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Mutation, Chemoprofiling, Dereplication, and Isolation of Natural Products from *Penicillium oxalicum*

Vidushi Abrol,[‖] Manoj Kushwaha,[‖] Divya Arora, Sharada Mallubhotla, and Sundeep Jaglan*

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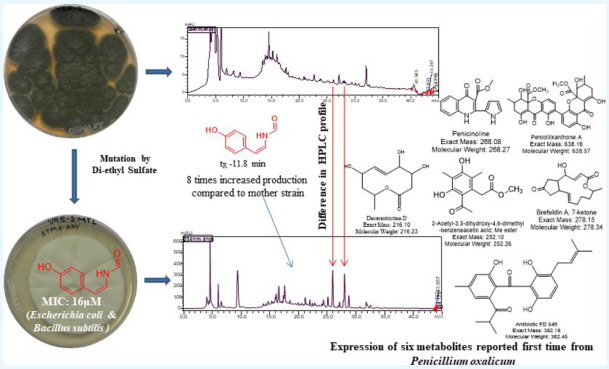
ABSTRACT: Diethyl sulfate (DES)-based chemical mutagenesis was applied on different fungal strains with the aim of diversifying the secondary metabolites. The mutant strain (VRE-MT1) of *Penicillium oxalicum* was subjected to dereplication (LCMS-based) and isolation of natural products, resulting in obtaining 10 molecules of bioactive potential. Metabolites, viz. tuckolide, methylpenicinoline, 2-acetyl-3,5-dihydroxy-4,6-dimethylbenzeneacetic acid, penicillixanthone A, brefeldin A 7-ketone, and antibiotic FD 549, were observed for the first time from *P. oxalicum*. The results of antimicrobial activity reveal that the compounds *N*-[2-(4-hydroxyphenyl)ethenyl]formamide, methylpenicinoline, and penipanoid A have potent antibacterial activity against *Bacillus subtilis* (ATCC 6633) with minimum inhibitory concentration (MIC) values of 16, 64, and 16 μM, respectively, and the compounds *N*-[2-(4-hydroxyphenyl)ethenyl]formamide, methylpenicinoline, and penipanoid A were found active against *Escherichia coli* (ATCC 25922), with MIC values of 16, 64, and 16 μM, respectively. Also, the metabolites *N*-[2-(4-hydroxyphenyl)ethenyl]formamide and tuckolide showed effective antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid scavenging assays. The mutant VRE-MT1 was found to have 8.34 times higher quantity of *N*-[2-(4-hydroxyphenyl)ethenyl]formamide as compared to the mother strain. The DES-based mutagenesis strategy has been found to be a potent tool to diversify the secondary metabolites in fungi.



INTRODUCTION

Studies revealed that fungal diversity and its biosynthetic clusters of genes possess unlimited metabolic potential for drug discovery. Filamentous fungi such as *Penicillium*, *Tolypocladium*, *Ganoderma*, and *Acremonium* are the most remarkable factories of chemicals and important products known today. It has been observed that many biologically active substances such as antimicrobials, antioxidants, mycotoxins, and antistress and anticancer compounds are reported, especially fungi have not been explored so much due to their cryptic pathways.[1,2] Recently, various techniques have been introduced to activate the metabolic capacities of microbial cultures for the expression of silent pathways.[2] The stimulation of hidden biosynthetic pathways for the production of natural products has become an important strategy for obtaining new compounds with high yields from fungi as these compounds generally form gene clusters and remain inactive under normal culture conditions.[3] In order to activate these silent metabolites, various strategies such as chemical epigenetics,[4] co-cultivation,[5] ribosome engineering,[6] and so forth have been followed for the introduction of important new compounds. Under the mandate of our research institute, we have explored important microbes to obtain the bioactive metabolites, and few successes have been already published.[7−12] Among different strategies, chemical mutagenesis has been exploited a little for finding potent bioactive molecules.[13,14] Therefore, chemical mutagenesis has been applied in our study using diethyl sulfate (DES). The effectiveness of DES is well documented, and it is an effective alkylating mutagen which ethylated the sixth oxygen atom of guanine and played a significant role in the mutation of fungi such as *Penicillium oxalicum*.[15−17]

P. oxalicum is an ascomycetous fungus, having economical importance in the era of pharmaceuticals. It is also known for the production of compounds with a variety of biological activities, including anti-microbial[18] and anti-tumor,[19] and various enzymatic compounds.[20] As part of the investigation of potent secondary metabolites, *P. oxalicum* grabbed our attention because this strain found as an important fungal strain exhibits high bioactive products, which may lead to the identification new chemical entities.[21] Further, the liquid chromatography−mass spectrometry (LCMS)-based derepli-

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cation technique was acclimatized to avoid re-finding known molecules.

Dereplication is an effective tool in drug discovery programs and is used as an initial screening process for rapid identification. Due to the presence of a pool of complex molecules of secondary metabolites, chemoprofiling of metabolites available in the crude extracts is a challenging task. However, the discoveries of secondary metabolites are hampered by re-isolation of known metabolites. Therefore, the dereplication strategy has been focused on the high-throughput screening of metabolites and identified the known compounds along with their chemical profiling at initial stages. There are numerous strategies applied for the rapid identification of secondary metabolites in which the most commonly used combined approach is LC−MS/MS.[22] The identification based on LC−MS and LC−MS/MS is quite sensitive and also provides structural data with molecular mass and formula, which can be easily searchable in a natural product database such as the dictionary of natural products (DNP).[23] Here, we have successfully obtained the mutants of *P. oxalicum*, having diverse metabolites of bioactive potential.

RESULTS

Effect of DES on Fungal Spores/Mycelia. Subsequent to the DES treatment, mycelia which differ in morphology were picked and finally four mutants (VRE-MT1, VRE-MT2, VRE-MT3, and VRE-MT4) of *P. oxalicum* were obtained (Figure 1).

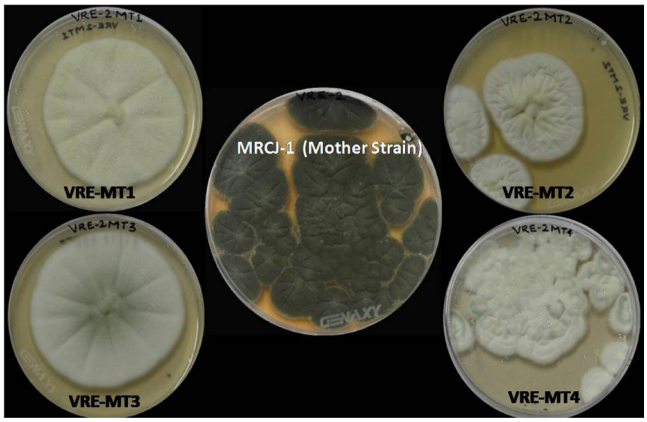

Figure 1. Mother strain (VRE-2) and mutants obtained (VRE-MT1, VRE-MT2, VRE-MT3, and VRE-MT4).

On sub-culturing the mutant strains to normal potato dextrose agar (PDA) medium plates, they maintained their morphological characteristics, which were different from those of the mother strain. When comparing the morphological characteristics, it was observed that the mutants keep their morphological characteristics intact even after multiple sub-culturing. It was noticeable that all the four mutants do not sporulate on PDA, whereas the mother culture sporulated (Figure 1).

Comparative Analysis and Screening of Mutants Based on HPLC Profiling. For the comparative metabolic analysis of mother and mutant cultures, HPLC-based screening was performed. Out of the four mutant strains, VRE-MT1 broth extract shows a difference of peak in the HPLC chromatogram, which reveals that it might significantly affect the metabolism of the mother culture after mutagenesis and raise the expectations to get new secondary metabolites or the reported metabolites with a high yield (Figure 2) (Figure S1). Therefore, fermentation and scale-up of VRE-MT1 were performed, and approximately 1 g of crude extract (dark brown oil) was obtained, which was used for semipreparative HPLC.

LCMS-Based Identification of Secondary Metabolites in VRE-MT1 (Mutant) of *P. oxalicum*. After confirming the variation in the HPLC chromatogram of VRE-MT1 in comparison with the mother strain MRCJ-1, the +ESI-LCMS chromatogram of the VRE-MT1 extract was analyzed. It was subjected to dereplication studies (Figure S2). This led to the identification of 10 natural products by searching through the DNP. After analyzing various parameters such as retention times (t_R), molecular formula, calculated mass $[M + H]^+$, observed mass (m/z), and Δ ppm error. The presence of metabolites was confirmed, viz. (1) *N*-[2-(4-hydroxyphenyl)-ethenyl]formamide ($C_9H_9NO_2$), Δ = −6.7 ppm, (2) methylpenicinoline ($C_{15}H_{12}N_2O_3$), Δ = −4.8 ppm, (3) tuckolide ($C_{10}H_{16}O_5$), Δ = −0.46 ppm, (4) penipanoid A ($C_{16}H_{13}N_3O_3$), Δ = −3.3 ppm, (5) 2-acetyl-3,5-dihydroxy-4,6-dimethylbenzeneacetic acid ($C_{13}H_{16}O_5$), Δ = 2.8 ppm, (6) penicillixanthone A ($C_{32}H_{30}O_{14}$), Δ = −2.5 ppm, (7) brefeldin A, 7-ketone ($C_{16}H_{22}O_4$), Δ = 0.00 ppm, (8) meleagrin A; Me ether ($C_{24}H_{25}N_5O_4$), Δ = 0.00 ppm, (9) penioxamide A ($C_{27}H_{35}N_3O$), Δ = 0.00 ppm, (10) antibiotic FD 549 ($C_{26}H_{26}O_5$), Δ = −2.8 ppm (Table 1) (Figures S3−S12).

Antimicrobial and Antioxidant Activity of Compounds. The antimicrobial activity reveals that the compounds *N*-[2-(4-hydroxyphenyl)ethenyl]formamide, methylpenicinoline, and penipanoid A showed potent antibacterial activity against *Bacillus subtilis* (ATCC 6633) with 16, 64, and 16 μM [minimum inhibitory concentration (MIC)], respectively and the compounds *N*-[2-(4-hydroxyphenyl)ethenyl]-formamide, methylpenicinoline, and penipanoid A were also found active against *Escherichia coli* (ATCC 25922), with 16, 64, and 16 μM (MIC), respectively (Table S2).

Further, all the isolated metabolites were analyzed for antioxidant activity. Here, the antioxidant results reveal that the metabolites *N*-[2-(4-hydroxyphenyl)ethenyl]formamide and tuckolide showed effective antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity with 18.53 and 79.17 μg/mL (EC_{50}), respectively (Table S3), and using 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical scavenging activity with (EC_{50}) 3.08 and 4 μg/mL, respectively (Table S4).

Isolation and Quantification of *N*-[2-(4-Hydroxyphenyl)ethenyl]formamide. The peak at t_R = 12.0 min was collected and dried under vacuum and further subjected to spectroscopic analysis; the isolated compound was identified as *N*-[2-(4-hydroxyphenyl)ethenyl]formamide (m/z 164.0717 [M + H]+, $C_9H_9NO_2$); the spectroscopic data of NMR and MS are available in the Supporting Information (Figures S3 and S14); *N*-[2-(4-hydroxyphenyl)ethenyl]-formamide showed potential pharmacological activity (Figure S13).The spectroscopic data are in accordance with reported literature.[24] ^{1}H NMR (400 MHz, D_2O): δ 7.95 (d, J = 18.6 Hz, 1H), 7.12 (dd, J = 15.1, 8.6 Hz, 2H), 6.76 (d, J = 8.6 Hz, 2H), 6.59 (d, J = 9.6 Hz, 1H), 5.78 (d, J = 9.6 Hz, 1H), ^{13}C NMR and ^{13}C-DEPT NMR (101 MHz, D_2O): δ 161.75, 155.05, 129.74, 129.55, 126.86, 117.43, 115.53, 113.97 (Figure S14).

The metabolite *N*-[2-(4-hydroxyphenyl)ethenyl]formamide was further used for quantification in mother and mutant strains. This unfolds that the amount of *N*-[2-(4-

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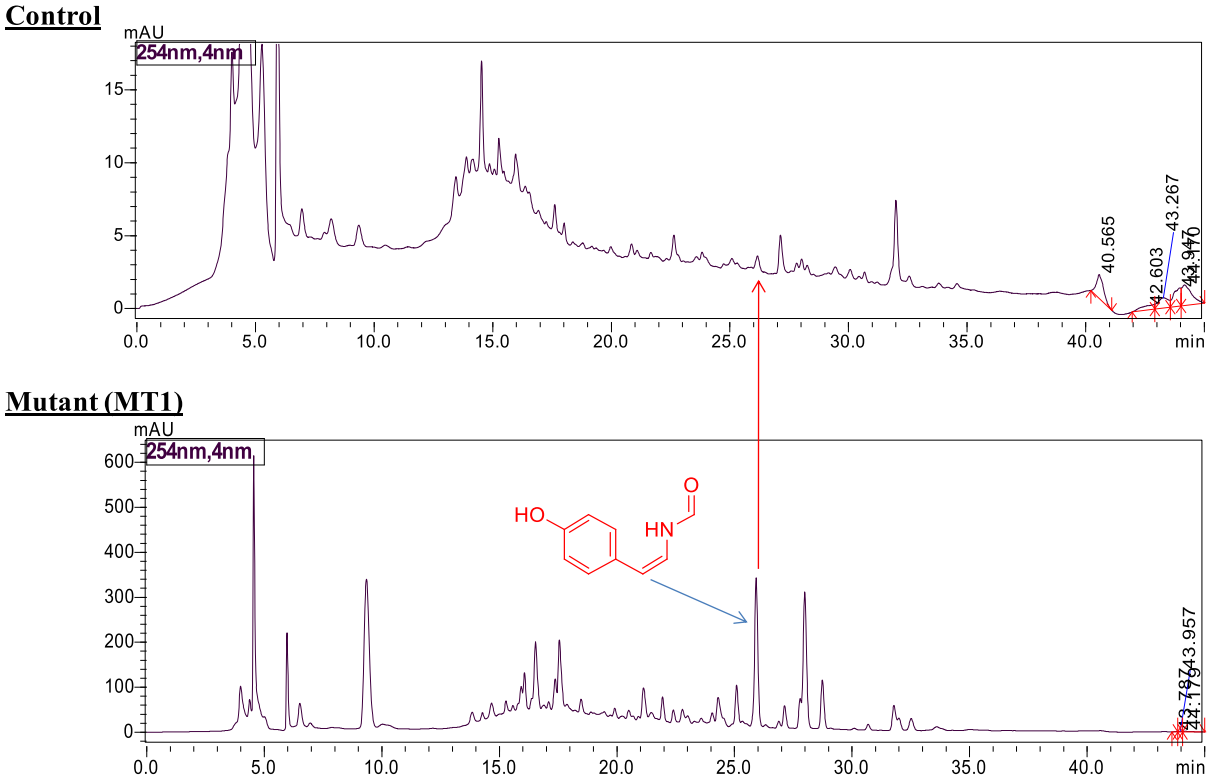

Figure 2. HPLC chromatogram: (A) control (*Penicillium oxalicum*) and (B) mutant (VRE-MT1).

hydroxyphenyl)ethenyl]formamide metabolite in the mother strain was 16 mg L^{-1} and in VRE-MT1, it was quantified as 134.2 mg L^{-1}. Therefore, this study revealed that as compared to the control, the yield of the *N*-[2-(4-hydroxyphenyl)-ethenyl]formamide compound was 8.34 times higher in the mutant strain VRE-MT1. The quantification data are shown in Figure S15.

DISCUSSION

The filamentous fungus, viz. *P. oxalicum,* produces a variety of secondary metabolites from antibiotics to mycotoxins.[25] There are few reports in the literature where the DES-based chemical mutagenesis have led to the expression of new metabolites and enhanced the efficacy of the fungal strains.[3,13,26−28] Here, we had successfully applied the DES-based chemical mutagenesis on *P. oxalicum* and obtained four mutants (VRE-MT1, VRE-MT2, VRE-MT3, and VRE-MT4). Among the mutants, VRE-MT1 displayed significant changes in the HPLC profiling as compared to the mother culture (MRCJ-1). For rapid identification of the secondary metabolites, we have used LC−MS-based dereplication workflow, in which the DNP was accessed, leading to the identification of 10 secondary metabolites from the crude extract of VRE-MT1 (Table 1). The DES-based strategy played its role, enabling the fungal strain to produce seven metabolites, viz. decarestrictine D, penipanoid A, penicinoline, *N*-[2-(4-hydroxyphenyl)ethenyl]-formamide, 2-acetyl-3,5-dihydroxy-4,6-dimethylbenzeneacetic acid, Me ester, penicillixanthone A, and antibiotic FD 549, which were found to be absent in the mother culture of *P. oxalicum*. Interestingly, except for penipanoid A, the above-mentioned six compounds were never been reported from the *P. oxalicum* species.

Since our aim was to explore the metabolites of the mutants and the mother strain via dereplication strategy, among those 10 metabolites, *N*-[2-(4-hydroxyphenyl)ethenyl]formamide was considered for the comparative quantitative analysis and characterization via NMR. The mutant strain (VRE-MT1) was found to produce an 8.34 times higher amount of *N*-[2-(4-hydroxyphenyl)ethenyl]formamide as compared to the mother strain. Despite being very less in quantity (≤1.5 mg), we were able to screen the compounds for bioactivities, and it was observed that the compounds *N*-[2-(4-hydroxyphenyl)-ethenyl]formamide, penipanoid A, and penicinoline show high anti-microbial activity and compounds *N*-[2-(4-hydroxyphenyl)ethenyl]formamide and decarestrictine D depict good anti-oxidant activity (Tables S2, S3). From the changes obtained after DES treatment, it can be concluded that the strategy works well on fungi and is potent to diversify the secondary metabolites. It is also noteworthy that the suppression of sporulation in mutants may occur due to the disruption of some essential genes, which regulates the sporulation in fungi. In future, it would be interesting to compare the mutant and the mother strain via transcriptomics, proteomics, and metabolomics approaches to decipher the changes obtained.

EXPERIMENTAL SECTION

Chemicals and Reagents. DES 98%, dimethyl sulfoxide (DMSO), ascorbic acid, DPPH, and LC−MS grade methanol, acetonitrile, and HPLC water were purchased from Sigma-Aldrich; amphotericin B, ciprofloxacin, streptomycin sulfate, 70% ethanol, formic acid (LC−MS grade), PDA, and potato dextrose broth (PDB) were procured from HiMedia Laboratories, India.

Microbial Strains. The fungal strain was obtained from the national microbial repository, that is, *P. oxalicum* (VRE-2) was procured from Col. Sir R. N. Chopra, Microbial Resource Center Jammu (MRCJ), India, under accession number

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Table 1. +ESI LCHRMS-Based Identification of Metabolites from *Penicillium oxalicum*

s. no	t_R	name of compound	molecular formula	calculated $[M + H]^+$	observed (m/z)	Δppm error	MRCJ-1 (control)	VRE-MT1	VRE-MT2	VRE-MT3	VRE-MT4	status of compound in genus (*Penicillium*)	status of compound in species (*oxalicum*)
1	3.4	decarestrictine D	$C_{10}H_{16}O_5$	217.1071	217.1072	−0.46	[a]	present	[a]	[a]	[a]	reported	no reports
2	4.0	penipanoid A	$C_{16}H_{13}N_3O_3$	296.103	296.1048	−3.3	[a]	present	[a]	[a]	[a]	reported	reported
3	5.9	penicinoline	$C_{15}H_{12}N_2O_3$	269.0921	269.0934	−4.8	[a]	present	[a]	[a]	[a]	reported	no reports
4	6.4	N-[2-(4-hydroxyphenyl)ethenyl]formamide	$C_9H_9NO_2$	164.0706	164.0717	−6.7	[a]	present	[a]	[a]	[a]	reported	reported
5	7.3	2-acetyl-3,5-dihydroxy-4,6-dimethylbenzeneacetic acid; Me ester	$C_{13}H_{16}O_5$	253.1076	253.1004	2.8	[a]	present	[a]	[a]	[a]	reported	no reports
6	7.8	meleagrin A; Me ether	$C_{24}H_{25}N_5O_4$	448.1977	448.1977	0	present	present	[a]	[a]	[a]	reported	reported
7	9.56	penioxamide A	$C_{27}H_{35}N_3O$	418.2854	418.2854	0	Present	present	[a]	[a]	[a]	reported	reported
8	16.7	penicillixanthone A	$C_{32}H_{30}O_{14}$	639.1714	639.1730	−2.5	[a]	present	[a]	[a]	[a]	reported	no reports
9	16.89	brefeldin A, 7-ketone	$C_{16}H_{22}O_4$	279.1595	279.1595	0	present	present	[a]	[a]	[a]	reported	no reports
10	24.39	antibiotic FD 549	$C_{23}H_{26}O_5$	383.1858	383.1869	−2.8	[a]	present	[a]	present	present	reported	no reports

[a]Absent.

MRCJ-1. The strain was cultured in PDB with constant shaking at 150 rpm for 7 days at 28 °C and on PDA in Petri dishes for 3−5 days at 28 °C. *Staphylococcus aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, one yeast, *Candida albicans* ATCC 24433, and *Aspergillus niger* ATCC 16404 used for antimicrobial activity screening were procured from American Type Culture Collection (ATCC).

Chemical Mutagenesis of the Fungal Strain. The fungal strain was grown on PDA to obtain a pure active culture. Fungal spores were harvested by pouring normal saline solution containing 0.01% tween 20. The chemical mutagenesis was performed on freshly inoculated fungal spores (5×10^6 CFU mL^{-1}) in the suspension. The suspension was made by adding DES in 20% (v/v) aqueous DMSO to get the final concentration of DES (5, 10, 20, 40, 80, 160, 320, 640 mM) (Table S1). The inoculated spores in the DES suspension solution were incubated at 4 °C for 32 days. Aliquots (100 μL) of the treated spores were taken on 1, 2, 4, 8, 16, and 32 days, spread on PDA plates, and incubated at 28 °C for 5−7 days. During this period, mutant colonies (different morphological characteristics) were picked and grown to obtain pure cultures that were visually distinct from the mother culture.

Fermentation and Extracts' Preparation. The freshly sub-cultured fungal strains (mother and mutant culture) were inoculated in 200 mL of PDB in Erlenmeyer flasks (500 mL) and incubated for 2−3 days at 28 °C with constant shaking at 150 rpm, which was used as the inoculum. Further, Erlenmeyer flasks containing 400 mL of PDB were inoculated with 10% of freshly prepared inoculums and were kept in a shaking incubator at 28 °C under 150 rpm in the dark condition for 10 days and finally 12 L of culture broth was obtained for further processing. The culture cell mass and filtrate were separated using a muslin cloth and were collected. The filtrate was extracted three times repetitively with EtOAc and then with chloroform two times and collected. The mycelia were homogenized twice with 10% MeOH and extracted with EtOAc thrice. The organic layer was separated and dried using a rotavapor. A total of 1 g of the crude extract was obtained; that is, 750 mg from broth and 250 mg from mycelia were collected separately.

HPLC Profiling of Crude Extracts of *P. oxalicum*. HPLC analysis of the crude extract was performed using a Shimadzu UFLC system. The chromatographic separation was achieved using a Merck RP18 HPLC column (4.6 mm × 250 mm, 5 μm). The mobile phase was composed of 0.1% formic acid (A) in water and acetonitrile (ACN) (B). The elution gradient program was followed: 0 min (20% B), 25 min (90% B), 35 min (90% B), 37 min (20% B), and 40 min (20% B). The total run time was 40 min; the flow rate was 0.7 mL/min. 10 μL of the sample volume was injected for HPLC analysis.

LC−MS Profiling of Crude Extracts of *P. oxalicum*. LCMS analysis of the crude extract was performed using an Agilent 1290 HPLC series equipped with an Agilent HRMS (UHD 6540) system. The analysis was performed in positive electrospray ionization mode with the mass range of m/z = 100−2000. The chromatographic separation was achieved using an Agilent Zorbax Eclipse RP18 HPLC column (4.6 mm × 100 mm, 3 μm). The same mobile phase as that used in HPLC profiling was used. The elution gradient program was followed: 0 min (10% B), 5 min (60% B), 15 min (60% B), 17 min (10% B), and 20 min (10% B). The total run time was 22 min, with the flow rate of 0.5 mL/min. 10 μL of the sample

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volume was injected for LCMS analysis. The MS scan parameters were as follows: capillary voltage, 3500 V; vaporizer, 200 V; gas temperature, 300 °C; drying gas, 12.0 L·min^{-1}; nebulizer pressure, 30 psi; fragmentor, 175 V; skimmer, 165.0 V; and octopole RF peak, 750 V. All the data processing was executed and evaluated using Agilent MassHunter qualitative software (version B.05.00, Agilent Technologies, Santa Clara, CA, USA).

LC−MS-Based Quantification of *N*-[2-(4-Hydroxyphenyl)ethenyl]formamide. The quantification of *N*-[2-(4-hydroxyphenyl)ethenyl]formamide was carried out using an Agilent HPLC 1290 series equipped with LCQQQMS (model no.6410B). *N*-[2-(4-hydroxyphenyl)-ethenyl]formamide was eluted through a 4.6 mm × 100 mm, 3 μm, Agilent Zorbax Eclipse, the column with a flow rate of 0.5 mL/min with the gradient program; eluent A was composed of 0.1% formic acid in water and eluent B was composed of ACN. The gradient program was followed: 10−70% of B in 0−15 min, 70% of B held for the next 5 min, and 70−20% of B in 20−21 min and held for 1 min at the initial composition of the mobile phase. The total run time was 22 min with a flow rate of 0.5 mL/min.

The calibration equation of *N*-[2-(4-hydroxyphenyl)-ethenyl]formamide was obtained by plotting the LC−MS peak area (y) *versus* the concentration [mg//L(ppm)]. Here, a five point calibration was included, that is, 0.04, 0.4, 4, 20, and 40 ppm; the line equation was, $Y = 84798.96x + 5893.56$, ($R^2 = 0.99$). The equation showed very good linearity over the range (Figure S15).

Antimicrobial Activity. Antibacterial and antifungal activities of compounds were performed using microdilution-based method (as per CLSI guidelines) against two Gram-positive bacterial strains (*S. aureus* ATCC 25923, *B. subtilis* ATCC 6633), two Gram-negative bacterial strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853), one yeast (*C. albicans* ATCC 24433), and one filamentous fungus (*A. niger* ATCC 16404). Antibacterial testing was performed in Mueller Hinton Agar (MHA) broth, whereas for antifungal testing, Sabouraud Dextrose Agar (SDA) broth was used. The stock solutions of the compounds were prepared in DMSO. The MIC of the compounds was determined by the preliminary twofold serial dilution experiment in a 96-well U-bottom microtiter plate. Amphotericin B and ciprofloxacin [16−0.03 μg/mL] were used as positive controls, that is, standard antifungal and antibacterial agents, respectively. The bacterial and fungal suspension of the overnight-grown bacteria and fungi was prepared in sterile normal saline solution, and the density was further adjusted to 0.5 Mcfarland. The bacterial cultures were then further diluted and added in a 100 μL volume to reach a final inoculum of 1×10^5 CFU/mL. For fungal cultures, 1×10^3 CFU/mL was used. The plates were kept in the incubator at 37 °C for 24 h for bacterial cultures and at 28 °C for 48 h for the fungal cultures. The plates were observed visually, and the minimum concentration of the compound showing no visual growth was recorded as MIC. Then, a loopful of inoculum was taken from all the wells showing no visual growth and streaked on the MHA or SDA plate for obtaining the mimimum bactericidal concentration and minimal fungicidal concentration, respectively.

Antioxidant Activity Using DPPH and ABTS Assays. *DPPH Free-Radical-Scavenging Activity.* The antioxidant activity of the compound was assessed by analyzing the DPPH free radical scavenging ability. The assay procedure comprised 0.53 mg/10 mL of DPPH in methanol incubated in the presence and absence of the test material for 30 min and read at 517 nm in the plate reader. The DPPH assay is based on the reduction of DPPH, a stable free radical. The stable free radical DPPH with an unpaired electron gives a maximum absorption at 517 nm (purple color). When DPPH reacts with antioxidants, its electrons become paired off in the presence of a hydrogen donor and it is reduced to DPPH-H and as a consequence, the absorbance decreased from the DPPH radical to DPPH-H for results in decolorization (yellow color) with respect to the number of electrons captured. The more the decolorization of DPPH, the more the reducing ability is. This test has been the most accepted for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (diphenylpicrylhydrazine; a non-radical) with the loss of this violet color. Ascorbic acid was used as a positive control.

Percentage antioxidant activity was calculated by using absorbance values of control containing DMSO + DPPH and the test samples, as shown below

$$\frac{(\text{O. D control} - \text{O. D test}) \times 100}{\text{O. D control}}$$

ABTS Radical-Scavenging Activity. The antioxidant scavenging activity was determined by the ABTS radical cation assay. In this assay, 7 mM ABTS solution was dissolved in 2.45 mM potassium persulfate. ABTS*$^-$ radical cations were generated after incubating the mixture at room temperature in the dark for 12−16 h. This gives a stable dark blue colored ABTS solution after diluting with ethanol at pH-7.4 till absorbance reaches 0.7 ± 0.05 at 734 nm. Then, 10 μL of each sample was mixed with 1 mL of ABTS solution and kept in the dark at room temperature for 5−6 min and was serially diluted to get accurate absorbance values at 734 nm. Ascorbic acid was used as a positive control.

Percentage antioxidant activity was calculated by using absorbance of the uninhibited radical cation solution (blank) according to the equation

$$\text{Inhibition of } A_{734\text{nm}}(\%) = \frac{(\text{O. D control} - \text{O. D test}) \times 100}{\text{O. D control}}$$

Semipreparative Isolation of Chemical Compounds from the Crude Extract of P. oxalicum. The fermented broth was terminated by adding 10% of methanol, followed by homogenization and centrifugation to separate the cell debris and filtrate. The supernatant was extracted thrice with an equal volume of ethyl acetate. The organic extract was concentrated under vacuum using a rotary evaporator. A total of 1 g of crude extract was obtained, out of which 100 mg of crude extract was dissolved in 1 mL of methanol, filtered through a 0.2 μm filter, and further, 100 μL was subjected to semipreparative isolation. LC separation was achieved by using a Shimadzu HPLC and a Merck RP18 HPLC column (9.4 mm × 250 mm, 5 μm). Semipreparative separation was performed at 2.0 mL min^{-1} under an elution gradient program in which mobile phase A was composed of 0.1% formic acid in water and mobile phase-B was composed of acetonitrile. The gradient system was applied as follows: 20−70% of B in 0−20 min, 70% of B in 20−30 min, 70% to 20% of B in 30−32 min and held for 3

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min; the total run time was 35 min. 100 μL of sample volume was injected for HPLC analysis.

Structure Elucidation of N-[2-(4-Hydroxyphenyl)ethenyl]-formamide. Characterization and structure elucidation were done by analyzing the data of MS, ^{1}H NMR, ^{13}C NMR, and HRESIMS by intercalating the pure compound with DMSO-d_6 in 5 mm NMR tubes..

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00141.

Analytical and bioactivity data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sundeep Jaglan − *Fermentation & Microbial Biotechnology Division, Indian Institute of Integrative Medicine (CSIR), Jammu 180001, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India;* orcid.org/0000-0002-5691-7980; Phone: 91-191-2585006-13 Ext. 293; Email: sundeepjaglan@iiim.res.in; Fax: 91-191-2586333

Authors

Vidushi Abrol − *Fermentation & Microbial Biotechnology Division, Indian Institute of Integrative Medicine (CSIR), Jammu 180001, India; School of Biotechnology, Shri Mata Vaishno Devi University, Katra 182320, India;* orcid.org/0000-0002-8607-0282

Manoj Kushwaha − *Fermentation & Microbial Biotechnology Division, Indian Institute of Integrative Medicine (CSIR), Jammu 180001, India*

Divya Arora − *Fermentation & Microbial Biotechnology Division, Indian Institute of Integrative Medicine (CSIR), Jammu 180001, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India*

Sharada Mallubhotla − *School of Biotechnology, Shri Mata Vaishno Devi University, Katra 182320, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c00141

Author Contributions

‖V.A. and M.K. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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