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Research Article

Different Methods Used For In Vitro Antitubercular Activity in Extracts of Flowers of *Jasminum Sambac* L. Ait.

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ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a global public health challenge, worsened by rising drug resistance and limited new therapeutics. Natural products derived from medicinal plants offer a valuable source of novel antimycobacterial agents. The flowers of *Jasminum sambac* (Oleaceae), traditionally used in various medicinal contexts, have demonstrated antibacterial, antifungal, antioxidant, and anti-inflammatory activities. (Rasheed et al., 2022; plant review literature) This article proposes a comprehensive framework for evaluating the in vitro antitubercular potential of *J. sambac* flower extracts. We detail methods of extraction (maceration, Soxhlet), phytochemical screening, and application of multiple in vitro mycobacterial drug susceptibility assays including the microplate Alamar Blue assay (MABA), resazurin microtiter assay (REMA), agar dilution / proportion method, automated liquid culture (e.g., BACTEC MGIT 960), and rapid colorimetric assays such as the Nitrate Reductase Assay (NRA) or tetrazolium-based assays. We discuss how minimum inhibitory concentration (MIC) values might be determined and compared with standard anti TB drugs (e.g., isoniazid, rifampicin). We analyze advantages and limitations of each method in the context of plant-extract screening, propose a hypothetical experimental design, and suggest how active extracts could be subjected to bioassay-guided fractionation. Finally, we recommend future directions including in vivo evaluation, cytotoxicity assays, standardization and chemical characterization, and adoption of emerging rapid screening technologies. This framework aims to facilitate discovery of novel plant-derived antitubercular candidates, leveraging the pharmacological potential of *J. sambac*.

INTRODUCTION

1.1. Tuberculosis: Global Burden and Drug Resistance

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Tuberculosis (TB) remains among the top infectious diseases worldwide, with millions of new cases and over a million deaths each year. The increasing prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* complicates TB control

and underscores the urgent need for new therapeutic agents. Conventional frontline drugs such as isoniazid and rifampicin remain effective in many cases but face limitations due to resistance, toxicity, long duration of therapy, and poor compliance.

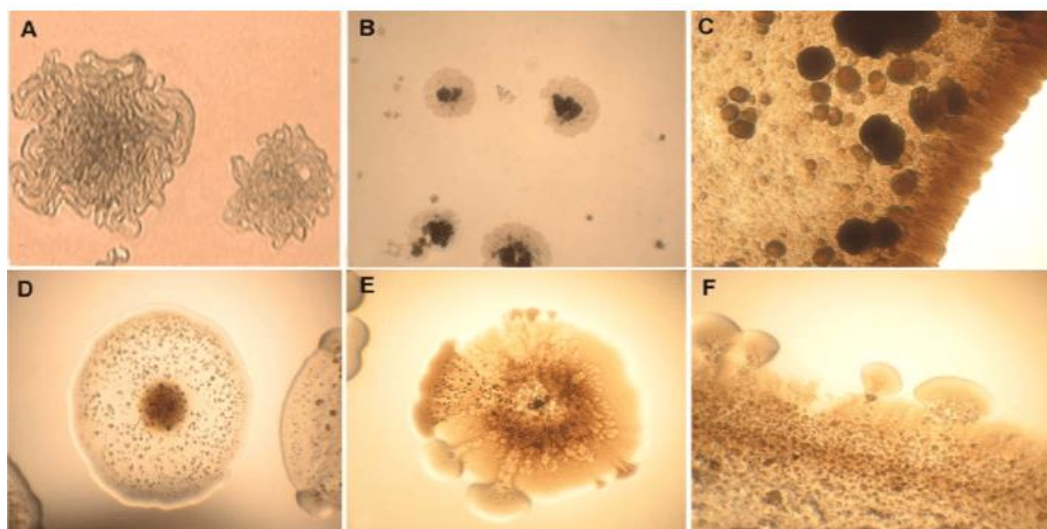


Fig no 1: *M. tuberculosis* Active colonies .in light microscope

1.2. Natural Products and Medicinal Plants as a Source of Novel Anti-TB Agents

Historically, natural products have provided important drugs across many therapeutic areas. Plant secondary metabolites including alkaloids, flavonoids, terpenes, phenolics, tannins, and saponins exhibit broad biological activities, among them antimicrobial effects. Medicinal plants thus represent a rich source for potentially novel compounds with antitubercular activity, especially relevant given the global need for affordable and diverse therapies.

1.3. Pharmacological Potential of *Jasminum sambac* Flowers

Jasminum sambac L. (commonly “mogra”) is a fragrant flowering shrub widely distributed across Asia. Several studies report that its extracts

(flowers, leaves) show antimicrobial, antioxidant, anti-inflammatory, and other bioactivities. For example, a recent study demonstrated that hot and cold extracts (ethanolic and aqueous) of *J. sambac* flowers inhibit several foodborne bacterial pathogens, with measurable MIC values. (Rasheed et al., 2022) Another review of *Jasminum* species highlighted antifungal and antimicrobial potential of flower and leaf extracts, including activity against skin-associated fungi. (Open Science Publications review) In addition, phytochemical analysis of *J. sambac* extracts reveal presence of phenolics, flavonoids, terpenoids, saponins, tannins and other secondary metabolites chemical scaffolds often associated with antimicrobial efficacy. (Development of *J. sambac* flower extracts for antioxidant/beauty applications) These findings suggest that *J. sambac* flower extracts could harbor compounds with activity against mycobacteria, motivating systematic evaluation.

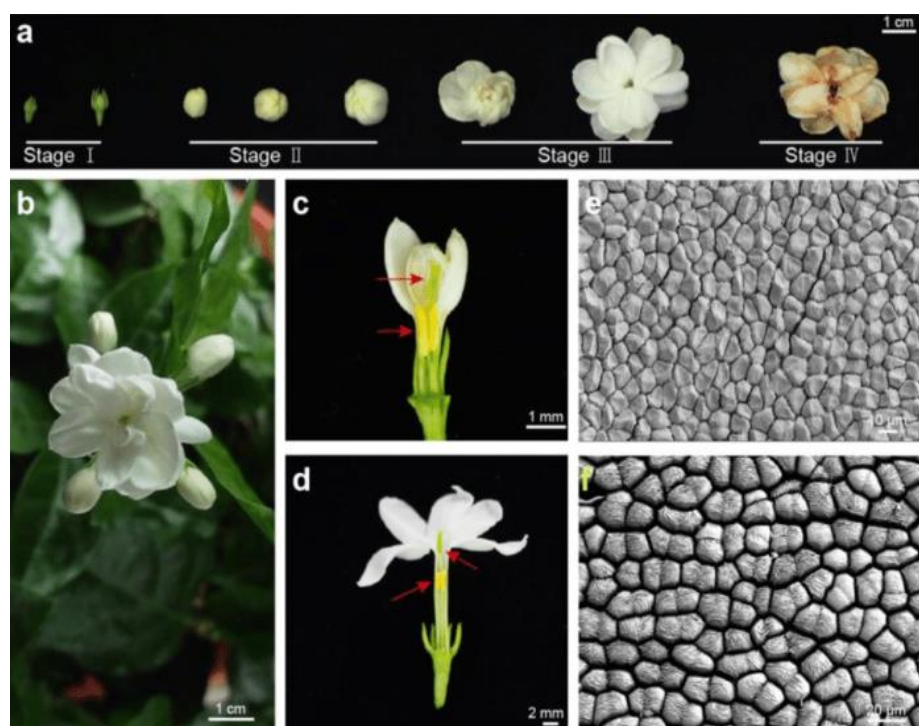


Fig no.2. Morphology of *Jasminum sambac* L. Ait.: flowering shrub (left) and close-up of flowers used for extraction (right).

1.4. Rationale and Significance

Given the global TB burden and urgent need for new antimycobacterial agents, exploring medicinal plants like *J. sambac* is scientifically justified. Demonstration of in-vitro antitubercular activity would open avenues for bioassay-guided

fractionation, chemical characterization, and eventual in-vivo testing. Moreover, plants accessible in low-resource and high-TB-burden regions could provide affordable, culturally acceptable therapies. For these reasons, a robust, multi-method in-vitro screening framework for *J. sambac* flower extracts is timely and necessary.

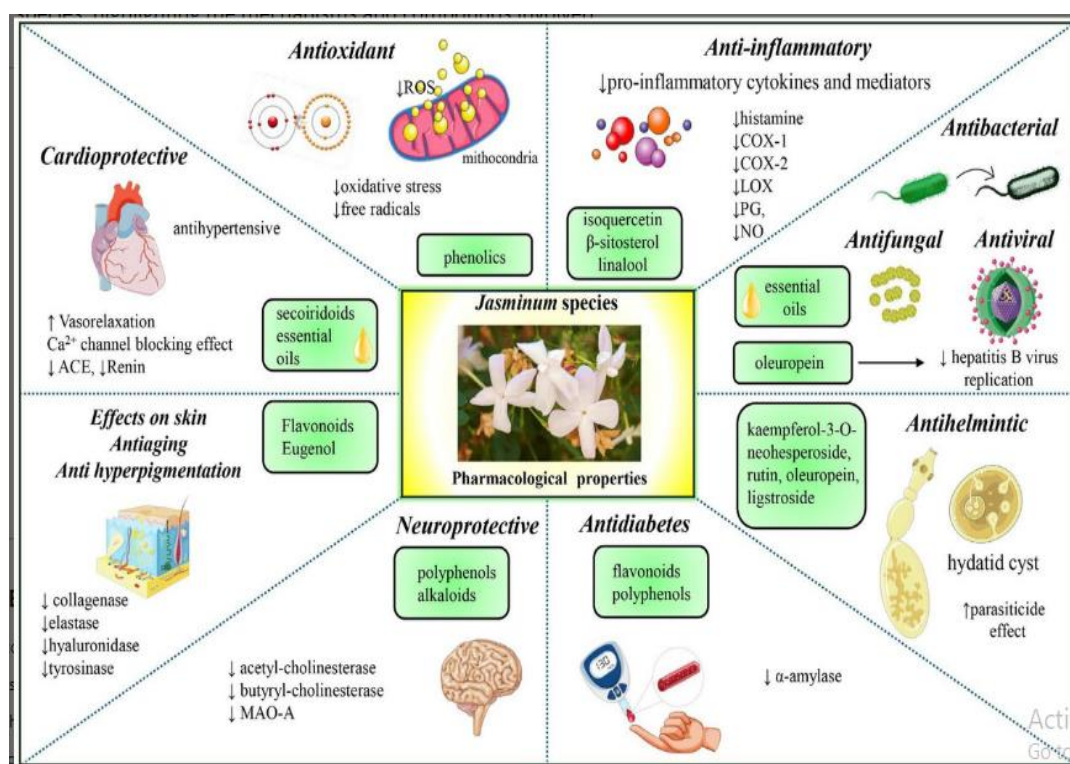


Fig no.3: Rationales and significance in other activity

2. Aim and Objectives

2.1.Aim:

To establish and describe a robust, multi-method in-vitro screening framework for assessing antitubercular activity of *Jasminum sambac* flower extracts, to identify potential lead extracts/fractions for further development.

2.2. Objectives:

1. Collect and prepare *J. sambac* flower material; perform solvent extraction using different techniques (maceration, Soxhlet) and solvents (polar and non-polar).
2. Conduct preliminary phytochemical screening to identify presence of major classes of secondary metabolites (e.g., flavonoids, phenolics, terpenoids, saponins, tannins).
3. Apply multiple in-vitro drug-susceptibility assays against *M. tuberculosis* (or surrogate

mycobacteria) including microplate assays (MABA, REMA), agar dilution / proportion method, automated liquid culture (MGIT 960), and colorimetric assays (NRA or tetrazolium-based), to determine MICs of extracts.

4. Compare MICs of active extracts with standard anti-TB drugs (e.g., isoniazid, rifampicin) under the same assay conditions.
5. Evaluate and compare the performance, advantages, and limitations of each assay method for screening plant extracts.
6. Propose steps for bioassay-guided fractionation, chemical characterization, cytotoxicity assessment, and eventual in-vivo testing of promising extracts/fractions.

3. MATERIALS AND METHODS:

3.1. Collection and Preparation of Plant Material:



- **Collection:** Fresh flowers of *J. sambac* will be collected at peak blooming from botanically authenticated plants. A voucher specimen will be deposited in a recognized herbarium.
- **Drying and Pulverizing:** Flowers will be washed with distilled water, air-dried in shade at ambient temperature (~25–30 °C) until constant weight (typically 7–10 days), then pulverized into coarse powder. Powdered material stored in airtight containers at 4 °C until extraction.
- **Maceration:** 50–100 g of dried flower powder soaked in 500–1000 mL of solvent (e.g., ethanol, methanol, chloroform, water) for 48–72 h at room temperature with occasional stirring. Filtrate collected and solvent removed by rotary evaporation under reduced pressure at 40–45 °C, yielding crude extract.
- **Soxhlet Extraction:** 30–50 g of powder subjected to Soxhlet extraction with chosen solvent (e.g., methanol, ethanol, chloroform) for 6–8 h, followed by concentration under reduced pressure.

3.2. Extraction Methods:

Multiple extraction protocols will be used to maximize recovery of a broad range of secondary metabolites:

- **Yield Calculation:** Final dry extract weight recorded; percent yield (w/w) calculated relative to dry plant material. Extracts stored at 4 °C.

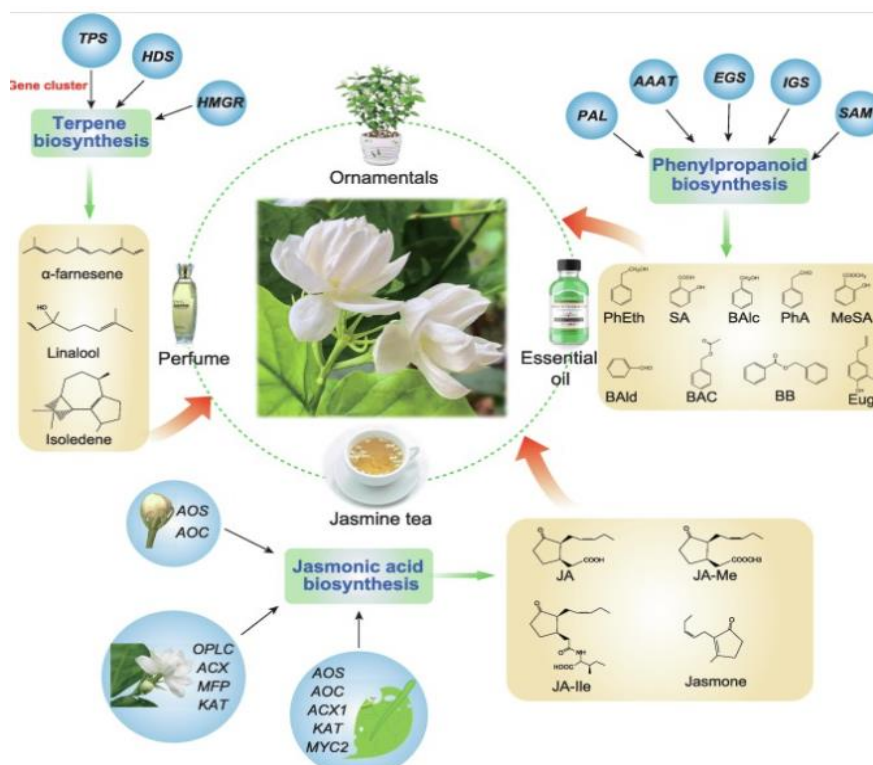


Fig no.4: Schematic diagram of genes, compounds, and applications in *J. sambac*

3.3. Phytochemical Screening:

Qualitative phytochemical assays will be performed on each crude extract to detect major classes of secondary metabolites:

- **Alkaloids:** Mayer's or Dragendorff's test.
- **Flavonoids:** Shinoda test or alkaline reagent test.
- **Tannins/Phenolics:** Ferric chloride test, lead acetate test.
- **Saponins:** Froth (foam) test.
- **Steroids/Terpenoids:** Liebermann–Burchard test, Salkowski test.
- **Glycosides:** Keller–Kiliani test (for cardiac glycosides) if desired.

Positive detection will guide prioritization of extracts for antimycobacterial screening. Further chemical characterization (e.g., HPLC, GC–MS, LC–MS, NMR) may be conducted on extracts/fractions showing activity.

3.4. In-Vitro Antitubercular Activity Assays:

Because *M. tuberculosis* is a BSL-3 pathogen requiring specialized containment, the study must be conducted in appropriate biosafety facilities. Alternatively, surrogate non-pathogenic mycobacteria (e.g., *M. bovis* BCG) may be used for preliminary screening, with later validation against *M. tuberculosis*.

The following assays are proposed:

3.4.1. Microplate Alamar Blue Assay (MABA):

- **Principle:** Based on metabolic reduction of resazurin (blue) to resorufin (pink) by viable mycobacteria. (Franzblau et al., 1998)
- **Procedure:** Use sterile 96-well microtiter plates. In each well: Middlebrook 7H9 broth supplemented with OADC, serial two-fold dilutions of plant extract (e.g., starting concentration 1–10 mg/mL, diluted down),

and a standardized inoculum of mycobacteria (e.g., $1-5 \times 10^5$ CFU/mL). Include growth control wells (no extract) and positive controls (standard drugs: isoniazid, rifampicin). Incubate at 37 °C for 7–14 days (depending on growth kinetics). After incubation, add Alamar Blue (resazurin) reagent (e.g., 10% v/v) and incubate additional 24–48 h. Observe color change (blue → pink) visually or spectrophotometrically. The MIC is defined as lowest concentration of extract that prevents color change. Perform each assay in triplicate.

- **Advantages:** Low cost, no requirement for expensive instrumentation, suitable for high-throughput screening, uses a non-toxic and stable redox indicator. (Franzblau et al., 1998)
- **Limitations/Considerations:** Plant extracts may contain colored compounds or redox-active constituents interfering with resazurin reduction or color reading; extracts may precipitate; clumping of mycobacteria may affect assay uniformity.

3.4.2. Resazurin Microtiter Assay (REMA) / Colorimetric Microplate Assays

- **Principle & Procedure:** Very similar to MABA. REMA is widely used for antimycobacterial drug-susceptibility testing. (Palomino et al., 2002). Use of resazurin as growth indicator in 96-well format, with serial dilutions of extracts or drugs, inoculation of standardized bacterial suspensions, incubation and colorimetric read-out.
- **Advantages:** Rapid (results in ~7–10 days), nonradioactive, cost-effective, suitable for screening multiple samples. (International Journal of Mycobacteriology evaluation)



- **Limitations:** Similar to MABA — interference by plant extract constituents; may need assay optimization (e.g., extract solubility, emulsification, adequate controls).

3.4.3. Solid Media Agar Dilution / Proportion Method

- **Principle:** Incorporating varying concentrations of plant extract (or drug) into solid medium (e.g., Middlebrook 7H10/7H11 agar or Löwenstein–Jensen medium), inoculating a defined bacterial inoculum, and incubating for extended periods (weeks). Colony formation indicates growth; MIC (or minimal inhibitory proportion) defined as lowest concentration preventing growth (or causing defined reduction in colony count). Solid proportion (agar dilution) method remains a “gold standard.” (Delamanid DST study using agar proportion as reference)
- **Advantages:** Direct observation of colony formation, accepted standard, less risk of interference by extract color or redox activity.
- **Limitations:** Very slow (3–5 weeks typical), labor-intensive, low throughput; not practical for screening many extracts or fractions.

3.4.4. Automated Liquid Culture System: BACTEC MGIT 960

- **Principle:** Uses fluorescence-based oxygen-quenching sensor growth of mycobacteria consumes oxygen, changing fluorescence; automated detection of growth; allows measurement of MIC by comparing growth kinetics in presence vs absence of drug / extract. (Chang et al.; multiple studies)
- **Procedure (modified for plant extracts):** Prepare MGIT 960 tubes supplemented with OADC; add defined concentrations of plant

extract; inoculate with standardized bacterial suspension; incubate in MGIT 960 instrument; monitor growth automatically. MIC defined as lowest extract concentration preventing tube positivity (or achieving growth suppression compared to control) within a defined time frame. For example, in DST of ciprofloxacin and ethionamide, results were obtained within 5–17 days (mean ~8.9 days).

- **Advantages:** Automated, relatively rapid compared to solid agar, good correlation with agar dilution (e.g., 80–98% agreement in various studies) higher throughput than solid media; does not require radioisotopes.
- **Limitations/Challenges:** Requires specialized and expensive instrumentation; plant extracts may have solubility issues in liquid medium; extract components may interfere with fluorescence read-out; need validation to ensure extract (not medium or extract precipitation) is responsible for growth inhibition.

3.4.5. Colorimetric Nitrate Reductase Assay (NRA) / Modified NRA (MONRA)

- **Principle:** Viable mycobacteria reduce nitrate to nitrite; nitrite detection (e.g., via Griess reagent) indicates growth. In presence of inhibitory compound (drug or extract), color change is suppressed. This can serve as a proxy for growth/inhibition. (Martin et al., 2001; early studies).
- **Procedure (proposed):** Use solid or liquid medium containing potassium nitrate and varying concentrations of plant extract. Inoculate with standardized bacterial inoculum. After incubation (e.g., 7–10 days for rapid liquid-based or 14–21 days for solid-based), add nitrate/nitrite detection reagents



(e.g., Griess reagent). Observe color change absence of colour change indicates inhibition. Include growth controls (no extract) and sterility controls.

- **Advantages:** Cheap, relatively rapid (results in ~9–10 days in many studies) compared to agar proportion (21–28 days). (Studies from India and Brazil) minimal equipment requirement suitable for resource-limited settings.
- **Limitations:** Not yet widely validated for plant-extract screening; background reduction (false positives/negatives) may occur due to reductive or oxidative compounds in extracts; requires careful controls; colorimetric read-out may be influenced by extract color.

3.4.6. Microscopic Observation Drug Susceptibility (MODS) Assay

- **Principle:** Based on direct microscopic observation of characteristic mycobacterial “cording” growth in liquid culture containing test compound/drug. Absence of cording in drug-containing wells indicates susceptibility. (Caviedes et al., classic MODS protocol)
Procedure (adapted for plant extracts): Prepare 24-well (or 48-well) microplates containing liquid medium (e.g., 7H9 + OADC + glycerol) with serial dilutions of extract; inoculate with standardized mycobacterial suspension; incubate at 37 °C in 5–10% CO₂; monitor wells every 1–2 days under inverted light microscope at ×40 magnification. Cording / acid-fast bacilli detection indicates growth. MIC defined as lowest concentration with no visible growth.
- **Advantages:** Faster than agar proportion (e.g., results often evident within 7–14 days); low-cost; no expensive instrumentation; direct

visualization avoids reliance on dyes or fluorescence. (Studies show MODS sensitivity/specificity comparable to automated or colorimetric assays)

- **Limitations:** Requires skilled microscopy; more subjective; risk of contamination; plant-extract precipitates may obscure observation; scalability limited; biosafety concerns remain.

4. Emerging / Novel Rapid Methods (e.g., Spectroscopy-Based AST):

Emerging approaches aim to reduce time-to-results dramatically. For example, a recent study demonstrated rapid, antibiotic-incubation-free TB drug resistance testing using few-to-single cell Raman spectroscopy combined with machine learning achieving classification of drug resistant vs susceptible mycobacteria within hours without requiring culture. While highly promising, such methods are still in proof-of-concept stage and not yet validated for plant-extract screening; moreover, they require specialized equipment and computational infrastructure. For now, they remain future tools for rapid screening once validated and adapted.

5. Comparison with Standard Drugs

In each assay, standard first-line anti-TB drugs (e.g., isoniazid, rifampicin) must be run in parallel under identical conditions (same medium, inoculum, incubation times) to provide reference MICs. This comparative approach allows estimation of relative potency of plant extracts and helps prioritize extracts/fractions for further development.

6. Statistical Analysis

All assays should be performed in at least triplicate (biological replicates) and, when possible, repeated independent times to ensure



reproducibility. MIC values ($\mu\text{g/mL}$ or mg/mL) will be reported as mean $\pm$ standard deviation (SD). For comparisons among extraction methods or assay types, statistical tests such as one-way ANOVA (for more than two groups) followed by appropriate post-hoc tests (e.g., Tukey's) may be used; a p-value < 0.05 may be considered statistically significant. For categorical data (e.g., susceptible vs resistant), measures of agreement (e.g., percentage agreement, kappa coefficient) may be calculated if comparing methods.

7. Hypothetical Results:

Because no published experimental data currently documents antitubercular activity of *J. sambac* flower extracts against *M. tuberculosis*, this section outlines expected outcomes and proposes how results should be presented in real experiments.

Table 1. Yield and Phytochemical Profile of *J. sambac* Flower Extracts.

Extract (Solvent / Method)	Yield (% w/w)	Phytochemicals Detected*
Ethanol (maceration)	e.g., 5–10%	Flavonoids, phenolics, tannins, saponins, alkaloids, terpenoids
Methanol (Soxhlet)	6–9%	Similar to ethanol; possibly more polar + moderately non-polar constituents
Chloroform (Soxhlet)	3–6%	Terpenoids, steroids, lipophilic compounds
Aqueous (maceration)	4–7%	Glycosides, saponins, polar phenolics / flavonoids

*Based on qualitative phytochemical assays.

Table 2. Hypothetical MIC Range of Active Extracts Against *M. tuberculosis* (via REMA / MABA).

Extract (Solvent)	MIC ($\mu\text{g/mL}$)	Remarks
Ethanol (maceration)	125–500	Moderate activity
Methanol (Soxhlet)	250–1000	Weak to moderate
Chloroform (Soxhlet)	>1000 (inactive)	Likely inactive or not soluble
Aqueous (maceration)	500–1000	Weak activity
Standard drug (isoniazid)	~ 0.05 – 0.1 (as per reference assay)	Positive control

These MICs are hypothetical; real data should be generated experimentally.

8. DISCUSSION:

8.1. Feasibility and Rationale for *J. sambac* Flower Extract Screening:

The known bioactivities of *J. sambac* antibacterial, antifungal, antioxidant support the hypothesis that its flower extracts may exert antimycobacterial effects. The presence of phenolics, flavonoids, terpenoids, saponins, and other secondary metabolites (as identified in prior phytochemical studies) suggests a chemical basis for potential inhibition of mycobacterial growth. (Rasheed et al., 2022; review literature) Moreover, in other medicinal plants, crude extracts have shown MIC values in the range of ~ 150 – $500 \mu\text{g/mL}$ against *M. tuberculosis* using colorimetric assays. (For example, leaves of *Solanum torvum* showed MIC $\approx 156.3 \mu\text{g/mL}$ against *M. tuberculosis* H37Ra) These data suggest that plant extracts, though less potent than conventional drugs, may serve as starting points (“leads”) for further fractionation and compound isolation.



Given the complexity of plant extracts, a screening approach that uses multiple in-vitro methods is justified: initial high-throughput colorimetric assays (MABA/REMA), followed by confirmatory assays (MGIT 960, agar dilution, or NRA), to avoid false positives/negatives and to validate activity robustly.

8.2. Strengths and Limitations of Different Assay Methods in Plant-Extract Context:

- **MABA / REMA:** Efficient for initial screening, but may give misleading results if plant extracts interfere (e.g., colored compounds reducing resazurin, or precipitate formation). Therefore, appropriate controls must be included: medium + extract (no bacteria) to check for color change; extract-free wells; solvent controls; and ideally spectrophotometric read-out rather than visual color assessment alone.
- **MGIT 960:** Offers automation, standardization, and speed; shows good concordance with traditional agar dilution (e.g., 80–98% agreement in drug susceptibility studies). However, its use with plant extracts is less well-documented; solubility of crude extracts, potential fluorescence interference, and stability over the long incubation period must be addressed. For any extract showing activity in MABA/REMA, performing MGIT 960-based MIC determination would strengthen evidence.
- **NRA / MONRA:** Provides a low-cost, relatively rapid alternative, especially in resource-limited settings. Studies have shown high sensitivity and specificity compared to conventional methods for standard drugs (e.g., INH, RIF) with results available in ~9–10 days. The main challenge is that plant extract components may interfere with nitrate reduction or reagent read-out; hence blank controls (medium + extract) and appropriate negative/positive controls are mandatory. If optimized, NRA could be a practical DST for plant extracts in low-resource labs.
- **MODS:** Useful when microscopy expertise is available; faster than solid agar; no reliance on dyes or fluorescence, which may be affected by extract components. However, subjective interpretation of cording, need for acid-fast staining, and risk of contamination or extract precipitation complicate its use.
- **Solid Agar Dilution / Proportion Method:** Remains the most direct, least artifact-prone method. However, its long turnaround time (weeks) and labor-intensive nature make it unsuitable for screening many extracts; better reserved for confirmatory testing of most promising extracts/fractions.
- **Emerging Rapid Methods (e.g., Raman Spectroscopy + Machine Learning):** Promise to change the landscape dramatically by enabling near-real-time susceptibility testing without long culture. A recent proof-of-concept study demonstrated classification of drug resistance in mycobacteria using Raman spectroscopy of few-to-single cells, achieving >98% accuracy on dried BCG samples and ~79% on sputum. Once validated and optimized for plant-extract screening (e.g., ensuring that extract components do not interfere with spectral signatures), such methods could revolutionize natural-product screening by drastically reducing time and resource requirements.

8.3. Proposed Workflow / Strategy for *J. sambac* Extract-Based TB-Drug Discovery:



1. **Extraction and Phytochemical Screening:** Prepare multiple extracts (polar to non-polar), carry out qualitative phytochemical analysis, record yield and chemical profile.
 2. **Initial High-Throughput Screening:** Use MABA or REMA to screen all crude extracts for activity against surrogate mycobacteria or (if BSL-3 available) *M. tuberculosis*. Include proper controls.
 3. **Confirmatory Assays:** For extracts showing promising MICs (e.g., ≤ 500 $\mu\text{g/mL}$), perform MIC determination using MGIT 960 and/or NRA, and (if feasible) solid agar dilution. Evaluate reproducibility, dose-response, and calculate MIC ($\mu\text{g/mL}$).
 4. **Cytotoxicity and Selectivity:** Conduct cytotoxicity assays (e.g., MTS, resazurin on mammalian cell lines) to compute a selectivity index ($\text{SI} = \text{IC}_{50}$ for mammalian cells / MIC for mycobacteria). Only extracts/fractions with acceptable SI should proceed further. (As done in previous plant-TB studies)
 5. **Bioassay-Guided Fractionation:** Fractionate active extracts (e.g., by chromatography) and repeat MIC testing to isolate more potent fractions / pure compounds.
 6. **Chemical Characterization:** Use chromatographic and spectroscopic methods (HPLC, LC-MS/MS, NMR) to identify active constituents.
 7. **In-Vivo Studies (if applicable):** For fractions/compounds with potent in-vitro activity and acceptable SI, proceed to animal model testing (e.g., murine TB model), pharmacokinetics, toxicity, etc.
 8. **Standardization & Quality Control:** Develop standard extraction and fractionation protocols; define marker compounds; test batch-to-batch consistency; evaluate stability.
 9. **Consider Advanced High-Throughput Methods:** As resources allow, consider integrating rapid AST methods (e.g., Raman spectroscopy-based) for large-scale screening of natural-product libraries.
- ### 9. Challenges and Key Considerations:
- **Biosafety:** Working with *M. tuberculosis* requires BSL-3 labs; if not available, surrogate mycobacteria (e.g., *M. bovis* BCG) may be used initially, but definitive results require MTB testing.
 - **Extract Complexity / Solubility:** Crude extracts contain complex mixtures; some constituents may precipitate, degrade, or interfere non-specifically. Solubility in culture medium and stability over the incubation period must be verified.
 - **Assay Interference:** Plant compounds may interfere with colorimetric or fluorescence read-outs; appropriate blank controls are essential.
 - **Reproducibility:** Variability between extract batches (due to plant material, environment, extraction) can affect results hence standardization is critical.
 - **Translation to In-Vivo Efficacy:** Even potent in-vitro activity does not guarantee in-vivo efficacy; factors such as bioavailability, metabolism, toxicity, and pharmacokinetics must be addressed.
- ### 10. CONCLUSION
- This article provides a scientifically grounded, comprehensive framework for in-vitro screening



of *Jasminum sambac* flower extracts for antitubercular activity. Given the known bioactivities and phytochemical richness of *J. sambac* plus the urgent need for new anti-TB agents such an approach is timely and justified. Employing a combination of assays (colorimetric microplate, automated liquid culture, nitrate reduction, microscopic observation, and traditional solid media) increases the robustness of screening and minimizes false-positive/negative results. While challenges (biosafety, extract complexity, assay interference) remain significant, careful experimental design with appropriate controls and stepwise validation can mitigate them. Promising extracts identified through this framework can proceed to bioassay-guided fractionation, chemical characterization, cytotoxicity testing, and possibly in-vivo evaluation, paving the way toward novel plant-derived anti-TB agents. Furthermore, as advanced rapid AST technologies become accessible (e.g., Raman spectroscopy-based), the throughput and speed of natural-product screening will likely improve, accelerating drug discovery.

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