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Metabolic Profiling and Biological Activities of Bioactive Compounds Produced by *Pseudomonas* sp. Strain ICTB-745 Isolated from Ladakh, India

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In an ongoing survey of the bioactive potential of microorganisms from Ladakh, India, the culture medium of a bacterial strain of a new *Pseudomonas* sp., strain ICTB-745, isolated from an alkaline soil sample collected from Leh, Ladakh, India, was found to contain metabolites that exhibited broad-spectrum antimicrobial and biosurfactant activities. Bioactivity-guided purification resulted in the isolation of four bioactive compounds. Their chemical structures were elucidated by ^{1}H and ^{13}C NMR, 2D-NMR (HMBC, HSQC, ^{1}H,^{1}H-COSY, and DEPT-135), FT-IR, and mass spectroscopic methods, and were identified as 1-hydroxyphenazine, phenazine-1-carboxylic acid (PCA), rhamnolipid-1 (RL-1), and rhamnolipid-2 (RL-2). These metabolites exhibited various biological activities like antimicrobial and efficient cytotoxic potencies against different human tumor cell lines such as HeLa, HepG2, A549, and MDA MB 231. RL-1 and RL-2 exhibited a dose-dependent antifeedant activity against *Spodoptera litura*, producing about 82.06% and 73.66% antifeedant activity, whereas PCA showed a moderate antifeedant activity (63.67%) at 60 μg/cm^2 area of castor leaf. Furthermore, PCA, RL-1, and RL-2 exhibited about 65%, 52%, and 47% mortality, respectively, against *Rhyzopertha dominica* at 20 μg/ml. This is the first report of rhamnolipids as antifeedant metabolites against *Spodoptera litura* and as insecticidal metabolites against *Rhyzopertha dominica*. The metabolites from *Pseudomonas* sp. strain ICTB-745 have interesting potential for use as a biopesticide in pest control programs.

Keywords: Phenazine, rhamnolipids, Ladakh, bioactivity, antifeedant, insecticidal

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In nature, many bacteria exhibit harmonious growth in different hostile environments ranging from polar regions to deep seas, deserts, and acidic hot springs, which are generally categorized as extremophiles [28]. Microorganisms that survive in such environments have gained a renewed interest among researchers globally in view of their biotechnological importance [34]. In the recent past, the isolation of microorganisms has shifted towards novel unexplored niches and/or extreme environments such as rain forests [42], marine sponges [20], and mangroves [27], giving a clue that a careful exploration of such new habitats may result in identifying novel bioactive compounds. Ladakh is a cold desert located in the Western Himalayas of the Indian subcontinent with an altitude of more than 12,000 m above sea level and ambient temperatures (+25 to −25°C). The survival of microorganisms in this extreme niche requires a special adaptation and hardiness against various stress factors; namely, substrate limitation, low temperatures round the year, and short-time intensive heating during the summer with UV irradiation [22]. There are very few reports on the biodiversity of this extreme environment [22, 26]; moreover, none of them describe studies on bioactive properties. This fact has stimulated and reinforced the importance of carrying out a systematic survey for bioactive metabolites from different microflora isolated from Ladakh, India.

Globally, about 10–30% of the produced grains are damaged every year owing to insect pests [30], which persist in a wide variety of agricultural, arboreal, and urban environments. Control and eradication of these pest problems are addressed through repeated application of synthetic insecticides [17], which in turn has led to the development of pest resistance through disruption of biological systems [35]. In the recent years, there has been a growing interest in the potential application of microbial metabolites as agrochemicals. These metabolites are expected to overcome the problems associated with insect pest resistance and are generally biodegradable and environment-friendly as compared with their synthetic counterparts [44]. The natural ability of certain bacteria and fungi to produce metabolites that may be toxic against insect pests prompted us to explore their bioactivity against insect pests. This activity is normally mediated *via* metabolites secreted in the environment [52]. These metabolites can affect several traits of insect biology, such as toxicity, mortality, feeding activity, suppression of reproductive behavior, and reduction of fecundity and fertility [44, 47]. From the viewpoint of new pest control strategies with low environmental impact, the search for microbial metabolites active against insect pests is promising. Very few reports exist on microbial metabolites exhibiting antifeedant and insecticidal activities [14, 35, 46]. The present study constitutes the metabolic profiling of surface-active bioactive metabolites from a *Pseudomonas* sp., strain ICTB-745, and the assessment of their antimicrobial, cytotoxicity, antifeedant, and insecticidal activities.

Materials and Methods

Bacterial Strain and Fermentation Conditions

The strain ICTB-745 was isolated from an alkaline soil sample collected at Leh, Ladakh, India. The pure culture is maintained in the culture collection of the laboratory with the accession number ICTB-745 and was identified as *Pseudomonas* sp. (GenBank Accession No. HQ916866). The isolate ICTB-745 was cultured aerobically in potato dextrose medium of pH 5.2 ± 0.2 at 30°C with agitation at 200 rpm for 4 days in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA). The fermented medium (5 l) was subjected to centrifugation (Sorvall RC 5C Plus; Kendro Lab Products, Ashville, NC, USA) at 8,000 rpm to recover the cell-free supernatant.

Extraction, Analysis, and Purification of Bioactive Metabolites

Bioactive compounds were extracted from the cell-free supernatant by absorption onto Diaion HP-20 (Supelco, Bellafonte, PA, USA) resin. The resin was washed with methanol yielding crude extracts. Analysis of the crude extract by thin-layer chromatography (TLC) on silica gel 60 plates (F_{254}; Merck, Darmstadt, Germany) revealed four spots. These extracts were concentrated and dried under reduced pressure on a rotary vacuum evaporator (Rotavapor R-205; Büchi, Switzerland) and further profiled on a silica gel (100–200 mesh) column (3 × 60 cm) with an ethyl acetate–hexane solvent system as a mobile phase. Spot 1 was eluted in an ethyl acetate–hexane mixture [15:85 (v/v)]. The same solvent mixture was continued until compound 1 was completely eluted and after drying gave a yellow solid. Spot 2 was eluted in an ethyl acetate–hexane mixture [30:70 (v/v)], and drying of the fractions resulted in compound 2 as yellow needles. The solvent system was further increased in a step-wise fashion to elute compounds 3 and 4 in ethyl acetate–hexane (40:60) and (50:50) solvent systems, which after concentration were bluish green and wine red colored oily liquids, respectively.

Physical Properties

Surface tension of the culture supernatant was measured by the Wilhelmy plate method using the K100MK2 Processor Tensiometer (Krüss, Hamburg, Germany). A computer-controlled Dosimat (Metrohm AG, Switzerland) automatically generated a series of concentrations. The corresponding measurements and their evaluation were performed with LabDesk software interfaced with the tensiometer. Critical micelle concentration (CMC) measurement using the CMC add-in feature of LabDesk software was done by plotting the concentration of the surfactant as a function of surface tension, and CMC was taken as the point where the slope of the curve abruptly changed [40]. The emulsification index (EI_{24}) was determined by adding 4 ml of culture supernatant to 6 ml of different compounds, such as *N*-hexadecane, tridecane, xylene, hexane, toluene, mineral oil, olive oil, and castor oil individually; the mixtures were vortexed at high speed for 2 min and then allowed to settle for 24 h before emulsion stability was measured [10]. The EI_{24} was estimated after 24 h as the height of the emulsified layer expressed as a percentage of the total height of the liquid column. The quantification of rhamnolipids in the supernatant was estimated by the orcinol method at 420 nm using rhamnose as the standard [8].

Structural Characterization

Spectroscopic studies were carried out for elucidating the structure of purified compounds. The UV spectra were measured by dissolving the samples in spectroscopic acetonitrile and recorded at 30°C on a UV–visible double-beam spectrophotometer (Lambda 25; Perkin-Elmer, Shelton, CT, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer (Bruker, Switzerland) in $CDCl_3$ at room temperature, and chemical shifts were represented in δ values expressed in ppm with tetramethylsilane as the internal standard. The Fourier transform infrared (FT-IR) spectrum was recorded using the Thermo-Nicolet Nexus 670 FT-IR spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI, USA) and spectra were collected at a resolution of 4 cm^{-1} in the wavenumber region of 400–4,000 cm^{-1}. The high-resolution mass spectra (HR-MS) were recorded on a QSTAR XL Hybrid ESI-Q TOF mass spectrometer (Applied Biosystems Inc., Foster City, CA, USA).

Antimicrobial Activity and *In Vitro* Cytotoxicity Testing

Antimicrobial activity of all the four compounds was determined using the microtiter broth dilution method according to the method described previously [25]. Cell lines used for *in vitro* cytotoxicity testing included MDA MB 231, HeLa, A549, and HepG2, obtained from the American Type Culture Collection, Manassas, VA, USA. Cytotoxicity assays were performed using the method previously described [48]. IC_{50} values (in μM) are expressed as the average of three independent experiments.

Antifeedant Bioassay

Two major agricultural pests, the castor semi-looper *Achaea janata* and the tobacco cutworm *Spodoptera litura* (Fab), were reared on fresh castor leaves (*Ricinus communis* L.) grown in the laboratory at 28 ± 2°C, 65 ± 5% relative humidity (RH), and a 16:8 (light:dark) h photoperiod. Antifeedant activities of the purified metabolites, except 1-hydroxyphenazine, profiled from *Pseudomonas* sp. strain ICTB-745 were tested against the third instar larvae of *A. janata* and *S. litura* by using the classical no choice leaf disc method. Stock solutions were prepared in methanol and a series of concentrations for each compound were prepared. Late second instar larvae of insect cultures were fed on castor leaves overnight. Newly molted third instar larvae were selected and starved for 3 h. Castor leaves were washed with distilled water, and leaf discs (10.6 cm^2 area) were cut after drying and placed in clean glass petri dishes (15 cm diameter) containing moist filter paper discs, which provided humidity inside and aided in retaining the freshness of the leaf discs. Different doses (10, 25, 50, 75, and 100 µg/cm^2 of leaf) of the purified metabolites were sprayed uniformly on both sides of the leaf discs and were air-dried at room temperature. Control leaf discs were treated with the same quantity of solvent only. In each petri dish, a pre-starved healthy third instar larva of *A. janata* or *S. litura* was introduced to assess antifeedant activity. The experimental containers were kept in an illuminated growth chamber at 28 ± 2°C. The food consumption by each larva was recorded by measuring the leaf area consumed after 24 h of both control and treated leaf discs using an AM-300 portable leaf area meter (ADC BioScientific Ltd., Herts, England). The antifeedant index was calculated from the formula: AFI (%) = (C – T) / (C + T) × 100, where C is the leaf area consumption of control discs and T is the consumption of treated discs [4]. For all the treatments, there were 30 replicates, and all the treatments and control were replicated three times.

Contact Toxicity Bioassay

Cultures of three stored-product pest insects, namely, the rice weevil *Sitophilus oryzae* L. (Coleoptera: Curculionidae), the lesser grain borer *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae), and the red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), were maintained in our laboratory for over 8 years without exposure to insecticides. *S. oryzae* was reared on sterilized whole wheat (*Triticum aestivum* L.), *R. dominica* on sterilized green gram (*Phaseolus mungo* L.), and *T. castaneum* on broken rice (*Oryza sativa* L.) grains. The cultures were maintained in an illuminated growth chamber set at 28 ± 1°C and 65 ± 5% RH and photo regime of 16:8 (light:dark) h. Adult insects, 1–7 days old, were used for fumigant toxicity tests. All experiments were conducted in the laboratory with colonies under the same environmental conditions.

The insecticidal properties of purified metabolites, except 1-hydroxyphenazine, profiled from *Pseudomonas* sp. strain ICTB-745 were evaluated against adults of three stored-product insects by fumigation assay [45]. Each of the purified metabolite in methanol (20 µg/ml) was applied to filter papers (Whatman No. 1; 2–3 cm diameter). The solvent was allowed to evaporate for 1 h prior to introduction to insects. Each filter paper (dried) was placed at the bottom of a petri dish (5.0 cm diameter), and 10 adults (7–10 days old) each of *S. oryzae*, *R. dominica*, and *T. castaneum* were placed in each petri dish and covered with a lid. The inner side of the lid was coated with vaseline to prevent the insects staying on the lid. Control insects received 100 µl of methanol alone. Tests were carried out at 28 ± 2°C and 65 ± 5% RH. Mortality was ensured by probing the insect body with a slender paintbrush. Dead insects were counted every 24 h for a total period of 72 h post-treatment. Five replicates were maintained per treatment, and tests were repeated 3 times on different dates each time, to avoid any day-to-day variation. Percentage mortality was calculated using the corrected formula of Abbott [2] for natural mortality in untreated controls.

RESULTS AND DISCUSSION

Metabolite Production and Characterization

The isolate *Pseudomonas* sp. strain ICTB-745 cultivated under submerged fermentation conditions produced about 1.7 g/l of biosurfactant after 96 h of incubation. The surface tension of the culture medium decreased to 27.933 mN/m after 96 h of incubation. Furthermore, the critical micelle concentration (CMC) of the crude biosurfactant was estimated as 40.830 mg/l, when the surface tension was 27.933 mN/m, and revealed excellent surface-active properties, which is in agreement with published reports on biosurfactants [1, 29]. The crude biosurfactant exhibited good emulsification activity (EI_{24} value), ranging between 50% and 60% with all hydrocarbons and oils studied (data not shown), and formed stable emulsions in the culture supernatant.

Purification of the Metabolites

The culture filtrate of strain ICTB-745 revealed the presence of four major spots in TLC. Compounds 1 and 2 were developed in a solvent mixture of ethyl acetate–hexane (70:30). Compound 1 was UV-active and visualized under UV light at 254 nm, whereas compound 2 was developed by spraying with anisaldehyde reagent followed by heating at 100°C for 2–3 min, and appeared as a yellowish-green colour spot. Compounds 3 and 4 were developed in a solvent mixture of ethyl acetate–hexane (90:10) and were developed by spraying with a phosphomolybdic acid reagent followed by heating at 100°C for 2–3 min; they appeared as green cone-shaped spots. All the four compounds were purified from the crude mixture sequentially in a single step by silica gel column chromatography. The four spots were identified as two phenazine pigments (*viz*., 1-hydroxyphenazine and phenazine-1-carboxylic acid), and two biosurfactants (rhamnolipid-1 and rhamnolipid-2). A significant difference in the concentrations of the secreted metabolites by strain ICTB-745 was observed, which could be attributable to the changes in the environmental variables like stress, nutrient availability, and the relationships with competing microflora that can cause modifications in the normal biosynthesis routes [51].

Structural Characterization of the Purified Metabolites

The chemical structure of the purified metabolites was elucidated based on the NMR, FT-IR, and mass spectral

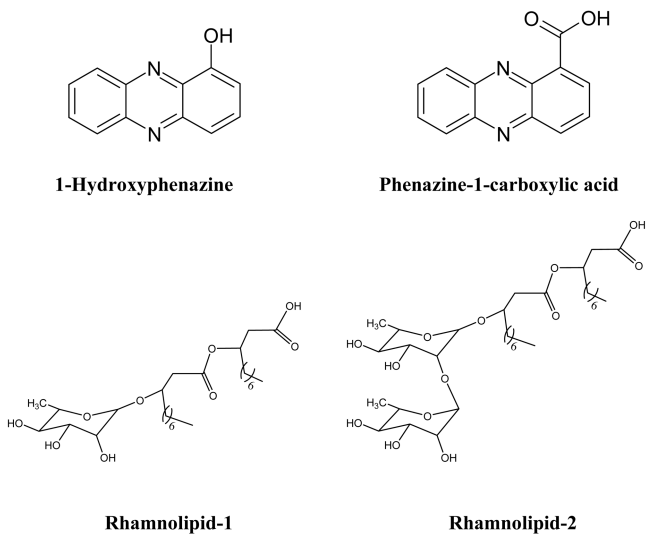

Fig. 1. Chemical structures of the identified metabolites profiled from *Pseudomonas* sp. strain ICTB-745.

data analyses. Spot 1 was isolated as a yellow solid (25 mg/l) with a UV λ_{max} (log ε) of 260 (2.86) and a molecular weight of 197 based on the positive mode of the ESI-MS. The molecular formula of $C_{12}H_9N_2O$ was deduced based on HR-ESI-MS data (Fig. S1), which showed a molecular ion peak at *m/z* of 197.0718 $[M + H]^+$ [calcd. for $C_{12}H_9N_2O$, 197.0714, $M + H]^+$. The FT-IR spectrum of spot 1 (Fig. S2A) showed $\nu = 3{,}443\ cm^{-1}$ as a broad stretching band, indicating the presence of a phenolic –OH group, and $\nu = 1{,}520\ cm^{-1}$ represented the C1 carbon (C-O) stretching. The ^1H NMR of spot 1 (Fig. S3A) showed a chemical shift at δ = 8.06–8.14 ppm corresponding to the phenolic hydroxyl proton attached to the phenazine moiety and all signals of the remaining aromatic protons were attached to the phenazine ring and attributable to δ = 7.17–7.22 (m, 1H -ArH), 7.73–7.77 (t, 2H, *J* = 4.91 -ArH), 7.88–7.87 (t, 2H *J* = 4.15 -ArH), and 8.18–8.29 (m, 2H, -ArH) ppm. The ^{13}C NMR ($CDCl_3$) of this compound (Fig. S4A) showed that the C1 carbon at δ = 151.649 ppm, which is bonded to the OH group, and the other carbon signals corresponded to chemical shifts δ = 108.87, 119.86, 129.15, 129.59, 129.62, 130.46, 130.52, 130.76, and 131.83 ppm, which matched with the phenazine basic structure. Based on the spectral data, spot 1 was identified as 1-hydroxyphenazine.

The molecular weight of the yellow needles (200 mg/l) of compound 2 was 225 based on the positive mode of the ESI-MS, and the molecular formula $C_{13}H_9N_2O_2$ was deduced on the basis of HR-ESI-MS (Fig. S5), which showed a molecular ion peak at *m/z* 225.0667 $[M + H]^+$ [calcd for $C_{13}H_9N_2O_2$, 225.3196, $M + H]^+$. The compound 2 had a UV λ_{max} (log ε) of 254 (2.93). The FT-IR spectrum of compound 2 (Fig. S2B) showed a broad band at $\nu =$ 3,406 cm^{-1}, which is attributed to the hydroxyl group, while the peak at 1,737 cm^{-1} is characteristic of the carboxylic C=O group. The peaks at 1,597, 1,561, 1,522, 1,467, and 1,422 cm^{-1} indicate -C=C- bonds. The chemical structure was further precisely elucidated by ^1H and ^{13}C NMR spectroscopy. The ^1H NMR spectrum of spot 2 (Fig. S3B) indicated an ABCD pattern in the aromatic ring, which is a typical feature observed among phenazine derivatives [37], while the ^{13}C NMR spectrum (Fig. S4B) in $CDCl_3$ displayed 13 well-resolved signals, suggesting a symmetrical structure for the molecule. The ^1H NMR spectrum exhibited multiplet peaks at the position of δ = 8.0–9.0, representing a heteroaromatic ring structure having nitrogen. Furthermore, the peaks at δ = 7.92–8.06 and 15.10–16.05 suggest the presence of seven methine groups and a carboxylic acid group, respectively. The resonance at δ = 8.92–9.02 corresponds to the proton at the position 4 of the basic phenazine ring, while the next peak at δ = 8.51–8.54 analogously represents the proton at position 2. The protons at position 3 resonated in the same region as those at positions 7 and 8 (δ = 8.31–8.35). Furthermore, the resonance at δ = 7.92–8.06 corresponds to the remaining two protons at positions 6 and 9 of the basic phenazine ring. The ^{13}C NMR spectrum showed peaks at δ 165.70 ppm and 128.26–143.27 ppm, suggesting the presence of a carbonyl group and aromatic rings, respectively. The peaks at δ 139.55–143.27 ppm represent the carbons at positions 4a, 5a, 9a, and 10a, while the resonances at δ 129.34–135.07 ppm correspond to the carbons at positions 2, 3, 4, 6, 7, 8, and 9 of the basic phenazine ring. The resonance at δ 128.26 ppm corresponds to carbon at position 1. Additionally, the specific assignment of the carboxylic group carbon attachment at position 1 was determined based on the 2D-NMR spectroscopies based on HMBC, HSQC, and DEPT-135 (Fig. S6–S8). The counts of proton and carbon from ^1H and ^{13}C NMR spectra revealed the presence of 8 protons and 13 carbons, which supported the molecular formula as deduced by HR-ESI-MS. The ^1H and ^{13}C NMR and 2D-NMR spectral data of spot 2 and their specific assignments correlated with phenazine-1-carboxylic acid (PCA). Phenazines are heterocyclic nitrogen-containing pigments produced naturally by microbes of different genera, including *Pseudomonas*, *Streptomyces*, *Nocardia*, and *Brevibacterium*, surviving in soil or marine habitats. The biosynthetic pathway of phenazine revealed that PCA is a precursor of 1-hydroxyphenazine, which is derived from shikimic acid. These compounds are reported to exhibit a broad spectrum of biological activities such as antibacterial, antimalarial, antitumor, and antiparasitic activities [9].

Spot 3 was obtained as an oily liquid (800 mg/l) with a UV λ_{max} (log ε) of 250 (3.21) and was eluted in an ethyl acetate–hexane (40:60) solvent system. The molecular weight of the oily compound was 527, based on the positive mode

of ESI-MS, and the molecular formula of $C_{26}H_{48}O_9Na$ was deduced based on HR-ESI-MS (Fig. S9), which showed a molecular ion peak at *m/z* 527.3186 $[M + Na]^+$ [calcd. for $C_{26}H_{48}O_9Na$, 527.3196, $M + Na]^+$. The FT-IR spectrum (Fig. S11A) of this spot showed that $\nu = 3{,}399$ cm^{-1} (broad –OH) and 1,737 cm^{-1} (C=O), indicating the presence of a carboxylic acid group. The wavenumbers $\nu = 2{,}928$ and 2,857 cm^{-1} (C-H) showed the presence of an aliphatic hydrocarbon chain. The ^1H NMR spectrum (Fig. S12A) of this spot in $CDCl_3$ showed the presence of a carbohydrate ring attached with the aliphatic chain. The signals observed at $\delta = 0.82–0.91$ (t, 6H $-CH_3$, $J = 6.50$) ppm corresponded to the methyl group attached to the aliphatic chain, $\delta = 1.17–1.26$ (m, 23H $-CH_2$), 1.44–1.65 (m, 4H $-CH_2$) ppm represented the methyline group in the same aliphatic chain and the methyl group of the carbohydrate ring, $\delta =$ 3.37–3.42 (t, 1H –CH $J = 9.10$), 3.47–3.48 (s, 2H –CH), 3.65–3.72 (m, 2H –CH), 3.79–3.91 (s, 1H –CH), 4.26–4.32(brs, 2H –OH), and 4.36–4.73 (brs, 1H –OH) ppm signals represented the hydroxyl group protons, and 4.84–4.92 (s, 1H –CH), 5.38–5.51 (m, 1H –CH) ppm were the protons attached to both the carbohydrate ring and aliphatic moiety, and $\delta = 2.36–2.46$ (d, 2H $J = 9.06$ $-CH_2$) and 2.49–2.60 (d, 2H $J = 9.18$ $-CH_2$) ppm is the methyline proton attached to carboxylic acid. ^{13}C NMR (Fig. S13A) in $CDCl_3$ having the signals at $\delta = 17.28$ ($-CH_3$) and $\delta = 67.91$, 70.45, 71.05, 71.32, and 73.45 (–CH) ppm were attributable to the methyl and ring carbons of the sugar moiety. The signals at $\delta = 95.37$, 96.16 ppm were the (–CH) carbons attached to the electron withdrawing group (–C=O). The presence of an aliphatic chain and a carbohydrate ring with the spectral data correlated to rhamnolipids and spot 3 was identified as rhamnolipid-1 (designated as RL-1).

Spot 4 was isolated as an oily liquid (400 mg/l) with a UV λ_{max} (log ε) of 260 (3.24) and was eluted in an ethyl acetate–hexane (50:50) solvent system. The FT-IR spectrum (Fig. S11B) of this spot showed that $\nu = 3{,}381$ cm^{-1} (broad –OH group) and $\nu = 1{,}733$ cm^{-1} (C=O), indicating the presence of a carboxylic acid group. The wavenumbers $\nu = 2{,}928$ and 2,857 cm^{-1} (C-H) indicated the presence of an aliphatic hydrocarbon chain. The molecular weight of the oily compound was 673, based on the positive mode of ESI-MS, and the molecular formula $C_{32}H_{58}O_{13}Na$ was deduced based on HR-ESI-MS (Fig. S10), which showed a molecular ion peak at *m/z* 673.3794 $[M + Na]^+$ [calcd. for $C_{32}H_{58}O_{13}Na$, 673.3775, $M + Na]^+$. The ^1H NMR spectrum (Fig. S12B) of this spot in $CDCl_3$ suggested that a carbohydrate ring was attached with the aliphatic chain. The signals observed at $\delta = 0.77–0.97$ (t, 6H, $J = 6.61$ $-CH_3$) ppm corresponded to the methyl group of the aliphatic chain, and $\delta = 0.99–1.415$ (m, 28H $-CH_2$) and 1.415–1.676 (m, 2H $-CH_2$) ppm corresponded to the methyline group of the aliphatic chain and methyl group of the sugar moiety. The signals with $\delta = 3.17–3.52$ (d 1H $J = 6.61$ $-CH_2$), 3.60–3.93 (m, 4H –CH), 4.00–4.32 (, 2H $J = 6.42$ –CH), and 4.57–4.99(m, 2H –CH) ppm represented the protons attached to both the carbohydrate ring and aliphatic moiety, $\delta = 5.53–6.71$ (brs, 5H –OH) ppm represented the hydroxyl group protons, and $\delta = 2.18–2.63$ (m, 4H –CH) ppm corresponded to the carbon protons bonded to carbonyl group. The ^{13}C NMR spectrum (Fig. S13B) in $CDCl_3$ having the signals at $\delta = 67.96$, 68.85, 70.60, 71.16, 72.59, 73.47, 77.19, 79.81, and 102.46 ppm represented the –CH groups present in the sugar moiety. Signals at $\delta = 94.55$ and 96.15 ppm were the (–CH) carbons attached to the electron withdrawing group (–C=O). The NMR spectra matched with the rhamnolipids moiety. Based on the ^1H and ^{13}C NMR and FT-IR data, the spot-4 was identified as rhamnolipid-2 (designated as RL-2).

Moreover, 2D-NMR spectroscopies based on HMBC, HSQC, and ^1H,^1H-COSY were employed for complete assignments of the glycolipid signals of both RL-1 and RL-2 (see Fig. S14–S19). In the ^{13}C NMR spectrum, spot 3 fractions revealed lipid tail signals of CH_2 from $\delta =$ 22.65 – 34.57 ppm and CH_3 at $\delta = 14.13$ ppm and ester and carboxylic signals at $\delta = 171.35$ ppm and $\delta = 173.76$ ppm. Similarly, spot 4 fractions showed lipid signals in the ^{13}C NMR spectrum at $\delta = 14.12$ and 17.53 ppm (CH_3) and resonance at $\delta = 22.63–34.60$ ppm corresponded to $-CH_2$ and ester and carboxylic signals at $\delta = 171.19$ ppm and $\delta = 173.50$ ppm (Fig. S13A and S13B). The ^1H,^1H-COSY spectra showed the presence of characteristic spin systems corresponding to the two α-rhamnose and two β-hydroxy fatty acid moieties. The interconnections and sequence was established from the cross-peaks within the multiple-bond ^{13}C-^1H correlation (HMBC) NMR spectrum, which was attributed to the interactions through three bonds *via* the bridging oxygen atoms. The HMBC spectrum also enabled the unambiguous assignment of the ^{13}C signals and a clear distinction between the two carbonyl carbons (Fig. S14 and S17). The HSQC NMR spectrum revealed the presence of one signal in RL-1 and two signals for RL-2 at $\delta = 5.0$ ppm, which shows the presence of ether linkages with the rhamnose sugar and the lipid chain (Fig. S15 and S18). The ^1H,^1H-COSY NMR spectrum showed the presence of only one rhamnose moiety in the case of RL-1, and the characteristic signal of H-2 of the RL-2 at 4.02 ppm was absent, while the characteristic signals of both fatty acid moieties were present (Fig. S16 and S19). In both RL-1 and RL-2, it was assumed that all sugar moieties had the normal absolute L-configuration. Integration of the 1D spectra provided the information on the length of the fatty acid chains in both types of molecules. The NOESY analysis (data not shown) showed important signals: the space connection was obtained by the ROESY cross-peak at $\delta = 4.673/4.071$ ppm, which confirmed the L-rhamnosyl (1'↔1)-hydroxy fatty acid linkage.

The HR-MS analysis under positive mode showed major molecular ion signals at *m/z* 527.3186 [M + Na^+] and 673.37 [M + Na^+], corresponding to the sodium adduct of spots 3 and 4, respectively (Fig. S9 and S10). These ion signals were consistent with the structures expected for 2-*O*-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid (Rha-C_{10}-C_{10}, a mono-rhamnolipid) and 2-*O*-α-L-rhamnosyl-α-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid (Rha-Rha-C_{10}-C_{10}, a di-rhamnolipid), respectively, where Rha indicates a rhamnose moiety. All these results support the observations established for another biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614 [32]. HR-MS spectra (Fig. S9 and S10) of both rhamnolipids also showed the presence of significant fragment ions. From the mass spectrum of RL-1, three significant fragment ions [*viz*., the sugar group (*m/z* = 163) and two fragments of aliphatic side chains (*m/z* = 171, 341)] were observed. In the mass spectrum of RL-2, four significant fragment ions [*viz*., two sugars together as a fragment (*m/z* = 310), the aliphatic moiety of RL-2 (*m/z* = 342), and the individual fragments (*m/z* = 170, 171)] were observed. Rhamnolipids are surface-active glycolipids produced by various *Pseudomonas* sp. and show potential application in bioremediation, oil recovery, cosmetics, and pharmaceutical sectors [3].

Antimicrobial Susceptibility Testing and Cytotoxicity

The results of the antimicrobial susceptibility tests of the purified metabolites profiled from *Pseudomonas* sp. strain ICTB-745 are shown in Table 1, which indicates that all the compounds possess significant antimicrobial activity against all the Gram-positive and Gram-negative bacteria, and *Candida albicans* tested. The MIC results (Table 1) indicated that the PCA exhibited a broad-spectrum antimicrobial activity. *Micrococcus luteus*, *Staphylococcus aureus, Klebsiella planticola*, and *Candida albicans* were the microorganisms that showed greater susceptibility and the estimated MIC value was 9.3 μg/ml. Based on the literature survey, PCA was first reported from a strain of *Pseudomonas aureofaciens* Kluyver [18] and was designated as tubermycin B, based on its antibiotic activity against *Mycobacterium tuberculosis* [21]. The biological role of a dimeric compound of PCA produced by *Pseudomonas fluorescens* 2–79 was reported to suppress the soilborne fungal pathogen *Gaumannomyces graminis* var. *tritici*, which is a causative agent for take-all, a wheat root disease, and enables the survival of the host in the rhizosphere [16]. PCA isolated from a marine *Pseudomonas* sp. also exhibited antimicrobial activity against nine bacterial strains that inhibited the settlement of barnacle larvae, reducing *Ulva lactuca* spore settlement and percent covering of germlings [33]. Moreover, phenazine compounds were reported to exhibit other biological activities including antifungal [23], enzyme-inhibitor [6], and as photosensitizers [11, 13]. Beyond these functional roles, Wang and Newman [49] have recently shown that phenazines also play an important physiological role in iron sequestration. Recently, PCA produced by *Pseudomonas* sp. M18G has been marketed as *Shenqinmycin* in China, which has gained a Pesticide Registration Certification (code LS20031381) as issued by the Chinese Ministry of Agriculture, owing to its high efficiency against various phytopathogens, low toxicity, and good environmental compatibility. It proved as an effective agent for the biocontrol of withering of watermelon sprout (*Fusarium oxysporum*) and pimiento epidemic disease (*Pythium capsici*) [19]. Similarly, rhamnolipids were reported to exhibit antifungal effects against several plant pathogens such as *Colletotrichum orbiculare*, *Magnoporthe grisea*, *Phytophthora capsici*, *Pythium aphanidermatum*, and *Rhizoctonia solani* [36] and also significantly inhibited the growth of harmful algal blooms [50]. Furthermore, the US

Table 1. Antimicrobial activity (MIC) of purified metabolites profiled from *Pseudomonas* sp. strain ICTB-745 against tested strains.

S. No.	Tested strains[a]	Minimum inhibitory concentration (MIC, μg/ml)[b]			
		1-Hydroxyphenazine	Phenazine-1-carboxylic acid	Rhamnolipid-1	Rhamnolipid-2
1	*Micrococcus luteus* MTCC 2470	18.7	9.3	37.5	75
2	*Staphylococcus aureus* MTCC 96	37.5	9.3	75	75
3	*Staphylococcus aureus* MLS-16 MTCC 2940	37.5	75	75	37.5
4	*Bacillus subtilis* MTCC 121	37.5	37.5	150	37.5
5	*Escherichia coli* MTCC 739	37.5	75	37.5	18.7
6	*Pseudomonas aeruginosa* MTCC 2453	37.5	37.5	75	37.5
7	*Klebsiella planticola* MTCC 530	37.5	9.3	75	75
8	*Candida albicans* MTCC 3017	75	9.3	150	150

[a]Microbial strains were procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.
[b]Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the compound that inhibited 90% of the growth when compared with that of the control growth. Tests were repeated three times and average values are shown.

Table 2. Cytotoxicity assay of metabolites profiled from *Pseudomonas* sp. strain ICTB-745 against various cancer cell lines.

Human cancer cell line type	ATCC No.	1-Hydroxyphenazine	Phenazine-1-carboxylic acid	Rhamnolipid-1	Rhamnolipid-2
		Cytotoxicity (IC_{50}, μM)[a]			
HepG2 (Human hepatocellular liver carcinoma cells)	HB-8065	–[b]	11.8	140	79
A549 (Human lung adenocarcinoma epithelial cells)	CCL-185	–	23	154	98
MDA MB 231 (Human breast adenocarcinoma cell line)	HTB-26	–	11.3	86	58
HeLa (Human cervical cancer cells)	CCL-2	–	8.2	123	88

[a]IC_{50} = Half maximal (50%) inhibitory concentration (IC) of antibiotic that is required for 50% inhibition of the target cell lines. Tests were repeated three times and average values are shown.
[b]No activity.

Environmental Protection Agency (EPA) on March 31, 2004 issued a rule regarding the establishment of an exemption from the requirement of a tolerance for rhamnolipid residues on all food commodities when applied or used as a fungicide [12].

Since no published cytotoxicity data on PCA and rhamnolipids could be sourced, the *in vitro* antitumor activity of both rhamnolipids and PCA were evaluated against a panel of human tumor cell lines in culture. Based on the cytotoxicity results (Table 2), the treatment of the compounds for 48 h against all the target human tumor cell lines resulted in dose-dependent inhibition of the cell growth, except for 1-hydroxyphenazine. The IC_{50} value represents the drug concentration (μM) required to inhibit 50% of cell growth. The compound PCA exhibited high cytotoxic potency as compared with rhamnolipids, which were moderately cytotoxic against all the cell lines tested. 1-Hydroxyphenazine showed no inhibitory activity against any of the tested cell lines. However, one hexa-substituted phenazine PD 116,152, which resembled lomofungin produced by a soil isolate, *Streptomyces lomondensis*, exhibited cytotoxicity against L1210 (lymphocytic leukemia) and HTC-8 (human colon adenocarcinoma) cell lines with IC_{50} values of 0.52 and 0.71 μg/ml, respectively, and a moderate antitumor activity was observed against P388 (lymphocytic leukemia) cell lines [41].

Antifeedant Activity

The antifeedant activities of the purified metabolites, except 1-hydroxyphenazine, profiled from strain ICTB-745 were tested against the third instar larvae of *S. litura* and *A. janata*. Based on antifeedant bioassay results, it is obvious that although both *A. janata* and *S. litura* are leaf tissue chewing insects, the larval responses towards the tested compounds differed. The exposure of larvae to treated leaf discs also influenced the pupal weight. Weights of the pupae of both the insects in treated discs are shown in Table 3. PCA exhibited high antifeedant activity, causing less food consumption of the test larva, which resulted in the decrease in pupal weight, and the pupal weight was least when compared with other compounds. The purified compounds exhibited significant antifeedant activity against *S. litura* (Table 4). RL-1 showed the highest antifeedant activity (82.06%) at a dosage of 60 μg/cm^2 area of castor leaf, whereas PCA was found to be less active (63.15%) among the compounds tested. The results suggests that the food consumption by *S. litura* larvae decreased after treatment with RL-1, and increasing the concentration of the

Table 3. Effects of the purified metabolites profiled from *Pseudomonas* sp. strain ICTB-745 on insect morphology.

Compounds	*Spodoptera litura*		*Achaea janata*	
	Pupal weight (μg)	Weight (μg/larvae)	Pupal weight (μg)	Weight (μg/larvae)
Crude supernatant	321.2 ± 8.163	5	435.8 ± 9.254	3
Phenazine-1-carboxylic acid	–[a]	2	400.0 ± 7.071	1.5
Rhamnolipid-1	299.6 ± 6.337	2.5	419.4 ± 5.325	2
Rhamnolipid-2	316.8 ± 9.367	2.9	409.0 ± 7.218	1
Control	350.0 ± 3.162	0	454.0 ± 5.099	0

Values are the mean ± SE.
[a]Insects were dead.

Table 4. Antifeedant index and larval weight gained by *Spodoptera litura* treated with purified metabolites profiled from *Pseudomonas* sp. strain ICTB-745.

Compounds	Property	Dosage (μg/cm^2)				
		10	20	30	50	60
Phenazine-1-carboxylic acid	Antifeedant index (%)	5.726 ± 0.183	1.980 ± 0.116	1.622 ± 0.043	21.686 ± 0.416	63.148 ± 0.079
	Larval weight gained (g)	0.070 ± 0.005	0.0614 ± 0.003	0.0168 ± 0.001	0.0434 ± 0.003	0.0186 ± 0.002
Rhamnolipid-1	Antifeedant index (%)	0.00 ± 0.0	13.220 ± 0.405	1.704 ± 0.044	81.084 ± 0.048	82.056 ± 0.697
	Larval weight gained (g)	0.134 ± 0.002	0.0476 ± 0.004	0.0336 ± 0.003	0.0366 ± 0.004	–[a]
Rhamnolipid-2	Antifeedant index (%)	5.040 ± 0.058	12.624 ± 0.356	25.192 ± 0.209	46.776 ± 0.201	73.552 ± 0.398
	Larval weight gained (g)	0.133 ± 0.002	0.0908 ± 0.001	0.0594 ± 0.005	0.0404 ± 0.002	–[a]

Values are the mean ± SE.
[a]Insects were dead.

extract resulted in increased reduction in food consumption. Most of the antifeedant compounds either completely prohibit the insect from feeding (deterrents) or allow the insect to initiate feeding but suppress further feeding activity (toxicants). The compounds that prevent insect feeding affect the peripheral nervous system and the suppression of further feeding owing to cellular, biochemical, and physiological processes [31]. RL-1 at a dosage of 50 μg/cm^2 per leaf disc seemed to possess both deterrent and toxicant properties. The rejection of the treated diet was evident at a higher dose (60 μg/cm^2), and it could be due to instantaneous suppression or rapid post ingestive feedback [5].

The antifeedant activity is significant, as it reduces the pest damage to crops by the deterrence in feeding without killing the pest. In the long run, insect populations are reduced through disrupted metamorphosis [39] and starvation deaths. The antifeedant activity can therefore be incorporated into other insect control methods in the integrated pest management (IPM) strategies. It was observed that these compounds interfered with the normal development and metamorphosis of *S. litura* and *A. janata*, which was manifested at different stages of the life cycle. In the case of RL-2, pupae of the exposed larvae were completely melanized, suggesting a neuro-endocrine involvement in this process. Antifeedant activity is also often associated with other biological effects against insects, such as larval growth inhibition, toxicity, and the adverse effects on metamorphosis [52]. The majority of the pupal instars died after pupation, indicating the potency of the tested bioactive compounds that persisted for a long time in the insect body and did not get metabolized fully during the larval stage or larval pupal molt. These and other effects may amplify the total biological activity of the compounds. The persistence duration of the antifeedant activity is also important. In earlier reports, it was observed that several plant constituents exhibited phagodeterrency or antifeedant activity against the agricultural crop pests [7]. Moreover, this is the first report of rhamnolipids effective as insect antifeedant against Lepidopteran pests. *S. litura* is a generalist polyphagous insect pest of worldwide importance, as it attacks more than 112 species of cultivated crops, causes severe losses to cotton, vegetables, and ornamentals, and is present in large numbers in tropical countries, even during rainy seasons [43]. Thus, the present study may acquire a special emphasis, as generalist herbivores are considered as less sensitive to deterrents than specialists [5, 38].

Insecticidal Activity

Spodoptera oryzae (rice weevil), *Rhyzopertha dominica* (lesser grain borer), and *Tribolium castaneum* (red flour

Table 5. Insecticidal activity of purified metabolites profiled from *Pseudomonas* sp. strain ICTB-745 against adults of *Sitophilus oryzae*, *Rhyzopertha dominica*, and *Tribolium castaneum* in filter paper bioassays.

Compounds	Dosage (mg/cm^2)	Mortality (%) mean ± SE		
		Sitophilus oryzae	*Rhyzopertha dominica*	*Tribolium castaneum*
Phenazine-1-carboxylic acid	0.1	–[a]	65 ± 1.26	–
Rhamnolipid-1	0.1	–	52 ± 1.01	–
Rhamnolipid-2	0.1	–	47 ± 0.82	–
Control	0.1	–	–	–

Values are the mean ± SE.
[a]No mortality was observed.

beetle) were selected in the present study, since they are widespread and very destructive primary pest insects of a variety of stored grains as well as stored products [15]. All the purified secondary metabolites, except 1-hydroxyphenazine, profiled from strain ICTB-745 were tested on adults of *S. oryzae*, *R. dominica*, and *T. castaneum* for their insecticidal activities. As shown in Table 5, the test insects showed high resistance to all compounds, except for the insect *R. dominica*, which showed susceptibility towards the compounds tested in the vapor-phase toxicity assays. It was observed that PCA, RL-1, and RL-2 were highly effective against *R. dominica*, showing 65%, 52%, and 47% mortality at 20 µg/ml after 72 h of treatment, respectively. PCA is a nitrogen-containing heterocyclic phenazine molecule having an active carboxylic acid functional group [9], whereas rhamnolipids are glycolipidic surface-active biomolecules constituting of both lipophilic and hydrophilic moieties [3]. We therefore hypothesize that the epicuticular waxes may assist in the uptake of these biomolecules through the host cuticle, or the compounds might be absorbed through the intersegmental membranes as the insect crawls over the contact surfaces. The major advantage of these microbial metabolites tested in the present study is their high toxicity towards *R. dominica*, which is a desirable characteristic for insecticidal preparations in the control of stored-product insect pests. Very recently, rhamnolipids produced by *Pseudomonas* sp. EP-3 have been reported to exhibit insecticidal activity against green peach aphid, *Myzus persicae* [24].

In conclusion, the secondary metabolites produced by *Pseudomonas* sp. strain ICTB-745 exhibited good antimicrobial and cytotoxicity activities in addition to significant antifeedant and insecticidal activities. We report for the first time the role of rhamnolipids as antifeedant metabolites against *Spodoptera litura* and as insecticidal metabolites against the stored-product pest *Rhyzopertha dominica.* It appears that the rhamnolipids possess more than one property against the insect species tested, denoting their efficiency as pest control agents. Being antifeedant to lepidopteran larvae and toxicant to stored-product insect pest, the bacterial metabolites are promising products for the insect pest management. Further studies are required to characterize the exact mode of action of the rhamnolipids against the insects *S. litura* and *R. dominica*. A field assessment of the insecticidal and antifeedant effects of the rhamnolipids may find their potential for the development of a natural plant protection agent that is environmentally safe and ecologically acceptable.

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