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Phytochemical analysis and evaluation of inhibitory potential of *Ricinus communis* leaf extract against novel *Bacillus* pathogens

Diptimayee Acharya¹, Puspanjali Parida¹, Himansu Sekhara Mohapatra², Subhasmita Mallik³, Jatindra Nath Mohanty^{3*} and Santi Lata Sahoo⁴

Abstract

Mastitis is a common and complex disease, mainly caused by aureus and non-aureus species, and *Enterobacteriaceae*. Recently, a new and serious group of Gram-positive bacteria, the sporulating bacilli, has emerged as a mastitis pathogen. The study aimed to identify and characterize novel *Bacillus* strains (HSM85, SLS01, and DA03) associated with bovine mastitis and to evaluate the antimicrobial efficacy of *Ricinus communis* (castor) leaf extract against these pathogens. In methodology, *Bacillus* strains were isolated from milk samples of mastitis-infected cows, and the antimicrobial activity of different solvent-based leaf extract fractions was assessed using MIC, MBC, killing and biofilm inhibition assays, and GC–MS analysis to identify bioactive compounds. In result we found, the chloroform (non-polar) fraction showed the highest antimicrobial activity, followed by the ethanol (polar) fraction. MIC and MBC values ranged from 25 to 200 and 25–400 µg/ml, respectively. Bacterial growth was significantly inhibited at ½ MIC, while control cells entered log phase within 4 h. Assays (MIC, MBC, MBC/MIC ≤ 4, killing, biofilm inhibition, and nucleic acid leakage) confirmed the bactericidal potential of *Ricinus communis* leaf extract against novel *Bacillus* strains. GC–MS analysis revealed 48–53 peaks across solvent extracts, identifying dominant compounds like phytol, phytol palmitate, and tetracontane, with some solvent-specific presence. *Ricinus communis* leaf extract showed strong bactericidal activity against emerging *Bacillus* strains causing bovine mastitis, with the chloroform fraction showing the highest efficacy. Its bioactive compounds and multi-target properties suggest its potential as a natural alternative for managing mastitis in dairy cattle.

Keywords *Bacillus*, Mastitis, *Ricinus communis*, Leaf extract, Antibacterial, GC–MS analysis

1 Introduction

Bovine mastitis is an inflammatory disease of the mammary gland with multiple causes. It affects dairy herds worldwide, causing damage to udder tissue and altering the physical, chemical, and biological properties

of milk. This disease leads to global economic losses exceeding 40 billion USD each year [1, 2]. The global losses in dairy industries due to mastitis are estimated to be greater than 40 billion USD [2, 3]. India faces a loss of 71,655.1 million INR due to this disease per annum [4]. The disease is broadly divided into two types on the basis of the development of symptoms: clinical and subclinical mastitis, respectively. Clinical mastitis can be easily detected by visible abnormalities like a swollen udder, fever, etc., in dairy cows, and the milk of the affected cows usually becomes watery with visible flakes and clots, whereas, in subclinical mastitis, milk production is reduced without any visible mark [5]. The

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economic cost imposed by mastitis on dairy producers can be divided into two types: direct and indirect costs [6, 7]. The direct cost is apparent in clinical mastitis; it comprises veterinary services, diagnostics, treatment, additional labor, and the disposal of milk in the course of treatment. However, financial loss due to subclinical mastitis generally goes unnoticed by the farmers, but includes a decline in milk production, premature culling, and reduced quality premiums [1]. The etiological studies showed that mastitis is predominantly caused by bacteria encompassing *Staphylococcus aureus*, non-aureus Staphylococci (*S. chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis*), *Streptococcus* spp., Enterobacteriales, and by some non-bacterial pathogens like *Mycoplasma bovis*, *Candida* spp., and *Prototheca zoofari* [8–11]. Besides *Staphylococcus aureus*, non-aureus Staphylococci (NAS) and Enterobacteriales, members of the genus Bacilli have gained increasing attention owing to their spore and toxin production ability. Earlier studies reported that the incidence of Bacillus species causing mastitis is rare in dairy cows [12]. However, their prevalence as the causal agents of mastitis has increased and turned out to be an important pathogen causing both clinical and subclinical forms of mastitis [13, 14]. The disease Mastitis is multi-etilogic in nature and is caused by a wide range of pathogens. Early detection of mastitis, identification of the causative pathogens, and knowledge of their antimicrobial susceptibility are of the utmost importance for the selection of an appropriate treatment regimen toward effective management [15, 16].

As bacterial pathogens are the principal cause of Mastitis, antibiotic therapy has been adopted as the mainstay of treatment for decades [17]. The efficacy of antibiotics used in the treatment of mastitis is falling off due to the emergence and dissemination of antimicrobial-resistant pathogens (AMR) in the environment, which substantially impair the therapeutic effectiveness of clinically important antibiotics for long-term utilization [18, 19]. The extensive use of antibiotics in the treatment of mastitis contributes to the increase in the number of antibiotic-resistant pathogens and also raises concerns over antibiotic residues in milk, which can trigger public health risk [20, 21]. Addressing the mitigation of the emergence of antimicrobial-resistant pathogens and to ensure the food security of milk products, it is indispensable to switch from conventional antibiotic therapy to more sustainable alternatives. Several studies have proved that Phytotherapy, which involves the use of plant-based medicines exhibiting antimicrobial properties, could be a potential alternative to the existing antibiotic therapy against many diseases of livestock and humans as well [22].

Ricinus communis L. (*R. communis*) is one of the most potent medicinal plants belonging to the family Euphorbiaceae [23]. It is an evergreen semi-woody small tree widespread in tropical, subtropical, and warm temperate climatic regions of the world [24, 25]. Medicinal preparation of the Ricinus plant has been used by various communities across the globe in the management of different illnesses [26]. In ethnomedicine, medicinal preparations from the different parts of the plant have been used in the treatment of various ailments such as liver problems, arthritis, muscle pain, warts, asthma, intestinal worms, jaundice, and bone diseases [27]. Seeds and leaves of *R. communis* are used in veterinary medicine to treat sprains, inflammations, and wounds [28].

Previous studies have revealed that bioactive ingredients of *R. communis* leaf extracts have antinociceptive, hepatoprotective, antibacterial, antifungal, cytotoxic, Antioxidant, Anti-Inflammatory, and larvicidal activities [29–34]. Castor oil is bestowed with antimicrobial, bone regeneration, antiulcer, laxative, and wound healing properties [35–37]. The root exhibits antifungal, anti-diabetic, antiasthmatic, analgesic, and anti-inflammatory activities [35, 36, 38], and the seed possesses antioxidant, toxicological, antiulcer, antifertility, and abortifacient properties [35, 36, 39].

This study aimed to evaluate the phytochemical profile and in vitro antibacterial effects of *R. communis* leaf extracts against three novel *Bacillus* pathogens, *Bacillus paranthracis* SLS01, *Bacillus safensis* HSM85, and *Bacillus stercoris* DA03, isolated from a mastitis-affected cow.

2 Materials and methods

2.1 Sample collection, isolation, and characterization of novel pathogenic strains

The milk sample was collected in sterile [15 ml] polypropylene tubes without causing stress or harm to the cow from Adaspur locality, Cuttack, Odisha, India. The sample was brought to the laboratory, cultured on nutrient agar [NA] plates, and analyzed microbiologically by spreading onto the pre-made Nutrient Agar [NA medium, HiMedia, India] plates using the spread plate technique, followed by incubation at 37 ± 1 °C for 24 h. From the pool of colonies, three morphologically different colonies evident on NA plates were picked, sub-cultured, and purified. The pure cultures were maintained, and a sample was stored at -80 °C as Glycerol stock. All three isolates were characterized morphologically and physiologically using the standard methods [40]. Gram-staining, motility, and growth at different pH levels and aerobic/anaerobic natures were examined in the nutrient broth (NB) and Tryptone soy broth [TSB] [HiMedia, India]. Growth of cultures at 18, 22, 30, 37, and 42 °C was examined in NB broth. Cell shape was examined under

a fluorescent microscope, EVOS M5000 [Invitrogen, USA]. Catalase activity was determined using 3% H₂O₂. Unless otherwise stated, all tests were carried out at 37 °C. Some biochemical tests were carried out using the VITEK-2 automated microbial identification system, Version 9.02 [bioMérieux, USA], with a BCL [Gram-positive spore-forming bacilli] reagent card having 63 different biochemical tests. Through the VITEK-2 automated biochemical microbial identification system, all the isolates were found susceptible to the antibiotic polymyxin B, as the bacterial isolates are motile.

Whole-cell DNA was extracted using the boiling lysis method [Kumar et al. 2011], and DNA concentration was estimated using a NanoDrop spectrophotometer [ND1000, Thermofisher, USA]. The whole-cell DNA [100 ng/ 25 µl PCR reaction volume] was used as a template for the amplification of 16S rRNA gene sequence in presence of 27F and 1492R primer pairs [10 pM each], and 1U Taq DNA Polymerase [GeneDirex, Inc] under reaction conditions: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1.5 min for 30 cycles, and final extension at 72 °C for 7 min. The amplified PCR products were purified and cloned in the pGEMT easy vector system II using E. coli DH5α host cells, and sequencing was done at the DNA sequencing facility, University of Delhi South Campus, Delhi, India. The obtained 16S rRNA gene sequences of strains HSM85, SLS01, and DA03 were subjected to BlastN analysis followed by multiple sequence alignment with validly published *Bacillus* species using ClustalW. The resulting multiply aligned sequences were corrected and edited, and approximately 1422 bp long nucleotide sequences of all the *Bacillus* sp. were taken for further analysis. The Neighbor-joining [NJ] method was used to construct a phylogenetic tree using Kimura 2-parameter model with bootstrap values based on 1000 replications using MEGA11 tool. The 16S rRNA gene sequence of *Aureibacillus halotolerans* strain S1203T [Ac. No. NR145627] was used as an outgroup. The 16S rRNA gene sequences of strains HSM85, SLS01, and DA03 were submitted to the NCBI GenBank database under accession numbers OR493439, OR490598, and OR593314, respectively.

2.2 Plant material and extraction of bioactive compounds

The leaves of *Ricinus communis* collected from Cuttack, Odisha, were washed first with tap water and then with distilled water and dried in an oven at 40 °C for 48–72 h. The dried leaves were initially ground in a kitchen grinder and then subjected to Geno/Grinder® 2010, a high-throughput homogenizer and cell lyser [Cole-Parmer, USA] in the presence of liquid nitrogen. The 5 g of homogenized tissue was suspended in 50 mL

of solvent [ethanol, methanol, chloroform, and acetone] and allowed for maceration for 48 h at room temperature [RT] to drive bioactive compounds in solvents [41]. The debris was removed by centrifugation at 7000 rpm, and the clear supernatant was collected in a fresh sterile 5.0 ml microcentrifuge tube. The solvents were evaporated using UVS400A Universal Vacuum System [Thermofischer, USA] at 40 °C under reduced pressure. The yield was weighted, and the percentage yield was calculated as described earlier [42].

2.3 Antibacterial assay

2.3.1 Agar well diffusion assay

The in vitro antibacterial activity of extracts from leaves obtained using ethanol, methanol, acetone, and chloroform was determined against bacterial strains HSM85, SLS01, and DA03 using the agar well diffusion method [43]. The culture [OD₆₀₀=0.5] grown at 37 °C, 200 rpm for 3–4 h was flooded on the pre-made Mueller Hinton Agar [MHA] plates [HiMedia, India] devoid of any additional nutrients and/or antibiotics. The excess culture was aspirated with the help of a micropipette, and the plates were dried in an aseptic condition for 10–15 min. Approximate 6 mm wells were made in the plates with the help of a flame-sterilized corkborer. The leaf extracts were dissolved in respective solvents at a final concentration of 5.0 mg/ml, and 100 µl [500 µg] of each extract was dispensed into the agar wells. An aliquot of 100 µl of each solvent [85% v/v] devoid of leaf extract was used as a negative control. Gentamycin 10 µl [10 mg/ml] was used as a positive control. Plates were incubated at 37 °C overnight, and the zone of bacterial growth inhibition was measured in millimeters.

2.3.2 Determination of relative percentage inhibition [RPI]

The relative percentage of growth inhibition of the test with respect to the positive control was calculated by using the formula [24]

$$\text{RPI}[\%] = \frac{100 \times [X - Y]}{[Z - Y]}$$

X: total area of the inhibition zone of the test.

Y: total area of the inhibition zone of the negative control.

Z: total area of inhibition zone of the standard drug [Positive control].

The total area was calculated by using area = πr^2 , where r is the radius of the zone of inhibition.

2.3.3 Determination of minimum inhibitory concentration

[MIC] and minimum bactericidal concentration [MBC]

The broth microdilution method was employed to determine the MIC of leaf extracts [44]. The leaf extracts

exhibiting strong antibacterial activities in the agar-well diffusion assay were further examined for MIC evaluation at 500, 400, 200, 100, 50, 25, 12.5, and 6.25 µg/ml concentrations. The leaf extracts at defined concentrations were added into the wells of a 96-well microtiter plate containing 0.1 ml 2X Mueller Hinton [MH] broth, followed by the addition of 10 µL [1×10^7 CFU/ml] log phase cells of each strain. Well devoid of culture, but with and without leaf extract, were taken as blank and reaction blank. The final volume of the media was adjusted to 200 µL with sterile distilled water. Media containing gentamycin [10 µg/ml] and bacterial cells were used as a positive control. The plates were incubated at 37 °C with 150 rpm for 24 h, and optical density was measured at 600 nm in the LX multimode plate reader [Agilent, USA]. The values obtained from wells containing bacterial cells in the media without leaf extract were subtracted from the bacterial growth in the media containing solvents. The final absorbance was compared with the test's OD₆₀₀, and the data were interpreted to evaluate the MIC value. MIC was defined as the lowest concentration [in µg/ml] of compound/ extract that inhibits microbial growth after 24 h incubation.

Minimum bactericidal concentration [MBC], the concentration of the plant extract at which no growth was observed, was evaluated by exposing bacterial cells [1×10^7 CFU/ml] to leaf-extract amended with 1X MH broth [final volume: 200 µl] containing pre-estimated MIC and 2MICs followed by incubation at 37 °C with 150 rpm for 16 h. Optical density was measured at 600 nm, and a loopful culture from each concentration was streaked onto a fresh MH agar plate devoid of any antibiotic or leaf extract, followed by incubation at 37 °C for 24 h. The growths were examined with respect to the used extract concentration.

2.4 Time-kill assay

The killing effect of leaf extract was evaluated by measuring the cellular growth in the presence of leaf extract [Conc: ½ MIC and MIC] [45]. The 50 ml MH broth containing bacterial cells [$\approx 1 \times 10^7$ CFU/ml] was supplemented with ½ MIC and MIC dose of ethanol, chloroform, methanol, and acetone extracts, followed by incubation at 37 °C for 18 h with agitation at 150 rpm. Media devoid of cells was used as a blank. Media with leaf extract and without cells was used as a reaction negative control. The standard drug, gentamycin, was used as a positive control. Media with cells without leaf extract was taken as a negative control. The assay was conducted at 37 °C with shaking at 200 rpm for 18 h. The 2 ml sample was withdrawn every 2 h, and the O.D. was measured at 600 nm. The O.D. was plotted against time, and the data were analyzed.

2.5 Biofilm production and inhibition assay

The biofilm formation properties of strains DA03, HSM85, and SLS01 were examined according to Stepanović et al. [2007] method [46], and the biofilm inhibition assay was performed in the presence of leaf extract in a 96-well microtiter plate to the method described by Olawuwo et al. [2022] with minor modification [47]. Tryptone soy broth [TSB] was used as a culture medium. The wells containing 0.1 ml TSB media were inoculated with 10 µl of seed culture [OD₅₉₅=0.5] and allowed to grow initially without leaf extract at 37 °C for 6 h under static conditions. Thereafter, the leaf extracts were added at a concentration of ½ MIC and MIC in the wells to be tested, followed by 22 h incubation under identical conditions. The total media volume of 0.2 ml was adjusted with fresh TSB media. Each experiment contains a blank [TSB without bacterial cells], negative control [TSB + culture], reaction control [TSB + leaf extract/antibiotic but without culture], and positive control [TSB + culture + standard drug]. Reaction control was taken to correct the background staining. After completion of the reaction, O.D. was measured at 595 nm using a microplate reader [Agilent, USA], and then the content of the wells was decanted. The plates were washed with sterile phosphate-buffered saline [pH 7.2] to remove non-adherent free cells and dried in the air by inverting on a paper towel under aseptic conditions for 15–20 min, followed by additional incubation at 60 °C for 1 h to heat-fix the well-attached cells. The biofilm formation in the microwells was quantified using crystal violet staining according to the method described previously [47].

2.6 Effect of leaf extract on nucleic acid leakage and whole cell proteins

The nucleic acid leakage was examined according to an earlier method [48]. The 5.0 ml TSB broth containing fresh cells [OD₆₀₀=0.5, cell conc adjusted: 1×10^7 CFU/ml] was embedded with ½ MIC and MIC dose of leaf extracts and incubated at 37 °C with agitation at 200 rpm for 24 h. Samples [2.0 ml] were withdrawn after 12 h by centrifugation at 10,000 rpm [Eppendorf, 5418R, Hamburg] for 10 min at 4 °C, and supernatant was used to measure the nucleic acid leakage at 260 nm using a NanoDrop spectrophotometer [ND-1000, ThermoFischer, USA]. Untreated cells were taken as a negative control, and cells with standard drugs were used as a positive control.

For protein inhibition assay, cells were allowed to grow initially for 6 h at 37 °C and then in the presence of leaf extract [conc: ½ MIC and MIC] for another 6 h at 200 rpm. Media devoid of leaf extract but with bacterial cells were used as a control. The cells were harvested by centrifugation at 7000 rpm for 10 min at 4 °C. The pellet

was resuspended in protein extraction buffer and subjected to sonication [10/10 s pulse on/off] for 10 min on ice. The cell debris was removed by centrifugation at 12,000 rpm at 4 °C for 15 min, and protein content was estimated using Bradford's assay at 280 nm in a UV–VIS spectrophotometer. Bovine serum albumin [BSA] was used to prepare a standard curve. The protein bands were resolved in a 12% SDS-PAGE gel at 120 V for 3–3.5 h after denaturing the protein with 1×loading dye (Gallagher, 2012) [49]. The gel was stained and de-stained, and protein bands were visualized in white light in the GelDoc [Invitrogen image system, USA].

2.7 Phytochemical analyses of leaf extracts by GC–MS

The phytochemical constituents of leaf tissue extracted with ethanol, methanol, chloroform, and acetone were determined through GC–MS using Shimadzu QP2010 Ultra using the Rtx-5Sil-MS column [Restek Corporation, USA]. A standard temperature program was followed, where isothermal heating was at 60 °C for 2 min, followed by a ramp rate of 5 °C/min to 250 °C for 15 min during injection. GC–MS was subjected to a total of 40 min. In order to determine the phytochemical constituents, chromatograms obtained from GC–MS were

analyzed by comparing them with the GC–MS spectra database NIST12 [National Institute of Standards and Technology].

2.8 Statistical and bioinformatics study

All the experiments were done in triplicate. Data were analyzed and represented as mean value, and standard deviation was calculated using GraphPad Prism Version 8.0.2 [GraphPad Software, USA]. MEGA 11, BlastN, ClustalW, and EMBL online pairwise sequence alignment tools were used for sequence analysis and phylogenetic studies.

3 Result

3.1 Phenotypic and molecular diagnosis of isolates

Three bacterial strains, SLS01, HSM85, and DA03, were isolated from the clinical mastitis-affected cow's milk sample collected from Adaspur locality, Cuttack, Odisha, India. Before sampling, the teats and udder of the cow were examined, and the stage of disease was confirmed as the clinical stage of mastitis by Dr. Jaya Prakash Bhanja, AVAS, Kantapada Veterinary Dispensary, Kantapada, Cuttack, Odisha, India. The differential biochemical characteristics of strains are shown in Table 1. All three

Table 1 Differential characteristics of bacterial strains isolated from mastitis affected cow milk

Characteristics	Strains		
	<i>Bacillus safensis</i> HSM85	<i>Bacillus paranthracis</i> SLS01	<i>Bacillus stercoris</i> DA03
Optimal temperature	37 °C	37 °C	37 °C
pH	7.0	7.0	7.0
β-xylosidase	–	–	+
Leucine arylamidase	+	+	–
β-galactosidase	–	–	+
L-pyrrolidiny arylamidase	+	+	–
α-galactosidase	–	–	+
Alanine arylamidase	+	–	–
β-N-acetyl-glucosaminidase	+	+	–
D-galactose	–	–	+
Methyl-α-D-glucopyranoside acidification	–	–	+
α-mannosidase	–	–	+
D-mannose	–	–	+
N-acetyl-d-glucosamine	+	+	–
L-rhamnose	–	–	+
β-glucosidase	+	–	+
Phosphoryl choline	+	–	–
α-glucosidase	+	+	–
D-tagatose	–	–	+
D-trehalose	+	–	+
Tetrazolium red	–	–	+

* Symbol: +, positive reaction; –, negative reaction

strains were motile, tolerant to 6.5% NaCl, Gram-positive, rod-shaped, aerobic, and resistant to polymyxin B. The optimum growth pH and temperature were observed at 7 and 37 °C, respectively. Colonies of HSM85 were translucent with a glossy appearance, serrated margin, and round shape, while SLS01 and DA03 were mucoid, opaque, translucent, and off-white with smooth margins. The 16S rRNA gene amplification with 27F and 1492R primer pairs produced a full-length amplicon of 1500 bp. BlastN analysis of 16S rRNA gene sequences of strains DA03, HSM85, and SLS01 showed that all isolates belonged to the genus *Bacillus*. The multiple sequence alignment of 16S rRNA gene sequences of all the strains was conducted with the nearest members using ClustalW, and the phylogenetic study was performed with the MEGA11 tool. Phylogenetic analyses revealed that strains DA03 and HSM85 branched deeply with *B. stercoris* JCM 30051 T [Ac. No. MN536904], *B. safensis* subsp. *osmophilus* BC09T [Ac. No. KY990920], respectively, while strain SLS01 clustered with *B. paranthracis*

Mn5^T [Ac. No. MACE0100012] and *B. nitratireducens* 4049^T [KJ812430] in the Neighbor-Joining [NJ] tree (Fig. 1). In the pairwise alignment of 16S rRNA gene sequences, strain DA03 shared 99.4% homology with *B. stercoris* JCM 30051^T [Ac. No. MN536904]; strain HSM85 exhibited 100% similarity with *B. safensis* subsp. *osmophilus* BC09^T [KY990920] while 16S rRNA gene sequence of strain SLS01 displayed 100% homology with *B. paranthracis* Mn5^T [Ac. No. MACE0100012] and 99.9% with *B. anthracis* Ames^T [Ac. No. AE016879].

3.2 Tissue extraction, percentage yield, and determination of antibacterial activity

The bioactive compounds from homogenized leaf tissue of *R. communis* were extracted by maceration with solvents [85%], namely ethanol, methanol, acetone, and chloroform. The highest extraction percentage yield was observed in methanol [8.47%], followed by ethanol [7.95%], chloroform [6.22%], and acetone [4.77%]. However, the results of agar-well diffusion assay revealed

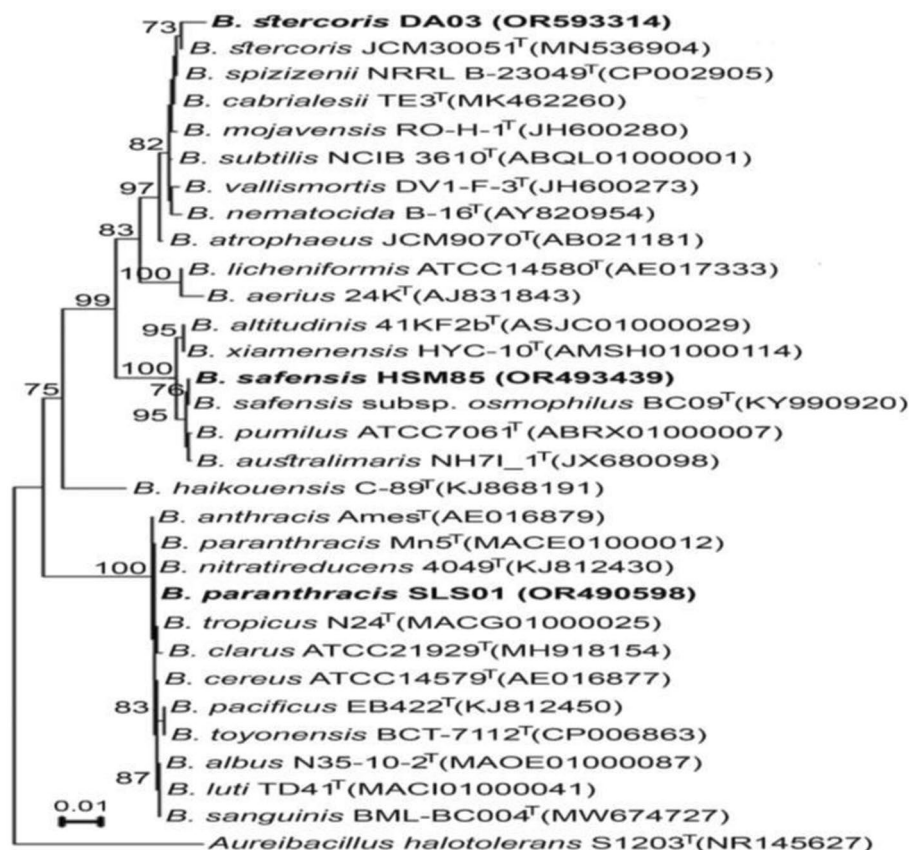


Fig. 1 NJ Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain DA03, HSM85 and SLS01 [Boldface] within the validly published members of genus *Bacillus*. *Aureibacillus halotolerans* S1203 [NR145627] was used as outgroup. Bootstrap value [> 70%], expressed as a percentage of 1000 replications, are shown at branching nodes. GenBank/ EMBL accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position. The tree was constructed using MEGA 11 tool

that the highest antimicrobial activity was displayed by chloroform against strain HSM85 with a zone of inhibition [ZOI] 15.94 mm, followed by ethanol against strain DA03 with ZOI 15.01 mm, acetone and methanol against strain DA03 with ZOI 13.081 and 12.07 mm, respectively. Strain SLS01 was maximally susceptible to the ethanol extract with ZOI 12.95 mm (Fig. 2). Data showed that the growth of all strains was significantly inhibited by the leaf extracts except HSM85, which was insensitive to the methanolic extract, while it was most susceptible to the chloroform extract. Compared to the standard drug gentamycin [positive control], all strains exhibited low susceptibility to the leaf extracts.

The results of the antibacterial activity of leaf tissue extracted with chloroform, ethanol, methanol, and acetone were compared with the positive control gentamycin [standard drug] for assessing relative percentage inhibition [RPI] (Fig. 3). The results of RPI exhibited that the chloroform extracts exhibited maximum [62.57%] RPI against strain HSM85, followed by SLS01 [30.12%] and DA03 [29.86%]. The ethanol leaf extract showed maximum RPI against DA03 [53.08%], followed by SLS01 [51.96%] and HSM85 [35.63%]. The methanol extract of *R. communis* displayed maximum RPI against

SLS01 [30.12%] next to DA03 [34.36%], while no inhibition of strain HSM85 was observed against the methanol extract. The acetone extract of *R. communis* leaf showed maximum [49.29%] RPI against SLS01, followed by HSM85 [42.17%] and DA03 [40.72%].

As shown in Table 2, the MIC and MBC values of the leaf extract ranged from 25 to 200 and 25–400 µg/ml, respectively, against all strains except strain HSM85, which displayed utter resistance to the methanolic extract; however, it was maximally susceptible to the chloroform extract with identical MIC and MBC value of 25 µg/ml. The MIC and MBC values of other extracts are shown in Table 2.

According to Mogana et al. [2020], the effect of the drug could be predicted as bacteriostatic or bactericidal by dividing the MBC by the MIC value, and if the ratio of MBC/MIC > 4, the effect would be bacteriostatic, and if the MBC/MIC ratio ≤ 4, the effect would be considered as bactericidal [50]. The MBC/MIC ratio of all the extracts is displayed in Table 2. The most promising antimicrobial activity of the chloroform extract was observed against HSM85 and SLS01, while the acetone extract was observed toward SLS01 and DA03. As shown in Table 2, the MBC/MIC ratio of all extracts ranged from 1 to 4,

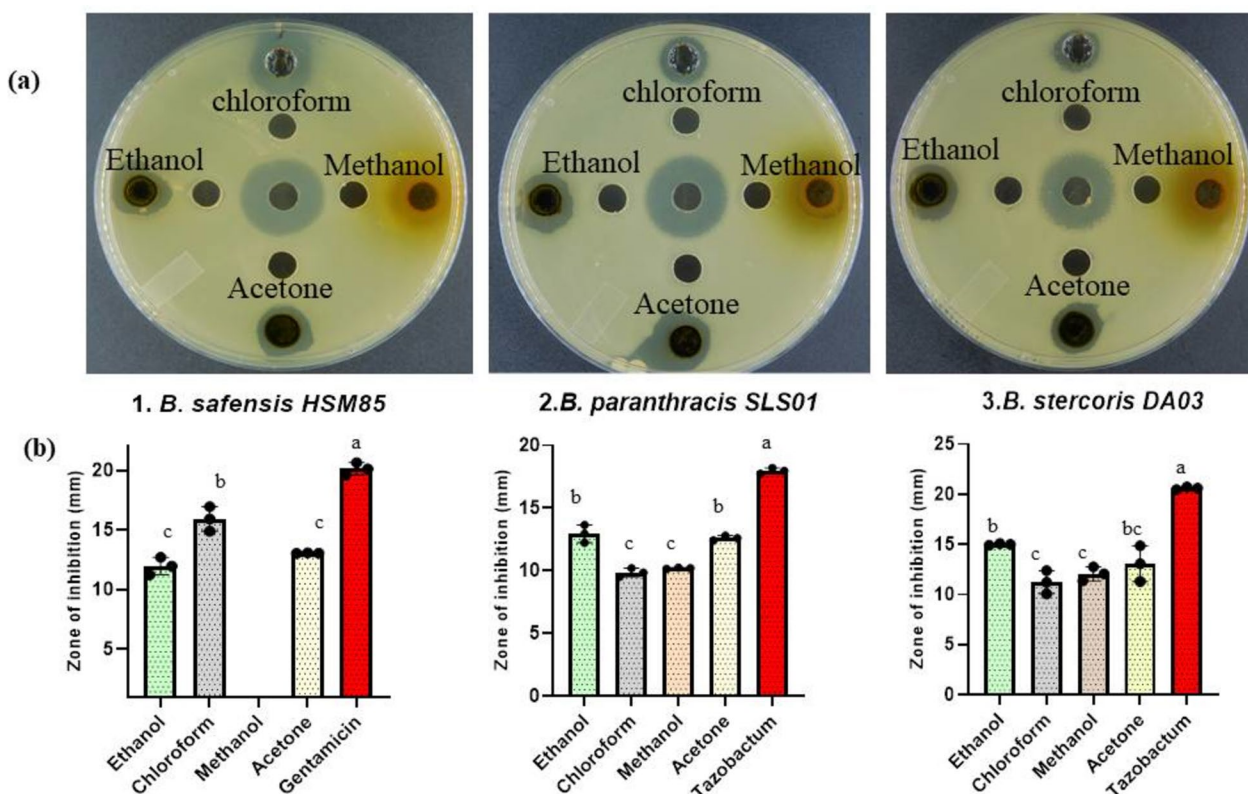


Fig. 2 Antimicrobial activity of *R. communis* leaf extracts on different strains of *Bacillus*. **a** Agar well-diffusion assay, **b** corresponding graphical data of antimicrobial activity of agar-well diffusion assay against respective strain

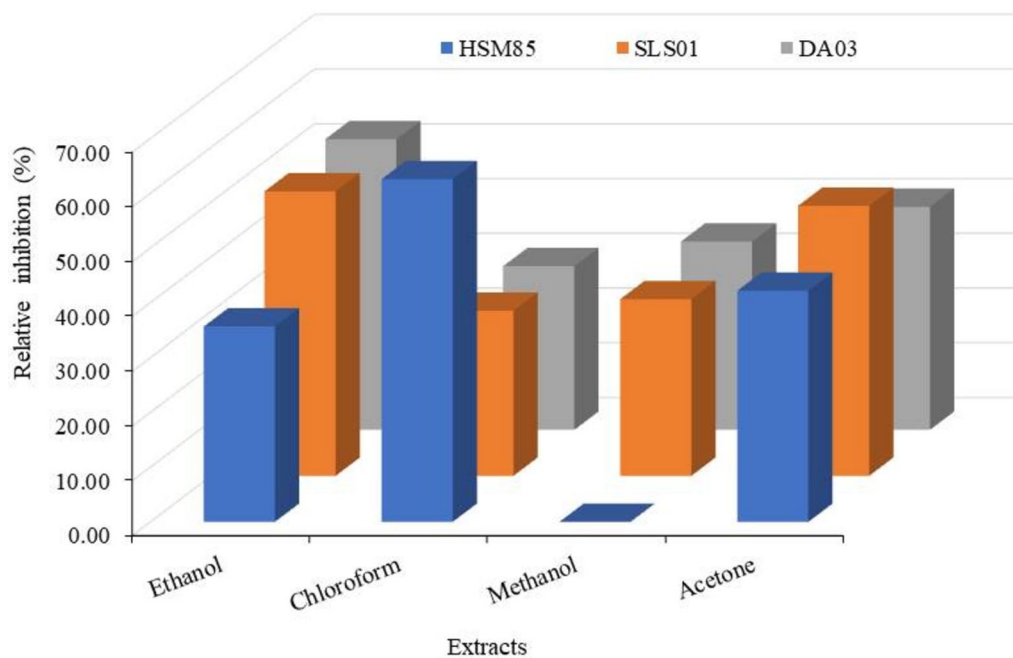


Fig. 3 Relative percentage inhibitions of ethanol, chloroform, methanol, and acetone extracts from leaf tissue of *R. communis* compared to gentamycin

Table 2 MIC and MBCs of *R. communis* leaf extracted with ethanol, methanol, acetone and chloroform solvents [85% v/v]

Strains	MIC and MBC [$\mu\text{g/ml}$] and MBC/MIC ratio of different extracts											
	Ethanol			Chloroform			Methanol			Acetone		
	MIC	MBC	MBC/MIC ratio	MIC	MBC	MBC/MIC ratio	MIC	MBC	MBC/MIC ratio	MIC	MBC	MBC/MIC ratio
<i>B. safensis</i> HSM85	50	100	2	25	25	1	ND	ND	0	50	100	2
<i>B. paranthracis</i> SLS01	100	200	2	50	50	1	200	400	2	100	100	1
<i>B. stercoris</i> DA03	50	100	2	50	200	4	100	200	2	100	100	1

* The values are expressed in $\mu\text{g/ml}$. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; ND, not detected

except methanol, which was insensitive to strain HSM85, suggesting that, irrespective of the solvents used for extraction, *R. communis* leaf was a potent inhibitor with strong bactericidal activity.

3.3 Time-dependent killing and biofilm inhibition assay

Data of time-dependent bacterial growth in presence of leaf extracts [conc: $\frac{1}{2}$ MIC and MIC] revealed that the cells devoid of leaf extracts [control] rapidly attained log phase within a short period [4 h] of incubation, whereas the leaf extract treated cell displayed lag phase throughout the incubation period of 18 h (Fig. 4). The results of the time-dependent killing assay were consistent with the results of agar-well diffusion, MIC, and

MBC assays. No bacterial growths were observed in the media supplement with MIC concentrations [test] and gentamycin [standard drug] used as positive control.

In the biofilm assay, maximum biofilm production was exhibited by strain SLS01. The order of biofilm formation was SLS01 > DA03 > HSM85. The biofilm formation of all three strains was strongly inhibited by all extracts except methanol, which was ineffective against the biofilm of strain HSM85 (Fig. 5). Results showed that biofilm synthesis of strain DA03 was reduced by 86.42% and 84.96% of SLS01 in the well embedded with methanol extract at a concentration of MIC dose. Figure 5 showed that the maximum inhibition [80.64%] of biofilm of HSM85 cells was recorded in the presence

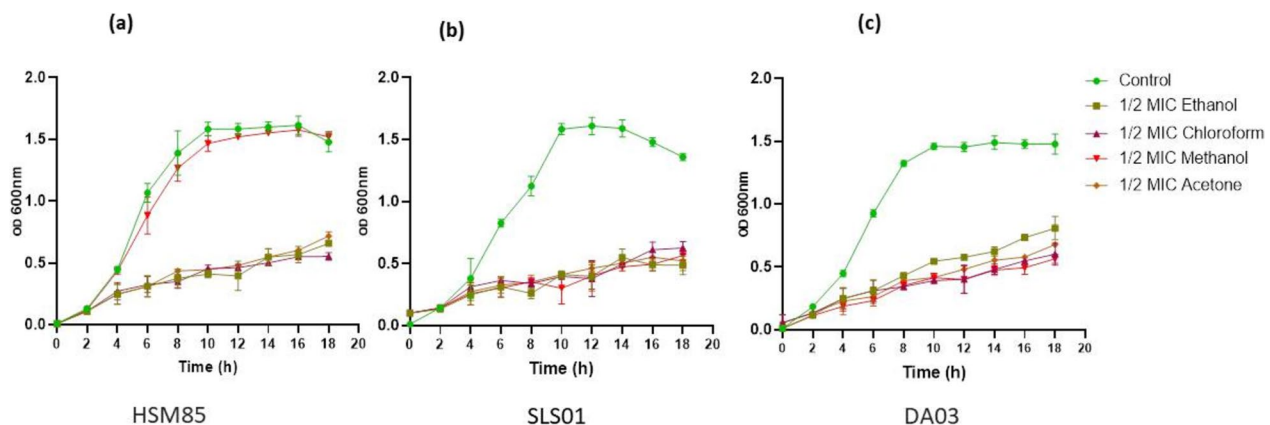


Fig. 4 Growth kinetics of treated [test] and untreated [control] cells of **a** strain HSM85, **b** strain SLS01, and **c** strain DA03. Negative control devoid of leaf extract [●], ethanol extract [■], methanol extract [▲], chloroform extract [▼], acetone extract [◆], and Positive control-Gentamycin [●]

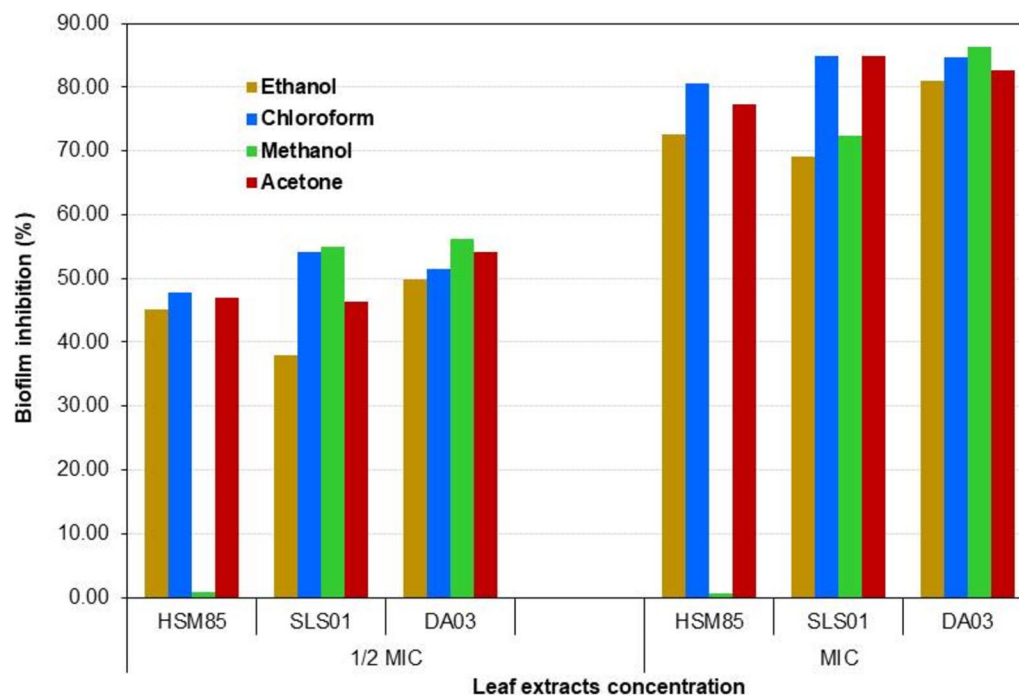


Fig. 5 Effect of 1/2 MIC and MIC dose of ethanol, chloroform, methanol and acetone extracts on biofilm formation on **a** strain HSM85, **b** strain SLS01 and **c** strain DA03. Bar represents mean value, error bar represents $\pm$ SD [n = 3]. Significant difference according to one way ANOVA, where asterisk indicates significant difference with p value [$*p < 0.05$, $**p < 0.01$, $***p < 0.001$]

of chloroform extract at the MIC dose of *R. communis* leaf.

3.4 DNA/RNA leakage assay and effect of leaf extract on cellular proteins

The leaf extract treatment resulted in the disruption of the cell wall and cell membrane, and thus leakage of

nucleic acid from bacterial cells. The results revealed liberation of nucleic acids from all the strains except HSM85, which was resistant to the methanol extract. Figure 6a shows that the maximum DNA/RNA liberation occurred from HSM85 cells treated with chloroform extract, whereas strain SLS01 and DA03 released maximum DNA/RNA in the presence of ethanolic extract (Fig. 6b-c).

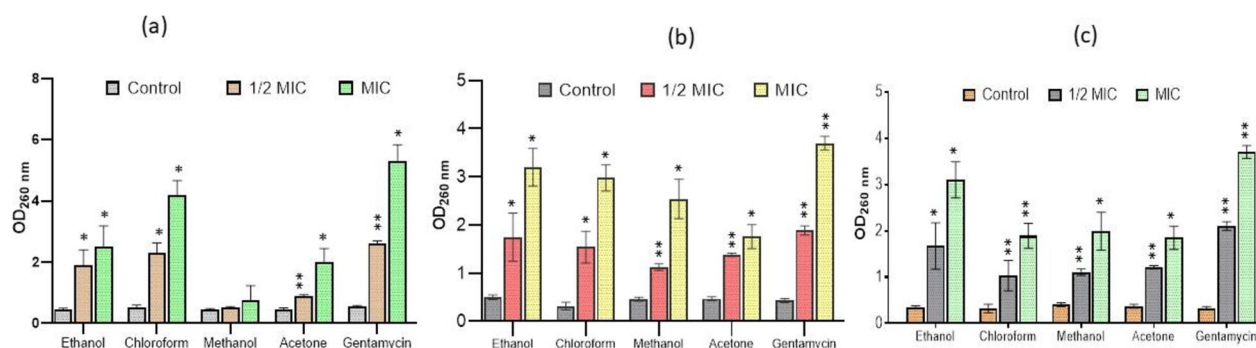


Fig. 6 Effect of leaf-extract [conc: 1/2 MIC and MIC] on nucleic acid leakage from **a** strain HSM85, **b** strain SLS01, and **c** strain DA03 bacterial strains. Bar represents mean value, error bar represents +SD [n = 3]. Significant difference according to one-way ANOVA, where asterisk indicates significant difference with p -value [$*p < 0.05$, $**p < 0.01$]

In order to evaluate the effect of leaf extract on the whole cell protein of strains, the total protein was extracted by sonication from treated cells and estimated using the Bradford assay. The data showed that the cellular proteins were strongly inhibited in the presence of leaf extracts compared to the control without leaf extract, except methanol, which was ineffective against strain HSM85 (Fig. 7). The proteins of treated cells were also resolved on 12% SDS-PAGE (Supplementary Fig. 1), which strengthened the results of other antimicrobial data.

3.5 Phytochemical profiling of *Ricinus communis* leaf extracts

A total of 197 compounds were identified from the four different fractions of *R. communis* leaf using the GC–MS library. Out of 197 compounds, 48 were detected in ethanol, 53 in methanol, 44 in acetone, and 52 in chloroform extract. GC–MS analysis revealed mainly the presence of terpenes [diterpenes and triterpenes], alkanes, saturated/unsaturated fatty acids, alcohols,

phytosterols, tocopherols, and traces of aliphatic hydrocarbons, ketones, aldehydes, sulfonamide derivative, etc. in the ethanol, methanol, acetone, and chloroform extracts. The principal [peak area: >5%] compounds in the methanolic extracts were phytol [21.8%], 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl [11.15%], 3-pyridinecarbonitrile, 1,2-dihydro-4-methyl [7.24%], 1,3,4,5-tetrahydroxy-cyclohexanecarboxy [6.25%]. In ethanolic extract, abundant phytochemicals are phytol [20.47%], Phytol palmitate [7.13%], γ -sitosterol [6.01%], Methyl commate B [5.07%], and vitamin E [4.89%]. The acetone-extract of *R. communis* leaf exhibited abundance of phytol [11.17%], tetracontane [7.94%], γ -sitosterol [5.5%], and phytol palmitate [5.16%], while the chloroform extract displayed abundance 1,2-benzenedicarboxylic acid, bis [2-methyl propyl] ester [9.44%], phytol [6.61%], and tetracontane [5.21%]. Besides major compounds, many compounds were observed in moderate and trace amounts. Some of the major phytochemicals of *R. communis* leaf extracts are presented in the heat map (Supplementary Fig. 2), and detailed composition of

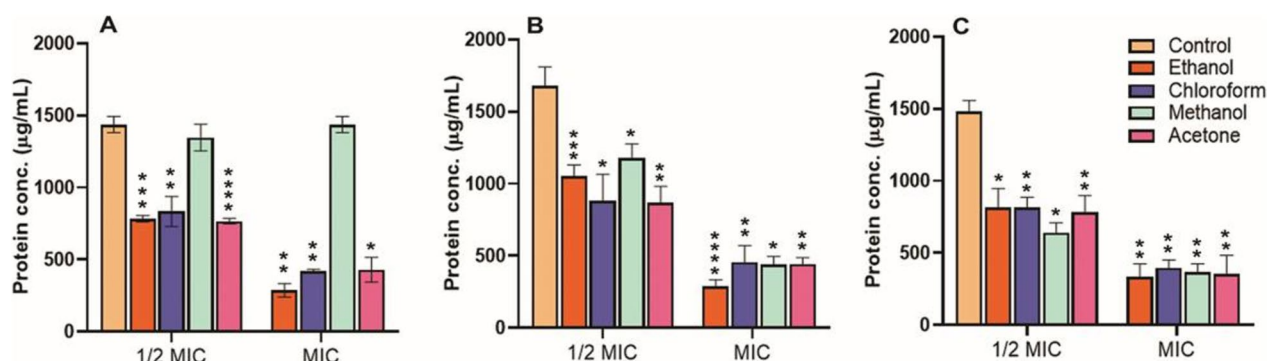


Fig. 7 Quantitation of whole-cell protein content of treated [test] and untreated [control] cells of **a** HSM85, **b** SLS01, and **c** DA03 strains. Dose: 1/2 MIC and MIC of leaf extracts. Bar represents mean value, error bar represents +SD [n = 3]. Significant difference according to one-way ANOVA, where asterisk indicates significant difference with p -value [$*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$]

phytochemicals fraction-wise with retention time, peak area percentage, molecular weight, and chemical formula are shown in Table 3.

4 Discussion

Mastitis is regarded as one of the most complex diseases, costing billions of dollars to the economy every year [51, 52]. Numerous microbes have been reported to be associated with the mastitis disease [52]. In the current study, three new, less common, but important groups of bacteria belonging to pathogenic *Bacilli* were isolated from the milk of a cow affected with clinical mastitis, and the efficacy of *R. communis* leaf was evaluated to manage them under in vitro conditions. Unlike non-sporulating common mastitis pathogens such as *Staphylococcus aureus*, non-aureus staphylococci, and Enterobacter, spore-forming bacteria possess extra advantages that allow them to survive in adverse conditions; thus, they are more dangerous than non-sporulating ones in terms of eradication. Discovery of new pathogens, as well as proper diagnosis of mastitis pathogens, could lead to better management of this devastating disease.

The newly isolated strains, DA03, HSM85, and SLS01, were picked from the pool of discrete colonies enumerated on an NA agar plate. The strains were purified and diagnosed phenotypically, biochemically, and also identified at the molecular level using the 16S rRNA gene sequence. All three strains were Gram-positive, aerobic, resistant to 6.5% NaCl and Polymyxin-B, biofilm producers, but biochemically different from each other's (Table 1). Based on 16S rRNA gene sequence phylogeny and pairwise sequence homology [%] performed with the 16S rRNA gene sequences of closest members, strains DA03, HSM85 and SLS01 were identified as *B. stercoris* JCM 30051^T [99.4%], *B. safensis* subsp. *osmophilus* BC09^T [100%], and *B. paranthracis* Mn5^T [100%], respectively. As far as is known, only a few papers reported *Bacillus* sp. [*Bacillus anthracis*, *Bacillus paranthracis*, and *Bacillus cereus*] as the pathogenic agents from bovine mastitis [53–55]. Based on phenotypic and genotypic data, the newly isolated strains differ from each other as well as from the reported closest members of the genus *Bacillus*. Results revealed that strains *B. stercoris* DA03 and *B. safensis* SLS01 have not been reported from any mastitis-infected sample. Whereas strain HSM85 was moderately different at the phenotypic level but shared 100% identity at the 16S rRNA gene sequence level with *B. paranthracis*. The stage of disease was diagnosed as a clinical form of mastitis. Recently, Sokolov et al. [2023] isolated *Bacillus paranthracis* from mastitis-infected cow milk carrying toxigenic genes in their genome [14]. Eid et al. [2023] reported *Bacillus cereus* as the most common pathogen from Egyptian dairy cows infected with

subclinical which was associated with the enterotoxins such as *hemolysin BL* [*hbl*] and *non-hemolytic enterotoxin* [*nhe*] genes associated with diarrhea [56]. Sousa [2021] reported 120 genes linked to stress resistance and pathogenicity in the genome of *B. paranthracis* BCCL01 [56].

To combat this devastating disease, several strategies/therapies have been proposed by researchers, which are chiefly based on antibiotics and non-antibiotic alternatives [1]. Multi-drug resistance, caused by over-/blind use of antibiotics, is a global challenge nowadays; therefore, application of other alternatives like herbal drugs or plant extracts, which are much safer for animal, human, and environmental health, has also received greater attention in the past decades. The application of herbal extract in cattle disease management has also been recommended by the National Mastitis Council [57, 58].

Since ancient times, several herbs have been in common practice for treating multiple diseases, and many more are being explored for their potential applications [59–61]. In the present study, we evaluated *R. communis* leaf as an antimicrobial agent to control the newly isolated mastitis gram-positive sporulating pathogens under in vitro conditions. *R. communis* is a semi-woody small tree that belongs to the family Euphorbiaceae and grows worldwide in tropical and temperate regions. The leaves and other parts of *R. communis* have been used for centuries in various parts of the world to treat wound infections, candidiasis, and other skin diseases as folk medicine. Scientifically, many workers have also proved and validated its pharmaceutical importance [55, 62]. We utilized acetone and chloroform [non-polar] along with ethanol and methanol, commonly used by earlier researchers, for the extraction of leaf tissue of *R. communis* [41, 60, 62]. In this study, the highest extraction yield [8.47%] and maximum number of compounds [52] from *R. communis* leaf were observed in the methanolic extract as reported by previous workers [55, 62]; however, the maximum antimicrobial activity was noted in the chloroform extract. A total of 197 peaks were recorded in the GC–MS chromatogram from four extracts. Experimental data indicated that *R. communis* leaf was rich in terpenes, fatty acids, long-chain hydrocarbons, unsaturated aliphatic hydrocarbons, alcohols, aldehydes, ketones, alkanes, esters, tocopherol, and sulfonamide derivatives. Alkanes, fatty acids, terpenes, and alcohols were observed as the most abundant compounds. The results of agar well diffusion assay revealed that chloroform extract was highly potent against strain HSM85 with a maximum ZOI of 15.95 mm and RPI of 62.57%. Whereas the strain HSM85 was utterly resistant to the methanolic extract. Strain DA03 displayed high susceptibility to the ethanolic and methanolic extracts with RPI 53% and 34.36%, respectively, whereas strain SLS01 was

Table 3 Percentage of phytoconstituents detected through GCMS from leaf-tissue extracted with ethanol, methanol, acetone and chloroform

Sl. No	Compound	Retention time	Compound % proportion in respective extract			
			Ethanol	Methanol	Acetone	Chloroform
1.	Phytol	20.47	21.80	11.17	6.61	
2.	Heneicosane	11.46	ND	4.65	11.86	
3.	Phytyl palmitate	7.13	ND	5.16	ND	
4.	γ -Sitosterol	6.01	1.82	5.50	ND	
5.	Methyl commate B	5.07	2.43	4.57	ND	
6.	Vitamin E	4.89	0.85	4.29	2.88	
7.	Tetracontane	3.88	ND	3.98	7.72	
8.	Stigmasta-5,22-dien-3-ol, [3. β ,22e]	3.80	1.07	ND	2.29	
9.	1,2-Benzenedicarboxylic acid, bis[2-methylpropyl] ester	3.51	ND	1.74	9.44	
10.	Ethyl Oleate	2.67	ND	ND	ND	
11.	Palmitic Acid, TMS derivative	2.67	0.71	4.65	3.27	
12.	Butyl tetratriacontyl ether	2.48	ND	ND	ND	
13.	2-methyloctacosane	2.25	ND	ND	6.08	
14.	Docosanoic acid, ethyl ester	1.99	ND	ND	ND	
15.	9,12-9,12-Octadecadienoic acid [Z,Z]-, octyl ester	1.62	ND	ND	ND	
16.	Ethyl 13-docosenoate[ethyl erucate]	1.56	ND	ND	ND	
17.	4,4,6a,6b,8a,11,11,14b-octamethyl-1,4,4a,5,6,6a,6	1.51	ND	ND	ND	
18.	1-hexadecene	1.51	ND	ND	ND	
19.	9,10,12,13-Tetrabromooctadecanoic acid	1.49	ND	0.83	ND	
20.	Bis[2-ethylhexyl] phthalate	1.32	ND	ND	ND	
21.	1-Heptadecene	1.32	ND	ND	ND	
22.	Neophytadiene	3.14	ND	1.87	1.69	
23.	Eicosane	1.12	ND	1.42	1.65	
24.	1-Decanol	0.87	ND	ND	ND	
25.	5-[7a-isopropenyl-4,5-dimethyl-octahydro-i	0.74	ND	ND	ND	
26.	Heptadecanoic acid, ethyl ester	0.74	ND	ND	ND	
27.	γ -Tocopherol	0.73	0.71	ND	ND	
28.	2[3H]-Naphthalenone, 3-[[[1,1-dimethylethyl]dimethylsilyl	0.71	ND	ND	ND	
29.	9-Octadecenoic acid, [E]-, TMS derivative	0.70	ND	1.48	1.53	
30.	Stigmast-5-en-3-ol, [3. β]	0.61	0.53	ND	4.07	
31.	Squalene	0.59	ND	ND	ND	
32.	9-octadecenal, [z]	0.43	ND	ND	ND	
33.	3-n-Butylthiophene-1,1-dioxide	0.38	ND	ND	ND	
34.	Cyclopentane, 1,2,4-trimethyl-, [1.alpha.,2.a	0.38	ND	ND	ND	
35.	Cyclononanone	0.36	ND	ND	ND	
36.	Hexadecanedinitrile	0.33	ND	ND	ND	
37.	Octadecanoic acid, trimethylsilyl ester	0.31	ND	ND	ND	
38.	1,3-Dimethylbutyl ethylphosphonofluoridoate	0.31	ND	ND	ND	
39.	1-Octanol, 2,7-dimethyl	0.12	ND	ND	ND	
40.	Dodecanoic acid, ethyl ester	0.11	ND	ND	ND	
41.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	ND	11.15	ND	ND	
42.	3-pyridinecarbonitrile, 1,2-dihydro-4-meth	ND	7.24	ND	ND	
43.	1,3,4,5-tetrahydroxy-cyclohexanecarboxy	ND	6.25	ND	ND	
44.	6-Octadecenoic acid, methyl ester, [Z]	ND	3.93	ND	ND	
45.	2-Amino-3-hydroxypyridine	ND	3.08	ND	ND	
46.	13-Docosenoic acid, methyl ester, [Z]	ND	3.05	ND	ND	
47.	Benzeneacetaldehyde	ND	2.68	ND	ND	

Table 3 (continued)

Sl. No	Compound	Retention time	Compound % proportion in respective extract			
			Ethanol	Methanol	Acetone	Chloroform
48.	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran		ND	2.05	ND	ND
49.	9-Octadecenoic acid [Z]-, 2,3-dihydroxypropyl ester		ND	1.91	ND	ND
50.	6,6-dideutero-nonen-1-ol-3		ND	1.76	ND	ND
51.	Methyl stearate		ND	1.64	ND	ND
52.	2-methoxy-4-vinylphenol		ND	1.63	ND	ND
53.	6. β ., 6. β .-Dibromo-6,7-methylenetesterone		ND	1.61	ND	ND
54.	Hexadecanoic acid, methyl ester		ND	1.48	ND	ND
55.	n-Hexadecanoic acid		ND	1.45	ND	ND
56.	4-[2,6,6-trimethyl-1,3-cyclohexadien-1-yl]-3		ND	1.36	ND	ND
57.	2-cyclohexen-1-one, 5-methyl-2-[1-methyle		ND	1.27	ND	ND
58.	1,3,5-Triazine-2,4,6-triamine		ND	1.23	ND	ND
59.	Ethyl [9z,12z]-9,12-octadecadienoate		ND	1.21	ND	ND
60.	9,12-Octadecadienoic acid, methyl ester		ND	1.65	ND	ND
61.	β -Amyrone		ND	0.97	ND	ND
62.	Propylamine, N-[9-borabicyclo[3.3.1]non-9-yl]-		ND	0.89	ND	ND
63.	2-acetyl-2-hydroxy- γ -butyrolacton		ND	0.89	ND	ND
64.	1,2,3,4,5-Cyclopentanepentol		ND	0.86	ND	ND
65.	Undecanoic acid		ND	0.79	ND	ND
66.	Benzene, 1-chloro-2-methoxy-		ND	0.76	ND	ND
67.	2,6,8-trimethylbicyclo[4.2.0]oct-2-ene-1,8-dio		ND	0.67	ND	ND
68.	17-octadecenoic acid, methyl ester		ND	0.64	ND	ND
69.	Hexadecanoic acid, 2-hydroxy-1-[hydroxymethyl]ethyl est		ND	0.59	ND	ND
70.	Decahydroquinoline-10-ol		ND	0.51	ND	ND
71.	2-hydroxy-3-[[9e]-9-octadecenoyloxy]propy		ND	0.48	ND	ND
72.	3-Cyclohexen-1-carboxaldehyde, 3-methyl-		ND	0.46	ND	ND
73.	Propyl tetratriacontyl ether		ND	0.46	1.33	ND
74.	2-[di-methyl amino]ethyl 1-adamantanecar		ND	0.46	ND	ND
75.	1,5-benzothiazepin-4[5 h]-one, 3-[acetyloxy]		ND	0.43	ND	ND
76.	2[4 h]-benzofuranone, 5,6,7,7a-tetrahydro-4		ND	0.42	ND	ND
77.	1-Methyl-4-phenyl-4-propionoxypiperidine		ND	0.42	ND	ND
78.	2,6-Bis[2-[dimethylamino]ethoxy]pyridine		ND	0.32	ND	ND
79.	5,8,11-Eicosatrienoic acid, [Z]-, TMS derivative		ND	0.29	ND	ND
80.	1[2H]-Naphthalenone, octahydro-5-hydroxy-4a-methyl-		ND	0.27	ND	ND
81.	1-[3-methyl-3-butenyl]pyrrolidine		ND	0.24	ND	ND
82.	Quinolin-6[7H]-one, 1,2,3,4,8,8a-hexahydro-1,4-ethano-		ND	0.23	ND	ND
83.	Heptanedioic acid, 4-methyl-, dimethyl ester		ND	0.21	ND	ND
84.	1-[n,n-dimethylsulfamoyl]-1 h-1,2,4-triazole		ND	0.20	ND	ND
85.	Tetratetracontane		ND	ND	9.1	ND
86.	Glycidyl oleate		ND	ND	5.15	ND
87.	9-Octadecenoic acid, 1,2,3-propanetriyl ester, [E,E,E]-o		ND	ND	4.97	ND
88.	Stigmasterol		ND	ND	3.75	ND
89.	5,5-Dimethyl-1,3-dioxane-2-ethanol, TBDMS derivative		ND	ND	2.36	ND
90.	1,2-Benzene dicarboxylic acid, bis[2-methylpropyl] ester		ND	ND	2.34	ND
91.	Stigmast-5-en-3-ol, oleate		ND	ND	2.14	ND
92.	D:A-Friedoolean-6-ene		ND	ND	2.13	ND
93.	Hexatriacontane		ND	ND	1.87	ND
94.	Stigmasta-7,22-dien-3-ol, acetate, [3. β .,5.alpha.,22E]-		ND	ND	1.83	ND
95.	13-96.Hexyloxacyclotridec-10-en-2-one		ND	ND	1.63	ND

Table 3 (continued)

Sl. No	Compound	Retention time	Compound % proportion in respective extract			
			Ethanol	Methanol	Acetone	Chloroform
96.	Stearic acid, TMS derivative		ND	ND	1.23	0.97
97.	Tetracosane		ND	ND	1.11	ND
98.	18,19-Bisnor-5-chole, 3,24-dihydroxy-		ND	ND	1.04	ND
99.	1-Decanol, 8-[[trimethylsilyl]oxy]-, trifluoroacetate		ND	ND	0.97	ND
100.	Heptadecane, 2,6,10,15-tetramethyl		ND	ND	0.83	ND
101.	Glycidyl palmitate		ND	ND	0.80	ND
102.	9-Octadecenoic acid [Z]-, 2-hydroxy-3-[[1-oxohexadecyl]o		ND	ND	0.74	ND
103.	Glycine, N-butoxycarbonyl-, propyl ester		ND	ND	0.70	ND
104.	Oleoyl chloride		ND	ND	0.64	ND
105.	Octadecanoic acid, 2-hydroxy-1-[hydroxymethyl]ethyl ester		ND	ND	0.62	ND
106.	13-Docosenoic acid, [Z]-, TMS derivative		ND	ND	0.52	ND
107.	[R]-6-Methoxy-2,8-dimethyl-2-[[4R,8R]-4,8,12-trimethyltr		ND	ND	0.39	ND
108.	1,5,6-trimethyl-2,3,3a,4,7,7a-hexahydro-1 h-in		ND	ND	0.28	ND
109.	Cholestan-16-one semicarbazone		ND	ND	ND	5.47
110.	Stigmast-5-en-3-ol, [3. β]		ND	ND	ND	4.07
111.	Phytyl dodecanoate		ND	ND	ND	3.95
112.	lup-20[29] -ene-3,28-diol, [3. β]		ND	ND	ND	3.21
113.	Eicosanoic acid, tetradecyl ester		ND	ND	ND	3.15
114.	1,4-di-o-acetyl-2,3,5-tri-o-methylpentitol		ND	ND	ND	2.83
115.	13-Octadecenal, [Z]		ND	ND	ND	2.08
116.	Phenol, 2,4-bis[1,1-dimethylethyl]-, phosphite [3:1]		ND	ND	ND	1.90
117.	6-Ethyl-3-decanol, TMS derivative		ND	ND	ND	1.85
118.	OCTADECANE		ND	ND	ND	2.41
119.	Bis[tridecyl] phthalate		ND	ND	ND	1.29
120.	STIGMAST-5-EN-3-OL, [3. β,24S]		ND	ND	ND	1.18
121.	Benzene, 1,3-bis[1,1-dimethylethyl]		ND	ND	ND	1.13
122.	Phenol, 3,5-bis[1,1-dimethylethyl]		ND	ND	ND	1.05
123.	Sulfurous acid, 2-ethylhexyl isohexyl ester		ND	ND	ND	0.81
124.	2-oxecanone, 10-methyl-, [+ -]		ND	ND	ND	0.77
125.	HEXADECANE		ND	ND	ND	0.65
126.	Undecane, 3,8-dimethyl		ND	ND	ND	0.64
127.	2-Methylhexacosane		ND	ND	ND	0.64
128.	Phosphorochloridic acid, dipropyl ester		ND	ND	ND	0.62
129.	9-Octadecenoic acid [Z]-, 2-hydroxy-3-[[1-oxo hexadecyl]o		ND	ND	ND	0.61
130.	Pentadecanal		ND	ND	ND	0.60
131.	Hexanedioic acid, bis[2-ethylhexyl] ester		ND	ND	ND	0.59
132.	Heptadecane		ND	ND	ND	0.53
133.	Octane, 2,3,6-trimethyl		ND	ND	ND	0.51
134.	Cyclononasiloxane, octadecamethyl		ND	ND	ND	0.45
135.	Silane, Trimethyl[[3,7,11,15-Tetramethylhex		ND	ND	ND	0.38
136.	Decyl octyl ether		ND	ND	ND	0.30
137.	Docosane		ND	ND	ND	0.18
138.	Nonane, 3,7-dimethyl		ND	ND	ND	0.18

* ND: Not detected

highly sensitive to acetone with RPI 42.17%. The data of MIC and MBC revealed that the non-polar [chloroform] extract of *R. communis* leaf showed maximum

antibacterial activity against all three strains, followed by ethanol [polar], and acetone. Contrary to earlier reports, the lowest antimicrobial activity was recorded in the

methanolic extract [55]. The chloroform extract of *R. communis* leaf exhibited significant antimicrobial activity among all the extracts, with MICs ranging from 25 to 50 µg/ml (Table 2). The lowest MIC and MBC value [25 µg/ml] of chloroform extract was observed against strain HSM85, while a moderate susceptibility with an MIC value of 50 µg/ml was found against strains SLS01 and DA03. Ethanol extract exerted maximum antibacterial effect on HSM85 and DA03 [50 µg/ml] and the lowest on SLS01 [100 µg/ml]. Compare to other extracts, methanolic extracts of *R. communis* leaf exhibited poor antibacterial activity against DA03 and SLS01 with MIC values of 100 and 200 µg/ml, respectively, possibly due to the absence or low percentages of antimicrobial compounds like heneicosane, tetracontane, stearic acid, 2-methyl octacosane, stigmasterol and palmitic acid [41, 63–65]. Strain HSM85 showed utter resistance to the methanolic extract. The resistance of HSM85 to methanolic extract could not be established; however, it may be due to the altered cell wall structure of strain HSM85. In methanol, ethanol, and acetone extracts, phytol was observed as a major compound with concentrations of 21.8, 20.47, and 11.17%, respectively, while its concentration was noted to be lowest in the chloroform extract. Phytol was noted as the 3rd abundant compound [6.61%] after heneicosane [11.86%] and tetracontane [7.72%], which were recorded as the first and second abundant compounds in the chloroform fraction of *R. communis* leaf. Phytol, an acyclic diterpene, has been demonstrated as a potent antioxidant, anti-diuretic, nematocidal, hepatoprotective, hypocholesterolemic, anticoronary, antiarthritic, anti-diabetic, anti-cancerous, anti-allergic, anti-inflammatory, biofilm inhibitor, antimicrobial, and immunostimulatory compound by several workers [66, 67]. The abundance of long-chain bioactive antimicrobial alkanes like heneicosane and tetracontane was noted in all the extracts of *R. communis* leaf except methanol, in which these were completely absent. In previous reports, palmitic acid, a long-chain saturated fatty acid found in all extracts, has been shown as a potent antimicrobial compound against both Gram-positive and Gram-negative bacteria, while stearic acid, an antimicrobial compound, was noted in the acetone and chloroform fractions only. The GC–MS phytochemical profile of all extracts revealed that the absence, presence, and percentage of phytoconstituents were directly related to the solvent used in the extraction. β-Amyrone, which has been reported as anti-inflammatory, antifungal, and antiviral, was exceptionally found in the methanolic extract of *R. communis* leaf [64], while stigmasterol, which is known to possess antimicrobial properties, was found in the acetone extract only [68]. The antimicrobial activity of *R. communis* leaf extract was comparable to the results of an earlier report [66].

In the time-dependent growth kinetics assay, except methanol, which showed no response to the strain HSM85, all extracts [dose: ½ MIC] of *R. communis* leaf arrested and kept the cellular growths in lag-phase throughout the incubation period of 18 h compared to the untreated cells [negative control] that entered in the log phase within 4 h and attained stationary phase in 10 h. The cells treated with the MIC dose and standard drug [positive control] failed to replicate. Biofilm formation, a characteristic feature of most pathogens that helps them in adhesion to the tissues and disease progression by providing them with an extra layer of protection from the antibiotics used in treatment, was noted in all three strains [69, 70]. Results showed that biofilm formation was strongly inhibited by all extractants except methanol, which was found insensitive to the strain HSM85. The data of biofilm inhibition was comparable with the previous study observed against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans* after *R. communis* leaf-extract treatments [41]. The nucleic acid leakage from the treated cells indicated that the bioactive phytochemicals of *R. communis* leaf can damage and disrupt the cellular membrane. Strain HSM85 showed utter insensitivity to the methanol extract, so no DNA/RNA concentration was detected in the cells treated with the methanol extract. Mogana et al. [2020] described that if the MBC/MIC ratio is > 4, the effect would be bacteriostatic, and if the MBC/MIC ratio is ≤ 4, the effect would be bactericidal [50]. In the current study, the MBC/MIC ratio of all extracts, except methanol, which was insensitive to strain HSM85, ranged from 1 to 4. The MBC/MIC ratio: 4 < of all extracts alluded that *R. communis* leaf was a potential bactericidal agent [Mogana et al. 2020] [50]. Based on the MBC/MIC ratio, the highest bactericidal activity was observed in the chloroform extract [1] against HSM85 and SLS01, and the lowest MBC/MIC ratio: 4 against DA03.

5 Conclusion

In brief, the current study reports the characterization of three novel bacillus pathogens from the milk of bovine mastitis and their in vitro management by using *R. communis* leaf. Based on 16S rRNA gene sequence analyses and molecular phylogeny, isolates HSM85, SLS01, and DA03 were identified as *B. stercoris*, *B. safensis*, and *B. paranthracis*, respectively. The GC–MS data revealed that the abundance and biological activity of the herbal extract greatly vary according to the solvents used for extraction. All extracts of *R. communis* leaf, except methanol, which showed no activity against the HSM85 strain, exhibited significant antibacterial activity against all three strains. The results of agar-well

diffusion, MIC, MBC, cell-killing, biofilm inhibition, protein inhibition assay and DNA/RNA leakage and MBC/MIC ratio suggest that the *R. communis* leaf extract could be used as a sustainable, safe, and effective alternative drug alone or as an adjunct to antibiotics in the management of mastitis caused by *Bacillus* pathogens. The GC–MS analysis of *R. communis* leaf revealed the highest percentage of phytol in the methanol; however, it showed poor antibacterial activity against SLS01 and DA03, while no activity against HSM85. In ethanol, acetone, and chloroform extracts, heneicosane and tetracontane were observed as major compounds along with phytol, which may be one reason for the high antimicrobial activity. However, this could be confirmed with further antimicrobial assays with purified compounds. In order to develop *R. communis* leaf extract as an antimicrobial compound, we are in the process of purifying and identifying the specific phytochemical with potent antimicrobial activity for the future.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43088-025-00666-2>.

Additional file1. Supplementary Fig. 1. SDS-PAGE showing whole cell protein profiling of treated [test] and untreated [control] cells of **a** HSM85, **b** SLS01, **c** DA03 strains. Dose of leaf extracts: ½ MIC.

Additional file2. Supplementary Fig. 2. Heat-map showing major phytoconstituents observed in leaf extracts of *R. communis* [color code represents % abundance of phytoconstituents].

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Author contribution

DA, PP, JNM, HSM, SS, wrote the main manuscript text, JNM, SM, SS, DA, PP prepared the figures. All authors have read and approved the manuscript.

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Data availability

No datasets were generated or analyzed during the current study.

Declarations

Competing interests

The authors state that they have no conflicts of interest, financial or otherwise.

Ethics Approval and Consent to Participate

Not applicable.

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