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Investigation of the antibacterial properties of *Moringa oleifera* extracts against *Xanthomonas citri* pv. *glycines* the incitant of bacterial pustules of *Glycine max*

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Abstract

Bacterial pustule disease, incited by *Xanthomonas citri* pv. *glycines* (*Xcg*), represents a significant phytopathological constraint limiting soybean productivity across major cultivation zones in India, with epidemiological reports documenting moderate-to-severe disease intensities causing considerable yield losses. The present study evaluated the in vitro antibacterial efficacy and in vivo prophylactic potential of *Moringa oleifera* Lam. extracts against *Xcg* under controlled conditions. Key physiological and biochemical parameters, including leaf number per plant, soluble protein, total soluble carbohydrate, and photosynthetic pigment content, were quantitatively assessed to determine host plant responses to pathogen challenge and extract treatment. In vitro bioassay results demonstrated that seed extract at 500 µg/mL elicited the most pronounced antibacterial activity, producing a zone of inhibition of 18.86 mm. Under greenhouse pot trial conditions, seed extract treatment significantly enhanced plant height, carbohydrate, chlorophyll, and carotenoid accumulation relative to inoculated controls. Soluble protein and carbohydrate concentrations exhibited a concentration-dependent decline at elevated extract doses, a trend similarly observed with the reference antibiotic streptomycin, suggesting potential phytotoxic effects at supraoptimal concentrations. At the highest evaluated concentration, seed extract formulation SE5 significantly reduced disease severity (DS) to 20.34% and achieved a percentage efficacy of disease control (PEDC) of 75.46%. Furthermore, the bioactive phenolic compound cinnamic acid, identified as a principal constituent of the seed extract, demonstrated a comparable zone of inhibition of 18.95 mm against *Xcg*, substantiating the mechanistic role of hydroxycinnamic acid-class phenolics in pathogen suppression. Collectively, these findings establish the phytotherapeutic potential of *M. oleifera* seed extracts and cinnamic acid as promising biopesticidal agents, warranting validation through large-scale field experimentation and integration into sustainable soybean crop protection and management strategies.

Keywords Soybean, Bacterial pustules disease, *Xanthomonas*, *M. oleifera*



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1 Introduction

Sustainable food production for a rapidly growing human population is one of the major challenges faced by the agriculture sector globally [1–3]. Therefore, the increased uses of pesticides and synthetic agrochemicals have become essential to maximize agricultural productivity. Despite their beneficial role in agriculture, they can be hazardous to humans and other non-targeted organisms [4, 5]. According to the Food and Agriculture Organization of the United Nations, an estimated 3.2 million tones of pesticides are used on crops each year [6]. In recent years, unrestrained use of commercial and synthetic agrochemicals in protection of crop has raised significant concern about environmental contamination and resistance enhancement in phytopathogenic microbes [7]. To address these issues, the development of bio-based/non-synthetic biocides for agriculture has become an important research direction.

Soybean [*Glycine max* (L.) Merrill] is significant and major pulse crop worldwide that delivers two-third of calories derived from agriculture [8] and accounts for half of the global demand for oil and vegetable protein. It is high in edible oil (18–22%) with major essential amino acids and proteins (38–42%). Its oil and food make up about 85% of the global soybean edibles including soy milk, tofu, soy sauce and tempeh [9, 10]. Federation of Indian Chamber of Commerce and Industry (FICCI) claims that India currently produces 25% of its oil and 43% of its oilseeds from soybean, making it the fourth-largest producer of soybeans in the world, behind the United States, Brazil, Argentina and China [11].

Despite all these aspects, soybean yield in India currently stands at around 1 t/ha to 3.5 t/ha, there is still an urgent need to increase production to meet the demand of ever increasing population [12]. Bacterial pustule disease, caused by the Gram-negative bacterium *Xanthomonas citri* pv. *glycines* (*Xcg*) of the family Xanthomonadaceae [13], represents a significant threat to soybean productivity. Unlike vascular pathogens, *Xcg* primarily colonizes the leaf mesophyll, where it induces characteristic hypertrophic pustules and chlorotic lesions. These symptoms severely impair the plant's photosynthetic capacity and can lead to premature defoliation, ultimately compromising crop yield. It was first identified and named as *Bacterium phaseoli* var. *sojense* [14]. The pathogen underwent reclassification to *X. campestris* pv. *glycines* [15], then *X. axonopodis* pv. *glycines* (*Xag*) [16] after DNA-DNA hybridization analysis, ultimately being named *X. citri* pv. *glycines* [13].

It is among the most serious diseases that reduces the yield and quality of the crop [17]. It limits the soybean production worldwide by 20–40%. This disease runs rampant in most soybean-growing fields and may cause yield loss as high as 53% under favorable conditions [18]. It may enter soybean leaves via natural openings such as stomata and wounds and proliferate in intercellular spaces [19]. Typical symptoms are small, light yellow colored pustules surrounded by chlorotic halos on the underside of soybean leaves [20]. The pustules spots range in size from small brown patches to large. Numerous management strategies, including the use of synthetic agrochemicals, commercial antibiotics and nanoformulations, have been studied [13, 17] but have side effects on overall plant growth and development. Therefore environment-friendly strategies and plant based products for disease management is necessary. Some studies on biocontrol investigations on stem rot by the fungus *Sclerotinia sclerotiorum*, root rot by the fungus *Phytophthora sojae* and cyst by the nematode *Heterodera glycines* were evaluated in

previous years [22], also bio based formulations were investigated [23] but plant based disease prevention and management studies of bacterial pustules disease of soybean have not been identified yet.

M. oleifera Lam. (Family: Moringaceae, commonly known as horseradish, drumstick, ben oil, miracle, and Mother's best friend), one of the 13 species of the monogeneric family Moringaceae, native to the sub-Himalayan lowlands in northwest India, shows remarkable medicinal potential [24]. Traditionally, *M. oleifera* has been associated with various medicinal benefits, including pain relief, controlling blood sugar levels, liver protection, anti-inflammatory effects, promotion of urination, reduction of pain sensitivity, prevention of artery hardening, blood pressure regulation, antioxidant properties and tumor growth inhibition [21, 22]. Different phytochemicals like phenols, omega fatty acids, flavonoids, carotenoids, kaempferol, terpenoids, tocopherols, tannins, vanillin, glycosides, β -sitosterol, triterpenoids, ascorbates, quercetin and sterols have been isolated from the flowers, leaves, roots, seeds and fruits [23, 24, 25–28]. In particular, this plant family is rich in compounds containing the simple sugar rhamnose and it is rich in a fairly unique group of compounds called glucosinolates (β -thioglucosideN-hydroxysulfates) and isothiocyanates (4- α -L-rhamnopyranosyloxy) benzyl isothiocyanate [29].

About 20% of all plants have undergone biological and pharmacological testing and a significant number of new antibiotics introduced in agriculture sector are from natural or semi-synthetic resources. For this, in the present investigation, we report for the first time the in vitro and in vivo efficacy of different plant parts methanolic extract against *Xcg* along with the antibiotic streptomycin, which is widely used for controlling this disease in soybean field. Our results convincingly establish effective control of bacterial pustule disease of soybean.

In search for potential phenolic compounds against bacteria several studies have done before, especially against the genus *Xanthomonas* and its strains as it damaged the crop at higher level. Many studies suggest that these compounds inhibited bacterial growth especially quercetin exhibited antibacterial potential against *Xcg* [30]. In another study, p-coumaric acid induced phenolic metabolites alleviated the disease symptoms caused by *Xanthomonas campestris* pv. *campestris* (Xcc) in cabbage [31]. Peach tree bacterial shot hole disease, a global concern, is caused by pathogen *Xanthomonas arboricola* pv. *pruni* (Xap) this bacteria was effectively controlled and inhibited by the derivatives of cinnamic acid [32].

2 Materials and methods

2.1 Chemicals

Folin–Ciocalteu's reagent, diphenyl-1-picrylhydrazyl (DPPH), sodium bicarbonate (Na_2CO_3), HCl, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride (FeCl_3), methanol, gallic acid, acetic acid, sodium acetate, nutrient broth (modified), Lethen agar (modified), certified seeds of soybean (JS 27), and Streptomycin were of analytical grade purchased from Himedia, India. 16s-rRNA was purchased from Merck. Molecular grade Tris–HCl buffer, EDTA, β mercaptoethanol, CTAB and Isoamyle alcohol were purchased from Sigma Aldrich.

2.2 Collection of plant material and preparation of extract

Various parts of *Moringa oleifera*, including the leaves, flowers, seeds, gum, and bark (Fig. 1A), were collected from the University College of Science campus at Mohanlal Sukhadia University (MLSU), Udaipur, Rajasthan. The botanical identity of the plant was verified against a herbarium sheet by Dr. S. L. Meena, Scientist E and Head of Office at the Botanical Survey of India (BSI), Jodhpur, India (Ref. No: BSI/AZRC/I.12012/Tech./2021-22 (PI.-Id.)/412; dated 14-12-2021). The collected plant parts, except gum, were washed thoroughly with sterile water, then shade-dried at room temperature and ground to coarse powder by using a blender. The materials kept in airtight jars until further experimentation. 50 g dried powder of each plant part was extracted with 250 mL methanol using a Soxhlet apparatus. While 5 g gum was kept in 25 mL methanol for 4 days at room temperature. The extract was filtered through a membrane filter using Whatman No. 1 filter paper. The filtrate was reduced through a rotary vacuum evaporator at 50 °C, 80 rpm to obtain the concentrated extract (Fig. 1B), which was then frozen and freeze-dried at – 20 °C until analysis.

2.3 Total phenolic content

Total phenolic content (TPC) of *M. oleifera* different extracts was determined by the Folin-Ciocalteu's method [33]. 2 mg of each extract was dissolved in 1 mL of methanol. The freshly prepared Folin-Ciocalteu's reagent (2.5 mL) was mixed with 500 µL of extract, and then 400 µL of sodium bicarbonate was added and kept for 1 min in a boiling water bath. For the calibration curve, gallic acid (30–300 µg/mL) was used, and

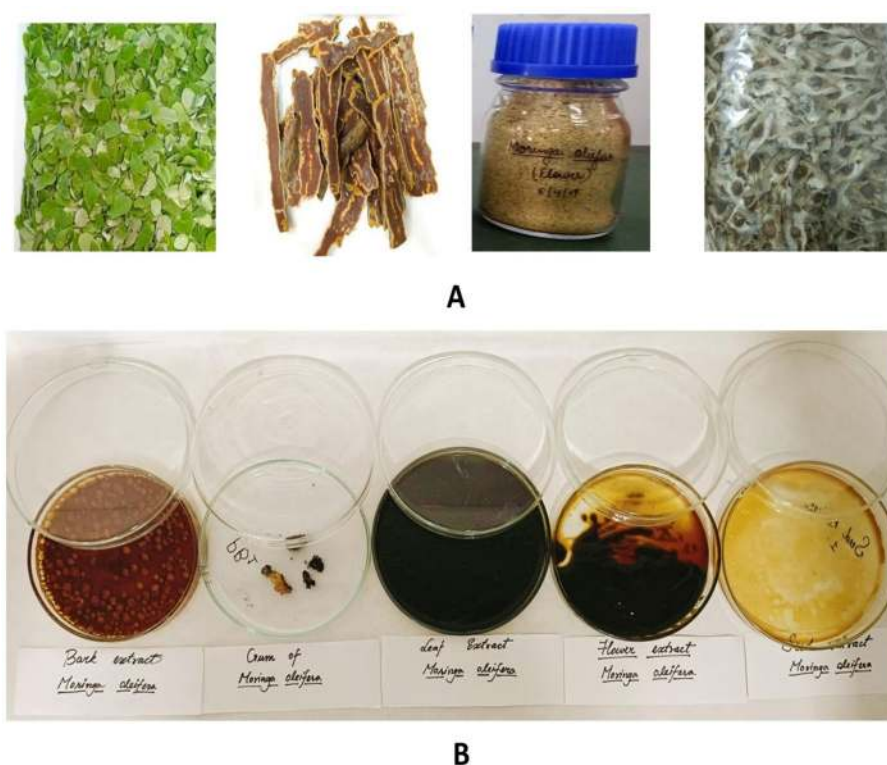


Fig. 1 (A) *M. oleifera* plant parts used for extraction (leaves, bark, flower powder, and seed). (B) Extracts obtained after evaporation through a rotary vacuum evaporator along with gum

data were recorded at 650 nm. TPC was expressed as mg gallic acid equivalent/g DW of extract.

2.4 Antioxidant activity

2.4.1 DPPH radical scavenging capacity

This antioxidant activity was calculated by DPPH method [34]. Different extracts of *M. oleifera* (2 mg/100 µL) were added to the reaction mixture, having 1 mL of DPPH, 1.5 mL of ethanol, and 500 µL of acetate buffer (pH 5.5). This reaction mixture was kept in a dark place at room temperature. After half an hour, the absorbance was measured at 517 nm. Methanol served as the blank. The radical scavenging activity was expressed in percentage using the equation.

$$\% \text{ scavenging activity} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_s = absorbance of sample solution, A_c = absorbance of control.

2.4.2 FRAP analysis

The ferric reducing power of different extracts was determined by the method given by Benzie and Strain [35]. The FRAP reagent was incubated for 30 min at 37 °C in a water bath. 100 µL of extract was added to the FRAP reagent (3 mL) and shaken vigorously. Absorbance was measured at 593 nm using a spectrophotometer. Results were expressed in µM Fe^{2+} /G, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (100–1000 µg/mL) was used as a standard for the calibration curve.

2.5 Isolation and purification of *Xcg*

The pathogen was isolated from the infected soybean leaves showing typical disease symptoms collected from soybean fields of RCA, MPUAT, Udaipur, Rajasthan, during 2023. The yellowish-brown infected portion of the leaves was subjected to the isolation of the pathogen (Fig. 3) on Lethen agar media incubated at 29.2 °C. The colony developed on the periphery was streaked on fresh media [36]. Pure cultures were established by single colony isolation and maintained in a refrigerator at 5 °C by sealing 48 h old cultures on media.

2.6 Pathogenicity and host range

Bacterium pathogenicity was confirmed on 30 days old soybean plants grown in 25 cm pots in a glass house. The plants were kept in humid chamber (30 ± 1 °C, 80–90% RH) for 24 h before inoculation for stomatal opening and to optimize high intercellular humidity in the surrounding tissues of natural openings. 48 h old bacterial culture was gently scrapped from slants and suspended in DDW. An inoculation (2×10^8 CFU/mL) was made by spray and pinpricks to runoff the methods. When the disease symptoms occur (Fig. 3), the pathogen is again isolated from the inoculated plant and compared with the original culture. Suitable controls were maintained, and pathogenicity was proved by following Koch's postulates.

2.7 Morphological and biochemical characterization of bacterium

The isolated bacterium was examined for cell flagellation, Gram staining, KOH solubility and catalase. Methyl red test, urease, and growth in NaCl concentration were also tested [37].

2.8 Molecular characterization and identification of the pathogenic bacterium

Genomic DNA from the pathogenic bacteria strain was extracted using the CTAB protocol following the procedure outlined by [38]. The obtained DNA was purified, quantified, and its quality assessed through agarose gel electrophoresis and spectrophotometric analysis using a Nanodrop (Thermo Scientific, USA). The final DNA sample was adjusted to a concentration of $50 \text{ ng } \mu\text{L}^{-1}$ and served as the template for PCR. The 16S rRNA gene was amplified with the universal primers 27F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), targeting approximately a 1465 bp fragment. PCR amplicons were separated on a 1% agarose gel, and the desired band was cut out and purified using a commercial gel extraction kit (Promega, USA) according to the manufacturer's guidelines. Bidirectional sequencing was performed to ensure complete gene coverage. The resulting sequences were analyzed using the BLAST nucleotide search tool available at the National Center for Biotechnology Information (NCBI) to determine the closest bacterial match.

2.9 Antibacterial activity of plant extracts

Antibacterial activity of different extracts from *M. oleifera* was evaluated by the agar well diffusion method with slight modifications given by [39]. Active cultures were prepared by transferring a loop full of cells from the stock cultures to test tubes containing nutrient broth for bacteria that were incubated for 24 h at $29 \pm 2^\circ\text{C}$. To prepare the samples for the antibacterial assay, 100–600 $\mu\text{g/mL}$ of each extract was dissolved in methanol and sonicated for 30 min. Pure methanol was used as a negative control, and a standard antibiotic, streptomycin (30 $\mu\text{g/mL}$), was used as a positive control. Petri plates containing 20 mL of Lethen agar medium were seeded with a 24 h old culture of bacterial strains. 100 μL of each test solution was poured into the respective wells and incubated for 24 h at $29 \pm 2^\circ\text{C}$. The antimicrobials present in the extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organism. The resulting diameter of the zone of inhibition was measured in millimeters (mm).

2.10 Pot experiment

JS27, a non-resistant variety of soybeans acquired from an authorized local vendor at Krishi Kendra, Udaipur, Rajasthan was used for plantation. The study was conducted from July 2023 to November 2024 to investigate the antibacterial effect of seed extract against *Xcg*. The experiment was conducted following a Completely Randomized Design (CRD) with three replicates. While bark extract also showed significant inhibition in the agar well plate method, it was not considered in pot experiment because it was partially dissolved in solvent, both water and methanol, and a thin extract layer was observed on leaves, so the penetration of the extract was blocked, and it clogged the natural openings. A pot experiment was performed in the greenhouse of the Botanical garden, Department of Botany, UCOS, MLSU, Udaipur, Rajasthan, India. For this, pots (33 cm height $\times$ 23 cm bottom $\times$ 30 cm top) were sterilized with 20% CuSO_4 solution. Approximately

8 kg of sandy soil per pot was autoclaved, cooled and filled into the pre sterilized pots. Before sowing, the seeds were soaked in broth, containing the pathogen for thirty minutes. Subsequent to sowing, the soil was inoculated with the pathogen by spraying 10 mL of water-suspended bacterial solution to ensure timely disease development. A total of eight pots were prepared for experimentation, with five allocated for assessing the impact of seed extract treatment against *Xcg*. Streptomycin (STRP) served as positive control, and *Xcg* inoculated diseased plant as negative control (NC). Both controls were compared with healthy plant (non treated-NT) and treatments.

In late July seeds were sown in the pre sterilized pots at a depth of 3–3.5 cm for optimal seed viability as below 5 cm seed viability reduces, and were allowed to grow under ambient atmospheric conditions. The plant was allowed to grow for 10 days above the surface without further inoculation. Approximately 35 days after sowing, plants achieved decent growth so first foliar spray was given to plants with 24 h old culture of *Xcg* [40]. Disease manifestation was observed after ten to twelve days. Following the onset, different concentrations of *M. oleifera* seed extract 100–600 µg/mL and STRP (30 µg) were sprayed on the potted plants after 48 h of the second foliar spray. Biochemical parameters were also measured after the second foliar spray. Plant height, leaf per plant, disease severity (DS) and percent efficacy of disease control (PEDC) were observed and recorded after 10 days of the last spray. Three replicates per concentration were maintained, and for each replicate 4 plants per pots were placed.

2.11 Biochemical analysis

2.11.1 Chlorophyll and carotenoids

The quantitative estimation of chlorophyll and carotenoids was performed by using the method of [41]. Fresh samples from healthy and inoculated plant leaves were collected after 10 days of inoculation from the greenhouse. 1 gram of plant leaves was chopped and homogenized to a fine pulp by using 85% acetone for 5 min and centrifuged for 10 min at 2000 rpm. The supernatant was made up to 30 mL with 80% acetone; aqueous acetone is used as a blank. The absorbance was measured at 665, 663, and 450 nm by using a spectrophotometer. Chlorophyll a, b, and total chlorophyll content were calculated by these formulas:

- Total chlorophyll (mg/g) = $(20.2A_{645} + 8.02 A_{663} \times V)$.
- Chlorophyll a (mg/g) = $(12.7A_{663} - 2.69A_{645} \times V)/AC1000 \times W$.
- Chlorophyll b (mg/g) = $(22.7 A_{645} + 4.68 A_{663} \times V)/A \times 1000 \times W$.
- Carotenoids (mg/g tissue) = $(10A_{450} \times V \times F)/2500 \times W$.

Where, A (absorbance) nm in a 1 cm cell, V = volume of extract in mL, W = fresh weight of the sample, F = dilution factor.

2.11.2 Determination of soluble protein content

Soluble proteins were estimated by the method given by [42] using bovine serum albumin as a standard. 1 g of fresh leaves was ground in 5 mL chilled phosphate buffer (0.1 M, pH 7.5) and centrifuged at 12,000 rpm for 15 min. Supernatant was used for analysis. 500 µL of extract was added to Coomassie Brilliant Blue reagent and vortexed for 30 s after color development; absorbance was noted by using a spectrophotometer at 595 nm, and the results were expressed in mg g⁻¹ dry weight through the calibration curve with bovine serum albumin.

2.11.3 Determination of total carbohydrate content

Estimation of carbohydrate content was carried out by the method given [43]. 5 g of powdered sample was homogenized in 4 mL of 80% ethanol, and the total volume was made up to 25 mL. This solution was subjected to a centrifuge for 10 min at 2500 rpm, and the supernatant solution was used for analysis. A suitably diluted aliquot was mixed with 4.5 mL of freshly prepared anthrone reagent and heated for 10 min. The absorbance was noted at 620 nm, and total carbohydrate content was calculated with the help of a calibration curve with glucose and expressed as mg g^{-1} dry weight of samples.

2.12 Disease assessment through DS and PEDC

Bacterial pustule disease was assessed through the observation of leaves. Lesion formation and its amalgamation are crucial for any plant protection and disease control strategy. Therefore, DS and PEDC were evaluated against bacterial pustule disease in soybean through visual observations. DS was evaluated by using a severity scale ranging from 0 to 5 (0 = no visible symptoms on leaves, 1 = a few individual lesions, 2 = many individual lesions, 3 = small diseased patches of lesions, 4 = medium sized patches of lesions, and 5 = large patches of lesions) provided by [44–46]. Further, DS and PEDC were calculated by the formula given by [37, 38].

$\text{DS} = \text{Sum of all individual disease rating} / \text{total number of leaf assessed} - \text{maximum rating} \times 100.$

$\text{PEDC} = \text{DS in control} - \text{DS in treatment} / \text{DS in control} \times 100.$

2.13 Isolation and HPLC analysis of phenolic compounds

M. oleifera seeds' dry powder (500 g) was exhausted in a reflux apparatus with a methanol: water 7:3 v/v (500 × 1 L) solvent system at 50 °C for 5 × 3 h. The extract was filtered and concentrated under vacuum at 45 °C. This extract was subjected to partition thrice with hexane for the removal of photosynthetic pigments and lipid substances. The remaining methanolic phase was concentrated in a vacuum with silica gel 60–120 mesh size to make silica slurry and stored until use. 100 gm of free flowing extract with silica slurry was eluted with a gradient of dichloromethane/methanol (100:00, 95:5, 90:10, 85:15, 80:20, 75:25, 50:50) and with pure methanol (100 mL each) to afford 55 fractions. After TLC analysis with DCM: Methanol (9:1), the column fractions were pooled in five groups (MOF 1–5). These pooled fractions were analyzed by sub-columns and visualized on TLC plates in a UV chamber.

HPLC analyses were performed with a Waters Alliance 2695 separation maintained at 25 °C with a PDA detector. Test samples (20 μL) were injected into a reverse phase column C18 (250 mm length, 4.6 mm, and 5 μm). The samples were dissolved in methanol and filtered with 0.45 μm nylon filters. 1 mL/min flow rate, 254 nm λ , mobile phase A 0.05% TFA in aCn, and B 0.05% TFA in water were used as chromatographic conditions. Gradient elution (T/A %) from 0/5, 30/25, 40/40, 50/63, 60/5, and 66/5 and 20 $\mu\text{g}/20 \mu\text{L}$ concentrations of samples were used. The compositions of the mobile phase were rising over the period of 20 min.

2.14 Antibacterial activity of isolated phenolic compound

Isolated phenolic compounds from the seeds of *M. oleifera* were evaluated by the agar well diffusion method given by [39] as described earlier in Sect. 3.9. Instead of seed

extract here, we used pure compounds, i.e., p-coumaric acid, quercetin, and cinnamic acid. For antibacterial activity, 50–300 µg/mL of each compound was dissolved in 1 mL of methanol and sonicated for 5 min. All experiments were performed in triplicates, and results were expressed in mm after measuring the zone of inhibition.

2.15 Determination of minimum inhibitory concentrations (MICs)

The MIC of seed extract and isolated phenolic compounds was determined by using the broth dilution method [47]. The test samples were dissolved in methanol and added to LB medium to get 1 mg/mL, which were serially diluted to 1, 0.5, 0.125, 0.06, 0.03, and 0.015 mg/mL, respectively. 50 µL of 24 h old *Xcg* suspension with 1.5×10^8 CFU/mL was transferred into each tube. All the tubes, along with the control, containing *Xcg* suspension in broth were incubated at 29 ± 1 °C for 24 h. The absorbance was noted at 600 nm by using a UV visible spectrophotometer. Results were expressed in percentage by using the formula given below $(Ab_c - Ab_s)/Ab_c \times 100$. Where Ab_c is control (broth with *Xcg*) and Ab_s is sample (broth with *Xcg* and compound) absorbance. The lowest concentrations of the samples, which did not show any bacterial growth, were determined as MICs and were expressed in mg/mL.

3 Statistical analysis

Data recorded from the experiments are expressed as mean $\pm$ standard deviation of triplicate determination ($n=3$). All the statistical analysis were accomplished by using graph pad prism10.4.2 software. The significant differences among treatments in pot experiment and antioxidant potential were determined by Tukey-Kramer HSD test at $p=0.05$. Dunnett's post hoc test was used for comparative analysis in zone of inhibition at $p=0.05$.

4 Results

4.1 Total phenol content

It was reported that the antioxidant activity of plant extracts is well correlated with the content of their phenolic compounds. Total phenolic content of the extracts was calculated using the standard curve of gallic acid. Phenol molecules possess a hydroxyl group (-OH), making them polar. This polarity allows them to dissolve readily in polar solvents. The highest total phenolic content was found in the bark extract (56.10 GAE/g), followed by seeds (42.00 GAE/g), leaves (33.13 GAE/g), and flower (13.6 GAE/g). The lowest total phenolic content (5.63 GAE/g) was obtained from gum extract (Fig. 2A). Results indicated that bark and seed extract contained a higher amount of phenols as compared to other extracts.

4.2 DPPH radical scavenging capacity

In the present study, the extracts of *M. oleifera* plant parts were investigated for antioxidant activity using the DPPH free radical scavenging assay. The antioxidant present in plant extract neutralizes the DPPH by the transfer of an electron or hydrogen atom. The methanolic extracts of different plant parts showed varying scavenging ability. Among these five, bark extract showed the highest free radical scavenging effect in percentage (74.70), followed by seed extracts (70.39), leaf extract (54.63), and flower extract (42.40) (Fig. 2C). Again lowest DPPH activity was observed in gum extract (10.61).

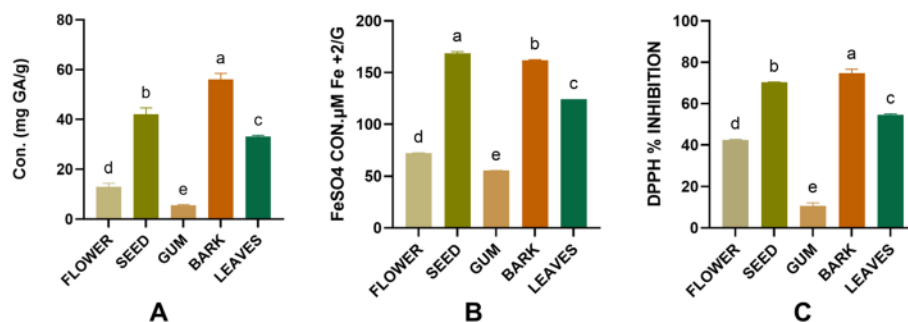


Fig. 2 Quantification of total phenolic content (A), FRAP (B), and DPPH free radical scavenging activity (C). Bar diagrams represent the mean $\pm$ SD for three replicates of each extract followed by significantly different letters ($P < 0.05$) as determined by the Tukey-Kramer HSD test

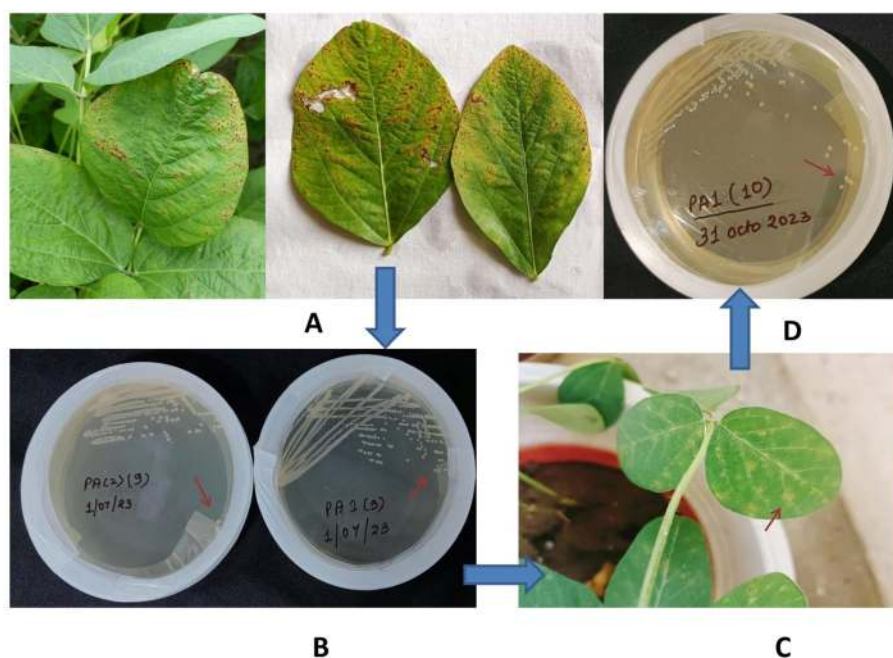


Fig. 3 Isolation of inoculum (A) Infected leaves collected from a soybean field (B) Pure *Xcg* colony (red arrow) on Letheen agar media after isolation (C) Disease symptoms on leaves in pot experiment induced through foliar application of *Xcg* (D) *Xcg* colony observed again on media from inoculated plant (red arrow denotes pure colonies isolated from the infected part of soybean leaves)

4.3 FRAP analysis

FRAP analysis was performed with a similar concentration used in DPPH activity by using the standard curve. Seed extract displayed the highest activity with 168.61 μ M Fe²⁺/g, followed by bark extract 162.11 μ M Fe²⁺/g and leaves extract 124.25 μ M Fe²⁺/g (Fig. 2). However, flower and gum extract did not show any significant results (Fig. 2B).

4.4 Isolation, morphological identification of test bacterium

The bacteria were isolated from naturally infected leaves of soybean (Fig. 3A) on Letheen agar media after 72 h of inoculation. Single semitransparent gummy, circular, pale yellow colonies were observed (Fig. 3B). When this purified bacterium inoculated on soybean plant leaves showed typical pustule symptoms as pale yellow spots near the interveinal area (Fig. 3C), they become large brown patches on leaves. All the inoculated plants

yielded the same bacteria on reisolation (Fig. 3D) within 15–17 days after inoculation with circular light yellowish-brown spots on leaves. Isolated bacteria were rod-shaped ($0.9 \times 1.6 \mu\text{m}$), gram-negative, capsulated with single polar flagella. These observations of morphological parameters indicated its similarity with the genus *Xcg*.

4.5 Characterization of test organism

4.5.1 Biochemical characterization

The isolated bacterium showed growth between 12 and 37 °C, pH 5.0–8.0, and NaCl concentration at 2.5–4.5%. It was KOH soluble and negative for urea hydrolysis. The bacteria did not utilize the carbohydrate but derived energy and produced acid from fructose, mannose, sucrose, dextrose, and mannitol. Based on biochemical characteristics, the host range and specificity of the bacterium for soybean, it was related to *X. citri* variant *glycine*.

4.5.2 Identification of pathogenic bacterial isolate using 16 S rDNA sequencing

The isolate was subjected to species identification using 16 S rRNA gene sequencing. Sequences of ribosomal genes were end trimmed, counted, assembled, and BLAST analyzed in the NCBI database. The curated sequence yielded a 1387 bp length that was used to determine the species identity of bacteria by the closest match in the NCBI database. Species identity of the isolates was established and is presented in Table 1. The sequence was submitted to GenBank with accession number (PX686996). Agarose gel image of PCR-amplified 16 S rRNA products from bacterium for soybean, identified as *X. citri* variant *glycine* has been provided as supplementary material.

4.6 In vitro antibacterial activity by different extracts of *M. oleifera*

The agar well plate method is widely used to evaluate the antimicrobial activity of different extracts. Contact and diffusion of the extract into the media are better in the well plate method. Evaluation of the antibacterial activity of *M. oleifera* plant extracts against plant pathogenic bacteria *Xcg* was illustrated in Fig. 4.

The results revealed that plant extracts were potentially effective in suppressing microbial growth of *Xcg* in a sequential manner; when the concentration of extract increased, the zone of inhibition also increased (Fig. 5). Therefore, it showed a larger sized inhibition zone. Each well is of the same diameter. The results were recorded after 24 h. Seed extract was the most effective extract for retarding microbial growth of pathogenic bacteria at 500 µg/mL concentration, showing an 18.86 mm zone of inhibition, followed by bark extract at 600 µg/mL (18.16 mm), flower extract 600 µg/mL (15.36 mm), leaf extract at 600 µg/mL (15.26 mm), and gum extract at 600 µg/mL (5.24 mm) (Fig. 4). It was observed that the methanol extract of leaves and flowers was slightly less active against the test organism. However, the reference plate of streptomycin showed a wider zone of inhibition as compared to the seed and bark extract.

Table 1 Identity based on the closest match of 16 S rDNA sequences

Isolate name	Seq. length (bases)	Max score	Total score	Query cover	E value	Identity %	Closest match accession no.	Closest match bacterial species	Assigned accession no.
Gm_ <i>Xcg</i>	1387	2562	5124	100%	0.0	100.00%	CP009040.1	<i>Xanthomonas citri</i> pv. <i>glycines</i>	PX686996

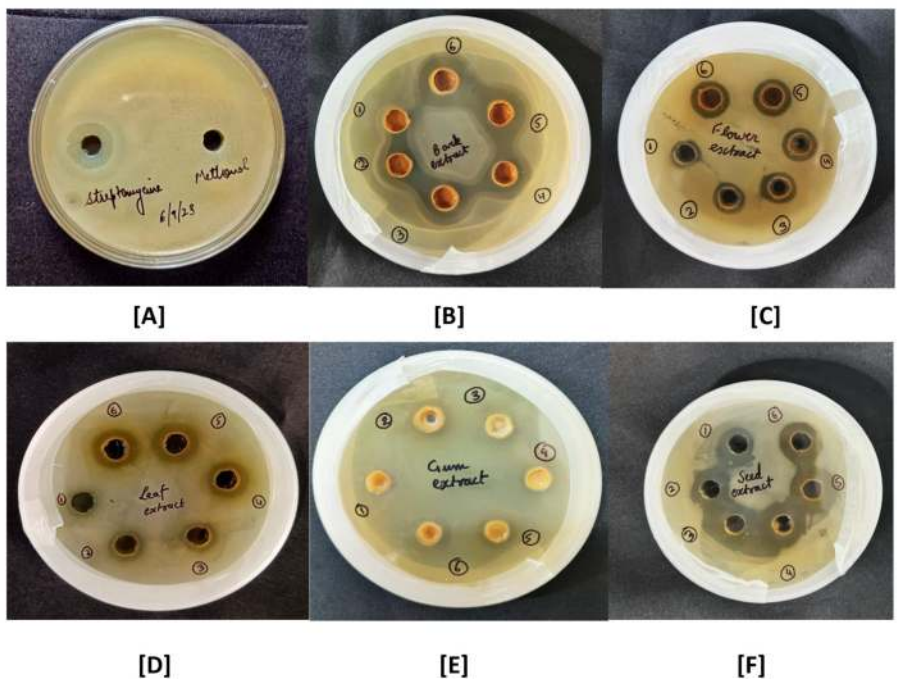


Fig. 4 Inhibitory impact of different concentrations of *M. oleifera* plant parts on cell growth of *Xcg* is indicated as the zone of inhibition. [B Bark extract, C flower extract, D leaf extract, gum extract, and F seed extract. Plat A represents the control STRP and methanol effect against *Xcg*

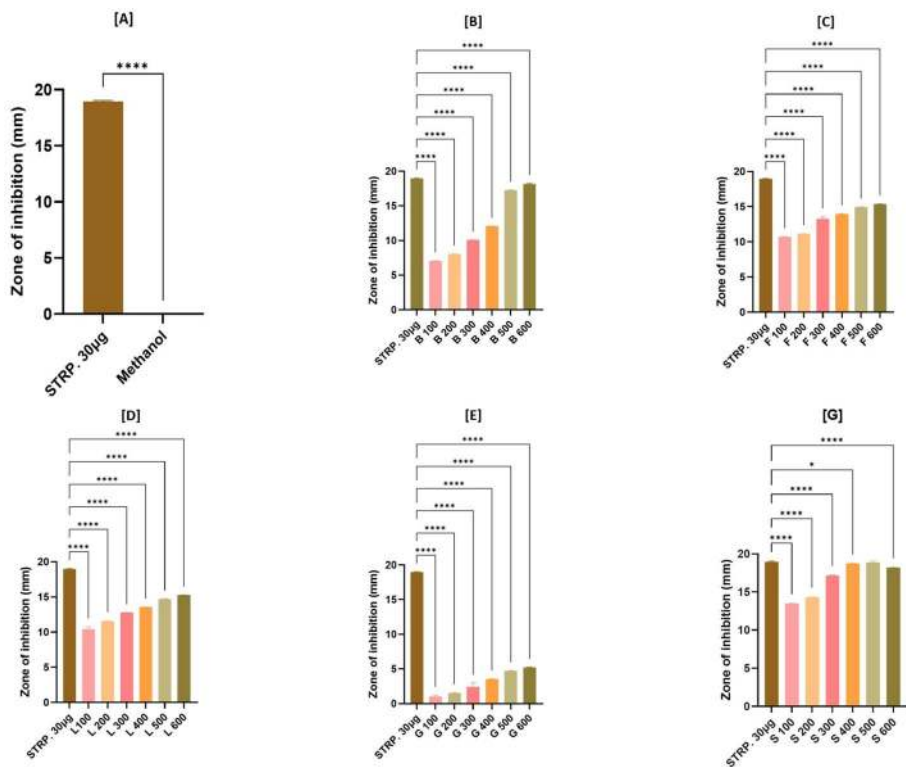


Fig. 5 Bar diagrams represent the effect of different *M. oleifera* plant part extracts against *X. pv. glycine* in the agar well plate method. Bar diagrams represent the mean $\pm$ SD for three replicates of each extract, followed by different asterisks that showed comparative significant differences ($p < 0.05$) as determined by Dunnett's post hoc test. Streptomyacin (STRP), B (bark), F (flower), L (leaves), G (gum), S (seeds)

4.7 Biochemical analysis

4.7.1 Chlorophyll and carotenoids content

The result of the preventive effect of seed extracts on chlorophyll and carotenoid contents is summarized in Table 2. Data revealed that seed extract was positively correlated with chlorophyll and carotenoid content. The maximum chlorophyll content was observed in SE4 (1.98 mg/g), followed by SE5 (1.96 mg/g), SE3 (1.66 mg/g), and SE1 (1.22 mg/g) as compared to STRP (1.81 mg/g). However, it was lower than healthy NT plants (2.12 mg/g) but higher than NC (1.13 mg/g). Similar results were observed in Chlorophyll a and b content. The highest weight was observed in SE4 (1.19 mg/g, 0.84 mg/g), followed by SE5 (1.14 mg/g, 0.82 mg/g), SE3 (1.01 mg/g, 0.76 mg/g), SE2 (0.97 mg/g, 0.72 mg/g), and SE 1 (0.91 mg/g, 0.67 mg/g). In control groups Ch a and b content was significantly different, where NT showed the highest content (1.33 mg/g, 0.97 mg/g) and NC showed the lowest (0.88 mg/g, 0.59 mg/g). While in carotenoids content extracts, results were positively correlated with it. SE4 showed the highest carotenoids (0.98 mg/g), followed by SE5 (0.97 mg/g), SE3 (0.89 mg/g), SE2 (0.81 mg/g), and SE1 (0.74 mg/g). As compared to STRP (0.94 mg/g) SE5 and SE4 were significant.

4.7.2 Protein and carbohydrate content

The result of the effect of seed extract prepared from *M. oleifera* on biochemical parameters, i.e. carbohydrate and protein content of Soybean, is summarized in Table 2. Data clearly indicated that seed extract increases carbohydrate and protein content of the host plant. The maximum carbohydrate content was observed in SE 4 (4.022 mg/g), followed by SE 5 (4.01 mg/g), SE 3 (3.34 mg/g), SE 2 (3.16 mg/g), and SE 1 (3.06 mg/g), except for NC (2.61 mg/g). Carbohydrate in STRP (3.94 mg/g) and healthy plant (5.08 mg/g) were observed, which were significant.

Similarly, protein content was increased as the concentration of seed extract increased. The highest protein content was observed in SE 4 (9.81 mg/g), followed by SE 5 (9.24 mg/g), SE 3 (7.76 mg/g), SE 2 (5.48 mg/g), and SE 1 (4.05 mg/g). All the treatments are significant as compared to streptomycin (8.43 mg/g) and healthy plant (13.95 mg/g) for protein, except NC (1.67 mg/g).

4.8 Plant height and leaf per plant

Results of the effect of seed extracts on the number of leaves per plant are given in Table 2; Fig. 6. The data indicated that there was a slight increment in the number of leaves as the extract concentration increased. The maximum increase in the number of leaves/plant was observed in treatments with SE 4 (24.33), followed by SE 5 (21.67), SE 3 (19.33), SE 2 (16.33) and SE 1 (15.00).

On the other hand data given in Table 2 indicated that there was a significant increment in the plant height due to the treatment of seed extracts. The maximum increase in plant height was observed in SE4 (50.33 cm), followed by SE5 (50.18 cm), SE3 (49.95 cm), SE2 (49.88 cm), and SE1 (48.76 cm). However seedling length and growth were found in SE4 and STRP, which indicated that SE4 had a good capacity to influence seedling growth as compared to other treatments. All the treatments are significant as compared to both control groups except NC (48.33 cm) for parameters, plant height and leaves per plant, as shown in Table 2.

Table 2 Estimation of growth parameters (leaf per plant and plant height) and biochemical parameters (Chlorophyll a, b, carotenoids, protein and carbohydrate) in soybean by different concentrations of seed extract against bacterial pustule disease

T	Leaf/plant	Plant height (cm)	Carbohydrate (mg/g)	Chlorophyll and carotenoids (mg/g) fresh weight			Protein (mg/g)
				Total ch	Cha	Chb	
SE1	15.00±1.00 ^e	48.76±0.56 ^d	3.06±0.13 ^d	1.22±0.04 ^{ef}	0.91±0.06 ^b	0.67±0.22 ^c	4.05±0.44 ^f
SE2	16.33±1.15 ^{de}	49.88±0.06 ^c	3.16±0.02 ^d	1.41±0.17 ^{de}	0.97±0.13 ^b	0.72±0.13 ^{ab}	5.48±0.44 ^e
SE3	19.33±0.58 ^{cd}	49.95±0.07 ^{bc}	3.34±0.03 ^c	1.66±0.09 ^{cd}	1.01±0.15 ^{ab}	0.76±0.09 ^{ab}	7.76±0.59 ^d
SE4	24.33±1.15 ^{ab}	50.33±0.05 ^{bc}	4.02±0.09 ^b	1.98±0.03 ^{ab}	1.19±0.19 ^{ab}	0.84±0.07 ^b	9.81±0.22 ^b
SE5	21.67±1.15 ^{bc}	50.18±0.04 ^{bc}	4.01±0.09 ^b	1.96±0.12 ^{ab}	1.14±0.06 ^{ab}	0.82±0.03 ^{ab}	9.24±0.50 ^{bc}
STRP	24.67±1.53 ^{ab}	50.49±0.05 ^b	3.94±0.04 ^b	1.81±0.01 ^{bc}	1.11±0.12 ^{ab}	0.81±0.01 ^{ab}	8.43±0.29 ^{cd}
NT	26.00±1.00 ^a	51.84±0.06 ^a	5.08±0.03 ^a	2.12±0.03 ^a	1.33±0.11 ^a	0.97±0.20 ^a	13.95±0.36 ^a
NC	13.33±1.53 ^e	48.33±0.51 ^d	2.61±0.15 ^e	1.13±0.13 ^f	0.88±0.08 ^{cd}	0.59±0.11 ^d	1.67±0.44 ^g

SE (1–5) seed extract concentration µg/500µl, NT (non treated healthy plant), STRP (streptomycin) and NC (negative control or diseased plant). The results are expressed as mean ±SD of three replicates. Different significantly different letters (*P* < 0.05) as determined by Tukey-Kramer HSD test



Fig. 6 Potential efficiency of seed extract against pustule disease of soybean in a pot experiment. **[A]** Represents the control group [healthy plant (NT) and streptomycin treated plant as positive control and *Xcg* inoculated plant as negative control (NC)]. **[B]** SE (1–5; 200–600 $\mu\text{g/mL}$) potential against *Xcg* inoculated plants in pots

Table 3 Effect of seed extract on bacterial pustule disease in pot experiment. The results are expressed as mean $\pm$ SD of three replicates. SE (1–5) seed extract concentration $\mu\text{g}/500\ \mu\text{l}$, NT (non treated healthy plant), STRP (streptomycin) and NC (negative control or diseased plant). Significantly different letters ($P < 0.05$) as determined by Tukey-Kramer HSD test

Treatment	DS (percentage)	PEDC (percentage)
SE1	79.99 ± 1.01^{ab}	3.50 ± 1.77^f
SE2	61.33 ± 1.99^b	26.01 ± 1.02^e
SE3	40.12 ± 1.34^c	51.60 ± 0.78^d
SE4	24.18 ± 0.98^d	70.83 ± 0.81^c
SE5	20.34 ± 0.58^e	75.46 ± 0.29^b
NT	0 ± 0.00^f	100.00 ± 0.00^a
STRP	19.89 ± 1.21^{de}	76.00 ± 0.95^{ab}
NC	82.89 ± 1.87^a	0.00 ± 0.00^g

4.9 Effect of seed extract on bacterial pustules disease in pot experiment

Lower disease incidence and higher PEDC of seed extract were explained in Table 3. Results positively indicated that the presence of seed extract reduced the occurrence and amalgamation of bacterial pustule disease. Lesion formation and its amalgamation were visually distinguishable in NT, which represents the control group or diseased plant. A significantly lower disease incidence and higher PEDC were observed in SE 4 and 5 along with STRP. Among extracts, SE5 strongly repels disease onset, its spread, and lesion formation with 75.46% of PEDC, which is comparatively good. This is almost similar to the STRP ability against disease (76.00%). However, the severity percentage in SE4 was also low (24.18%), and it also has a good ability to hold the disease spread with 70.83% PEDC. Lower concentrations of seed extract could not hold the spread; that's why the severity

percentage was higher. SE3 moderately controls the disease onset with 51.60% PEDC, but it cannot be considered because it does not hold the spread.

4.10 Isolation of phenolic compounds and HPLC analysis

In order to obtain the antibacterial phenolic compounds from the seeds of *M. oleifera*, repeated column chromatography and TLC were performed. For their purity, HPLC analysis was used, which revealed the presence of three major phenolic compounds. The fraction number 2 gives quercetin (MOF 2.3.1 with solvent ratio toluene: ethyl acetate: formic acid 5:4:1). p-coumaric acid and cinnamic acid were isolated from fractions 5.4 and 5.7. Cinnamic acid was eluted with ethyl acetate: n-hexane (70:30), and p-coumaric acid was isolated with the solvent system of chloroform: ethyl acetate (60:40). HPLC showed a single compound profile with the highest peak area percentage of quercetin at 16.34 RT, p-coumaric acid at 13.18 RT, and cinnamic acid at 17.20 RT (Fig. 7).

Among all three compounds, the peak area percentage of cinnamic acid was highest at 99.13%. The remaining two phenolic compounds also had good percentage areas, in which p-coumaric acid had 98.04% and quercetin had 90.67%. However, minor peaks in quercetin were also observed, indicating that impurity in isolated quercetin at a certain level.

4.11 In vitro antibacterial activity of isolated phenolic compounds

Results indicated that all three isolated phenolic compounds effectively inhibited *Xcg* at certain levels but cinnamic acid was the most effective among them, as shown in Figs. 8 and 9.

It inhibited *Xcg* in a dose dependent manner with a wider zone of 18.95 mm at 300 ug/mL. While p-coumaric acid performed less against *Xcg*, as it has a below 10 mm zone of inhibition that is 8.70 mm. Quercetin had 15.88 mm diameter which is good but not preferable as compared to cinnamic acid and streptomycin (19.02 mm). Bar diagram results showed that each isolated compound inhibited *Xcg* in a dose dependent manner. All data were significantly different except for cinnamic acid because at higher concentrations the compound depicts a wider zone, which was almost similar to the reference drug STRP.

4.12 MIC interpretation of seed extract and isolated phenolic compound

In addition, contrary to qualitative methods, the MIC value was determined to assess the degree of resistance to the extract or compound. Here, Table 4 indicates the MIC values of seed extract and its phenolic compounds. Among all three phenolic compounds, cinnamic acid had the best ability to combat *Xcg in vitro*. It showed the lowest MIC value with 0.0075 mg/mL. Seed extract and quercetin also have good MIC values of 0.3 mg/mL and 0.015 mg/mL, but p-coumaric acid did not show better results, as it showed a high MIC value among all test samples of 0.06 mg/mL. However, MIC itself does not provide exact information on the exposure pattern of *Xcg* to all test samples and its concentration that must be achieved relative to the MIC to ensure a sufficient antibacterial response.

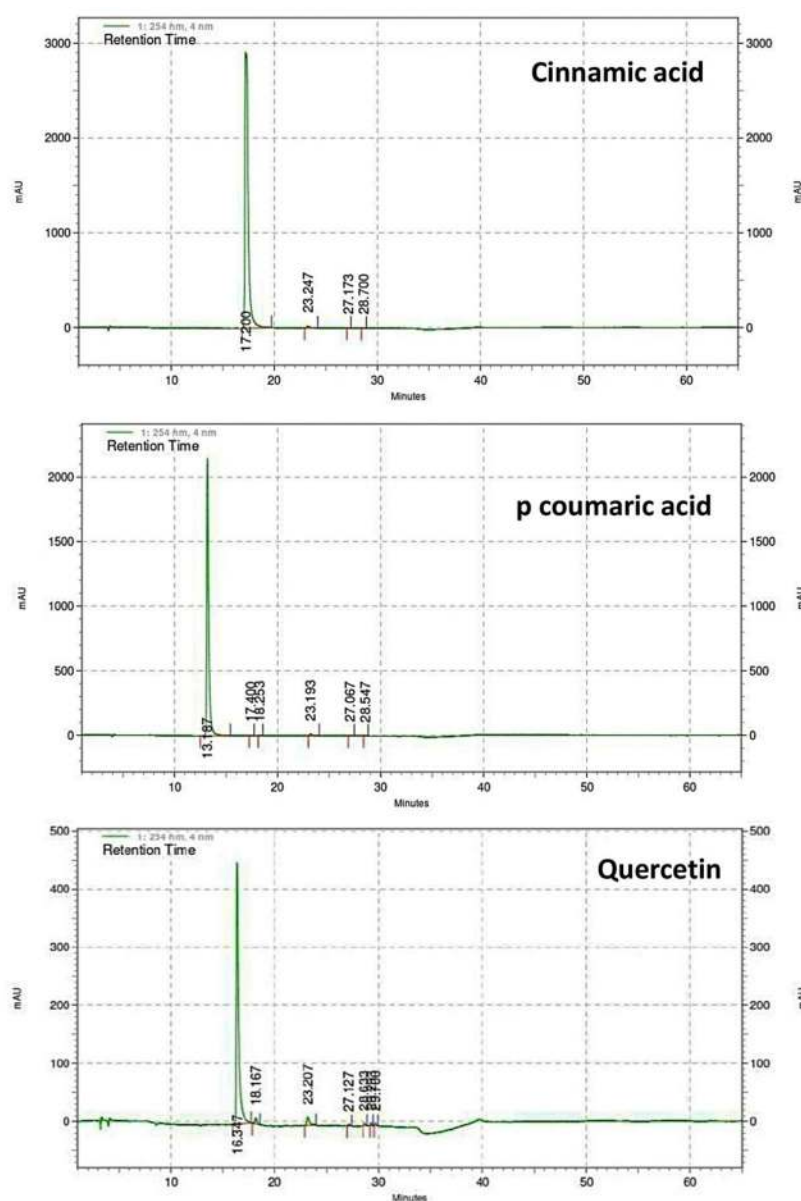


Fig. 7 HPLC chromatograms of isolated phenolic compounds from *M. oleifera* seed extract, showing retention time at 254 nm

5 Discussion

The soybean harvest is a significant crop, with its seeds being essential for oil and protein feed. Globally, it is a crucial food crop, yet it is susceptible to various microbial diseases triggered by fungi, phytoplasma, viruses, and bacteria, leading to a significant reduction in overall production worldwide. A recent report of production losses by diseases in soybean in the USA and Canada from 2015 to 2019 was 45 USD per acre (111 USD per hectare) [48–50]. Bacterial pustule disease is one of them, which causes severe damage to its yield and overall production [17]. Soybean yield reduction from microbial diseases and its management continue to demonstrate the need for scientific research, as well as agribusiness and farmer education about it. Disease management by different commercially available chemicals can control the severity, but there are several negative impacts

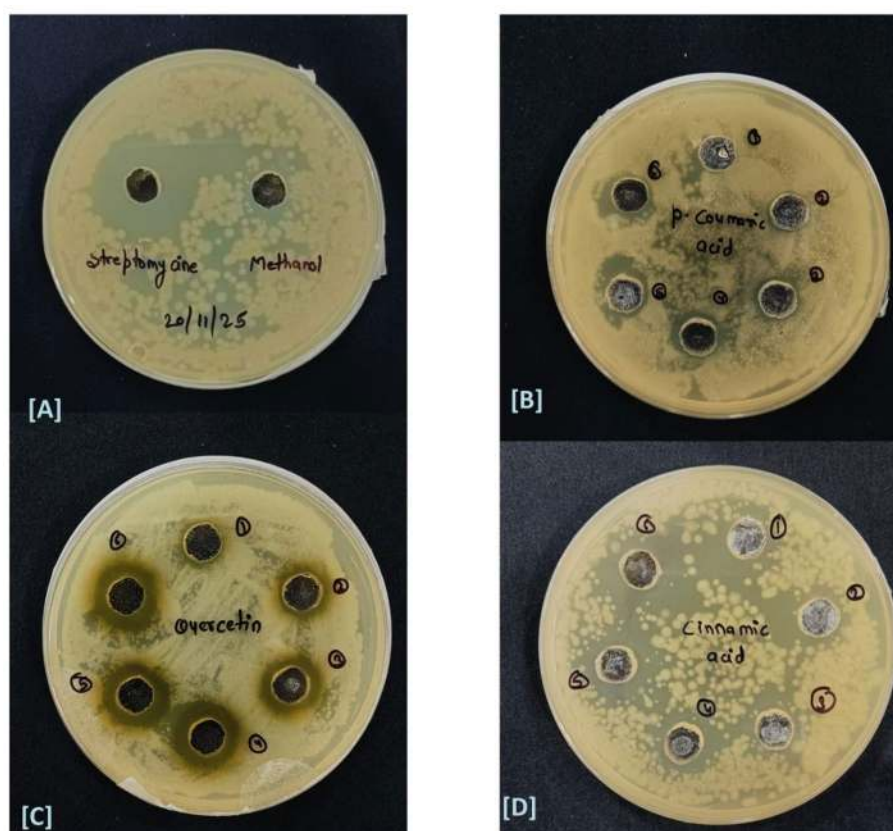


Fig. 8 Zone of inhibition for the test organism *Xcg* by the isolated phenolic compound from the seed extract of *M. oleifera* (Plates **B**, **C**, and **D** represent the antibacterial activity of p-coumaric acid, quercetin, and cinnamic acid). Plate **A** represents antibacterial activity of standard drug STRP and methanol

on the soybean crops physiologically and morphologically, and it also affects the quality of seeds. Entering in the food chain is another concern, as it damages other populations as well as the environment. In the quest of sustainable agriculture, biocontrol agents have emerged as environmentally friendly alternative to synthetic chemicals for managing plant pathogens. Several reports are available related to natural plant based and integrated approaches for bacterial disease control and management [36–38]. In the search for a potent biocontrol agent against bacterial pustule disease, we examined the efficacy of *Moringa oleifera* in eliciting a defense reaction against *Xcg* in vitro, while simultaneously promoting sustainable soybean growth. Given that, all parts of the *Moringa* tree are biologically valuable; its efficacy is attributed to a rich profile of biomolecules. The most significant of these include phenolic compounds, alkaloids, glucosinolates, isothiocyanates, folates, tannins, fatty acids, saponins, and carotenoids [51, 52].

Total phenolic content of different plant parts was positively correlated with their antioxidant capacities and effectiveness against plant pathogens. There are many differences in extract effectiveness as compared to other work done before. In one study, *M. oleifera* bark and leaf methanolic extract gave 44.03 and 32.83 mg GAE/g [53]. But in our study methanolic bark and leaf extract gave slightly higher TPC with 56.10 and 33.13 mg GAE/g. In another study, ethanolic extract of *M. oleifera* seeds through Soxhlet extraction gave only 11.85 mg GAE/g [54], while in our study SE gave 42 mg GAE/g. These discrepancies can be due to the selection of extraction protocols, plant geographical

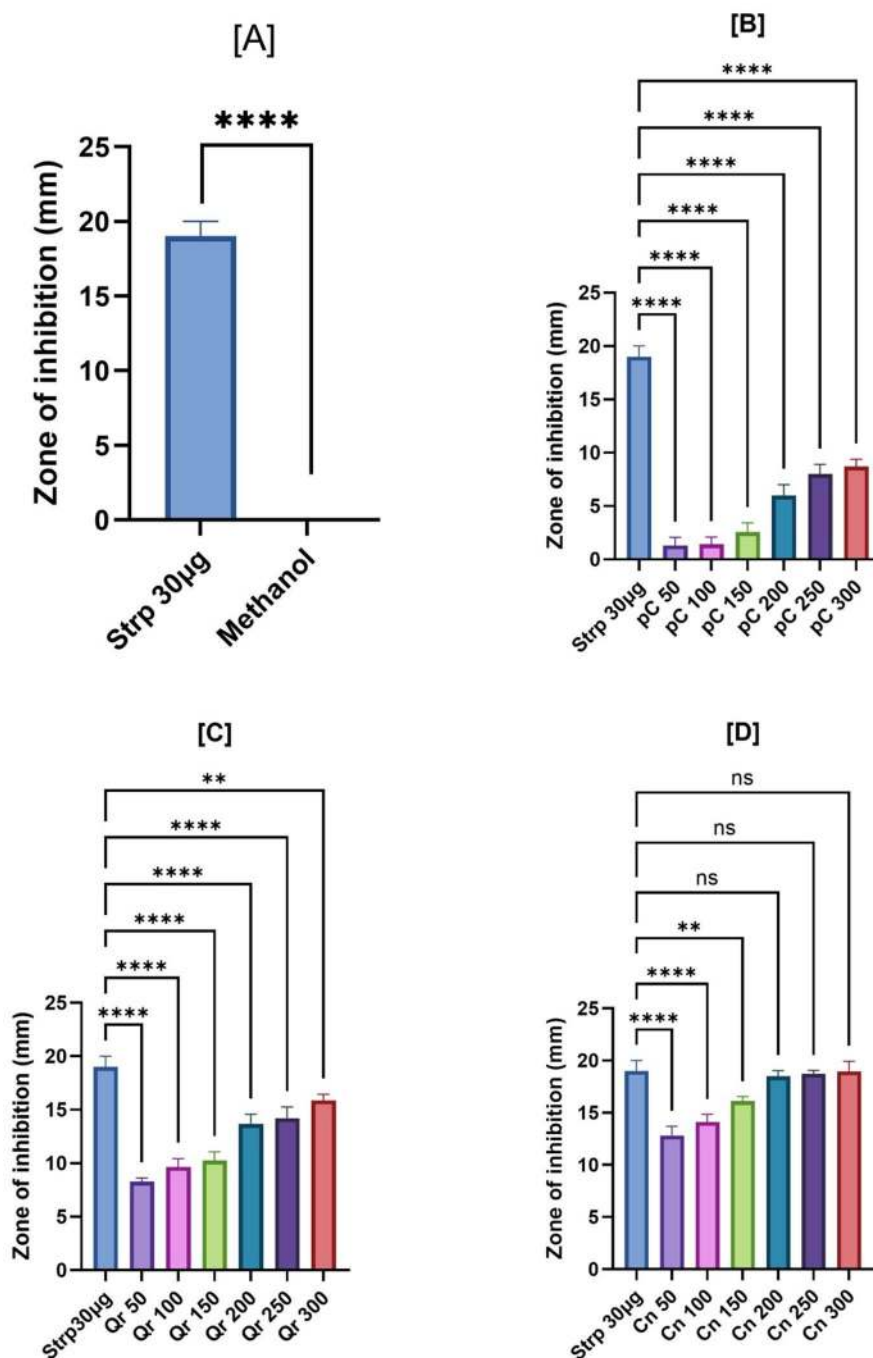


Fig. 9 Bar diagrams represents the effect of isolated phenolic compounds on *Xcg* along with standard streptomycin (STRP) in agar well plate method. It represents the mean $\pm$ SD, $n=3$ of each compound followed by different asterisks showed comparative significant difference ($p < 0.05$) as determined by Dunnett's post hoc test. Here pC (p-coumaric acid), Qr (quercetin), and Cn (cinnamic acid)

condition, and the number of replicates in the experiment. Variations in the bioactive substance composition of the extract and its amount are other factors, as they may differ depending on the solvent used. Antioxidant analysis revealed a positive correlation between TPC. SE showed good FRAP ($168.61 \mu\text{M Fe}^{+2}/\text{g}$) and DPPH (70.39%) results. Bark extract also showed good results in DPPH activity (70.39%). But overall antioxidant capacity was better in SE.

Table 4 MIC values of seed extract and isolated phenolic compounds from seed extract of *M. oleifera*

Test sample	MIC (mg/mL)
Seed extract	0.3
Cinnamic acid	0.0075
p-coumaric acid	0.06
Quercetin	0.015

Comparing the zone of inhibitions of various *M. oleifera* plant part extracts to the positive control, data revealed an interesting pattern of effectiveness. Among all the treatments, SE was recorded with a significant zone of inhibition in the agar well plate method; it inhibited *Xcg* with 18.86 mm, which is much wider as compared to other extracts. The STRP showed a slightly wider zone with an 18.94 mm diameter. Whereas these results do not imply that the seed extract of *M. oleifera* is more effective against *Xcg* than the control antibiotic, they only demonstrate that methanolic seed extract has notable antibacterial activity in vitro. Previous studies have indicated that organic solvent extracts of *M. oleifera* had better antibacterial activity against many plant pathogens and *Xanthomonas* strains. In one study SE of *M. oleifera* successfully inhibited *E. coli*, *S. aureus*, *P. aeruginosa*, etc. with a wider zone of inhibition ranging from 16.2 to 32 mm [55]. In other work, green synthesized silver and zinc nanoparticles from *M. oleifera* leaves were examined against bacterial leaf spot in chili plants caused by *Xanthomonas campestris* pv. *vesicatoria* (Xcv), their solo applications inhibited Xcv and gave 18.45 mm (AgNPs) and 12.25 mm (Zn NPs) [56], which is good as compared to leaf extract results. But in our study, SE suppressed *Xcg* in a better way as compared to nanoparticles, as it gives an 18.86 mm diameter. However, our test pathogen is not similar, but both bacteria are gram negative in nature.

In view of strong antibacterial activity against *Xcg* in Petri plate experiments, we further explored in-vivo efficacy of SE and its effect on biochemical parameters of soybean against pustule disease incidence. SE 4 considerably enriched the overall health of soybeans in the pot experiment as compared to STRP treated plants. However, STRP also showed good results in the agar well plate method, but in the pot experiment, protein, chlorophyll, and carbohydrate quantities were low as compared to non treated (NT) healthy plants and SE 4, which indicated that streptomycin might have negatively impacted the plant's own defense system, growth, post germinative development, and weakened the plant's overall defense capacity. Whereas increased sugar content in treated plants, especially in SE4, can be directly correlated with enhanced photosynthesis (1.98 mg/g total chlorophyll content). Because reactive oxygen species are byproducts of photosynthesis and phenolic compounds can help in regulating ROS levels, potentially influencing photosynthesis efficiency [47–49, 57–59]. Increased photosynthesis activity is positively correlated to building carbohydrates. Protein synthesis also increases with photosynthesis via translation initiation stimulation [60]. In our experiment, data clearly support that SE4 positively increased protein (9.81 mg/g) and carbohydrate content (4.02 mg/g). Earlier scientists revealed the use of *M. oleifera* dried leaf extract for growth improvements in soybeans, in which they suggested that it improved photosynthetic pigments by 7–10% through foliar application [61]. In other work, again on *M. oleifera* dried leaf extract foliar application, data provided that it revitalizing soybean yield component (37%) and carotenoids (8.33%). However, they did not conduct

the research against any stress condition, and correlative analysis of different plant part extract but it supported the importance of *M. oleifera* as an herbal potential to aggravate in a physiological manner.

In another experiment conducted in search of disease control efficacy, the pattern of lesion formation and its amalgamation were visually observed. Data revealed that SE significantly elevates the symptoms of pustule disease at higher concentrations, SE4 (70.83%) and SE5 (75%) PEDC. The diseased plant (NC) showed a higher disease incidence (82.89%). STRP positively control DS with a 19.89% severity index. These findings could be explained by SE biocontrol efficacy and influence plant defense reactions by induction of phenol synthesis and trigger defense mechanism in response to *Xcg*. Earlier *M. oleifera* leaf, bark, and seed extracts were evaluated against (*Xcc*) in *Brassica napus*; after foliar applications (60% concentration), seed extract showed lower mean leaf defoliation (1.59 cm), which was the best result among all to suppress *Xcc* in the field [62]. In another study methanolic extract of *M. oleifera* leaf seems to be most effective against *Xcc* on radish plants with 80% reduction of necrosis area [63]. However, we did not evaluate the growth parameters like plant height, root length, root weight, number of pods, seeds weight, etc. and defense related enzymes like peroxidase, polyphenol oxidase, superoxide dismutase, and phenylalanine ammonia lyase, but our information regarding the interaction of SE with soybean plants corroborates the assumption that it plays an important role in amending the biochemical response and increasing the activity of antioxidant enzymes.

The antimicrobial activity of phenols is well known against a variety of bacteria, fungi, and viruses. The defense response of seed extract might be due to the presence of phenolic compounds, which can stimulate plant immune response by enhancing its antioxidant defense enzyme system and contribute to its resilience and adaptability towards environmental challenges, including microbial stress. SE contains a wide array of beneficial phenolic compounds such as gallic acid, ferulic acid, caffeic acid, catechin, epicatechin, vanillin, quercetin, cinnamic acid, p-coumaric acid, etc. [64]. and function as signaling compounds that trigger the development of systemic acquired resistance (SAR) and activation of defense genes.

In support of above the experimental results of SE, we further successfully isolated three phenolic compounds, Quercetin, cinnamic acid, and p coumaric acid, and evaluated their ability against *Xcg* in an in-vitro manner. For antibacterials, the most commonly used efficacy surrogate is the MIC because it directly measures the drug's potency in vitro and indirectly measures in vivo activity. After comparative examination of MICs of isolated phenolic compounds and SE extract of *M. oleifera*, results clearly indicated that SE showed good results with 0.3 mg/mL MIC. All three phenols also had good MIC values, which support the role of phenolic acids of SE in the inhibition of *Xcg* in both in-vitro and in-vivo experiments. Some studies suggested that application of genistein, an isoflavonoid, increased antioxidant capacity, decreased H_2O_2 level, and increased accumulation of phenolic compounds in leaves of *Xcg* inoculated soybean plants [19]. In another study conducted against *Xcg*, amino acid cystein showed a 2.5 mM MIC and its foliar application on soybean was able to reduce pustule disease severity in soybean [65]. Here, cinnamic acid showed a 0.0075 mg/mL MIC, which is the best among all three phenolic compounds, therefore supporting the biocontrol efficacy of SE, mainly due to it. A study was performed against *Xap*, a pathogen for peach bacterial shot hole

disease. Results clearly indicated that cinnamic acid derivatives especially methyl caffeate, strongly repelled *Xap* with 0.0625 MIC value [32]. However, SE showed higher MIC as compared to its phenolic compounds. It is because of the presence of several other molecules like lipids, proteins, carbohydrates, and chlorophyll in SE, as they can also dissolve in a similar solvent, methanol. Methanol is known as a universal solvent and shows affinity not only towards phenols but also against the above mentioned molecules. Therefore, it increased the amount of the extract.

In the soybean plant, flagella biosynthesis genes *flgB* and *flgK*, transcriptional regulator *LcrX*, type II and III effector systems, and *OmpA* are responsible for *Xcg* virulence in soybeans [17]. So, *M. oleifera* SE and phenolic compounds, especially cinnamic acid, might mitigate the signaling cascade and resist the bacteria fighting against the above mentioned virulence factors. Our results demonstrated that SE and cinnamic acid were able to directly inhibit *Xcg* but it might also suppress this bacterium by acting as a signaling molecule in soybean plant to induced resistance to the pathogen. Here, we explored only the in-vitro potential of phenolic compounds and SE efficacy through a pot experiment. However, concerning the effect of SE in field trials, its large scale assessment and cross validation of its phenolic compounds, especially cinnamic acid, must be evaluated as biocontrol agent. Although the mechanistic approach of SE and specific phenols in defense pathways related to *Xcg*, virulence factor analysis and its physiology related signaling pathway should be explored before commercial use.

However, we only target phenolic compounds from SE, but it also contains phenylpropanoids such as 4-hydroxyphenyl acetate, 3-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, 3-hydroxy-1-(4-hydroxyphenyl)-propyl-1-one, erythro-guaiacylglycerol and threo-guaiacylglycerol [66]. They can be involved in the synthesis of cell wall strengthening compounds, such as lignin and suberin, so it is positively correlated with fortifying plant core structure against environmental stress and preventing the pathogen penetration in the cortical region [67]. They also function as signaling compounds that trigger the development of systemic acquired resistance (SAR) and activation of defense genes [68]. Glucosinolates and their derivative byproduct (isothiocyanates), also present in the seeds of *M. oleifera*, can act as antibacterials. While it is not influenced by photosynthesis directly but indirectly through their impact on plant physiology and stress response [69].

Disease and agribusiness management, related to soybean needs scientific rationale and understanding. Our results provide soybean breeders, scientists, and management agencies to inform and prioritize research related to pustule disease, its biocontrol, and herbal management. Therefore, antimicrobial rich SE and its phenolic compound, cinnamic acid from *M. oleifera* plant, can act as environmentally friendly by eliminating phytopathogenic microbes, especially *Xcg*. Such a sustainable approach has the capacity to revolutionize agriculture and reduce reliance on chemical pesticides.

6 Conclusion

Moringa oleifera is recognized for its diverse profile of primary and secondary metabolites. Our study demonstrated that the seed extract possesses high total phenolic content and robust antioxidant activity, which translated into significant antibacterial efficacy against *Xanthomonas axonopodis* pv. *glycines* (*Xcg*) in both in vitro and in vivo systems. These results suggest that the antibacterial potential of the extract is primarily driven

by its phenolic constituents, specifically quercetin, cinnamic acid, and p-coumaric acid. Consequently, this extract holds promise as a cost-effective, eco-friendly phyto-formulation for managing bacterial pustule in soybean. However, further research is required to isolate and characterize additional bioactive constituents using advanced spectroscopic and chromatographic techniques, such as HPLC and NMR. Establishing the precise molecular mechanism of action is essential to validate these findings. Given the escalating global demand for sustainable agriculture and chemical-free food production, adopting plant-based formulations is imperative to bolster plant immunity and ensure sustained crop productivity.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s42452-026-08836-2>.

Supplementary Material 1

Acknowledgements

The authors are highly thankful to the Ministry of Education and SPD-RUSA Rajasthan for the Infrastructure support created under funds received from the RUSA 2.0 projects. All the authors acknowledge their host institute for infrastructure support.

Author contributions

Conceptualization, F.K. and J.A.; methodology, F.K., A.T. and J.A.; investigation, F.K., A.T. and J.A.; resources, F.K., B.K. and J.A.; data curation, F.K., A.J., K.P.S. and J.A.; writing—original draft, F.K., A.T. and J.A.; writing—review F.K., A.J., B.K., K.P.S. and J.A.; visualization, F.K. and J.A.; supervision, J.A. All figures have been made by F.K; Revision, final update and editing, R.K., F.K. and J.A. All authors have read and agreed to the published version of the manuscript.

Funding

No Funding support provided to this work.

Data availability

All data underlying the results are available as part of the article and no additional source data are required.

Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval

Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

Not applicable. This study did not involve human participants, and therefore, informed consent to participate was not required.

Consent to publish

The authors give their consent for the publication of identifiable details, which can include figures and tables, to be published in the Journal.

Received: 6 September 2025 / Accepted: 12 May 2026

Published online: 30 May 2026

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