

Antimicrobial Properties of the Endophytic Fungus *Penicillium roqueforti* 12Ph Isolated from the Plant *Peganum harmala*

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Abstract

Peganum harmala L. is a medicinal herb native to Central Asia and Iran and has long been used in traditional medicine in many countries for the treatment of various diseases. Endophytic microorganisms inhabit plant tissues without causing apparent harm to their host and have attracted considerable scientific attention due to their ability to produce biologically active secondary metabolites, including antimicrobial compounds. In the present study, we investigated the antibacterial activity of secondary metabolites produced by the endophytic fungus *Penicillium roqueforti* 12Ph isolated from *Peganum harmala*. A total of fourteen fungal isolates were obtained from the plant tissues, among which isolate 12Ph demonstrated the strongest antimicrobial activity against pathogens, including *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Candida albicans*. Molecular genetic identification based on sequence analysis confirmed that the isolate belongs to the species *P. roqueforti*. Preliminary phytochemical screening revealed the presence of several classes of bioactive compounds, including terpenoids, steroids, flavonoids, tannins, cardiac glycosides, and alkaloids. Further chemical characterization using gas chromatography–mass spectrometry (GC–MS) showed that the crude extract of *P. roqueforti* 12Ph and its chromatographic fractions contained compounds belonging to different chemical classes such as alkanes, fatty acids, and amino-containing compounds. The major detected constituents included methyl hexadecanoate, n-hexadecanoic acid, and 9,12-octadecadienoic acid (Z, Z). These findings suggest that the endophytic fungus *P. roqueforti* 12Ph associated with *P. harmala* may represent a promising source of antimicrobial secondary metabolites with potential pharmaceutical applications.

Keywords: antimicrobial activity, endophytic fungi, *Penicillium roqueforti*, *Peganum harmala*, differential fractionation, thin-layer chromatography, GC–MS, MRSA, *Candida albicans*

Резюме

Peganum harmala L. е лечебна билка, произхождаща от Централна Азия и Иран, и отдавна се използва в традиционната медицина в много страни за лечение на различни заболявания. Ендофитните микроорганизми обитават растителните тъкани, без да причиняват видима вреда на своя гостоприемник, и са привлекли значително научно внимание поради способността си да произвеждат биологично активни вторични метаболити, включително антимикробни съединения. В настоящото изследване изследвахме антибактериалната активност на вторичните метаболити, произведени от ендофитната гъба *Penicillium roqueforti* 12Ph, изолирана от *Peganum harmala*. Общо четиринадесет гъбични изолата бяха получени от растителните тъкани, сред които изолат 12Ph демонстрира най-силна антимикробна активност срещу патогени, включително метицилин-резистентен *Staphylococcus aureus* (MRSA) и *Candida albicans*. Молекулярно-генетичната идентификация, базирана на анализ на последователността, потвърди, че изолатът принадлежи към вида *P. roqueforti*. Предварителният фитохимичен скрининг разкри наличието на няколко класа биоактивни съединения, включително терпеноиди, стероиди, флавоноиди, танини, сърдечни гликозиди и алкалоиди. По-нататъшна химическа характеристика с помощта на газова хроматография-маспектрометрия (GC-MS) показва, че суровият екстракт от *P. roqueforti* 12Ph и неговите хроматографски фракции съ-

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държат съединения, принадлежащи към различни химични класове, като алкани, мастни киселини и аминокъдържащи съединения. Основните открити съставки включват метил хексадеканонат, п-хексадеканова киселина и 9,12-октадекадиенова киселина (Z, Z). Тези открития показват, че ендофитната гъба *P. roqueforti* 12Ph, свързана с *P. harmala*, може да представлява обещаващ източник на антимикробни вторични метаболити с потенциални фармацевтични приложения.

Introduction

Most plant extracts are used in traditional medicine as antibacterial, antioxidant, anti-inflammatory and antidiabetic treatments (Mukhammedov *et al.*, 2026). One of them is *Peganum harmala* L. (family *Zygophyllaceae*). It is a perennial herb widely distributed in Central Asia, the Middle East, and parts of North Africa (Cowan, 1999). The plant has long been recognized as an important medicinal species and has been widely used in traditional medicine in many countries for the treatment of various ailments (Sharaf *et al.*, 1997). Modern pharmacological studies have confirmed that *P. harmala* possesses a broad spectrum of biological activities, including antitumor, insecticidal, and antimalarial properties (Saini, 2009). In addition, extracts and bioactive constituents of this plant have demonstrated wound-healing potential, antioxidant activity, immunomodulatory effects, and hepatoprotective properties (Hamden *et al.*, 2008). Several studies have also reported cytotoxic, antibacterial, antifungal, and antiviral activities associated with compounds derived from *P. harmala* (Darabpour *et al.*, 2011).

In recent years, increasing attention has been directed toward endophytic microorganisms associated with medicinal plants. Endophytes are microorganisms that colonize internal plant tissues without causing apparent harm to their host. These microorganisms form complex ecological relationships with plants and are capable of producing a wide range of secondary metabolites with significant biological activities. Many of these metabolites possess antimicrobial, anticancer, antioxidant, and anti-inflammatory properties. Because of their metabolic diversity, endophytic microorganisms are increasingly regarded as promising and sustainable sources of novel natural products for pharmaceutical applications (Nazir *et al.*, 2024; Gulyamova *et al.*, 2026).

At the same time, the emergence and rapid spread of antimicrobial resistance (AMR) has become one of the most serious global public health challenges. The growing prevalence of antibiotic-resistant bacterial pathogens significantly increases morbidity and mortality rates worldwide and places an increasing burden on healthcare sys-

tems. In particular, Gram-positive pathogens such as *Staphylococcus aureus* have developed resistance to multiple antibiotics. *Methicillin-resistant S. aureus* (MRSA) is considered one of the most problematic pathogens in both hospital and community settings (Golban *et al.*, 2025). According to recent global health reports, antimicrobial resistance was associated with nearly five million deaths in 2019, with approximately 1.27 million deaths directly attributable to drug-resistant infections (Ortega *et al.*, 2025). These alarming statistics highlight the urgent need for the discovery of new antimicrobial agents capable of combating resistant pathogens.

Endophytic fungi have historically been an important source of biologically active compounds with therapeutic potential. Many widely used pharmaceuticals, including antibiotics, immunosuppressants, and cholesterol-lowering agents, have originated from fungal secondary metabolites. Several studies have demonstrated that endophytic fungi isolated from medicinal plants can produce compounds with significant antimicrobial activity (Ouzid *et al.*, 2020). These findings suggest that the microbial community associated with *P. harmala* may represent a valuable reservoir of antimicrobial metabolites.

Among endophytic fungi, species of the genus *Penicillium* are particularly notable for their remarkable biosynthetic potential and their ability to produce diverse secondary metabolites. Some strains of *P. roqueforti* isolated from plants have been reported to synthesize biologically active compounds such as indole-3-acetic acid (IAA), especially when associated with plants growing under environmental stress conditions such as heavy metal contamination (Ikram *et al.*, 2018). However, despite the well-known metabolic capabilities of *Penicillium* species, the antimicrobial potential of endophytic *P. roqueforti* strains remains insufficiently explored.

Therefore, the aim of the present study was to investigate the antibacterial activity of secondary metabolites produced by the endophytic fungus *P. roqueforti* 12Ph isolated from *P. harmala*. To identify and characterize the biologically active compounds, several analytical approaches were employed, including differential fractionation,

thin-layer chromatography (TLC), and gas chromatography–mass spectrometry (GC–MS). Particular attention was given to determining which fractions and chemical constituents contribute to antimicrobial activity against clinically relevant and opportunistic pathogenic microorganisms.

Materials and Methods

Isolation and cultivation of fungal strains

Endophytic fungal strains used in this study were previously isolated from healthy tissues of *P. harmala* collected from natural populations in Uzbekistan. Plant tissues were surface sterilized following the protocol described by Strobel *et al.* (1996) to eliminate epiphytic microorganisms. Sterilized tissue segments were placed onto Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol (100 mg/L) to suppress bacterial growth and incubated at 28°C until fungal colonies emerged.

Individual colonies were subcultured repeatedly on fresh PDA plates to obtain pure cultures. The purified isolates were maintained on PDA slants at 4°C and routinely subcultured every four weeks to preserve viability. Before metabolite extraction, fungal isolates were transferred onto fresh PDA plates and incubated for 5–7 days at 28°C to obtain actively growing cultures for inoculation into liquid medium.

Isolation of crude extracts of secondary metabolites

Isolates of endophytic fungi were grown in liquid Czapek-Dox nutrient medium on a shaker to enhance aeration in the liquid for 7 days at a temperature of 28°C at 180 rpm (Marcellano *et al.*, 2017; Abdulmyanova *et al.*, 2020).

The metabolites from the biomass of endophytic fungi were extracted by the Hazalin method (Hazalin *et al.*, 2009) with a slight modification. In this procedure, 5 g of biomass was ground in a Potter homogenizer, transferred into a flask, and mixed with ethyl acetate at a 1:20 ratio. The mixture was shaken at room temperature on a shaker at 150 rpm. Afterwards, the mixture was filtered through paper to remove solid residues, and Na_2SO_4 was added at a concentration of 40 µg/mL to remove any remaining water. The resulting ethyl acetate extract was then dried using a rotary evaporator, and the obtained dry mass (3 mg/mL) was dissolved in dimethyl sulfoxide (DMSO).

To isolate secondary metabolites from the fungal culture broth, ethyl acetate was added to the broth in a 1:1 ratio, mixed, and allowed to stand.

Using a separation funnel, the aqueous layer was separated from the ethyl acetate layer. The collected ethyl acetate extract was then dried using a rotary evaporator, and the resulting dry mass (3 mg/mL) was dissolved in DMSO.

Determination of antibacterial activity of crude extracts

The antibacterial activity of the crude extracts was evaluated using the agar well diffusion method (Melvin and Weinstein, 2018), with minor modifications introduced to optimize the procedure. The test microorganisms from “Collections of Important Microorganisms for Industry” of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan: *MRSA* 97, *Staphylococcus aureus* 96, *Escherichia coli* 86, *Pseudomonas aeruginosa* 75, *Bacillus subtilis* 91, and *Candida albicans* 84 were used. All microbial suspensions were adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). A volume of 0.5 mL of each bacterial suspension was evenly spread onto the surface of meat-peptone agar plates and allowed to be adsorbed for 15 minutes. Wells with a diameter of 10 mm were aseptically punched into the agar using a sterile cork borer. Subsequently, 100 µL of the crude extracts prepared in DMSO at a concentration of 3 mg/mL were added to each well. As positive controls, antibiotic discs (Ceftriaxone – 30 µg/disc (third-generation cephalosporin) and as a negative control, DMSO were used. The Petri dishes were incubated at 37°C for 24 hours, after which the diameters of the inhibition zones were measured in millimeters.

Molecular genetic identification of endophytic fungi

Molecular identification of endophytic fungi was performed by amplification of the ribosomal DNA (rDNA) region using the polymerase chain reaction (PCR) method. PCR amplification was carried out using the GenePak™ PCR Core lyophilized reagent kit (IsoGene Laboratory LLC, Russia). The reaction mixture contained a Master Mix with lyophilized Taq DNA polymerase, MgCl_2 , deoxynucleotide triphosphates (dNTPs), and an optimized buffer system. Each reaction included 20 pmol of forward and reverse primers. The following universal primers were used for amplification (Martin and Rygiewicz 2005): NSA3 (5'-AAACTCTGTCGTGCTGGGGATA-3'), NLC2 (5'-GAGCTGCATTCCCAAACAACCTC-3'). PCR amplification was performed using a Veriti 96-well Thermal Cycler (Applied Biosystems).

Determination of the qualitative composition of secondary metabolites

The qualitative chemical analysis of crude extracts isolated from endophytic fungi was carried out using the method described by Visweswari *et al.*, 2013) In this procedure, chemical compounds were identified by differential color reactions.

Tannins and phenolic compounds: to detect the presence of tannins and phenolic compounds, 2 mL of extract was taken, and 2–3 drops of 1% FeCl_3 solution were added. The iron ions in the solution react with tannins to produce a blue-black or green-black color, and with phenols to produce a violet color.

Flavonoids: NaOH and HCl were used to detect flavonoids. A few drops of 20% NaOH solution were added to 2 mL of extract, resulting in an intense yellow-orange color. Subsequent addition of a few drops of diluted HCl caused the yellow-orange color to disappear. The formation and disappearance of this yellow-orange layer indicated the presence of flavonoid-class compounds in the extract.

Saponins: To detect saponins, 1 mL of dry extract was dissolved in 5 mL of hot distilled water (60°C) and shaken for 5 minutes until a stable foam formed. The presence of saponins was confirmed if the foam persisted for the next 30 minutes.

Terpenoids: For terpenoid detection, 0.5 mL of extract dissolved in ethyl acetate was mixed with 2 mL of chloroform and 3 mL of concentrated H_2SO_4 . Formation of a reddish-brown color at the interface indicated the presence of terpenoids.

Anthraquinones: To determine anthraquinones, 2 mL of extract was mixed with 4 mL of n-hexane and shaken. Separation into two layers was observed. The upper layer was separated and mixed with 4 mL of 10% diluted ammonia, and the color of the lower layer was observed. A violet-pink color indicated the presence of anthraquinones.

Cardiac glycosides: To detect cardiac glycosides, 1 mL of extract was mixed with 1 mL of cold acetic acid, followed by the addition of 1 drop of 3% FeCl_3 in methanol. Concentrated H_2SO_4 was then carefully added along the test tube wall, and the color of the lower layer was observed. A blue-green coloration indicated the presence of cardiac glycosides.

Purification of extract by differential fractionation

Secondary metabolites in ethyl acetate extracts of endophytic fungi were fractionated using a differential fractionation method, following the

scheme proposed by Kumar and Kaushik (2013) with some modifications.

For this procedure, 5 mL of ethyl acetate was added per gram of 7-day-old fungal biomass, homogenized, and placed on a shaker at 180 rpm at room temperature to allow extraction. Then, the obtained ethyl acetate extract was mixed with an equal volume of distilled water and divided into two portions. First portion: The ethyl acetate fraction was dried and then dissolved in a 1:1 mixture of methanol and n-hexane. The solution was partitioned into methanol and hexane fractions. Second portion: An equal volume of butanol was added to the aqueous fraction, mixed, and separated into butanol and aqueous fractions.

The aqueous, butanol, methanol, and hexane fractions were dried using a rotary vacuum evaporator. The resulting dry masses were dissolved in DMSO for further use.

Thin layer chromatography (TLC)

For TLC, silica gel plates measuring 10×10 cm and 10×15 cm (Sigma-Aldrich) were used (Suleimana *et al.*, 2009). The chromatographic separation was performed in a standard chromatographic chamber using the following two eluent systems:

- Weakly polar, acidic: Ethyl acetate:chloroform: formic acid (4:5:1)
- Non-polar, basic: Benzene: ethyl acetate: ammonium hydroxide (90:10:1)

From extracts at a concentration of 25 mg/mL, 25 μL was applied onto the start line of the plates. After development in the chromatographic chamber, the plates were dried, and the separated compounds were visualized using the following methods:

- Under ultraviolet (UV) light at 254 nm
- Spraying with 20% phosphomolybdic acid in ethanol
- Spraying with 10% H_2SO_4 in methanol and heating at 110°C

The separated compounds were scraped off individually. For antibacterial activity testing, they were dissolved in DMSO, and for chemical characterization, they were dissolved in ethyl acetate. To isolate the sub-fractions from the TLC plates, the sub-fraction bands were first marked with a pencil under ultraviolet light, after which each fraction was carefully scraped off with a blade and collected separately. Subsequently, ethyl acetate was added to the sorbent silica gel in the test tubes as a solvent in order to extract the compounds from each sub-fraction (Fig. 1).

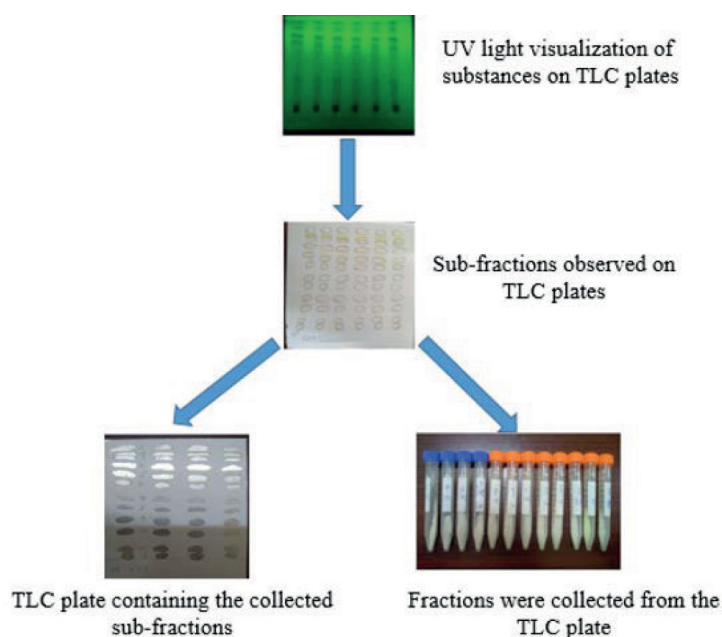


Fig. 1. Collection of sub-fractions from the TLC plates

Gas Chromatography–Mass Spectrometry (GC-MS)

The GC-MS analysis of ethyl acetate extracts of fungi was performed using an Agilent 5873 GC-MS system equipped with an HP-5 MS capillary column (30 m × 0.25 mm × 250 μm, 5% diphenyl) (Kaur *et al.*, 2020). An electron ionization (EI) system with an ionization energy of 70 eV was used for GC-MS analysis. Ultra-pure helium gas was employed as the carrier gas at a constant flow rate of 1 mL/min. The ion source, transfer line, and injector temperatures were set at 230°C, 250°C, and 290°C, respectively. The oven temperature was programmed from 50°C to 150°C at a rate of 3°C/min, held isothermally for 10 minutes, and then increased to 250°C at 10°C/min. Liquid samples (1/100, w/v in ethanol) were injected at 1 μL. The mass spectral scanning range was 29–450 m/z, with a solvent delay of 2 minutes.

The components of the extracts were identified by comparing their relative retention times on GC and mass spectra with the NIST MS Search Library Software, version 2.0.

Statistical analysis

All experiments were performed in triplicate, and the data were expressed as mean ± SEM (n = 3). Statistical differences were evaluated using an ANOVA test at p < 0.05.

Results and Discussion

*Isolation of fungal endophytic isolates from *P. harmala* and the screening for antimicrobial activity*

The plant *P. harmala* was collected from the Samarqand region, Uzbekistan, aseptically pack-

aged, and delivered to the Institute of Microbiology. In total, 14 isolates were isolated from different parts of the plant (Table 1).

Fourteen morphologically distinct endophytic fungal isolates were recovered from different tissues of *P. harmala*. The successful isolation of numerous endophytes confirms that this medicinal plant harbors a diverse fungal community. Similar fungal diversity has been reported previously in medicinal plants growing under arid and semi-arid environmental conditions, where endophytes contribute to plant adaptation and defense through the production of biologically active secondary metabolites.

Identified secondary metabolites of industrial importance in both the biomass of the fungus *Tirmania nivea* and the liquid nutrient medium in which this fungus was grown. In addition, it is known from scientific sources that aeration is of great importance for the growth of microorganisms, including fungi, and for the production of secondary metabolites (Khaled *et al.*, 2021).

The antimicrobial activities of the culture fluids and biomass of the isolates were studied by the agar diffusion method. The results show that no extracts of culture fluids had noticeable activities, whereas extracts of biomass had remarkable activities (Table 2).

During the experiments, some isolates lost their abilities to grow in a media. However, the majority of fungi showed moderate or significant antimicrobial activity against test pathogens. Among all isolates, strain 12Ph exhibited the broadest antimicrobial spectrum, showing inhibitory activity against both Gram-positive bacteria and the oppor-

Table 1. Fungal endophytic isolates from *P. harmala*

Plant name	Parts of plant	Isolates
<i>P. harmala</i>	root	1Ph, 2Ph, 3Ph
	stem	4Ph, 5Ph, 6Ph 7Ph, 8Ph
	leave	9Ph, 10Ph, 11Ph, 12Ph, 13Ph, 14Ph

Table 2. Antibacterial effect of secondary metabolites of endophytic fungi in biomass against test microorganisms

Isolates	Test microorganisms/Inhibitory zone(mm)				
	<i>E. coli</i> 86	<i>P. aeruginosa</i> 75	<i>S. aureus</i> 96	<i>B. subtilis</i> 91	<i>C. albicans</i> 84
1Ph	-	-	14±0.28	-	-
4Ph	19±0.22	20±0.23	20±0.28	20±0.24	-
5Ph	-	-	-	-	-
6Ph	-	-	13±0.21	-	-
7Ph	-	-	15±0.22	-	-
8Ph	30±0.32	30±0.31	30±0.28	28±0.29	-
10Ph	19±0.35	20±0.36	20±0.24	20±0.23	-
12Ph	24±0.22	20±0.21	30±0.32	28±0.34	26±0.31
13Ph	-	-	25±0.31	15±0.28	-
14Ph	-	-	15±0.23	-	-
Ceftriaxone, 30 µg/disc	30±0.33	28±0.32	30±0.35	30±0.34	-

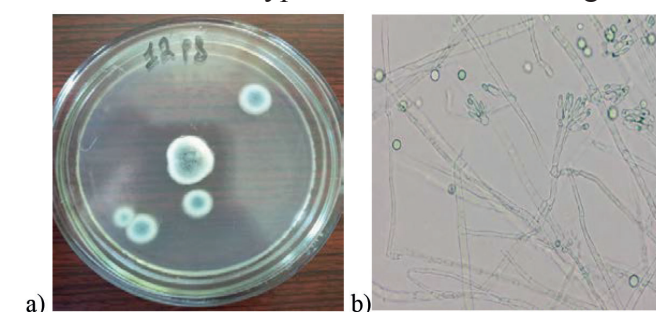
tunistic yeast *C. albicans*. This broad-spectrum activity justified its selection for detailed chemical investigation. Therefore, the 12Ph isolate was chosen for the next stage of our experiment. The Minimal Inhibitory Concentration (MIC) of the extract of the 12Ph strain was investigated, and the results were published (Yusupov, 2022). The results varied with respect to different pathogens. The lowest values were observed against the opportunistic pathogens *S. aureus* 96, *B. subtilis* 91, and *C. albicans* 84, all at 25 µg/mL. Against *E. coli* 86 and *P. aeruginosa* 75, relatively high MIC values were obtained: 100 µg/mL and 400 µg/mL, respectively.

Identification of endophytic fungi by classical and molecular genetic methods

The 12Ph isolate was cultured in a Petri dish using the macrocolony method on agar medium, and the growth morphology was examined visually and under a light microscope (Fig. 2). It was found that its colony was spreading and had smooth edges. After 5 days of growth, its diameter was about 4- 5 cm. The aerial mycelium was white and fluffy in the first days of growth, and changed to a blue-gray color as spores began to form.

Substrate mycelium was stained dark gray after 5 days. The color of the reverse side of the colony is dark green on the 7th day of growth, and the exudate is transparent on the 7th day of growth. The color of the aerial and substrate mycelium of the strain changes with age. When studying the micro-

scopic characteristics of the isolate, basidiophores were detected in its mycelium. Based on these and other noted features, it was determined that this isolate has a structure typical of the *Penicillium* genus.

**Fig. 2.** Macrocolony (a) and microscopy (b) of isolate 12Ph (1000x)

The study used one of the modern identification methods (The Internal Transcribed Spacer (ITS)) region sequencing to identify the most active endophytic fungal isolate 12Ph. The data obtained were analyzed using the “BLAST” program in the National Center for Biotechnology Information (NCBI) international database, according to which the 12Ph isolate was 99.68% similar to *P. roqueforti*. Based on these results, the 12Ph isolate was entered into the NCBI international database under the name *P. roqueforti* 12Ph (MZ424749).

Phytochemical analysis of *P. roqueforti* 12Ph fractions

During our experiments, we investigated various properties of the crude extract of *P. roqueforti* 12Ph, in particular its antibacterial activity against

conditionally pathogenic microorganisms, MIC, inhibitory effect on biofilm formation, and related characteristics. The next task is to identify the compounds present in the crude extract that are responsible for these properties. Scientific literature reports that several analytical methods are used to study the chemical composition of extracts obtained from biological objects (Suleimana *et al.*, 2009; Visweswari *et al.*, 2013).

The crude extract contains numerous compounds that differ in nature and polarity. Since the nature and polarity of the compound(s) responsible for the desired biological activity are unknown, it is necessary to perform solvent screening to isolate them (Murugan *et al.*, 2017). Taking this into account, the study of the extract composition was initiated by fractionating the antibacterial secondary metabolites extracted from the fungal biomass with ethyl acetate, based on their polarity, by dissolving them in a series of solvents with different polarities. For this purpose, the crude extract was obtained from 7-day-old cultured biomass using ethyl acetate (Fig. 3). The obtained fractions were dissolved in DMSO, and their antibacterial activity was evaluated together with the crude extract at a concentration of 1 mg/mL (Table 3).

As shown in Table 3, the compounds responsible for antibacterial activity in the crude extract were transferred predominantly into the methanolic fraction. However, a slight activity against *S. aureus* was also observed in the butanolic fraction. The migration of antibacterial compounds into the methanolic fraction can be explained by the similarity between their degree of polarity and that of methanol.

Naturally, under such conditions, these compounds were not transferred into hexane, which is a nonpolar solvent. The appearance of activity in the butanolic fraction can be hypothetically

explained by the fact that when ethyl acetate was mixed with water, the major compounds remained in the ethyl acetate phase, while a certain portion migrated into the aqueous phase and was then readily transferred into butanol upon mixing.

Based on these results, further studies were conducted to determine the nature of the compounds present in the biologically active fractions by subjecting them to various qualitative reactions. In this process, qualitative color reactions specific for different chemical classes, including terpenoids, steroids, flavonoids, tannins, cardiac glycosides, alkaloids, saponins, phenols, and anthraquinones, were performed (Table 4).

As shown in Table 4, the ethyl acetate crude extract obtained from the *P. roqueforti* 12Ph strain contains compounds belonging mainly to five chemical classes: terpenoids, steroids, flavonoids, cardiac glycosides, and alkaloids. In the methanolic fraction obtained by differential fractionation of the crude extract, terpenoids, flavonoids, and cardiac glycosides were identified. In the butanolic fraction, only flavonoids gave positive color reactions. No compounds were detected in the hexane and aqueous fractions using the applied color reactions.

These findings suggest that flavonoid-like compounds may contribute to the observed antibacterial activity. Similar results have been reported in several scientific studies. In particular, Mwanga *et al.* (2019) reported that *Fusarium* sp. isolated from the roots of *Moringa oleifera* contained 380.31 mg/g of flavonoids and exhibited antibacterial activity against human pathogens. Tang *et al.*, (2020) also demonstrated high antibacterial activity in flavonoid-producing endophytic fungi isolated from *Conyza blinii* H. Lév. Furthermore, a review article by Xie explains the mechanisms by which flavonoid compounds inhibit the proliferation of pathogenic microorganisms (Xie *et al.*, 2015).

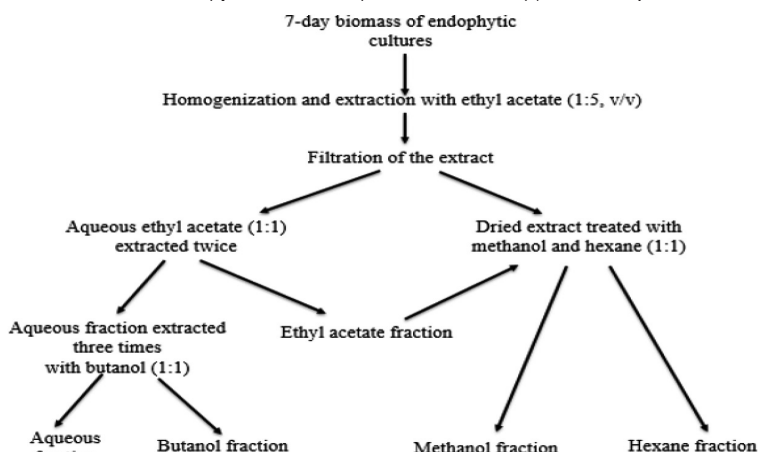


Fig. 3. Differential fractionation scheme of the secondary metabolite extract obtained from the biomass of *P. roqueforti* 12Ph using ethyl acetate

Table 3. Antibacterial activity of fractions obtained by differential fractionation of the crude extract of *P. roqueforti* 12Ph

Fractions	Zone of Inhibition (mm)		
	<i>MRSA</i> 97	<i>S. aureus</i> 96	<i>C. albicans</i> 84
Total extract	18±0.22	23±0.25	20±0.24
Hexane fraction	-	-	-
Methanolic fraction	15±0.20	16±0.21	22±0.21
Butanolic fraction	-	15±0.20	-
Aqueous fraction	-	-	-

Table 4. Phytochemical reactions of the crude extract of *P. roqueforti* 12Ph and its various fractions

Fractions	Terpenoids	Steroids	Flavonoids	Tannins	Cardiac Glycosides	Alkaloids	Saponins	Phenols	Anthraquinones
Total extract				-			-	-	-
Hexane fraction	-	-	-	-	-	-	-	-	-
Methanolic fraction		-		-		-	-	-	-
Butanolic fraction	-	-		-	-	-	-	-	-
Aqueous fraction	-	-	-	-	-	-	-	-	-

Based on the obtained results and the cited literature, it can be concluded that the crude extract of *P. roqueforti* 12Ph contains compounds belonging to the chemical groups of terpenoids, steroids, flavonoids, cardiac glycosides, and alkaloids, and that the antibacterial activity is most likely exhibited by flavonoid compounds that are transferred predominantly into the methanolic fraction.

Thin-layer chromatography of the methanolic fraction of the P. roqueforti 12Ph extract

One of the most widely used methods for the preliminary purification and fractionation of crude extracts obtained from biological objects is TLC. This method is relatively simple, cost-effective, can be performed in a short time, and at the same time is highly efficient. Using this technique, compounds migrate different distances on a silica gel

plate depending on their molecular mass, polarity, and the compatibility of the eluent system. This, in turn, enables the separation of complex mixtures of compounds into individual fractions (Lade *et al.*, 2014).

Based on these considerations, in the next stage of our study, we aimed to separate the methanolic fraction obtained from the crude extract of the *P. roqueforti* 12Ph strain into the most biologically active subfractions using the TLC method. To select a suitable mobile phase system, a literature survey revealed that three different solvent systems have been commonly used for fractionating antibacterial compounds (Suleimana *et al.*, 2009). Considering that our extract was obtained using ethyl acetate and that the fraction was dissolved in methanol, it became evident that in the ethyl acetate/methanol/

water solvent system, the compounds present in the methanolic fraction would not be resolved into sub-fractions. Therefore, the different solvent systems were used in this study (Fig. 4A). The separation of secondary metabolites with different Rf values was observed in both solvent systems. However, in the chloroform/ethyl acetate/formic acid system at a ratio of 5:4:1, the separation of secondary metabolites was optimal, and a greater number of sub-fractions were obtained. In total, 12 sub-fractions with Rf values of 0.93, 0.85, 0.80, 0.74, 0.65, 0.55, 0.46, 0.36, 0.25, 0.16, 0.07, and 0.01 were separated.

To identify the secondary metabolites separated from the methanolic fraction obtained from the crude extract of the *P. roqueforti* 12Ph strain using the chloroform/ethyl acetate/formic acid (5:4:1) solvent system, in addition to visualization under ultraviolet light at 254 nm (Fig. 4B), detection methods involving spraying the TLC plates with methanolic solutions of H₂SO₄ and FeCl₃ were also employed. The specificity of these methods lies in the fact that the subfractions are stained in different colors on the plate, which allows inference about the chemical groups to which the compounds belong.

From the images obtained, it was determined that the highest number of Rf values (12) was detected by visualization of the TLC plates under UV light at 254 nm. When treated with a 3% methanolic solution of FeCl₃, the sub-fractions developed a blue coloration, indicating their affiliation with flavonoids. This, in turn, confirms the results obtained from the phytochemical screening of the fractions produced by the differential fractionation method.

To evaluate the antibacterial activity of each obtained sub-fraction, an agar diffusion assay was performed. In this experiment, *MRSA*, *S. aureus*, and *C. albicans* were used as test microorganisms (Table 5).

The experimental results indicate that the compounds responsible for the antibacterial activity of the crude extract were distributed among several subfractions to varying degrees. In particular, the subfractions with Rf values of 0.937, 0.850, 0.800, 0.737, 0.650, and 0.550 showed activity only against *C. albicans*. Among these, the subfractions with Rf values of 0.800, 0.737, and 0.075 exhibited the highest inhibitory effects (18 ± 0.21 , 17 ± 0.20 , and 18 ± 0.20 , respectively). Subfractions with Rf values of 0.075 and 0.012 showed inhibitory activity not only against *C. albicans* but also against *MRSA* and *S. aureus*. Sub-fractions with Rf values of 0.250, 0.162, 0.075, and 0.012 demonstrated antibacterial activity against both pathogenic bacteria. Analyzing the results, the methanolic fraction contains different compounds with almost the same antimicrobial activities. While they were in different sub-fractions, they possessed moderate activity. However, when these compounds were in one methanolic extract, they had a synergetic effect and inhibited microbial growth more effectively.

Based on these observations, it can be inferred that compounds with antibacterial and antifungal activities were separated into distinct sub-fractions. Valle *et al.*, (2016) reported similar findings in a TLC study of ethanolic extracts from the leaves of *Piper betle* L., in which fractions with Rf values of 0.92, 0.86, 0.76, 0.53, 0.40, 0.25, 0.13, and 0.013

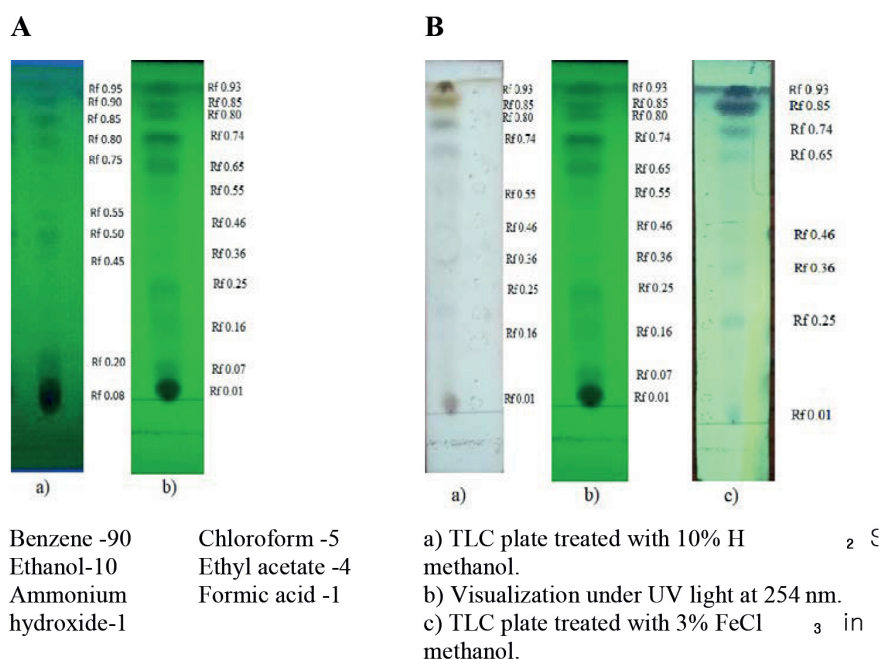


Fig. 4. Fractionation of the Methanolic fraction into subfractions using different solvent systems (A), and Visualization of methanolic fraction subfractions using different detection methods (B)

were obtained. Among them, fractions with Rf values of 0.86 and 0.13 exhibited antibacterial activity against antibiotic-resistant Gram-positive bacteria, including *MRSA* and vancomycin-resistant *Enterococcus*. Additionally, the compound with an Rf value of 0.86 showed inhibitory activity against antibiotic-resistant Gram-negative bacteria, such as carbapenem-resistant *Klebsiella pneumoniae* and metallo- β -lactamase-producing *Acinetobacter baumannii*.

To conclude, the TLC method is highly effective for the separation and preliminary purification of antibacterial compounds. Furthermore, the sub-fractions obtained by this method can be used for subsequent structural elucidation of the compounds they contain.

Gas chromatography–mass spectrometry of P. roqueforti 12Ph sub-fractions

GC-MS analysis was performed to characterize the chemical constituents present in the biologically active methanolic fraction and its TLC sub-fractions. The chromatographic analysis identified several compounds belonging predominantly to fatty acids, hydrocarbons, and nitrogen-containing metabolites (Tables 6 and 7).

To identify the specific compounds responsible for the antibacterial activity in the sub-fractions, each sub-fraction was further analyzed by GC-MS (Table 7).

Among these metabolites, n-hexadecanoic acid, 9,12-octadecadienoic acid, and carbendazim have previously been reported to exhibit antibacterial or antifungal activities. The occurrence of these

compounds is therefore consistent with the antimicrobial activity observed in the bioassays. Similar metabolites have also been identified in bioactive extracts of other endophytic fungi (Pu *et al.*, 2010; Sahani and Thakur, 2019; Kaur *et al.*, 2020).

The data from Tables 6 and 7 suggest that the methanolic fraction of the crude extract of *P. roqueforti* 12Ph and its sub-fractions, which exhibit antibacterial activity, are rich in compounds belonging to various chemical classes, including alkanes, fatty acids, amino group-containing compounds, and others. These metabolites may contribute to ecological interactions between the fungus, its host plant, and competing microorganisms. Numerous studies in the literature have reported the antibacterial activities of these compounds. For example, Shaaban *et al.* (2021) reported the antibacterial activity of the methyl ester of hexadecanoic acid, which was identified in the sub-fraction with an Rf value of 0.075. The study showed that this compound exhibited high antibacterial activity against clinical isolates of bacteria resistant to multiple antibiotics. Similarly, another paper reported that n-hexadecanoic acid and 9,12-octadecadienoic acid (Z, Z), identified in the subfractions with Rf values of 0.737, 0.075, and 0.012, demonstrated bacteriostatic effects against *S. aureus*, *E. coli*, and *Salmonella* sp. (Pu *et al.*, 2010).

Moreover, literature evidence indicates that carbendazim, identified in the subfraction with an Rf value of 0.850, exhibits high cytotoxic activity against 12 human cancer cell lines (Laryea *et al.*, 2010) and also possesses antifungal activity (Nagaraju *et al.*, 2019).

Table 5. Antibacterial activity of sub-fractions

Sub-fractions, №	Rf values	Inhibition zone, mm		
		<i>MRSA</i> 97	<i>C. albicans</i> 84	<i>S. aureus</i> 96
1	0.937	-	12±0.15	-
2	0.850	-	14±0.18	-
3	0.800	-	18±0.21	-
4	0.737	-	17±0.20	-
5	0.650	-	14±0.17	-
6	0.550	-	14±0.17	-
7	0.462	-	-	-
8	0.362	-	-	12±0.15
9	0.250	12±0.15	-	12±0.15
10	0.162	12±0.14	-	12±0.14
11	0.075	12±0.14	18±0.20	13±0.15
12	0.012	12±0.15	13±0.15	12±0.15

Table 6. GC-MS analysis of the methanol fraction of *P. roqueforti* 12Ph

Peak №	Retention Time (min)	Library/ID MassHunter\Library\NIST17.L	Similarity (%)
1	3.757	3-Methyl-1-butanol acetate	64
5	8.706	10-Methylundecanoic acid methyl ester	94
6	9.269	Dodecanoic acid	64
7	12.064	Methyl tetradecanoate	96
8	12.539	Pentadecanoic acid	38
9	14.378	Hexadecanoic acid methyl ester	91
10	14.713	n-Hexadecanoic acid	87
15	16.137	9,12-Octadecadienoic acid (Z,Z)-	76

Table 7. GC-MS analysis of the sub-fractions of the methanolic fraction of *P. roqueforti* 12Ph

Rf value	Peak №	Retention time	Library/ID MassHunter\Library\NIST17.L	Similarity (%)
0.937	1	3.757	1-Butanol, 3-methyl-, acetate	64
	12	7.281	Hydroxylamine, O-decyl-	83
0.850	1	3.757	1-Butanol, 3-methyl-, acetate	64
	11	5.796	Allyl undecyl oxalate-	78
	16	8.603	Carbendazim	64
0.800	1	3.757	1-Butanol, 3-methyl-, acetate	64
	7	5.184	Diaziridine, 3-ethyl-3-methyl-	64
	12	5.795	Allyl hexadecyl oxalate	72
	14	6.233	2,3,4,5,6-Pentamethylacetophenone	81
0.737	1	3.757	1-Butanol, 3-methyl-, acetate	64
	14	14.691	n-Hexadecanoic acid	64
0.650	1	3.757	1-Butanol, 3-methyl-, acetate	64
	4	4.940	1-Ethyl-2,2,6-trimethylcyclohexane	68
0.550	1	3.757	1-Butanol, 3-methyl-, acetate	64
	17	6.232	2,3,4,5,6-Pentamethylacetophenone	76
	18	7.279	Undecane, 2,6-dimethyl-	64
0.462	1	3.757	1-Butanol, 3-methyl-, acetate	64
	5	5.182	Decane	64
0.362	1	3.757	1-Butanol, 3-methyl-, acetate	64
	13	5.794	Isobutyl nonyl oxalate-	64
0.250	1	3.757	1-Butanol, 3-methyl-, acetate	64
	5	8.706	Methyl 10-methylundecanoate	94
	6	9.269	Dodecanoic acid	64
0.162	7	12.064	Methyl tetradecanoate	96
	8	12.539	Pentadecanoic acid	38
0.075	9	14.378	Methyl hexadecanoate	91
	10	14.713	n-Hexadecanoic acid	87
0.012	1	3.757	1-Butanol, 3-methyl-, acetate	64
	2	4.498	2,2,2-Trifluoroethyl 9-octadecenoate	74
	15	16.137	9,12-Octadecadienoic acid (Z,Z)-	76

From the obtained results and literature analysis, it can be concluded that the endophytic fungus *P. roqueforti* 12Ph, isolated from *P. harmala*, produces a chemically diverse crude extract containing several compounds previously reported to possess antibacterial activity. This studied strain can serve as a promising source of biologically active compounds for pharmaceutical applications.

Conclusion

The present study demonstrates that the medicinal plant *P. harmala* harbors a diverse community of endophytic fungi with considerable antimicrobial potential. Among the isolated endophytes, *P. roqueforti* 12Ph exhibited the strongest antibacterial and antifungal activities against clinically relevant and opportunistic pathogens, including *MRSA* and *C. albicans*.

The biologically active compounds were predominantly recovered from the fungal biomass by ethyl acetate extraction, and differential fractionation indicated that antimicrobial activity was mainly associated with the methanolic fraction. Thin-layer chromatography enabled the separation of several bioactive sub-fractions, while GC–MS analysis identified multiple compounds, including fatty acids, hydrocarbons, and nitrogen-containing metabolites that have previously been reported to possess antimicrobial properties.

Overall, these findings demonstrate that the endophytic fungus *P. roqueforti* 12Ph represents a promising source of bioactive secondary metabolites with antimicrobial potential. The study expands current knowledge of endophytic fungi associated with *P. harmala* and supports their potential as valuable resources for the discovery of novel antimicrobial agents.

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