

## Combined Antimicrobial Activity of *Chromolaena odorata*, *Azadirachta indica* Leaf Extract, against *Streptomyces scabiei* (Plant pathogen) and *Paenibacillus polymyxa*, and their interaction

Hannah Leah Lijo<sup>1</sup>, and Sandheep P.N.<sup>2\*</sup>

### ABSTRACT

*Streptomyces scabiei* is a gram-positive soil dwelling actinobacteria, that causes the scab disease in many plants, especially in potatoes. This causes black blotches on the tuber that negatively impacts the economic and market worth of potatoes. Plant species like *Chromolaena odorata* and neem are known for their antibacterial potential against common human microflora and agricultural pathogens. Aiming to find natural solutions to plant pathogens, the current study analyzes, the antimicrobial effect of these plant extracts at different concentrations using well diffusion, MIC and MBC. Combination study was also performed to test the synergistic antimicrobial effect against *Streptomyces scabiei*. In addition, another soil dwelling bacteria, *Paenibacillus polymyxa*, known for its plant growth promoting potential was also tested against the plant pathogen to understand microbe-microbe interactions through cross streak assay. Both plant extracts showed promising antimicrobial effect against *Streptomyces scabiei*, however, they showed very less antimicrobial potential against the plant growth promoting bacterium. Moreover, cross streak assay showed that both the bacterium coexisted together. Hence, further studies can be conducted to formulate a biofertilizer containing the two-plant extract in optimum concentrations and *Paenibacillus polymyxa* to prevent scab disease and also enhance plant growth.

**Keywords:** Well diffusion, MIC, MBC, Phytochemical analysis, FTIR analysis, GC-MS analysis.

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### INTRODUCTION

The potato, or *Solanum tuberosum* L., is an essential and adaptable vegetable crop of major global significance. It is the world's fourth-largest crop and is essential for maintaining food security. According to estimates from the Food and Agriculture Organization (FAO), more than 17 million hectares of land were used to grow more than 359 million metric tons of potatoes in 2020 (Kumar *et al.*, 2024), (Wei *et al.*, 2022). However, a number of phyto pathological diseases have become dangerous to their healthy growth. Potato scab, also known as common scab (CS), caused by a soil dwelling bacterium, *Streptomyces scabiei*. It

causes huge economic losses worldwide (Li *et al.*, 2024), and alone was estimated to have generated around 15.3–17.3 million Canadian dollars in economic losses in Canada in 2002. Due to the higher occurrence of common scab disease up to 32% of the total produced potatoes were thrown because of the low-grade shipment, and it is challenging to eradicate the disease once it enters the field (Naher *et al.*, 2024).

Previous studies have revealed that CS is independently caused by several different *Streptomyces* spp, including *S. acidiscabies*, *S. turgidiscabies*, *S. europaeiscabiei*, *S.*

*stelliscabiei*, and *S. bottropensis* (Shi *et al.*, 2019). This pathogen produces a family of phytotoxins called thaxtomin. They are cyclic dipeptides (2,5-diketopiperazines) containing 4-nitrotryptophan and phenylalanine residues. Thaxtomin A (ThxA), the most prominent member of the family, has been confirmed to be associated with *Streptomyces* pathogenicity. ThxA biosynthesis genes are found on a movable pathogenicity island (PAI) and are known to inhibit cellulose production, promote plant cell hypertrophy, and induce programmed cell death in plant cells (Shi *et al.*, 2019).

This study focuses on *Streptomyces scabiei* which is a saprophytic, filamentous, gram-positive bacterium, responsible for causing common scab (CS) in several underground crops like potatoes, beets, and radish, potatoes being most commonly affected. This causes a tremendous reduction in tuber quality and market value of potatoes (Lin I 2018; Wei *et al.*, 2022). The color of scab lesions can vary from brown to black. *Streptomyces scabiei* develop a large number of latent spores that can withstand environmental stress for extended periods of time. The resistant property of this bacterium has been one of the major challenges to develop effective treatment against potato scab (Merz and Falloon, 2009).

Actinobacteria, particularly *Streptomyces spp.* is responsible for the production of wide range of antibiotics, bioactive molecules that promote growth of plants and are antagonistic to several plant pathogens (Peng *et al.*, 2020). However, *Streptomyces scabiei* was found to cause a disease called common scab mainly infecting potato plants. It is found to be highly resistant to antibiotic treatment in the spore form and, worldwide, significant economic losses are caused by the absence of viable therapies for this disease (Lin *et al.*, 2018; Abdelrhim *et al.*, 2021). Research is being undertaken to identify plant extracts like myrrh, garlic, cayenne, barberry, frankincense, wild indigo root, and lavender as antimicrobials for the treatment of CS (Gutierrez *et al.*, 2022).

Medicinal plants like *Chromolaena odorata* has potential antimicrobial effect against

opportunistic bacteria like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and enteric bacteria like *Shigella flexneri*, *Escherichia coli*. Phytochemical screening showed the presence of terpenoids, steroids, flavonoids, alkaloids, saponins, tannins (Odutayo *et al.*, 2017). In addition, the neem tree (*Azadirachta indica*) is a well-known medicinal plant in Southeast Asia that contains over 140 bioactive compounds, the most prevalent being, azadirachtin, nimbin, and nimbidine. Medicinal plants are known to contain antimicrobial compounds, particularly against skin infecting bacteria (Daud *et al.*, 2019). Further, *Paenibacillus polymyxa* is yet another soil dwelling, spore forming, non-pathogenic bacterium possessing plant growth promoting traits like phosphate solubilization and nitrogen fixation. It also produces many bioactive compounds like Indole Acetic Acid, cytokinin (Hanphanphoom and Krajangsang, 2016).

This study aims to find the antimicrobial effect of each plant extract against *Streptomyces scabiei*. Their combined antimicrobial effect was also studied to check for the presence of synergistic effect against the pathogen. In addition, interaction between *Streptomyces scabiei* and *Paenibacillus polymyxa* was studied to test their combined effect which can help make a formulation ideal for the potato plant growth and disease prevention in future studies.

## MATERIALS AND METHODS

### Materials

#### Plant Samples

*C. odorata* leaves were collected from Kerala, Thiruvalla district, Nellimala in January 2025. *Azadirachta indica* leaves were collected from Karnataka, Bangalore, Ganganagar market in February and were certified at Central Ayurveda Research Institute, Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Govt. of India, Bengaluru, Karnataka.

#### Bacterial Strains

Bacterial strains were obtained as Agar Deeps from Kristu Jayanti (Deemed to be University), Bangalore, Karnataka, India. In 2023, rhizosphere

soil was taken from the medicinal garden at Kristu Jayanti (Deemed to be University) and *Streptomyces scabiei* as well as *Paenibacillus polymyxa* were isolated from them. Yeast Malt Extract Agar was used for cultivating *Streptomyces scabiei* and Nutrient agar for *Paenibacillus polymyxa*.

Morphological characteristics and 16S rRNA gene sequencing were used to confirm identification (GenBank Accession No.: OR835680 for *Streptomyces scabiei*). The strain was kept as glycerol stocks in the freezer, was revived in Yeast Malt Agar, and transported as Agar Deeps to Christ (Deemed to be University). Nutrient Agar was used for the cultivation of *Paenibacillus polymyxa*. Morphological, biochemical and 16S rRNA gene sequencing were used to confirm identification (GenBank Accession No.: PP316968 for *Paenibacillus polymyxa*). The strain was kept as glycerol stocks in the freezer, was revived in Nutrient Agar, and transported as Agar Deeps to Christ (Deemed to be University), Bangalore Central campus, Karnataka, India and stored at 4 degrees Celsius.

#### Sample preparation

The leaves were washed and shade dried for 20 days. Dried plant parts were then collected, blended and weighed before extraction (Odutayo *et al.*, 2017).

#### Ethanolic extraction

Plant material of 20g was then extracted with 200 mL 90 percent (Hanphanphoom and Krajangsang, 2016) ethanol using conventional Soxhlet's extraction technique. Next, the ethanolic extract was dried using rotary evaporator (Cao *et al.*, 2025). The powder obtained is suspended in 5 percent ethanol to obtain different dilutions. The same method was followed for Neem leaf extraction. The percent yield was calculated as;

$$\text{Percent yield} = \frac{\text{Extract weight}}{\text{Dried plant weight}} \times 100$$

#### Agar well diffusion

The agar well diffusion method (using Yeast Malt Extract agar) was used to identify the inhibitory zone. Streptomycin was used as

positive control and 5 percent lab grade ethanol were used as negative controls. Using a 0.5 McFarland standard, bacterial strain cultures were adjusted in Yeast Malt Extract broth to reach a final concentration of  $10^8$  CFU/mL. To produce a confluent growth, 0.1 mL of the test bacteria's standardized inoculum was spread on the surface of sterile Yeast Malt Extract Agar plates. Wells were bored using a 9mm cork-borer, and 50µL of 25mg/mL, 50mg/mL, 75mg/mL and 100mg/mL extracts were added to each well. The zones of inhibition were evaluated for the triplicates after these plates were incubated for 48 hrs at 37°C (Balouiri *et al.*, 2016; Hanphanphoom and Krajangsang, 2016).

#### MIC against *Streptomyces scabiei* and *Paenibacillus polymyxa*

MIC of plant extracts was found by microdilution method using 96 well microtiter plate. Each well was filled with 100 µl of sterile Yeast Malt Broth. The plant extract of 100mg/ml concentration was diluted 10 times by two-fold dilution. Bacterial culture of 100 µl was added to the wells and incubated for 48 hours (Djeussi *et al.*, 2013). One set of wells were maintained as blank where; broth was added in the plant dilutions instead of the culture. The absorbance values were obtained using microtiter reader and MIC values were calculated by subtracting from the absorbance values from the blanks.

#### MBC (Minimum Bactericidal Concentration)

Following analysis of MIC values, wells that showed MIC value and higher concentrations were sub cultured to find the MBC. The dilution of plant extract that showed no bacterial growth was considered as MBC (Djeussi *et al.*, 2013).

#### Cross streak assay

This was done to check the interaction between *S. scabiei* and *P. polymyxa*. *S. scabiei* was streaked and incubated for 24 hrs, following which *P. polymyxa* was streaked in a different direction (Gomaa *et al.*, 2020), perpendicular to the first. It was further incubated for 24 hrs.

**Phytochemical analysis**

Extract concentration of 10mg/mL was used for phytochemical analysis. Alkaloids, tannins, flavonoids (Nwanekezie *et al.*, 2023), Cardiac glycosides (Maheshwaran *et al.*, 2024), Test for terpenoids (Salkowski's test) to each well. The zones of inhibition were evaluated for the triplicates after these plates were incubated for 48 hrs at 37°C (Balouri *et al.*, 2016; Hanphanphoom and Krajangsang, 2016).

**Test for Phenols**

About 1 mL of extract was mixed with few drops of 1% FeCl<sub>2</sub>. Phenols are indicated by a deep blue or green color (Abdelrhim *et al.*, 2021).

**Test for Saponins (Foam test)**

About 1 mL of extract was mixed with 5 mL of water and mixed vigorously to observe the formation of foam that persisted for 10 minutes to 15 minutes (Nwanekezie *et al.*, 2023).

**FTIR analysis**

FTIR analysis was performed for the extract dried by rotary evaporation after FTIR analysis. An interferogram was produced and spectral analysis of the interferogram was done.

**GC-MS analysis**

To determine the phytochemical components found in the ethanolic extracts of *C. odorata* and *A. indica*, a Gas Chromatography–Mass Spectrometry (GC-MS) investigation was performed. Using accepted techniques for detecting volatile compounds, the analysis was carried out at Aknowlytiq Academy in Bengaluru. With a Shimadzu GCMS-QP2010 system and an RTX-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness; Restek, USA), GC-MS analysis was carried out. At a rate of 10°C per minute, the oven temperature was planned to rise from 60°C (with a 2-minute hold) to 300°C, with a 10-minute final hold. At a steady flow rate of 1.0 mL/min, helium was employed as the carrier gas, and the injector temperature was set at 250°C. Operating in electron impact (EI) mode, the MS's scan range was 40–600 m/z. Mass spectra were compared to the NIST 20 collection in order to identify

the chemicals.

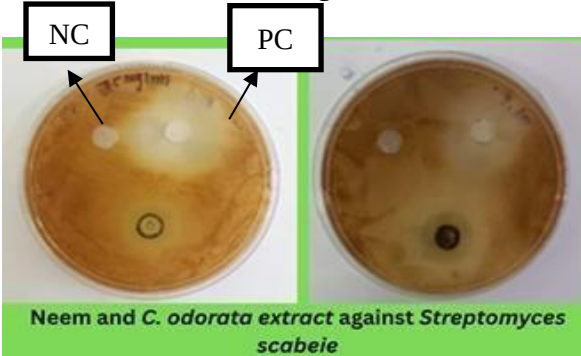
**RESULTS AND DISCUSSION**

The plant material was prepared and extracted according to the protocol mentioned above.

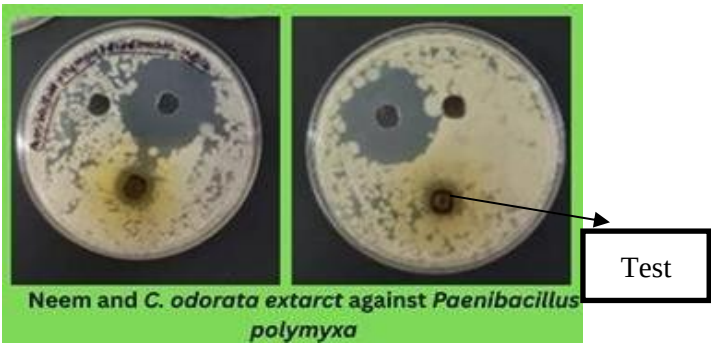
**Table 1.** Extraction Yield of plant extracts

| Plant extract              | Dried plant weight (g) | Extract weight (g) | Extraction Yield (%) |
|----------------------------|------------------------|--------------------|----------------------|
| <i>Azadirachta indica</i>  | 18                     | 1.96               | 10.8                 |
| <i>Chromolaena odorata</i> | 20                     | 2.1                | 10.5                 |

The well diffusion assay show that *A. indica* exhibits stronger inhibitory effects against *S. scabiei* than *C. odorata*. However, both extracts had much weaker activity against *P. polymyxa*, demonstrating selective toxicity-a desirable attribute for biocontrol agents.

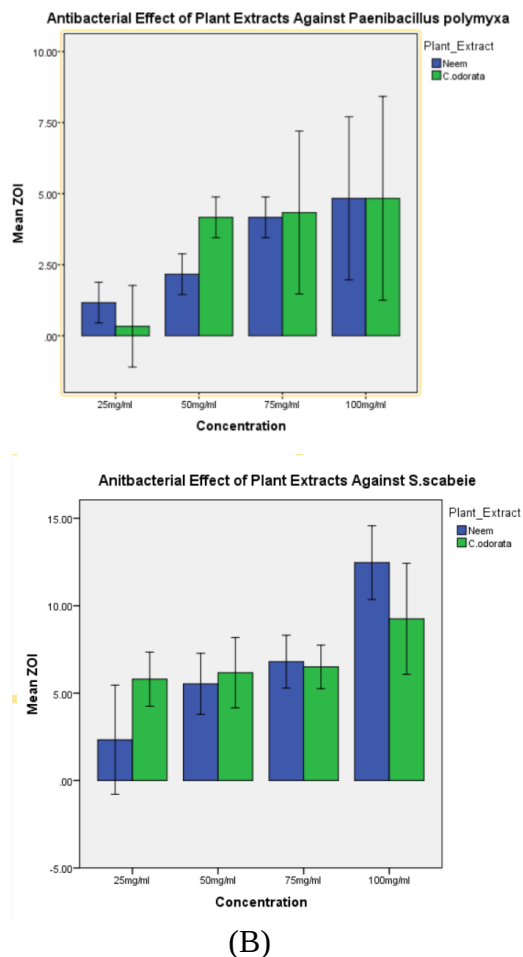


**Figure 1.** Well diffusion results of plant extracts against *S. scabiei*; PC: Positive Control, NC: Negative control



**Figure 2.** Well diffusion results of plant extracts against *P. polymyxa*

(A)

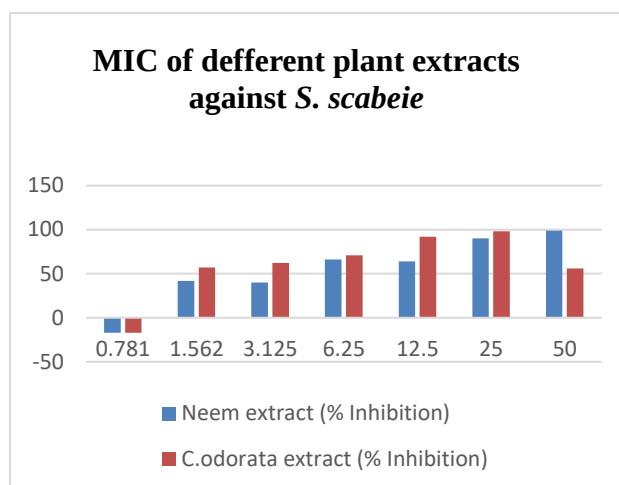


**Figure 3.** (A) Antibacterial effect of plant extracts against *S. scabiei*; (B) Antibacterial effect of plant extracts against *P. polymyxa*.

Figure 3. (A) indicates that *Azadirachta indica* and *Chromolaena odorata* showed antimicrobial activity against *Streptomyces scabiei* with the ZOI (Zone of Inhibition) measuring to be above 10 mm. With increasing concentration of the plant dilution, the ZOI increased. However, Figure 3. (B) indicates that both the plant extracts showed very minimal antimicrobial potential against the PGP bacteria. The ZOI against *P. polymyxa* was found to less than or equal to 5 mm at the highest concentration.

#### Analysis of MIC (Minimum Inhibitory Concentration) values

The MIC values confirmed the findings from the agar diffusion assay. From Figure 4., we can conclude that Neem showed a lower MIC (6.25 mg/mL) compared to *C. odorata* (12.5 mg/mL), indicating that neem is



**Figure 4.** Analysis of MIC of plant extracts

approximately twice as potent in inhibiting the growth of *S. scabiei*. In both the extracts, % inhibition increases with an increase in concentration, showing that the antibacterial response is dose-dependent. Very low concentrations, such as 0.781 mg/mL, show negligible or even negative inhibition, which might indicate insufficient antibacterial activity at the lowest dose. The inhibition gradually increases from 40% at 1.562 mg/mL to almost 100% at 50 mg/mL with a consistent upward trend. Strong inhibitions at 25 mg/mL (90%) and 50 mg/mL (100%) were obtained, showing the potent activity at higher concentrations. The MIC for neem seems to be within a range of 12.5-25 mg/mL. It displays relatively stronger inhibition in the mid-range concentrations compared to neem. Maximum inhibition of 98% was observed at 25 mg/mL and the MIC for *C. odorata* is approximately 12.5- 25 mg/mL.

#### MIC of Neem extract and *Chromolaena odorata* in combination against *Streptomyces scabies* using microdilution.

The plant extracts MIC, were found to be 6.25mg/mL and 12.5mg/mL for Neem and *C. odorata* respectively when tested individually against *Streptomyces scabiei*. They were tested in combination to check the synergistic effect.

**Table 2.** Analysis of combined effect of *C. odorata* and neem extract against *Streptomyces scabiei*

|                                        |      |
|----------------------------------------|------|
| MIC A Alone (mg/m L)                   | 6.25 |
| MIC B Alone (mg/m L)                   | 12.5 |
| MIC A in Combination (mg/mL)           | 1.56 |
| MIC B in Combination (mg/mL)           | 25   |
| FIC A (MICA Combination/ MIC A Alone)  | 0.25 |
| FIC B (MICB Combination / MIC B Alone) | 2.00 |
| FICI (FIC A + FIC B)                   | 2.25 |

Where A= *Azadirachta indica*;  
B= *Chromolaena odorata*

According to (A. Alqahtani et al., 2022), the combination defined synergy if  $\sum F.I.C. \leq 0.5$ , additive if  $0.5 < \sum F.I.C. \leq 1$ , indifference if  $1 < \sum F.I.C. \leq 4$  and antagonism as  $\sum F.I.C. > 4$ . From Table 2.,  $\sum F.I.C$  of the plant extracts was found to be 2.25, indicating indifferent interaction in combination.

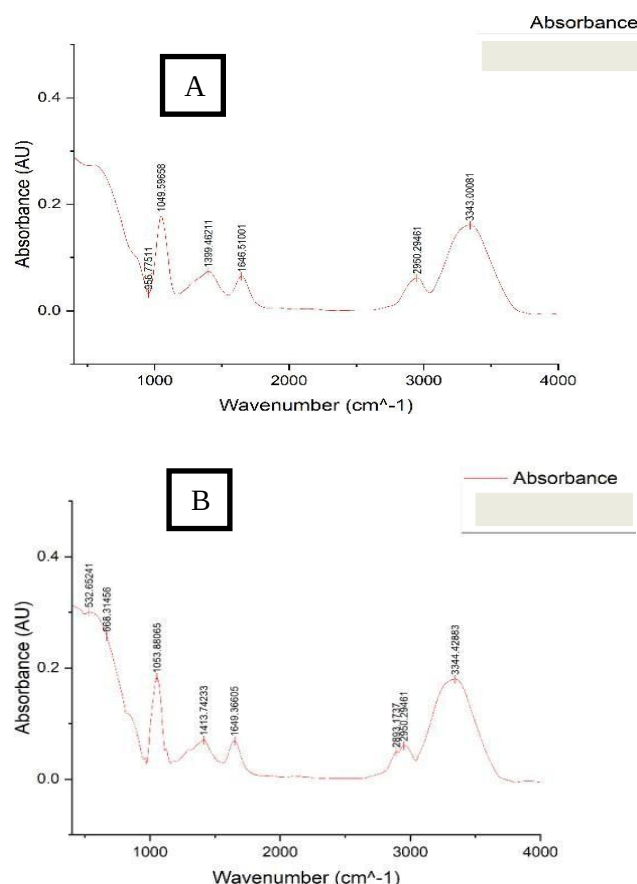
**Table 4.** Results for phytochemical analysis of plant extracts

| Phytochemical Test  | Neem | <i>C.odorata</i> |
|---------------------|------|------------------|
| Test for alkaloids  | +    | +                |
| Test for flavonoids | +    | +                |
| Test for glycosides | +    | +                |
| Test for phenols    | +    | +                |
| Test for tannins    | +    | +                |
| Test for terpenoids | +    | +                |
| Test for saponins   | —    | —                |

Table 3., indicates the compounds presence of certain phytochemicals in the plant extracts. According to (M. Takó et al., 2020), numerous bacteria can be inhibited in their development and activity by plant-derived phenolics, including tannins, flavonoids, stilbenes, and phenolic acids. It can be concluded broadly that the antimicrobial effect of *Azadirachta indica*

## Qualitative phytochemical analysis of plant extracts

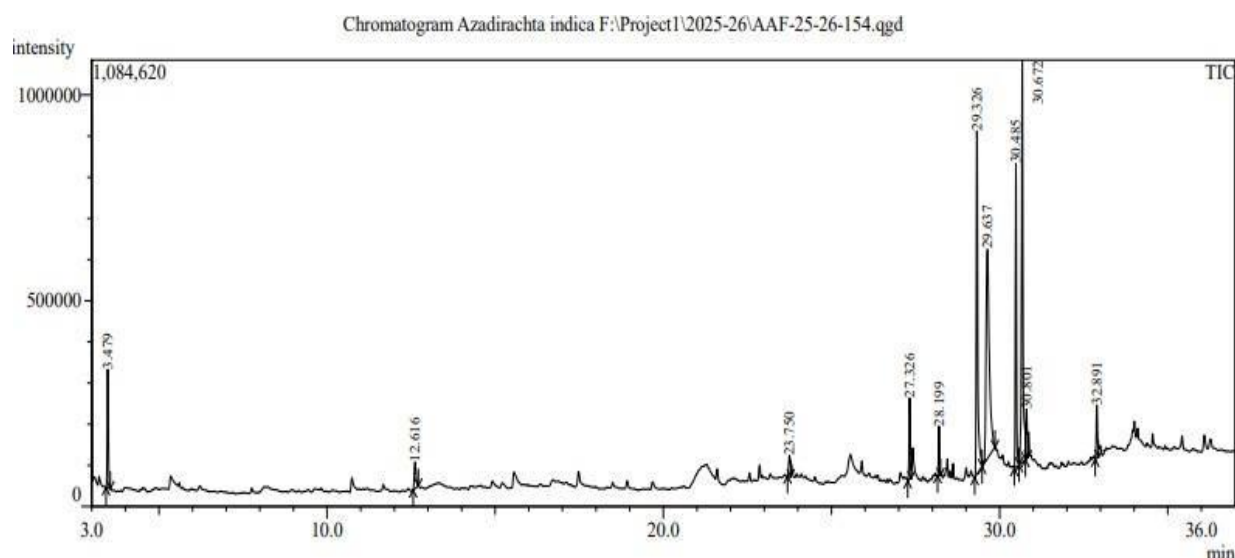
### FTIR analyses of plant extracts

**Figure 5.** (A) FTIR analysis of *Azadirachta indica* leaf extract; (B) FTIR analysis of *Chromolaena odorata* leaf extract**Table 3.** Compounds identified by FTIR

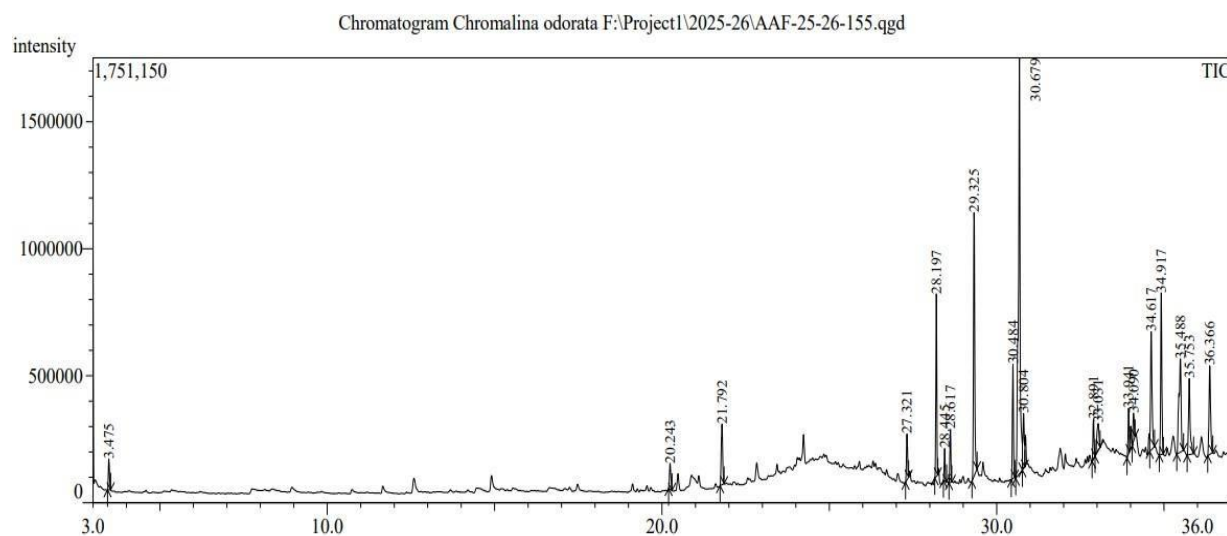
| Peak Position (cm <sup>-1</sup> ) | Functional Group                                     | Assignment                                   |
|-----------------------------------|------------------------------------------------------|----------------------------------------------|
| 3844–3330                         | O–H / N–H stretching                                 | Alcohols, phenols, amines                    |
| 2950–2850                         | C–H stretching (alkanes)                             | Saturated hydrocarbons                       |
| 1740–1640                         | C=O or C=C stretching                                | Ketones, esters, carboxylic acids, aromatics |
| 1450–1375                         | C–H bending or symmetric COO <sup>-</sup> stretching | Methyl/methylene groups or carboxylates      |
| 1200–1000                         | C–O stretching                                       | Alcohols, ethers, esters                     |
| 900–600                           | C–H out-of-plane bending (aromatic)                  | Aromatic rings / alkenes                     |

Table 4., indicates the peak position corresponding to specific functional groups. The plant extracts were found to contain the above-mentioned compounds after comparing the peaks. Hence, we can conclude that the presence of compounds like phenols, alcohols, carboxylic acids majorly contribute to the antimicrobial effect of the plant extracts (Takó *et al.*, 2020).

#### GCMS analysis of plant extracts



**Figure 7.** GC-MS analysis of *Azadirachta indica*



**Figure 6.** GC-MS analysis of *Chromolaena odorata*

**Table 5.** Major GC-MS analyzed compounds in the plant extracts

| Compound                                   | RT (min) | Area % |
|--------------------------------------------|----------|--------|
| <b><i>Chromolaena odorata</i></b>          |          |        |
| 9,12,15-Octadecatrienoic acid              | 30.679   | 26.73  |
| n-Hexadecenoic acid                        | 29.325   | 11.67  |
| 5,6,7,4'-Tetrahydroxyflavone               | 35.488   | 7.37   |
| Squalene                                   | 34.917   | 7.05   |
| Isopropyl linoleate diacetate              | 34.617   | 6.52   |
| <b><i>Azadirachta indica</i></b>           |          |        |
| Arabino-Hex-1-enitol, 1,5-anhydro-2-deoxy- | 29.637   | 26.58  |
| 9,12,15-Octadecatrienoic acid (Z, Z, Z)-   | 30.672   | 22.98  |
| n-Hexadecenoic acid                        | 29.326   | 18.69  |
| Phytol                                     | 30.485   | 12.45  |

Plant material extraction and analysis are critical for the development, updating, and quality control of herbal formulations. Table 5., analyzes the major peaks obtained from GC-MS analyses of the two plant extracts. GC-MS analysis showed the presence of bioactive compounds in *Azadirachta indica* and *Chromolaena odorata*. Some of them are tetra decanoic acid, squalene, and hexadecenoic acid which are examples of phytocompounds that have been found as antioxidants, 5-alpha-reductase inhibitors, antifibrinolytic, hemolytic, and antibacterial. Squalene, sometimes called triterpene, is a phenolic chemical are found in both latex and plant resins. They are recognized to have antibacterial and insecticide properties (Godlewska-Żyłkiewicz *et al.*, 2020). Further, octadecanoic acid has been reported to have both antimicrobial and antifungal effects. Phytol (3,7,11,15-tetramethylhexadec-2-EN-1-OL) is a key component of branched chain unsaturated terpenes produced by chlorophyll

metabolism in plants and can inhibit microbes (Ghaneian *et al.*, 2015). 5,6,7,4'-Tetrahydroxyflavone was also found to have anti-inflammatory and antioxidant activities (Wang *et al.*, 2023). It can be concluded that the antimicrobial effect of both the plant extracts against *Streptomyces scabiei* attributes to the presence of the above-mentioned compounds.

## CONCLUSION

Overall, the study shows that *Azadirachta indica* and *Chromolaena odorata* ethanolic extracts have strong antimicrobial activity against *Streptomyces scabiei*. Overall, *Azadirachta indica* showed better antimicrobial effect. However, *Chromolaena odorata*, a potential weed, showed significant antimicrobial effect. Additionally, at lower concentrations, these extracts have no effect on *Paenibacillus polymyxa*. Although the genus *Streptomyces* is known to inhibit the growth of other bacteria, cross steak assay demonstrated no interaction between the two bacteria; *Streptomyces scabiei* and *Paenibacillus polymyxa*. This demonstrates the suitability for the preparation of a bioformulation combining *Paenibacillus polymyxa*, and the two plant extracts. The antimicrobial potential of the plant extracts is supported by the presence of a variety of bioactive compounds that were found through phytochemical screening and FTIR, GC-MS. In order to control scab disease and advance plant health, the study establishes the foundation for creating a synergistic plant-microbe-based biofertilizer.

## Conflict of Interest

The authors have no Conflict of Interest

## Contribution of Authors

Both authors contributed to the idea and design of the study. Hannah Leah Lijo, performed the experiments and analyzed data using SPSS and Excel. Dr. Sandheep P.N. supervised the study, analyzed the manuscript, corrected and approved it.

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Hannah Leah Lijo, and Sandheep P.N.\*

<sup>1,2</sup>Christ University, Bangalore Central Campus, Hosur Road, Near Dairy Circle, Bangalore, Karnataka-560029, India

<sup>1</sup>Email id: hannahlijo2003@gmail.com,

\* Corresponding Author-Email id: sandheep.pn@christuniversity.in.