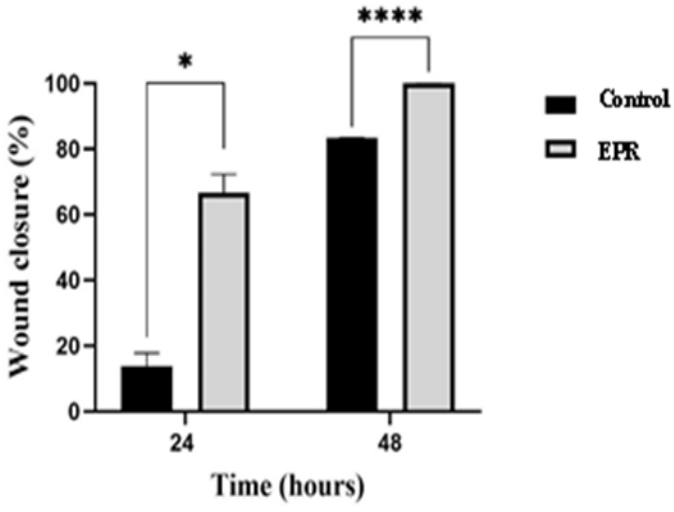

Figure 3. Improvement of the *in vitro* wound healing process by EPR. Data represented mean ± standard deviation (n=2), two-tailed unpaired *t*-test.

4. Discussion

The antibacterial effects of endophytic fungi against different bacterial species have been investigated in previous studies [39]. A peptide produced by the endophytic fungus *Aspergillus tamarii* showed marked antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis* [40]. Ethyl acetate extract of three endophytic *Aspergillus* strains showed antibacterial effects on gram-negative bacteria, such as *Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhimurium,* and *Escherichia coli*, and on gram-positive bacteria, such as *Bacillus subtilis, Bacillus cereus,* and *Staphylococcus aureus* [41]. The current study focused on the anti-*pseudomonas aeruginosa* activity of *P. rubens,* as an endophytic fungus isolated from *Cucumis sativus* leaves for the first time. The findings of the current study showed that the extract of the endophytic fungus *P. rubens* (EPR) has shown potential antipseudomonal activity. Previous studies have documented specific bioactive compounds in *P. rubens* extracts. Rubensteroid A, a steroid from *P. rubens* was reported to have an antibacterial effect [30]. Another study reported a strain of *P. rubens* with promising production of phenoxymethyl penicillin [22]. The marine-derived *P. rubens* BTBU20213035 was found to produce a secondary metabolite with an antibacterial effect [27]. One proposed mechanism of action involves the inhibition of peptidoglycan biosynthesis by β-lactam antibiotics, such as penicillin-derived compounds, leading to bacterial cell wall destabilization and lysis [22,25,42]. Additionally, secondary metabolites such as citrinin, which target bacterial efflux pumps, could reduce the ability of *Pseudomonas aeruginosa* to expel toxic compounds and increase susceptibility to antimicrobial agents [43].

The anti-inflammatory activity of the extract was investigated by detecting the effect of EPR treatment on TNF-α gene expression in LPS-stimulated WI38 cells, where a marked decrease in TNF-α gene expression was detected in EPR-treated cells, compared to the control LPS-stimulated ones. In agreement, secondary metabolites, such as sorbicillinolides, isolated from the marine-derived *Penicillium rubens*, showed anti-inflammatory properties [10,44]. These sorbicillinolides showed a significant anti-neuroinflammatory through the inhibition of the production of nitric oxide and prostaglandin E2 [44]. Also, a study on *Penicillium* sp. HFF16 from the rhizosphere soil of *Cynanchum bungei* Decne reported that indole-terpenoids isolated from the fungus had anti-inflammatory activities through an inhibitory action on nitric oxide, TNF-α, and IL-6 production [12]. Another study on *Penicillium bialowiezense* reported the anti-inflammatory effect of spiroditerpenoids isolated from the fungus, through suppression of the expression of pro-inflammatory mediators, such as IL-6, IL-12, (IL)-1β, TNF-α, prostaglandin E2, and nitric oxide [11]. Further mechanistic studies on EPR are still needed to investigate its effect on other factors that may affect the inflammation process, in addition to TNF-α.

In addition, the wound healing effect of EPR was investigated *in vitro* on WI38 cells, where a significant enhancement of healing was recorded. In agreement, it was reported that nanocellulose-based anthraquinone from *Penicillium flavidorsum*, a marine fungus, had a positive outcome in the context of wound healing progress in a rat model [14]. Another study showed the promotion of diabetic wound healing in mice by a secondary metabolite of *Penicillium purpurogenum*, an endophytic fungus [15]. Notably, the scratch wound healing assay was employed to investigate the effect of cytochalasin H, isolated from an endophyte, on cell migration in A549, a human lung adenocarcinoma cell line, where cytochalasin H treatment decreased the migration ability of the carcinoma cells [45].

Further studies are still needed for the purification and identification of the compounds responsible for the biological activities in EPR extract, using techniques such as LC-MS. Also, the antibacterial effect may be tested against a wider range of bacterial spectrum or resistant strains.

5. Conclusion

The current study aimed to investigate the anti-*Pseudomonas aeruginosa*, wound healing, and anti-inflammatory activities of the dried ethyl acetate extract of *P. rubens* isolated from cucumber leaves for the first time. Further research may be needed to purify, determine, and characterize the bioactive compounds in the extract, to which the reported biological activities are attributed. Further studies may also include *in vivo* efficacy and toxicity studies, which may help develop EPR as a new therapeutic or cosmeceutical candidate.

Declarations

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Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Competing Interest: The authors declare that they have no competing interest.

Author Contributions: Conceptualization, M.F., D.E, M.E.; Data curation, M.F., D.E, M.E.; Investigation, M.F., D.E, M.E.; Methodology, M.F., D.E, M.E.; Project administration, D.E, M.E.; Supervision, M.F.; Writing – original draft, M.F., D.E, M.E.; Writing – review & editing, M.F., D.E, M.E. All authors read and approved of the final manuscript.

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